Generation and passaging of airway epithelial organoids from hPSC-derived lung progenitors –
Kotton Lab

INTRO
This protocol describes an approach to establish three-dimensional (3D) culture of purified PSC-derived lung and drive them towards an airway epithelial phenotype. Cells are resuspended in 3D Matrigel matrix and cultured in media driving activation of FGF signaling via FGF2 and FGF10 and containing corticosteroids and cyclic-AMP drives cells to form epithelial spheres containing differentiated airway cell types.

REAGENTS
• Growth factor-reduced Matrigel matrix (Corning, cat. no. 356234)
• Complete Serum Free Differentiation Medium (cSFDM)
  — 375 mL 1x Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, cat. no. 12200-036)
  — 125 mL Ham's F12 (e.g. Invitrogen, cat. no. 11765-054)
  — 5 mL B27 with retinoic acid (ThermoFisher, cat. no. 17504044)
  — 2.5 mL N2 (ThermoFisher, cat. no. 17502048)
  — 500 μL ascorbic acid, 50 mg/mL
    o Ascorbic acid can be prepared by diluting stock powder (e.g. Sigma A4544-25G) in sterile, tissue-culture grade water to a final concentration of 50 mg/mL
  — 19.5 μL monothioglycerol, 500 μg/mL (Sigma, cat. no. M6145-25ML)
  — 3.75 mL Bovine Albumin Fraction V, 7.5% solution (Gibco, cat. no. 15260-037)
  — 5 mL Glutamax (Gibco, cat. no. 35050)
  — 1 mL primocin (Invivogen, cat. no. ant-pm-2)
  — Filter sterilize (for example, with Millipore Steritop Sterile Vacuum Bottle-Top Filters, cat. no. SCGPS01RE) and store for up to one month at 4°C, kept away from light.
• 10x cAMP/IBMX Stock
  — 50 mL cSFDM base
  — 21.5 mg 8-Bromoadenosine 3',5'-cyclic monophosphate sodium salt (cAMP, Sigma-Aldrich, cat. no. B7880-100MG)
  — 500 uL 0.1 M IBMX (3-isobutyl-1-methylxanthine, Sigma, cat. no. I5879)
  — Filter sterilize and store at 4°C for up to 1 year
• 250 μg/mL recombinant human FGF2
  — 1 mg recombinant human FGF basic protein (R&D Systems cat. no. 233-FB-025)
  — 4 mL 0.1% BSA
    o Prepare 0.1% BSA in PBS by diluting 13 μL Bovine Albumin Fraction V, 7.5% solution in 1 mL PBS.
    — Filter sterilize.
    — Aliquot and store at -80°C for up to 1 year.
    — After thawing individual aliquots, store at 4°C for up to 1 week.
• 10 μg/mL recombinant human FGF10
  — 25 μg recombinant human FGF10 protein (R&D Systems cat. no. 345-FG-025)
  — 2.5 mL 0.1% BSA
    o Prepare 0.1% BSA in PBS by diluting 13 μL Bovine Albumin Fraction V, 7.5% solution in 1 mL PBS.
    — Filter sterilize.
    — Aliquot and store at -80°C for up to 1 year.
    — After thawing individual aliquots, store at 4°C for up to 1 week.
**100 μM dexamethasone**
- Prepare 1 mM stock:
  - Dexamethasone powder (Sigma, cat. no. D4902-25MG)
  - 63.7 mL molecular biology grade ethanol
  - Store at -20°C for up to 2 years.
- Prepare 100 μM stock:
  - 500 μL 1 mM dexamethasone stock
  - 49.5 mL molecular biology grade ethanol
  - Aliquot and store at -20°C for up to 1 year. This is the working concentration.

**10 mM Y-27632 (Y)**
- 10 mg Y-27632 dihydrochloride (Tocris, cat. no. 1254)
- 3.1 mL sterile, tissue-culture grade water
- Filter sterilize (for example, with EMD Millipore Sterile Disposable Vacuum Filter Units, cat. no. SCGP00525).
- Aliquot and store at -80°C for up to 1 year.
- After thawing individual aliquots, store at 4°C for up to 1 month.

**Airway Differentiation Medium:**
- 45 mL cSFDM base
- 5 mL 10x cyclic AMP/IBMX stock
- 50 μL 250 μg/mL rhFGF2
- 500 μL 10 μg/mL rhFGF10
- 25 μL dexamethasone
- 50 μL 10 mM ROCK inhibitor

**2 mg/mL dispase II**
- 100 mg Dispase II, powder (ThermoFisher, cat no. 17105041)
- 50 mL DMEM
- Dissolve and filter sterilize. Store at 4°C for up to 2 weeks or aliquot and freeze at -20°C for up to 6 months.

- 0.05% Trypsin-EDTA (e.g. Gibco, cat. no 25-300-062)
- Fetal bovine serum (FBS, e.g. Gibco, cat. no. 10082139)
- DMEM (Gibco cat. no. 11995-065)

**PROTOCOL**

Begin this protocol after generating hPSC-derived NKX2-1+ lung progenitors and purifying by flow cytometry (related protocols: Human Lung Differentiation, CD47/CD26 sorting of lung progenitors)

**A) Establishing three-dimensional airway epithelial organoid culture**

1. Spin down single cells post-sort for 5 minutes at 4°C and 300 x g.
2. Resuspend cells at a concentration of 400 cells/μL in undiluted Matrigel matrix and replate in Matrigel drops (size can vary but typically 20-25uL per well of a 12 well plate or 50 – 100 μL per well of 6 well plate).

Prior to resuspending cells, Matrigel should be thawed and kept cold (on ice) to prevent polymerization. For ease of thawing, it is convenient to aliquot the Matrigel to be used in 3D culture into 500 μL – 1 mL aliquots. Take care when resuspending cells not to introduce bubbles into the Matrigel and to efficiently disperse the cell pellet into single cells distributed throughout the Matrigel.
Smaller Matrigel drops can be used but are not as robust at generating organoids as cells can settle through the Matrigel and attach to the bottom of the plate.

If more cells are required downstream, cells can also be replated in several small (50 – 100 μL) drops in a 6-well plate or a p100 dish.

Cells should be plated in one drop per well of a 12-well plate.

3. Allow drops to solidify for 15-20 minutes at 37°C.
4. After drops have fully polymerized, add Airway Differentiation Medium carefully to wells.

Add enough media to ensure that drops are fully covered, typically 1 – 2 mL per well of a 12 well plate.

5. Cells will begin to form epithelial spheres (“organoids”) after several days to one week of culture and will continue to proliferate and expand until the drop is filled with cells.

B) Passaging airway epithelial organoids

1. Aspirate media and add 2 mg/mL dispase to well to cover droplet (typically 1 mL/well) and incubate at 37°C for 20 minutes to 1 hour, until Matrigel is fully dissolved.

Dislodging the Matrigel pellet with a pipette prior to incubation and gentle pipetting 3-5 times after 10 min can facilitate dissociation.

2. Using a p1000 pipette, transfer dissociated organoids to a new 15-mL conical tube and add an equivalent volume of DMEM.
3. Spin down spheres for 1-2 minutes at 4°C, 300 x g.

If organoids have not formed a pellet after this time, spin for an additional 1-2 minutes.

If organoids are particularly large, they can be allowed to settle to the bottom of the conical instead of this centrifugation step. This is particularly useful if there is a lot of debris in the Matrigel drop, as this will not settle and is aspirated with the supernatant.

4. Aspirate supernatant and add 1 – 2 mL 0.05% trypsin per dissociated drop.

For example, if 3 drops were originally dissociated, add 3 mL trypsin.

5. Transfer trypsin and cells to a well of a 6-well plate and incubate for 8 – 10 minutes at 37°C.

Cells will begin to visibly dissociate from the spheres. Allow cells to incubate with trypsin until they are entirely dissociated; they will not survive being mechanically dissociated by pipetting so most of the dissociation should be enzymatic. If cells have not dissociated after 12 minutes in trypsin, collect the cells, spin them down, and resuspend in fresh trypsin for an additional 3 – 4 minutes.

6. While cells are dissociating, prepare “stop medium” by adding 50 mL FBS to 450 mL DMEM.
7. Collect dissociated cells in a new 15 mL conical tube and add an equivalent volume of stop medium.
8. Spin down cells for 5 minutes at 4°C and 300 x g.
9. Resuspend cells in 1 mL DMEM and count using an automated cell counter.
10. Spin down cells and resuspend at a concentration of 400 cells/μL in undiluted Matrigel matrix and replate in 50 – 100 μL drops, allow drops to solidify for 20 minutes to 1 hour at 37°C, and add Airway Differentiation Medium carefully to wells.
This protocol also is effective to prepare a viable single cell solution of cells for flow cytometry or sorting from airway organoids. For this approach, spin down cells and resuspend in FACS buffer for staining or other downstream analysis.

**VERSION HISTORY**

<table>
<thead>
<tr>
<th>Date</th>
<th>Changes</th>
<th>Author</th>
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<tbody>
<tr>
<td>2017-08-03</td>
<td>Drafted by Katie McCauley based on Current Protocols in Stem Cell Biology manuscript draft</td>
<td>KBM</td>
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<tr>
<td>2018-04-03</td>
<td>Update to cell concentration and droplet size</td>
<td>FH</td>
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<tr>
<td>2019-03-27</td>
<td>Update to cell concentration and droplet size and duration of Matrigel gelling.</td>
<td>FH</td>
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<tr>
<td>2022-1-19</td>
<td>Primocin changed to 1mL</td>
<td>CB</td>
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