Primary Culture Preparation (1.18.13)

Solutions:
DMEM/F12 serum free
DNase 10mg/ml
Trypsin (0.5g/L Trypsin/EDTA 0.2g/L in saline)

Wash buffer (collagenase buffer):
Hepes 10mM (MW 238.3, 2.38g/1Liter)
5% FBS
RPMI 1640 (Gibco Cat #61870)
Pen/Strep ({100 U,100} /ml)

Collagenase:
Sigma Blend L 0.5mg/ml (Sigma, Cat # C-8176)
or
Collagenase IV 2mg/ml (Sigma, Cat# C-5138)

Growth Media:
Insulin 5ug/ml
Hydrocortisone 1ug/ml (100% Ethanol)
(Roche 855-731) Mouse EGF 10ng/ml
FBS 10%
Pen/Strep ({100 U,100} /ml)
Gentamicin 50ug/ml
Glutamine (if needed)
DMEM/F-12
to 500 ml

GENERAL NOTE: Before pipetting cells or transferring cells to new plastic tubes, it is best to coat pipettes and tubes with 3% BSA/PBS (filter sterilized) to minimize cell adhesion to plastic. In smaller preps, this becomes more important.

1) Weigh a falcon tube containing approximately 30mL wash buffer. Isolate #4 and 5 glands only and remove the lymph node from the #4 gland (you should get about 0.3g/mouse). Place the tissue in the tube with wash buffer. Weigh the tube after tissue collection and determine the amount of tissue that was collected. This prep will produce 4-6 million single cells from each 8-week-old donor mouse.

2) Remove the glands from the tube and chop the tissue for 2-5 minutes with #10 scalpels in a 150mm tissue culture plate. Don’t chop too long in one place on the plate as shards of plastic will end up in your tube and will interfere with the collagenase step. Stop when it looks like a slurry (subjective).

3) Make the collagenase buffer by adding 2.5mg/ml of Sigma Collagenase (preferred collagenase- cat# C-5138) to the wash buffer. Add 5ml of the collagenase media to each gram of tissue. Filter this solution (with 2um syringe filter).
4) Incubate the tissue with collagenase for ~1.5 hours at 37C, shaking at ~150RPM. The solution should have a homogenous pink color and there should not be large clumps of tissue remaining after the incubation. Check for floating pieces of fat and gland near the top. Shake until gone.

5) Add RT Hanks (with Ca/Mg) until conical is full. Shake well. Spin the collagenase/cell mixture at 600rcf for 15 minutes to pellet the organoids. Pour off supernatant into second conical tube (coated with BSA-PBS) and shake vigorously again. The initial pellet will likely have a layer of DNA on top and you need to lyse the DNA so that organoids won’t get caught in the supernatant during later spins. Use 50ul of DNase (10ug/ul) in 10-15ml of Hanks for 3-5 minutes, gently shaking periodically. Fill first conical with Hanks and shake vigorously. Spin both conicals for 10 minutes at 600 rcf. The pellets will have a layer of red blood cells covering it. The RBCs will be washed away and gradually disappear from the pellet during the differential centrifugation. Aspirate the top layer of fat from each tube.

6) Aspirate the second conical and pour off the supernatant from the first conical into the second conical and add Hanks to the first conical, shaking both vigorously to break up the pellet. Wash the cells 4-6 times in HANKS (with Ca/Mg) by differential centrifugation. This involves centrifuging the cells at between 450-500 rcf (1400 rpm) for only 30 seconds at top speed (do first spin for 45 sec for good measure). The idea is to pellet the organoids and not the single cells. You will need to figure out the optimum speed and time to perform this with your centrifuge since the weight of different rotors can affect how well this works. You can monitor this by watching the layer of red blood cells disappear from the pellet as you perform this technique. You can also examine the organoids under the microscope to make sure you are isolating organoids and not single cells (e.g. take out 100 ul samples in 48-well plate after each step). In the end at least 90% of the prep should be organoids and not single cells. You will lose a lot of cells during this procedure but it is critical to obtain a clean epithelial prep. If you want to isolate mammary fibroblasts you can pool all of the supernatants from the differential centrifugations and pellet the fibroblasts by centrifuging for 10 minutes at 600rcf.

5) Wash pellet once with PBS. Resuspend the organoid pellets in 25-50% trypsin sol’n [0.05% Trypsin/ EDTA (Gibco 25300)] and PBS and incubate at 37c. I use 2 mls of trypsin solution and 4 mls of PBS per 4-6 mice. Place 2-3 mls of cells in each well of a 6 well plate. You can watch the organoids break up under the scope during this procedure. Trypsinize for about 3 minutes, then pipet cells up-and-down with a p1000 vigorously to help break up the organoids. Repeat every 3 minutes until most of the organoids are dissociated. Not all of the organoids will break up but many will. Allow trypsinization to occur up to 20 minutes. If you trypsinize for longer you risk killing cells. If you have a large prep, it is worthwhile to spin organoids in the centrifuge after 15 minutes of trypsinization and add Hanks+2% serum to the single cell supernatant. Retryptsinize spun down organoids for up to another 15 minutes. Due to cell lysis the cell/trypsin mixture can become viscous as DNA is released. Add Hanks + 2% serum to neutralize trypsin.

6) Pellet cell/trypsin mixture at 450 rcf for 3 minutes.
7) If there is a fluffy white pellet or cloud about your cells, carefully aspirate the supernatant but do not get close to the DNA. If the DNA is aspirated it will take most of your cells with it. If there is no pellet, skip the next step.

8) Resuspend the cell/DNA pellet with 5-10 mls serum-free DMEM/F12 and add DNase I to a final volume of 100ug/ml. Slowly mix by inverting tube for 2-3 minutes RT to break-up the DNA.

9) Filter the cells through a 40um cell strainer to remove clumps.

10) Count cells with trypan blue.

11) Spin down.

12) Freeze back at 4e6 cells per vial in freeze media (70% DMEM/F12, 20% FBS, 10% DMSO).