Murine Gastrointestinal Crypt Isolation & Plating
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Reagents	Equipment
Fresh 1X Sterile PBS	Clean cell cultures dishes
Fresh EDTA in PBS	15mL and 50mL conical tubes
Advanced DMEM/F12 (Gibco)	70um cell strainer
Matrigel matrix (Corning)	
Pre-warmed culturing medium	

Crypt Isolation

DUODENUM

- □ Keep 1X PBS and **2mM** EDTA/PBS on ice during procedure
- □ Euthanize adult mouse with CO_2 followed by cervical dislocation
- □ Dissect open mouse, locate start of small intestine, cut ~1cm after stomach, remove ~10cm of proximal duodenum. Move to dish of cold 1X PBS.
- □ Using a sterile syringe, tubing, and tip, flush intestine with cold 1X PBS until cleared of stool and debris
- □ Remove any extraneous connective tissue on outside of intestinal section
- □ Using small scissors (blunt tip work best), filet intestinal section to expose epithelium
- □ Wash intestinal section in dish of PBS to remove residual stool and debris
- □ With a sterile new razor blade, dice into ~5mm pieces
- □ Place diced intestine into 50mL conical tube with ~25mL cold 1X PBS
- □ Swirl to wash, allow pieces to settle, and decant; repeat at least 10X until PBS is clear
- □ Decant PBS, pipet off residual. Add 25mL fresh 2mM EDTA
- □ Place tube horizontally on ice, and rotate for **15 minutes**.
- □ Allow pieces to settle, decant EDTA, and replace with 25mL fresh 2mM EDTA.
- □ Place tube horizontally on ice and rotate for **25 minutes**.
- □ Remove tube from ice, shake vigorously for **1 minute** to release crypts from tissue. Solution will become very cloudy.
- □ With a 10mL serological pipet, pipet up and down 25x while mixing.
- □ Pass solution through 70um filter into new 50mL conical tube.
- □ Spin at 300 x g for **5 minutes**.

COLON

- □ Keep 1X PBS and **5mM** EDTA/PBS on ice during procedure
- □ Euthanize adult mouse with CO_2 followed by cervical dislocation
- □ Dissect open mouse, locate cecum, and remove entire colon from cecum to rectum. Move to dish of cold 1X PBS.
- □ Using a sterile syringe, tubing, and tip, flush intestine with cold 1X PBS until cleared of stool and debris
- □ Remove any extraneous connective tissue on outside of intestinal section
- □ Using small scissors (blunt tip work best), filet colonic section to expose epithelium
- □ Wash intestinal section in dish of PBS to remove residual stool and debris
- □ With a sterile new razor blade, dice into ~5mm pieces
- □ Place diced intestine into 50mL conical tube with ~25mL cold 1X PBS
- □ Swirl to wash, allow pieces to settle, and decant; repeat at least 10X until PBS is clear
- □ Decant PBS, pipet off residual. Add 25mL fresh 5mM EDTA
- □ Place tube horizontally on ice, and rotate for **15 minutes**.
- □ Allow pieces to settle, decant EDTA, and replace with 25mL fresh 2mM EDTA.
- □ Place tube horizontally on ice and rotate for **30 minutes**.

□ Remove tube from ice, shake vigorously for **1 minute** to release crypts from tissue. Solution will become very cloudy.
□ With a 10mL serological pipet, pipet up and down 25x while mixing.
□ Pass solution through 70um filter into new 50mL conical tube.
□ Spin at 300 x g for **5 minutes**.

STOMACH

□ Keep 1X PBS and **10mM** EDTA/PBS on ice during procedure.
□ Euthanize mouse with CO_2 followed by cervical dislocation.
□ Dissect open mouse, locate, and remove stomach. Move to dish of cold 1X PBS.
□ Dissect stomach, open along greater curvature, and wash in 1X PBS.
□ Remove serosal muscular layer, pinning if necessary.
□ With a sterile new razor blade, dice desired sections into ~5mm pieces
□ Place diced gastric gland into 50mL conical tube with ~25mL cold 1X PBS
□ Swirl to wash, allow pieces to settle, and decant; repeat at least 10X until PBS is clear
□ Decant PBS, pipet off residual. Add 25mL fresh 10mM EDTA
□ Place tube horizontally on ice and rotate for **2 hours**. Replace with fresh 10mM EDTA halfway.
□ Remove EDTA and add dissociation buffer (43.4mM sucrose and 54.9mM D-sorbital in PBS).
□ Shake vigorously for **2 minutes**.
□ With 10mL serological pipet, pipet up and down 25x while mixing.
□ Pass solution through 70um filter into a new 50mL conical tube.
□ Spin down at 300g for **5 minutes**.

Crypt Plating

The following steps should be performed in a biosafety hood

□ Aspirate EDTA, resuspend pellet in 20mL Advanced DMEM/F12.
□ Spin at 300 x g for **5 minutes**. Repeat 2x for a total of 3 washes.
□ Add appropriate volume of Matrigel matrix to 15mL conical tube.
50uL of Matrigel per well in a 24 well plate
□ Add 1uL crypt pellet per 50uL Matrigel expecting to plate.
Plating concentration typically 50-100 crypts per well, 1uL of crypt pellet is ~50-100 crypts. For 12 wells, add 12-15uL of crypt pellet to 625uL Matrigel
□ Suspend crypts into Matrigel with P1000, avoiding bubbles
□ Plate 50uL of Matrigel suspension into each well. Incubate at 37**°**C for **10 minutes**.
Allows Matrigel to polymerize. For dense cultures, 15-20 minutes is appropriate.
□ Add 500uL pre-warmed media to each well. Incubate organoids at 37°C/5% CO2. Feed after 4 days.
□ Feed and passage as needed.
Feed every 2-3 days, passage every 7-10 days.

Organoid Passaging

Typically confluent cultures will split 1:3 or 1:4

- □ Aspirate medium from wells
- □ Add 500uL of Cell Recovery Solution to each well. Resuspend Matrigel dome in solution and transfer to 15mL conical tube.
- □ Incubate on ice for 1 hour.
- □ Invert tube several times as organoids have likely sunk to the bottom. Spin at 500 x g for 5 minutes at 4C.
- □ Aspirate supernatant, careful to not disturb pellet.
  Note: In some cases, after incubation, Matrigel may not be completely dissolved resulting in a pellet gradient dispersed in Matrigel at the bottom of the tube. Resuspend organoids and matrix in tube with P1000, and re-spin.
- □ Add fresh Matrigel to tube based on how many wells that will be generated.
- □ Bend/kink a P1000 tip and mechanically break up organoids in Matrigel at least 50x. This process fragments the organoids, with each fragment resulting in formation of a new organoid.
  Note: Set the pipet 150-200uL lower than Matrigel volume before breaking up to prevent bubble generation.
- □ Seed 24-well plate with 50uL droplets into each well. Place in 37C/5% CO2 incubator for at least 10 minutes to polymerize Matrigel.
- □ Add 500uL culturing medium to each well. Feed every 2-3 days and passage as needed.