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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 proved to be promising to unravel the mechanisms involved in the 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) could generate enteroids. Here, we describe methods to establish gastric, small intestinal, and colonic epithelial organoids (Basic Protocol 1) and the generation of Lgr5⁺ single cell-derived epithelial organoids (Basic Protocol 2). We also describe the imaging techniques used to characterize those organoids (Basic Protocol 3). This in vitro model constitutes a powerful tool for studying stem cell biology and intestinal epithelial cell physiology throughout the digestive tract.

Keywords

Gastrointestinal stem cells; 3-dimensional cell culture; organoids; Lgr5 cell sorting; Imaging

INTRODUCTION

The intestine is organized into crypt-villus units which are lined by 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

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give rise to all intestinal epithelial lineages *i.e.* enterocytes, enteroendocrine cells, 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 tip 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 transformed and cancer-derived intestinal epithelial cell lines, have proved to be important tools for the study of intestinal physiology and have been useful experimental systems to elucidate mechanisms of proliferation, barrier function, epithelial nutrient and ion transport. However, none of these clonal cell cultures reflect the morphological and functional intestinal epithelium. In contrast, primary cell cultures which allow maintenance of a more physiological environment for the epithelial cells have proved to be promising (Simon-Assmann et al., 2007).

Recently, *Sato et al.* establish 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., 2011; Sato et al., 2009). These 3-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 (Spence et al., 2011; Tait et al., 1994), we collectively term these 3-dimensional structures epithelial organoids. More specifically, epithelial organoids from the stomach are gastroids, from the small intestine are enteroids (Stelzner et al., 2012), and 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 stomach, small intestine and colon crypts as well as the generation of $Lgr5^{+ve}$ single cell-derived epithelial organoids. In this methodological review, we also emphasize the imaging modalities that could be used to characterize this system and the possible experimental strategies carried out by this model.

BASIC PROTOCOL 1: Enteroids derived from small intestinal crypts

In this section, we describe a protocol for the isolation and culture of primary small intestine crypts into 3-dimensional units called enteroids. This method is the basis for other epithelial organoid cultures which will be presented as Alternate Protocol 1 (gastric) and Alternate Protocol 2 (colon) (Figure 1). This basic protocol outlines the isolation process and culture of small intestinal crypts as well as the maintenance of the enteroids over time.

Materials

Animals

Murine C57BL6/J strain (Jackson laboratory) aged from 6 to 8 weeks is used for intestinal crypts culture.

Reagents and solutions

Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (PBS).

Crypt chelating buffer (see recipe).

MatrigelTM, basement membrane matrix, growth factor reduced (GFR), phenol red-free (R&D Systems®).

Murine recombinant Wnt3a (R&D Systems®, 1 000× stock ; 100 µg/mL in sterile PBS/0.1% BSA).

Murine recombinant R-spondin 1 (R&D Systems®, 1 000× stock ; 1 mg/mL in sterile PBS/0.1% BSA).

Murine recombinant Noggin (R&D Systems®, 1 000× stock; 100 µg/mL in sterile PBS/0.1% BSA).

Human recombinant EGF (R&D Systems®, 10 000× stock ; 500 µg/mL in sterile PBS/0.1% BSA).

Minigut media (see recipe).

Complete crypt culture medium (see recipe).

Dissociation buffer (see recipe).

Freezing medium (see recipe).

Isopropyl alcohol.

Y27623 compound (Sigma-Aldrich®, 10 mM in ultrapure H_2O , sterile filtered with 0.22 µm filter).

Equipment

CO_2 incubator.

70% ethanol.

Tissue forceps.

Surgical scissors.

10 mL syringe with 18 gauge needle.

50 mL and 15 mL polypropylene conical tubes.

70 µm cell strainer.

24-well plate.

1 mL syringe with 27½ gauge needle (insulin syringe).

Cryovials.

Freezing container (Mr. Frosty, Nalgene®).

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 CO₂ 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 CO₂, immediately followed by cervical dislocation.
- 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 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 PBS using a 10 mL syringe mounted with an 18 gauge needle. The needle is placed into the lumen and the flushing proceeds until the removal of chyme is complete.
- 6 Cut the dissected intestine open lengthwise and chop with a razor into 2–4 cm pieces in ice-cold PBS. Place it in a 15 ml conical tube filled with 10 ml ice-cold PBS.
- 7 Gently invert the tube 4 times and discard the supernatant. Add 10ml of ice-cold PBS.
- 8 Remove the tissue with forceps and cut into <5 mm pieces, then place it into a 15 mL conical tube filled with 5 mL of crypt chelating buffer.
- 9 Bury the tube on ice horizontally. Gently shake the tube for 30 minutes on an orbital rocker.
- 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 minutes depending on the tissue type i.e. duodenum or ileum (see troubleshooting section). With tube oriented perpendicular to the ground, shake by hand at 2 to 3 cycles per second 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 (Figure 2).

The cell suspension can be observed under a microscope to check the crypts and villi enrichment.
- 13 Filter the solution through a 70 μ m 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 dissociation buffer.
- 14 Centrifuge the crypts fraction at 150 g for 10 minutes at 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 PBS.
- 16 Count the number of crypts per 10 μ L drops from the crypts suspension. The total number of crypts correspond to the number of counted crypts times 1000. Take the corresponding volume out of the crypts suspension to plate 200–500 crypts per well, and transfer to 5 mL round-bottom tube.
- 17 Centrifuge the crypts fraction at 150 g for 10 minutes at 4°C.

Small intestinal crypt culture

- 18 Mix the MatrigelTM with the growth factors on ice. Per 50 μ L of MatrigelTM, add 0.5 μ L of R-spondin 1 (1 μ g/mL), 0.5 μ L of Noggin (100 ng/mL) and 0.25 μ L of EGF (50 ng/mL).
- 19 Using pre-chilled pipette tips, resuspend the crypts pellet in MatrigelTM supplemented with growth factors (200–500 crypts/50 μ L MatrigelTM).
- 20 Apply 50 μ L of MatrigelTM suspension per well on the pre-warmed plate. Slowly eject the MatrigelTM in the center of the well.
- 21 Place the 24-well plate in a CO₂ incubator (37°C, 5% CO₂) for 20 minutes to allow a complete polymerization of the MatrigelTM.
- 22 Overlay the MatrigelTM with 500 μ L of basal minigut media.
- 23 Culture the plate in the CO₂ incubator (37°C, 5% CO₂) (Figure 3C).
- 24 Every 4 days, replace the media with fresh complete minigut media.

Enteroids culture passaging

- 25 Enteroids can be passaged 7 to 10 days after seeding. Prepare all the reagents before the beginning of the experiment. Thaw the MatrigelTM on ice and pre-incubate a 24-well plate in a CO₂ incubator at 37°C.
- 26 Remove media and add 1 mL of ice-cold PBS to each well. Break up the MatrigelTM by pipetting back and forth several times with P-1000 tips.

- 27 Remove the Matrigel™ suspension with a 1 mL syringe equipped with a 27½ gauge 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 PBS.
- 29 Centrifuge and resuspend the dissociated enteroids in Matrigel™ as in steps 18 to 24. Usually, 1 well of enteroids could be split to 3 to 4 wells.

Freezing the enteroids

- 30 Enteroids can be frozen 2 to 3 days after passaging. Remove the media and add 1 mL of ice-cold PBS to each well. Break up the Matrigel™ by pipetting back and forth several times with P-1000 tips.
- 31 Transfer the suspension from 2 to 3 wells into a 5 mL round-bottom tube filled with ice-cold PBS.
- 32 Centrifuge at 150 g for 10 minutes at 4°C, and resuspend enteroids in freezing media using 1 mL per collected 3 wells.
- 33 Place 1 mL of enteroids in freezing media in a labeled cryovial. The cryovial is placed in a freezing container containing 500 mL of isopropyl alcohol.
- 34 Transfer the freezing container to a –80°C freezer for 24 hours then, transfer cryovial to a 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 CO₂ incubator at 37°C.
- 36 Thaw the cryovial at 37°C in a water bath. The thawing is complete when the media becomes liquid.

Do not let the media warm up, this could affect the efficiency of the culture.
- 37 Suck out the cryovial solution and transfer into a 15 mL conical tube filled with 5 mL ice-cold basal media without growth factors.
- 38 Centrifuge and resuspend the enteroids in Matrigel™ as in steps 18 to 22. Usually, 1 cryovial of enteroids could be split to 2 well.
- 39 Overlay the Matrigel™ with 500 µL of basal minigut media supplemented with 10 µM of Y27623 compound (Sigma-Aldrich®, 1:1000 stock dilution).

Y27623 compound is only added after the seeding.
- 40 Culture the plate in the CO₂ 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 the basic protocol 1, we delineate the steps specific to the fundic or antral tissue isolation and the culture of gastroids from this area.

Materials

Animals

Murine C57BL6/J strain (Jackson laboratory) aged at least 6 weeks is used for gastroid culture.

In addition to basic protocol 1, the following additional reagents are required.

Reagents and solutions

Human recombinant FGF10 (PeproTech, 1000× stock ; 100 µg/mL in sterile PBS/ 0.1% BSA).

Human [Leu15]-Gastrin I (Sigma-Aldrich®, 1000× stock ; 10 µM in sterile PBS/ 0.1% BSA).

N-Acetylcysteine (Sigma-Aldrich®, 1000× stock; 500 mM in sterile PBS).

Y-27632 (Sigma-Aldrich®, 1000× stock ; 10 mM in sterile PBS).

In addition to basic protocol 1, the following additional equipment are required.

Equipment

Dissecting microscope.

Micro-dissecting curved scissors.

2 pairs of #7 fine point curved forceps.

Isolation of fundic and antral gland

- 1** See basic protocol 1, step 1–3.
- 2** Make an incision into the abdominal cavity just cranial to the external genitalia. Extend the incision to the rib cage by cutting 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 PBS.
- 4** Pin opened stomach (luminal side down) on the silicon dish filled with ice-cold PBS.

- 5
 - a. [For isolation of fundic glands] Under the dissecting microscope, strip the serosal muscle in the fundic region using micro-dissecting curved scissors and fine point curved forceps (Figure 4A).
 - b. [For isolation of antral glands] Under the dissecting microscope, strip the serosal muscle in the antral region using 2 pairs of fine point curved forceps (Figure 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 (5 mM EDTA).
If there is trouble with tissue dissociation, use 10 mM EDTA.
- 8 Bury the tube on ice horizontally. Gently shake the tube for 2 hr on an orbital rocker.
- 9 Gently invert the tube, allow the fragments to settle at bottom of tube and discard the supernatant. Fill with 5 mL dissociation buffer.
- 10 With tube oriented perpendicular to the ground, shake in hand for 1 to 2 minutes at 2 cycles per second to dissociate epithelium.
- 11 Follow step 15–16 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 Wnt3a (100 ng/mL), 0.5 µL of R-spondin 1 (1 µg/mL), 0.5 µL of Noggin (100 ng/mL), 0.5 µL of FGF10 (100 ng/mL), 0.5 µL of gastrin (10 nM), 1 µL of n-Acetylcysteine (1 mM), 0.25 µL of EGF (50 ng/mL) and 0.5 µL of Y-27632 (10 µM : only add for fundus).
- 13 Using pre-chilled pipette tips, resuspend the gland pellet in Matrigel™ supplemented with growth factors (200–500 glands/50 µL Matrigel™).
- 14 Apply 50 µL of Matrigel™ suspension per well on the pre-warmed plate. Slowly eject the Matrigel™ in the center of the well.
- 15 Place the 24-well plate in a CO2 incubator (37°C, 5% CO2) for 20 minutes to allow a complete polymerization of the Matrigel™.
- 16 Overlay the Matrigel™ with 500 µL of basal minigut media.
- 17 Culture the plate in the CO2 incubator (Figure 3A–B).
- 18 Every 4 days, remove media and replace with fresh minigut media supplied with growth factors as described in step 13.

Gastroid culture passaging

- 19 Same procedure is applied as described in basic protocol 1.

Freezing and thawing the Gastroid

- 20 Same procedure is applied as described in basic protocol 1.

ALTERNATE PROTOCOL 2: Colonoids derived from colonic crypts

In this third 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.

Materials

Solutions, reagents and equipments listed in the basic protocol 1 are used for this protocol.

Isolation of colonic crypts

1. Prepare all the reagents and samples as described in basic protocol 1, steps 1–3
2. Make an incision into the abdominal cavity and extend the incision to the rib cage by cutting abdominal musculature on both sides. Grasp the duodenum and cut the intestine from the stomach at the pyloric sphincter. Pull gently the intestine out of the abdominal cavity. Cut the proximal colon from the cecum and the distal colon at the anal margin.
3. Prepare the colon before the crypt isolation as described in basic protocol 1, steps 5–11.
4. Shake the tube for 8 minutes. Tube oriented perpendicular to the ground, handshake it at 2 to 3 cycles per second to dissociate epithelium from the basement membrane.

Layer the tube containing the intestine with tissues to keep it cool.

5. Remove remnant intestinal tissue, freed of crypts and villi and follow steps 14–18 in the basic protocol 1 (Figure 2).

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

6. Mix the MatrigelTM with the growth factors on ice. Per 50 μ L of MatrigelTM, add 0.5 μ L of Wnt3a (100 ng/mL), 0.5 μ L of R-spondin 1 (1 μ g/mL), 0.5 μ L of Noggin (100 ng/mL) and 0.25 μ L of EGF (50 ng/mL).
7. Terminate the seeding of the colonic crypts following the basic protocol 1, steps 7–25 (Figure 3D).

Maintenance, passaging as well as freezing procedures are listed in basic protocol 1.

BASIC PROTOCOL 2: Lgr5-GFP^{+ve} gastrointestinal stem cell sorting and culture

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 (Figure 5). The strategy for isolating and culturing gastric (antral) and colonic Lgr5-GFP positive cells is identical to the small intestine, with the addition of tissue-specific growth factors as described in the alternative protocols, above.

Materials

Animals

Lgr5-GFP⁺ires-CreER C57BL6/J mouse (Jackson laboratory) aged from 6 to 8 weeks is used for single stem cell culture.

Reagents and solutions

Dissociation media, TryPLE Express (Invitrogen™).

Jagged-1 Fc chimera peptide (R&D Systems®, 1 000× stock; 1 mM/mL in sterile PBS).

N-acetylcysteine (Sigma-Aldrich®, 1 000× stock; 0.5 M in ultrapure water, sterile filtered with 0.22 µm filter).

7-Aminoactinomycin D (Invitrogen™, 100× stock; 1 mg/mL in sterile PBS, Catalog #A1310, Ex/Em (nm): 548/649).

APC-Annexin V (Invitrogen™, Catalog #A35110, Ex/Em (nm): 650/660).

CHIR99021 (Stemgent®, 4000× stock, 10 µM in DMSO).

Thiazovivin (Stemgent®, 4000× stock, 10 µM in DMSO).

Equipments

MACS C-Tubes (Milteny-Biotec®)

GentleMACS™ Dissociator (Milteny-Biotec®)

40 µm cell strainer

FACS tube with 35 µm mesh cap

Cell sorter (BD FACSAria™ II; Beckman-Coulter MoFlo™ XDP)

96-well plate

Isolation of small intestinal crypts

- 1 Isolate crypts from Lgr5-GFP⁺ires-CreER mouse as described in basic protocol 1, steps 1–13.

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.

- 2 Tube oriented perpendicular to the ground, handshake it at 2 to 3 cycles per second to dissociate the crypt fraction another 3 minutes to promote the crypts dissociation.

- 3** Centrifuge the crypts fraction at 50 xg for 5 minutes at 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 10uM 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 minutes in a water bath at 37°C.

The dissociation program consists of 4 rotation cycles of 15 seconds 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 digestion solution for 60–90 minutes, with gentle trituration every 10–15 minutes, and changing the TryPLE solution once after 30–45 minutes.
- 6** After the incubation, run again the program “m-intestine-1” on the GentleMACS dissociator.
- 7** Transfer the dissociated crypts into a 50 mL conical tube filled with ice-cold minigut media supplemented with 0.5 mM N-acetylcysteine (1/1000 stock dilution) and 10uM Y27632 (1:1000 stock dilution).

The minigut media without the growth factors is used at this step.
- 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 at 500 xg for 5 minutes at 4°C and gently aspirate the supernatant.
- 10** Resuspend the pellet in 1 mL ice-cold minigut media supplemented with 0.5 mM N-acetylcysteine (1:1000 stock dilution) and 10uM Y27632 (1:1000 stock dilution) and 1% BSA. Count the number of cells with a hemacytometer and dilute the cell suspension to a concentration around 2 to 5×10⁶ cells/mL.

A Trypan-blue assay 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 minutes prior the sorting.

An annexin-binding buffer is not necessary as the minigut media contain 1 mM CaCl₂.

Lgr5-GFP⁺ cell sorting

- 12 A 100 µm nozzle is set up on the sorter. The fluidic on the machine is set up to reach at least 80% of 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, in doublet discrimination: the single cell population is plotted against forward scatter (FSC) vs side scatter (SSC) and SSC Height vs Area or FSC Height vs 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⁺ population is defined as the brightest population and is gated on the third part of the GFP histogram (Figure 5A).

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

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

- 13 Lgr5-GFP⁺ cells are sorted into minigut media 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% β-mercapto-ethanol.

Single cell-sorted culture

- 14 Centrifuge the sorted cells at 500 xg for 5 minutes at 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 Jagged-1 Fc chimera peptide (1 µM), 0.1 µL of Wnt3a (100 ng/mL), 0.1 µL of R-spondin 1 (1 µg/mL), 0.1 µL of Noggin (100 ng/mL) and 0.05 µL of EGF (50 ng/mL).
- 16 Using pre-chilled pipette tips, resuspend the cell pellet in Matrigel[™] supplemented with growth factors (200–500 cells/10 µL Matrigel[™]).

- 17 Apply 10 μ L of MatrigelTM suspension per well on the pre-warmed 96-well plate. Slowly eject the MatrigelTM in the center of the well.

To avoid any spreading of the matrigel on the bottom of the well, 2 μ L of plain MatrigelTM could be spotted before the MatrigelTM suspension.

- 18 Place the 96-well plate in a CO₂ incubator (37°C, 5% CO₂) for 10 minutes to allow a complete polymerization of the MatrigelTM.
- 19 Overlay the MatrigelTM with 100 μ L of basal minigut media supplemented with 2,5 μ M CHIR99021 (1:4000) and 2,5 μ M Thiazovivin (1:4000).
- 20 Culture the plate in the CO₂ incubator.
- 21 Every 2 days, aspirate the media and replace it with fresh complete minigut media.

1 μ M Jagged-1 Fc chimera peptide is on day 0 and 2 after sorting.

BASIC PROTOCOL 3: Imaging of the gastrointestinal epithelial organoids

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

Materials

8 well Lab-Tek chamber with #1.0 Borosilicate coverglass (Thermo Sci)
 4% paraformaldehyde (PFA)
 Antibodies
 NH₄Cl (50 mM)
 0.1 % Triton-X100
 Hoechst 33342 (10 μ g/mL: Invitrogen)
 Fetal Bovine Serum (FBS)
 Methylene Blue solution, 2% in PBS.
 OCT (Tissue-Tek)

Equipment

Inverted Confocal Microscope (Zeiss LSM710)
 CO₂ module S/temperature module S/humidifier S unit (PeCon incubation system)
 Heating insert P-labtek S1 (PeCon incubation chamber)
 EC plan-neofluaro 10x/0.3 (dry) or Plan-Apochromat 20x/0.8 (dry) objective lens
 C-Achroplan NIR 40x/0.8 (water) objective lens
 Paraffin embedding station

Live imaging

- 1 Passage epithelial organoids following steps 26–30 in basic protocol 1.
- 2 Apply 25 µl Matrigel/epithelial organoid suspension into 8 well chamber (split 1/2 well from original plate to 8 wells of this chamber).

Do not put more than 20 organoids in a well to keep media fresh
- 3 Add 400 µl complete minigut media per well, and culture at 5% CO₂/37°C until imaging.
- 4 Set 5% CO₂ and 37 °C PeCon incubation chamber through confocal microscope computer.
- 5 Insert 8 well chamber to the PeCon incubation unit.

Do not remove cover from chamber to avoid media evaporation.
- 6 Set up optical configurations, example (ZO-1/RFP enteroids created from mouse gifted by Dr. Turner) as described below (MOVIE 5,6,7).
- 7 Turn on 560 nm laser with 560 nm dichroic filter and set 565 – 650 nm for emission. Also turn on TPMT transmitted light channel.
- 8 Find enteroid which has bright RFP signal, followed by optimization of laser power and detector gain while keeping pinhole (30–50 µm for Plan-Apochromat 20x/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 hour).
- 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 30 minutes, then start (see troubleshooting).

Whole mount staining

- 11 Passage epithelial organoids following steps 26–30 in basic protocol 1.
- 12 Apply 25 µl Matrigel/epithelial organoids mixed into 8 well chamber (split 1 well from original plate to 4 well of this chamber).
- 13 Add 400 µl complete minigut media per well, and culture at 5% CO₂/37°C until staining.
- 14 Remove media, add 200 µl PBS (room temperature) and leave for 5 minutes.

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

- 15 Remove PBS and add 200 µl 4% PFA (room temperature) and leave for 30 minutes.
- 16 Repeat step 14 (wash step) twice
- 17 Add 200 µl NH₄Cl (50 mM in PBS: room temperature) and leave for 30 minutes.

This step will quench autofluorescence (coming from shed cells and debris in the lumen, specifically at 488 nm excitation wavelengths), but if there is fluorescent 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-X100 (0.1 % in PBS) and leave for 30 minutes (see troubleshooting).
- 20 Repeat step 14 (wash step) twice
- 21 Add 200 µl 5% BSA or serum and leave for 60 minutes
- 22 Repeat step 14 (wash step) twice
- 23 Add 200 µl 1st antibody made in PBS (see troubleshooting) and leave for overnight at 4 C.

Often, higher concentrations of antibodies are required than used for 2D staining of tissue sections.
- 24 Repeat step 14 (wash step) five times
- 25 Add 2nd antibody made in PBS (see troubleshooting) and leave overnight at 4 °C.

Often, higher concentrations of antibodies are required than used for 2D 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 minutes
- 28 Repeat step 14 (wash step) twice
- 29 Observe staining by confocal microscope using long distance objective lens (C-Achroplan NIR 40x/0.8 (water) objective lens)

Processing for frozen and paraffin-embedded sections

- 30 Remove media and resuspend matrigel containing epithelial organoids in ice-cold PBS.
- 31 Transfer resuspended epithelial organoids to an eppendorf tube.

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

- 32 Spin at 100 xg for 1 minute and gently discard the supernatant.
- 33 Fix the epithelial organoids for 20 min at 4°C in 500 µL 4% PFA.
- 34 Spin at 100 xg for 1 minute and remove the PFA.
- 35 Wash epithelial organoids with PBS and spin at 100 xg for 1 minute.
- 36 Resuspend in 100 µL Methylene Blue solution for 20 minutes at room temperature.
The Methylene Blue staining facilitates visualization of organoids in OCT or paraffin.
- 37
 - a. Frozen sections: Wash with PBS and resuspend in 30 % sucrose at 4°C overnight. Spin at 100 xg for 1 minute and remove sucrose. Embed in OCT in 1×1 cm cryo-mold.
Let the epithelial organoids settle for 30–45 min before freezing.
 - b. Paraffin embedded sections: Wash with PBS and resuspend in 70 % ethanol.
Process the epithelial organoids manually through the dehydration steps. Spin at 100 xg for 1 minute 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

Please refer to Table 1 for an alternate view of key media solutions and reagents.

Chelating buffer

Freshly prepare 500 mL of crypt chelating buffer from stocks of 0.5 M Ethylenediaminetetraacetic acid, pH8 (EDTA; Sigma-Aldrich®) and PBS 1x. The buffer is stored at room temperature.

For intestinal crypt isolation, the following volumes of EDTA and PBS, should be mixed: 2 mL and 498 mL. The final 2 mM EDTA solution can be stored at 4 C.

For gastric gland isolation, the following volumes of EDTA and PBS, should be mixed: 5 mL and 495 mL. The final 5 mM EDTA solution can be stored at 4°C.

Dissociation buffer

2 g D-sorbitol (54.9 mM) and 3g Sucrose (43.4 mM) dissolved in 200 mL PBS and can be stored at 4°C.

Minigut medium

Prepare 100 mL of culture medium from Advanced DMEM/F12 supplemented with 2mM GlutaMax, 10 mM HEPES, 100 U/mL penicillin/100 µg/mL streptomycin and 1× N2 and 1× B27 supplements (Life Technologies™). The following volumes of these are, respectively, for 100 mL of medium: 94 mL, 1 mL, 1 mL, 1 mL, 1 mL and 2 mL. Divide into 10 mL aliquots in 15 mL conical tubes and freeze.

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

Complete minigut medium

Minigut media should be mixed with 1 µg/mL R-spondin1 (1:1000 stock dilution), 100 ng/mL Noggin (1:1000 stock dilution) and 50 ng/mL EGF (1:10 000 stock dilution). The medium is prepared freshly before the crypt culture or media changing.

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

Wnt3a recombinant protein is unstable and has to be added prior to the experiment.

Freezing medium

Prepare freezing medium by combining Advanced DMEM/F12 (Life Technologies™) with 10% DMSO (Sigma-Aldrich®) and 10% decompemented Fetal Bovine Serum (Life Technologies™). The following volumes of these are, respectively, for 10 mL of medium: 8 mL, 1 mL and 1 mL.

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) which 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 varied from cysts lined by 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 et al.* described the establishment of a 3-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. 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 et al (Barker et al., 2010; Sato et al., 2009; 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). Altogether, those studies demonstrate the utility of epithelial organoid cultures to test 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 *et al.*, used enteroids to evaluate the dynamics of intestinal drug transport. In this report, they investigated the physiological function 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 recapitulates 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 hours after tissue collection. The tissue has to be maintained in PBS 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 PBS 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 substituted by Wnt3a, R-spondin and Noggin conditioned media. Wnt3a-expressing L-cell line is commercially available (ATCC). Other groups developed R-spondin1 (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⁺ 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).

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

For a good efficiency of the 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.

Immunofluorescence—Some 1st or 2nd antibody will be taken up by the paneth cell (Figure 6) and may appear as non-specific 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 up to 0.5 %. In addition, antibodies can be diluted in PBS-0.1 % triton X (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 organoid is plated in the chamber (step 2) [*AU: Not sure what protocol “step 2” refers to?]. This also increases efficiency of the staining.

Anticipated Results

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 hours after the seeding in Matrigel™. The crypt budding occurs usually 3 to 4 days after seeding. The passaging can be done after 7 days, depending of the organ considered. All the gastro-intestinal epithelia-organoids present all the differentiated lineages which could be observed by immunofluorescence imaging. All the gastrointestinal epithelial organoids present all the differentiated lineages which could 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).

Anticipated Results; Basic Protocol 2—Figure 5B shows a typical example of isolated single from the different regions. After isolation, the single cell should balloon-up after 48 hours post-splitting. The first buds usually appears around day 10 and undergo an extensive budding beyond this day. Sorted cells express GFP, however the GFP expression may vary during the growth and a mosaic expression appears in established enteroids.

Anticipated Results; Basic Protocol 3: Live imaging—Movies 5–7 show growth of ZO1-RFP tagged enteroids from day 3 to 6, while movies 2–4 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; and the enteroid first seals itself and then begins budding.

Anticipated Results: Basic Protocol 3: Processing for frozen and paraffin-embedded sections—Figure 6 shows nuclei (blue)/E-cadherin(green)/ZO-1 (red)/paneth (pink) immunofluorescence in enteroids. ZO-1 was endogenously tagged with RFP, while high concentration of secondary antibody resulted in binding to Paneth cells.

Time Considerations

Crypts isolation—Preparing the solutions takes ≈15 min; dissection of mice about 15 to 30 min depending of the number; crypt isolation from 30 minutes to 1 hour; crypt seeding 30 minutes.

Lgr5-GFP⁺ cell sorting—Preparing the solutions takes ≈15 min; dissection of mice about 15 to 30 min depending of the number; crypt isolation ≈45 minutes; cell dissociation ≈30 minutes; Antibody staining ≈45 minutes; sorting ≈25minutes; single cell seeding ≈20minutes.

Live imaging—As long as low laser power is used, epithelial organoids should stay healthy. However, bleaching of fluorescence may occur easily 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 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Workflow of gastric glands and crypts dissociation and generation of epithelial organoids in culture

Gastrointestinal tissues are processed differently according to their location. Every tissue undergoes isolation of the glands for the stomach or the crypts for the small intestine and colon by EDTA chelation. 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.

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.

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 hours 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 hours later. The closing crypt forms an enterosphere which undergoes an extensive budding by 7 days. (D) Colonic crypts are plated in Matrigel™ after isolation. The crypt closes and forms a colonoid after 7 days.

Figure 4. Dissection process of stomach

The stomach (from cytosolic YFP expressing mouse) is opened lengthwise and stretched into a silicon-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 brightfield, glands can be observed following removal of the muscle layer as individual light spots. Loss of muscle structure can be seen using YFP fluorescence.

Figure 5. 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 MatrigelTM. 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 in epithelial organoids is dynamically mosaic.

Figure 6. Imaging of the organoids

Confocal imaging and 3D 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.

Table 1

[*CE: Please have authors decide how they want to work with these tables; I grouped them all as one and called them out in the Recipes and Solutions, but this may not be ideal.]

Minigut medium					
Reagent Name	Supplier	Cat No.	Solvent	Stock Solution	Final Conc.
Advanced DMEM/F12	Invitrogen	12634-028	-	-	-
HEPES 1M	Invitrogen	15630-080	-	1M - Liquid	10mM
GlutaMAX	Invitrogen	35050-061	-	100X	1X
Pen/Strep	Invitrogen	15140-148	-	100X	1X
N2 Supplement	Invitrogen	17502-048	-	100X	1X
B27 Supplement	Invitrogen	17504-044	-	50X	1X
N-Acetylcysteine	Sigma-Aldrich	A9165-5G	-	Powder	1mM
BSA, Fraction V	Fischer	BP1600	-	Powder	1%

Growth factors, Hormones, Inhibitors...					
Reagent Name	Supplier	Cat No.	Solvent	Stock Solution	Final Conc.
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	100 µg/ml	100 ng/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	DPBS	10 mM	10 µM
CHIR99021	Stemgent	04-0004	DMSO	10 µM	2.5 uM
Thiazovivin	Stemgent	04-0017	DMSO	10 µM	2.5 µM
[Leu15]-Gastrin I human	Sigma	G9145	DPBS	10 µM	10 nM
Human recombinant FGF10	PeptoTech	100-26	DPBS	100 µg/ml	100 ng/ml
n-Acetylcysteine	Sigma	A7250	DW	500 mM	1 mM

Crypt's isolation reagents				
Reagent Name	Supplier	Cat No.	Solvent	Final Conc.
DPBS Ca ²⁺ , Mg ²⁺ free	Thermo Sci	SH3002802	-	-

Crypt's isolation reagents					
Reagent Name	Supplier	Cat No.	Solvent	Stock Solution	Final Conc.
EDTA	Sigma-Aldrich	431788	DW	0.5 M	2mM

-FACS Antibodies			
Company	Antigen	Conjugate	Catalog #
Invitrogen	Annexin V	AF647	A23204
Invitrogen	7AAD	-	A1310

-Immunofluorescence antibodies			
Company	Host	Antigen	Fluorescence Catalog #
Santa Cruz	Rat	E-cadherin	sc-59778
Invitrogen	Goat	rat IgG	Alexa 633 A21094
Invitrogen	Goat	F(ab') ₂ rabbit IgG	Alexa 488 A11070