Organotypic Raft Cultures

Materials:

1. Cotton Pads - #740-E, 8 x 10 in., 25/pack. Cut into 1 x 1 in squares. Autoclave in jar.
2. Sterile Forceps - Autoclave in paper bag.
3. Transwell Inserts - Order from Fisher (Costar #3450)
4. Organogenesis Trays (BD Biosciences #355467)
5. Rat Tail Collagen Type 1 - Upstate Biotechnology Inc. #08-115.
6. C8:0 - 1,2-dioctanoyl-*sn*-glycerol (Sigma #D1912)

Medias:

1. Fibroblast Media:
   - F12 media
   - 10% FBS
   - 1X pen/strep
2. Keratinocyte Plating Media:
   - F media (3:1 F12/DMEM) = 0.66 mM Ca^{2+}
   - 0.5% FBS
   - 1X (hydrocortisone, cholera toxin, insulin, adenine, pen/strep)
   - 1.88 mM Ca^{2+} (add 305 μl 1M $CaCl_2$ to 250 ml of media)
3. Cornification Media 1:
   - F media (3:1 F12/DMEM) = 0.66 mM Ca^{2+}
   - 5% FBS
   - 1X (hydrocortisone, cholera toxin, insulin, adenine, pen/strep)
   - 1.88 mM Ca^{2+} (add 305 μl 1M $CaCl_2$ to 250 ml of media)
4. Cornification Media 2:
   - F media (3:1 F12/DMEM) = 0.66 mM Ca^{2+}
   - 2.5% FBS
   - 1X (hydrocortisone, cholera toxin, insulin, adenine, pen/strep)
   - 1.88 mM Ca^{2+} (add 305 μl 1M $CaCl_2$ to 250 ml of media)
5. 10X F12 media:
   - Dissolve 1 packet Ca^{2+} free F12 in 90 mls ddH_20
   - Add 1.176 g of sodium bicarbonate ($NaHCO_3$)
   - pH to 7.2
   - Add 23 μl of 1M $CaCl_2$
   - Filter sterilize and store at -20°C

Preparation of Collagen Raft:

1. Trypsinize fibroblasts. Count and make final concentration $7.5x10^5$ cells/ml of Fibroblast media.

2. Prepare collagen premix (for 25 ml):

a. 10X F12 media	2.5 ml
b. 10N NaOH	6 μl
c. Pen/strep	250 μl
d. FBS	2.5 ml
e. collagen solution	20 ml

   Mix carefully. Avoid bubbles.

3. Plate 1 ml of collagen premix per Transwell insert. Tilt to coat bottom. Allow to gel ~5 minutes in the hood.

4. Add 600 μl of $7.5x10^5$ cells/ml of fibroblasts ($4.5x10^5$ cells) to remaining collagen mixture. Mix thoroughly. Avoid bubbles.

5. Layer 2.6 ml of fibroblast/collagen solution over each 1 ml gel. Allow to gel ~30 minutes in incubator.

6. Add ~20 mls Fibroblast media to outer well (or enough to submerge the collagen raft. Note: Once raft starts to float, you have enough media. The media will rise through the transwell membrane and through the raft itself.). Incubate in 5% CO_2, 37°C incubator.

7. Gels should contract to appropriate shape within 4-7 days. You do not need to change the media during this time.

Plating of Keratinocytes:

Day 0:

1. Aspirate media from outer well. Carefully remove media from inner Transwell by using a pipette. Layer 150 μl of 1.4 x 10^6 cells/ml (7 x 10^5 cells) onto collagen raft.

2. Incubate for 2 hours to allow cells to attach. Carefully add ~20 ml Keratinocyte Plating media to outer well.

Day 2:

1. Aspirate media from outer well. Carefully remove media from inner Transwell by using a pipette.
2. Feed cells with ~20 mls Keratinocyte Plating media to outer well.

Day 4:

1. Aspirate media from outer well. Carefully remove media from inner Transwell by using a pipette.

2. Sterily lift transwell insert and place 2 cotton pads on the risers of each outer well of the 6 well Organogenisis plate. Place transwell insert on the cotton pads.

3. Add 10-11 mls Cornification Media 1 containing 10 μM C8:0 (to induce productive HPV life cycle) to each outer well.

Feed cells every other day with 10 mls Cornification Media 1 containing 10μM C8:0. At about Day 9, appearance of skin equivalent should be dry. Full stratification occurs by Day 15. Full HPV life cycle peaks ~Day 16. For long experiments, switch to Cornification Media 2 containing C8:0 for feeding post-Day 15.

BrdU Labeling:

1. Aspirate media from outer well. Carefully remove media from inner Transwell by using a pipette.

2. Add 10 ml Cornification Media 1 containing 10 μM C8:0 and 10 μM BrdU to each outer well.

3. After 8 hours, fix in 4% formalin (see Elsa's Raft Fixation/Preparation for Histology Protocol).