**Mouse Intestinal Epithelial Cell Isolation and Flow Cytometry Prep**

Prepare ahead of time:

* Set large centrifuge to 4 degrees
* Dissecting tools
* Dishes for flushing tissues
* Flushing apparatus (10ml syringe fitted with 18G needle and polyethylene tubing
* (BD Intramedic Cat#: 427435) for inserting into intestine and flushing)
  + Normal syringe also fine
* Ice cold 5 mM EDTA in PBS (60 ml per mouse)
  + 30mL per epithelial fraction
* Ice cold PBS (60ml per mouse plus some for flushing)
* 50 mL Falcon tubes
* 10X TryplE
* Phenol red free- DMEM or RPMI
  + *Prepare and pre-warm* ***4x*** *TrypLE in DMEM/RPMI in water bath*
* Room temperature DMEM complete
* 70 μm cell strainers (BD Falcon Cat#: 352350)
* FACS tubes
* FACS buffer
* DAPI viability stain

Cell Isolation

1. Sacrifice mice and isolate desired section of intestine.
2. Flush intestines with PBS to remove debris and cut open along longitudinal axis.
   1. Optional: Cut into 2-3 inch-long pieces.
3. Rinse in ~30mL **ice cold PBS** by shaking in a 50mL conical tube.
4. Transfer intestines (optional: scrape villi and also collect/transfer) to another 50mL conical tube containing **30mL ice cold 5mM EDTA in PBS**.
   1. **Note:** Two glass slides can be used to scrape villi off of intestinal sections in order to mechanically isolate them. After scraping transfer all intestinal tissue back into EDTA by rinsing slides in the 50mL conical tube before incubation.
5. Rotate or rock tubes containing intestines in EDTA at **4ºC for 20 minutes**, shaking every 5-7 minutes for about 30 seconds.
6. After 20-25 minutes of incubation, **shake tubes vigorously for 30-60 seconds** before removing intestines and spinning down the epithelial fraction in EDTA at **800rpm for 5 minutes at 4ºC**.
   1. Optional: Removed intestines can be put into an additional 30mL cold 5mM EDTA in PBS and digested for 10-15 more minutes to collect a second epithelial fraction.
7. Carefully decant EDTA supernatant and re-suspend cell pellet in **20mL** of **pre-warmed 4x TrypLE**.
   1. Note: TrypLE can be diluted from 10x to 4x in phenol red-free DMEM or RPMI.
   2. Optional: wash with 10mL cold PBS before trypsinizing.
8. Place cells in **37ºC water bath** to digest for **30 minutes**, gently shaking/rocking tubes every **7-10 minutes**.
9. After 30 minutes incubation, neutralize with **15-20mL** of room temperature **DMEM complete**.
10. Spin neutralized cells at **1200rpm for 5 minutes**.

Antibody Staining:

1. Resuspend cells in 100ul FACS buffer. Add 1ul Sca\_1\_APC antibody.
2. Incubate for 20 mins on Ice.
3. Add 1ml FACS buffer and spin down.

Live\_Dead:

1. Prepare post-FACS collection tubes with 5mL of 50%FBS+50%DMEM complete with **Roc inhibitor** added
2. Re-suspend cells in **1-2mL FACS buffer** with **DAPI** and pass through another 70 micron filter before transferring to 40 micron filtered FACS tubes.
   1. Keep cells on ice until sorting.

Cell Recovery:

1. Collect flow sorted cells in 50%DMEM+50%FBS with Roc inhibitor (1:1000), keeping cells on ice.
2. Spin down at 1200rpm for 5 minutes.
3. Aspirate media and continue on to desired downstream applications:
   1. Organoid formation (plate with Roc inhibitor added to media)
   2. Cell hashing antibody staining protocol