McCaffrey Lab - Isolation of Primary Mouse Mammary Epithelial Cells

1. Euthanize mice with Isoflurane and cervical dislocation (see animal SOP 301_01, Section 5.3).
2. Isolate 2\textsuperscript{nd}, 3\textsuperscript{rd}, 4\textsuperscript{th}, and 5\textsuperscript{th} pairs of mammary glands.
   - Clean outside of mouse with 70\% ethanol.
   - Pin each paw to a surgical platform.
   - Cut through the skin layer along the ventral midline (from anus to neck).
     Make ~1 cm cuts along each leg and peel the skin back.
   - Grab the mammary fat pads with forceps and gently pull, cutting it away from the underlying skin.
   - It is easiest to remove the lymph node while the 4\textsuperscript{th} mammary gland is still in the mouse.
3. Collect all glands in a 10 cm petri dish. Keep dish covered.

**Perform all subsequent steps in a tissue culture hood.**

4. Mince the glands using scissors. Mince the glands until they are the consistency of sludge (about 2-5 minutes of continuous scissoring).
5. Transfer the minced glands to a 50 ml Falcon tube containing pre-warmed Digestion Medium.
   - Use 10 ml Digestion Medium for 1-2 mice and 25 ml for 3-4 mice and 50 ml for 5-8 mice.
6. Incubate for 1-1.5 hrs at 37°C, with constant mixing at 650 rpm (Thermomixer), until the digestion mixture is homogeneous and no chunks remain.
7. Centrifuge at 1000 rpm for 5 min at room temperature. Fat cells will float to the top of the tube.
8. Aspirate fat cells and supernatant.
9. Resuspend the epithelial cell pellet in 5 ml warm DMEM/F12.
10. Centrifuge at 1000 rpm for 5 min at room temperature.
   - If cells are in a sticky clump, resuspend the pellet in 5 ml DMEM/F12 containing 2U/ml DNase. Mix/swirl gently for 1 minute. (This step is rarely necessary – only if white gooey clumps of cells are present). Add 0.5 ml calf serum to inhibit DNaseI and centrifuge at 1000 rpm for 10 min at room temperature. Remove the supernatant and continue to step 13.
11. Resuspend cell pellet in 10 ml PBS with 5\% calf serum and transfer to a 15 ml tube.
   - Make up 50 ml of PBS with 5\% serum.
12. Centrifuge for 15 sec at 1500 rpm at room temperature. Supernatant is mainly single cells (fibroblasts, blood cells, single epithelial cells etc.).
13. Carefully aspirate the supernatant.
14. Repeat step 13-14 for a total of 5 times to obtain pure epithelial ‘organoids’. The cell preparation should be close to 100\% epithelial organoids with few single cells.
15. Resuspend the pellet in DMEM/F12 to remove serum, and then centrifuge 15 sec at 1500 rpm.
16. A) **For growing 3D organoids:** resuspend the organoids in 50% Matrigel/50% DMEM/F12 and plate on a 50% Matrigel cushion in an 8-well cover glass dish.
   - Use 60 ul for the bottom and 60 ul (i.e. total of 120 ul) for the top Matrigel pads. Incubate at 37°C for 30 minutes to allow the Matrigel to set for each the bottom and top cell-containing layers.

16. B) **For MEC monolayers:** plate the epithelial organoids in a TC dish. Organoids will settle down on the plastic and spread out forming distinct epithelial islands. In 2-3 days or more the dishes will be confluent. Change the medium every 2-3 days.

16. C) **For Mammosphere cultures:** see protocol on Mouse Mammospheres.

16. D) **For infecting MECs with lentivirus:** continue to step 17.

17. Resuspend organoids in 0.5 ml 0.05% Trypsin/EDTA for 12 minutes with frequent mixing. Inhibit Trypsin with 0.5 ml calf serum and pipette up and down with a P1000 to break up cell clumps.
18. Pass cells through a 45 um strainer and collect single cells in a 50 ml tube. Wash the strainer with 2 ml DMEM/F12 and collect with the cells.
19. Centrifuge at 1000 rpm for 5 minutes.
20. Aspirate the supernatant.
21. Resuspend cells in Mammosphere growth medium (0.5 ml/mouse used).
22. Count cells.
   - Mix 10 ul of cells with 10 ul of trypan blue/PBS and count cells with haemocytometer.

**Subsequent steps must be performed in the lentivirus room**

23. Transduce cells with lentivirus by centrifugation at 300 x g for 3h at room temp.
24. Resuspend cells and transfer to a TC dish with warm growth medium.
25. To passage cells, add 1 ml of 0.05% trypsin (with EDTA) and incubate at 37°C. Tap the dish and examine cells every 2-3 min under the microscope. As soon as cells detach, add 5 ml DMEM/F12 with 10% FCS to inhibit trypsin. Centrifuge the cells at 1000 rpm for 2 min at room temperature. Resuspend the pellet in MEC medium. For the first two passages, split the cells 1:1 or 1:2. Later the cells can be split 1:3 or 1:5, but don’t get too greedy – these cells are finicky. I try to keep the split low because if the cells are too sparse they tend to change morphology (long spindly extensions).
Reagents:

Digestion Medium (make fresh just before gland collection).
1. Dissolve Collagenase A in DMEM/F12.
2. Sterile by filtration through a 0.2 um syringe filter.
3. Add the rest of the components.

<table>
<thead>
<tr>
<th>Digestion Medium</th>
<th>Volume/weight</th>
<th>Stock Concentration</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM/F12</td>
<td>10 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>100 ul</td>
<td>100X</td>
<td>1X</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 ul</td>
<td>50 mg/ml</td>
<td>50 ug/ml</td>
</tr>
<tr>
<td>Collagenase A</td>
<td>20 mg</td>
<td></td>
<td>2 mg/ml</td>
</tr>
<tr>
<td>Nystatin</td>
<td>50 ul</td>
<td>120,000 U/ml</td>
<td>600 U/ml</td>
</tr>
</tbody>
</table>

Mammosphere Culture Medium (make fresh weekly)

McCaffrey Lab Mammosphere Medium:
1. 50 ml DMEM/F12 (with Penicillin/Streptomycin)
2. 20 ng/ml EGF
3. 20 ng/ml bFGF (FGF2)
4. 1 ml mouse KO supplement