Isolation of primary mammary epithelial cells from the mouse

**Isolation of mammary epithelial organoids.**

1. Isolate #3, #4, and #5 pairs of mammary glands from donor mice. Make sure to remove the #4 lymph nodes and to avoid the muscle in the #3 glands.
2. Weigh a 50ml conical tube containing 30ml of DMEM/F12 or HBSS.
3. Collect glands in the pre-weighed 50ml conical tube. Obtain a weight after collecting the glands and subtract from the original weight. For every 1g of tissue, you want to use a 10ml volume of digestion media (1mg/ml Type I Collagenase in DMEM/F12 without any additives. Alternatively, you can use HBSS with calcium and magnesium.
4. Place glands in a glass Petri dish and mince the tissue between two feather scalpels (size #21). Mince until the tissue resembles a paste. Minced tissue should be ~1x1mm in diameter. This step is imperative and will determine efficiency of the digestion.
   - Alternatively, you can use the McIlwain tissue chopper and chop the tissue in three segments, rotating 90 degrees each time.
5. Digest tissue in the calculated volume of digestion media, in a clean autoclaved 125ml (or appropriately sized for your volume) Erlenmeyer flask with beveled bottom with a foil lid. Digestion occurs for approximately 2 hours in a 37°C incubator set to rotate @125rpm.
6. Every 30 minutes, pipette the mixture up and down to break up any aggregated clumps. **After 2 hours, the digest should not have any undigested pieces remaining at this point.**
   **OPTIONAL:** If you notice an incomplete digest, sometimes sheared DNA (caused from the mince or digestion) can accumulate, resulting in an inefficient digest and tissue clumping. To resolve this, add 5μl of DNaseI (Stem Cell Technologies, 1mg/ml) per 10 ml of digestion buffer during the last 30 minutes of the digest.
7. Transfer suspension to a 15ml conical tube (depending on amount of starting material, you might have to use multiple 15 ml tubes) and centrifuge @1500 rpm for 5 minutes. For epithelial organoid isolation, proceed to step 9.
8. **For isolation of fibroblasts...** Transfer the SUPERNATANT to a new tube and centrifuge at 800 × g for 10 min. The pellet will contain fibroblasts. Resuspend in 10 mL of DMEM with 10%FCS and transfer to desired culture dishes. The cells can be grown in DMEM with 10% fetal calf serum (FCS) with penicillin and streptomycin as antibiotics. Plate the cells for one hour to allow the fibroblasts to adhere to the plate (they tend to adhere quickly), leaving behind extraneous cells. Wash and then replenish with growth media.
9. Aspirate the supernatant and resuspend the pellet in 10 ml 1X DPBS without calcium and magnesium. If you have multiple tubes, resuspend/combine the pellets in 10 ml back into one tube. Perform ~3 short centrifugation steps to enrich for mammary organoids, while depleting red blood cells and other stromal cells.
   - To do this, centrifuge the organoids at 450xg (~1500rpm) for 7 seconds and hit the brakes. Allow the centrifuge to ramp up to 1500rpm, count to 7, and then stop the centrifugation. Aspirate/remove the supernatant and resuspend the pellet in another 10ml of 1X DPBS. Repeat the pulse centrifugation ~2-3 times, or until you observe a clear supernatant. At this stage you should have enriched for mammary epithelial organoids in the pellet.
To obtain a single cell suspension:

1) Resuspend organoids in pre-warmed 0.25% Trypsin-EDTA and transfer to one well of a 6-well dish. This step will dissociate mammary organoids into a single cell suspension. Place in incubator for ~5 minutes, pipette up and down every two minutes with a P1000 pipette and monitor dissociation under the microscope until you see complete dissociation of organoids into single cells.
   - Generally, 2ml of Trypsin is used for up to 15 mice. If using more than 15 mice, then I would suggest scaling up the Trypsin depending on the size of the pellet.
   - ** Addition of DNase can be used during the Trypsin step (refer to Stem Cell Technologies protocol).

2) Neutralize Trypsin with 5ml of growth medium containing 10% FBS and filter the suspension through a 40µm filter. If you have a lot of cells, then use multiple filters to avoid clogging the filter and losing cells.

3) Centrifuge at 1000rpm for 5 minutes, wash pellet twice with Growth Medium, and resuspend pellet in 3-6 ml of mammary growth media, depending on the size of the pellet. Count cells and proceed to desired application.

MEC growth media:
DMEM/F-12, 10%FBS, 5µg/ml Insulin, 1µg/ml Hydrocortisone, 10ng/ml EGF, PenStrep

Mammosphere media:
DMEM/F12 with 20 ng/mL basic fibroblast growth factor, 20 ng/mL epidermal growth factor, B27, 100 µg/mL gentimycin, antibiotic-antimycotic, all from Invitrogen

Lentiviral transduction of primary mammary epithelial cells (PMECs)

1) Transfer up to 1 million PMECs to individual wells of a 24-well ultra low adhesion plate, add viral particles at an MOI-30 (titered in 293-T cells), and top off with MEC growth medium so that the final volume is 800 µl per well. Mix gently by pipetting up and down.

2) The PMECs will aggregate overnight at 37 °C in the virus-containing medium.

3) After 16-20 hours, wash the PMEC aggregates twice with 10 ml of HBSS (with Ca2+ and Mg2+) to dilute out any unbound virus. Resuspend the pellet so that there are 20,000 MECs / µl of HBSS. Alternatively, use 50/50 resuspension mix of HBSS/Growth Factor Reduced Matrigel to aid engraftment.

4) Inject 10 µl of aggregates (equivalent to 200,000 PMECs) into each cleared fat pad.