**Gina Duronio, Sethi Lab, 2/2021**

**Adapted from Zhong Wu, Bass Lab 2016/9/26**

**Organoids with primary mouse stomach epithelial cells**

**Protocol**

* Generation of mouse stomach organoids

Isolation of stomach epithelial cells from murine stomach

* 1. Remove the glandular stomach. Open the stomach longitudinally and roughly remove the contents onto a paper towel.
  2. Rinse the tissue with ice-cold PBS, wash by vigorous shaking.
  3. Dissect out fat and connective tissues carefully by using fine scissors and forceps under a dissection microscope. Bring the dish to the biological hood.
  4. Mince the tissue with fine scissors into <1-mm2 fragments.
  5. Add 1 ml of collagenase solution by using a 1,000-µl pipette and suspend the tissue fragments in the solution.
  6. Incubate the Petri dish in the cell culture incubator and pipette the tissue mixture vigorously every 5–10 min by using a 1,000-µl pipette.
  7. Filter the tissue mixture through a 70-µm cell strainer in a 50-ml centrifuge tube. Wash the strainer with 9 ml of washing medium.
  8. Transfer the filtrated cell solution to a 15-ml centrifuge tube, Centrifuge the tube at 20g for 5 min at room temperature.
  9. Aspirate the supernatant. Add 500 µl–1 ml of washing medium and suspend the pellet, Centrifuge the tube at 200g for 5 min at room temperature.
  10. Aspirate the supernatant completely. Place the tube on ice. Suspend the epithelial units in Matrigel (15-30 µl per well).
  11. Incubate the plates in the cell culture incubator to polymerize the Matrigel. Turn the plates upside down to avoid the attachment of epithelial units to the surface of the plate.
  12. Add 500 µl of 50% conditioned medium to the well. Change the medium at least every 2 days.
* Culture and passage for maintenance

1. Grow the spheroids in the 24-well plate for 3 d.
2. Wash the well with 500 µl of PBS-EDTA and aspirate.
   1. 1mL for 6 well plate
3. Add 200 µl of trypsin-EDTA to the well. Scratch and suspend Matrigel with a 1,000-µl pipette and transfer to 15mL tube.
   1. 1mL TyrpLE per well for a 6 well plate
4. Incubate the plates in the cell culture incubator or tubes in the water bath for 2–10 min.
5. Add 600–800 µl of washing medium and dissociate the spheroids by vigorous pipetting.
   1. *Optional* depending on how broken up you want the spheres, you can also pipette a few times before adding any media to neutralize the trypsin.
6. Add 4-5 ml of washing medium; transfer to a 15mL tube if you trypsinized in the plate.
7. Centrifuge the tube at 200g for 5 min at room temperature (or 1500RPM for 3 mins). Aspirate the supernatant, leaving ~100 µl left over or getting as close to the Matrigel pellet as possible.
   1. Can do an extra wash and spin in media to disperse more Matrigel if necessary.
8. Aspirate the supernatant completely. Place the tube on ice. Suspend the spheroids in Matrigel (15-30 µl per dome, ~100uL per well of a 6 well plate).
9. Carefully pipette Matrigel suspension as domes into desired # of wells.
10. Incubate the plates in the cell culture incubator to polymerize the Matrigel (3-10mins). Turn the plates upside down to avoid attachment of cells to the surface of the plate.
11. Add 50% WRN conditioned medium to the wells, pipetting carefully to not disturb the domes (2mL per well in a 6 well plate). Change the medium at least every 2 d.

* Thaw organoids

1. Thaw -80˚C stored organoids (stored in freezing media 20% FBS, 10% DMSO) in 37˚C water bath for no more than a few minutes, DMSO toxic).
2. Thaw Matrigel, keep on ice.
3. Add 5 mL washing medium.
4. Centrifuge at 200 g for 5 min.
5. Aspirate supernatant with aspirator
6. Add 500 µL washing media and re-suspend cells.
7. Centrifuge at 200 g for 5 min.
8. Aspirate supernatant.
9. Suspend organoids in Matrigel on ice (try not to generate bubbles).
10. Dispense 15~30 µL of cell-Matrigel suspension in the center of well.
11. Invert well plate and incubate at 37˚C upside-down for 10-15 min.

Add 500 µL culture medium to each well.

* Freeze organoids
  1. Grow organoids for 2-3 days
  2. Scratch and suspend Matrigel in culture medium.
  3. Centrifuge at 200 g for 5 min.
  4. Aspirate supernatant.
  5. Re-suspend in 500 µL freezing medium/well.
  6. Transfer to cryotubes. Freeze at -80˚C in cryofreezing container overnight (contain isopropanol).
* Preparing organoids for infection.

1. Grow organoids in a 24 well for 2-3 days.
2. Scratch and suspend Matrigel in culture media. transfer organoids suspension to a 15 ml tube. Combine suspension from multiple wells depending on the assay scale.
3. Centrifuge the tube at 200g for 5min. aspirate supernatant, add 5ml PBS resuspension, centriguge at 200g for 5min.
4. aspirate supernatant, resuspend organoids in 200ul trypsin-EDTA, incubate the tube in 37℃ for 2-10min.
5. add 1ml washing media and dissociate organoids by vigorous pipetting. dilute cell suspension with 4ml washing media, and centriguge at 200g for 5min.
6. Resuspend cells in 250uL lentiviral solution (concentrated by LentiX, with 8ug/ml polybrene, 10uM Y27632).
7. transfer the suspension into a well on a 48 well plate.
8. seal the plate with parafilm and perform spinoculation by centrifuging the plate at 600g 32℃ for 1hr (pre-warm centrifuge to 32℃).
9. incubate the plate at 37℃ for 6hr to allow transduction
10. add 1ml conditioned medium to the well, resuspend and transfer to a 1.5ml or 15ml tube.
11. centrifuge the tube at 200g for 5 min.
12. aspirate supernatant carefully, suspend cells in 15~30ul Matrigel and plate in a well of a 24 well plate. Invert well plate and incubate at 37˚C upside-down for 10-15 min.
13. Add 500 µL containing 10uM Y27632 culture medium to each well. Change media after 2d and select with appropriate antibiotic.

* Orthotopic organoid implantations

1. Organoids are suspended in PBS or culture media, with a cell number of 0.5~1 x 106, with or without equal volume of matrigel mixture, to a total volume of 80-100uL.
2. Mice were anestheized with Isoflurane, and an incision was made through the left upper abdominal pararectal line and peritoneum. the stomach wall was carefully exposed.
3. Resuspensed organoids were slowly injected into stomach wall, creating a single bubble of cells beneath the outlayer of stomach and avoiding too much spread of the cells.
4. The abdominal wall and skin were closed with 5-0 Ethicon sutures (ET-661G).

* Subcutaneous organoid implantations
  1. Organoids are resuspended in fresh PBS or culture media to a concentration of 1~2.5x106 , add equal volume of matrigel mixture, to a total volume of 200uL, and mix carefully with pipette. (Note: the cell number required depends upon the aggressiveness of the organoids).
  2. Slowly pull up 200uL of cell/matrigel mixture using an insulin syringe. Inject cells in to the flanks of mice. To do this, pinch the skin of the mouse and pull the skin away from the body of the mouse. inject slowly, creating a single bubble of cells beneath the skin and avoiding too much spread of the cells.
* organoids ATAC-seq sample preparation

1. Grow the organoids in the 6-well plate for 3-4d.

2. Wash the well with 2ml of PBS and aspirate.

3. Add 2.5 ml of trypsin-EDTA to the well. Scratch and suspend Matrigel with a 1,000-µl pipette. Transfer Matrigel and trypsin-EDTA to 15ml centrifuge tube.

4. Incubate the centrifuge tube in 37°C water bath incubator for 10-15 min.

5. Add 1.5-2 ml of washing medium/base medial and dissociate the organoids by vigorous pipetting.

6. Centrifuge the tube at 1500rpm for 3 min at room temperature. Aspirate the supernatant, leaving ~100 µl left over. Add 500ul-1 ml of PBS and resuspend the cells. Count organoids.

7. 150k organoids suspends in 10% DMSO in FBS.

For washing media: Add penicillin (100 units ml−1), streptomycin (0.1 mg ml−1), l-glutamine (2 mM) and FBS (10%, vol/vol) to DMEM/F12 with HEPES.

For base media: Add penicillin (100 units ml−1), streptomycin (0.1 mg ml−1), l-glutamine (2 mM) and FBS (20%, vol/vol) to Advanced DMEM/F12.