Mouse Colon Organoid Culture

Base Culture Media:

- 500 mL Advanced DMEM/F-12 12634010
- 5 mL 200 mM L-Glutamine (2 mM final) 25030081
- 5 mL HyClone™ Pen / Strep Solution SV30010
- 1 mL 500 mM N-Acetyl-L-Cysteine (NALC) (1 mM final) A9165-25G
- 5 mL 1M HEPES (10 mM final) H3375-25G
- 1 mL B-27 (50×) 17504044, 500 µL N2 (100×) 17502048 supplements to make 50 mL aliquots

Complete Culture Media:

- Murine R-spondin-1* (500-800 ng / mL final); 315-32
- Murine Wnt-3a* (100 ng / mL final; 315-20
- Murine EGF (50 ng / mL final); PMG8041
- Murine Noggin* (50 ng / mL final); 250-38
- Nicotinamide (10 mM final) N0636
- Rho-associated protein kinase inhibitor (Y-27632) – only for thawing organoids (10 µM final), Y0503

* Growth factors can be replaced by addition of ~20% L-WRN-conditioned media (L-WRN - a cell line that stably expresses mWnt-3A, hR-Spondin-1, hNoggin - Takahashi, Y. *et al.*)

Additional Materials and Instruments:

- Growth Factor Reduced Matrigel 354230
- PBS, Ca^{2+}/Mg^{2+}-free 10010056
- Sterile 1.5 mL Eppendorf tubes 30120086
- Cell culture plates (12-well plate) 92412T
- Recovery™ Cell Culture Freezing Medium 12648010
- Cryovials 377267
- Mr. Frosty freezing container 479-3200
- Tabletop centrifuge

Organoid Thawing

Day 0

1. Slowly thaw cryovials with organoids at 37 °C in water bath.
2. Transfer organoids into a sterile 1.5 mL Eppendorf and top up with 0.5 mL ice-cold PBS.
3. Spin down briefly (10 sec) using a benchtop centrifuge and carefully remove the supernatant.
4. Wash the organoids with 1 mL ice-cold PBS and centrifuge again.
5. Remove supernatant and resuspend the cells in X µL of Matrigel (volume depends on the size of the organoid pellet).
6. Plate 3×25 µL of organoid / Matrigel droplets per well (12-well plate format).
7. Incubate the plate at 37 °C for 5-10 mins to allow the Matrigel to polymerise.
8. Add 1 mL of complete culture media (with Y-27632) and incubate the cells at 37 °C, 5% CO_2.

Day 1

NOTE: Fragmented organoids will close up to form organoids, media change is required to remove Y-27632.

1. Remove media and replace with fresh complete media (without Y-27632).
2. *Optional: Organoids can be passaged to remove cell debris to clean up the culture.*

Organoid Passaging and Maintenance

1. Remove media from the wells and add 1 mL ice-cold PBS.
2. Mechanically break up the Matrigel droplets by P1000 pipetting (23G x 5/8" needles can also be used to finer passage the organoids).
3. Transfer PBS / Matrigel / organoids into a 1.5 mL Eppendorf and spin down in a benchtop centrifuge.
4. Remove supernatant and wash the cells with 1 mL ice-cold PBS. Centrifuge again and repeat wash.
5. Remove supernatant and resuspend cells in X* µL Matrigel to seed Y* number of wells.
6. Incubate the plate at 37 °C for 5-10 mins to allow the Matrigel to polymerise.
7. Add 1 mL of growth media and incubate at 37 °C, 5% CO_2.
8. Change media every 2-3 days and passage the organoids every 4-7 days.

**Organoids are split into fragments that may or may not contain stem cells that will develop into new organoids. It is therefore not possible to count the number of viable stem cells before seeding. Thus, the number of wells to seed after passage is determined from the size of the pellet.*

Organoid Freezing and Storage:

1. Harvest cells as described above.
2. Resuspend cell pellets in Cell Freezing Medium (0.5 mL / cryovial).
3. Transfer the cryovials to a Mr. Frosty and store at -80 °C for 24 hours.
4. Transfer the cryovials to liquid nitrogen storage.