Human Organoid Core (formerly Translational Tissue Modeling Laboratory)

Culture of Primary Human Lung Organoids

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MATERIALS

- Cell lifter (Corning 3008)
- 6-well culture dishes (USA Scientific CytoOne CC7682-7506): pre-warm in incubator; (Alternative: 12/24/48/96w USA Scientific CytoOne CC7682-7512/7514/7548/7596)
- BSA-coated plastic ware: 0.1% BSA in PBS (BSA: Sigma A8806; sterile-filtered) with 100 µg/mL Primocin (InvivoGen #ant-pm-2); store at 4°C for up to 6 months. Coat at room temp for the below times or overnight at 4°C. Store coated plasticware for up to 30 days at 4°C, but do not let dry.
  Note: Recommended to minimize loss of precious tissue/crypts/enteroids due to adherence to surfaces.
- DPBS (no Ca, Mg; Gibco 14190250)
- DPBSpg: containing 100µg/ml Primocin (InvivoGen ant-pm-, (1X)), 50µg/ml gentamicin (Invitrogen 15750060 1X)
- 0.25% Trypsin-EDTA (Gibco 25200), 0.05% Trypsin-EDTA (Gibco 25300), or TrypLE Express (Gibco 126050)
- Deactivating Serum (DS): DMEM/F12 (Corning 10-092-CV) + 10%FBS (Corning 35-015-CV) + 10 mM HEPES (Fisher 15630080) +10 µM Y27632 (Fisher 125450)
- Matrigel Matrix Basement Membrane (Corning #354234). Dilute to 8 mgs/mL with DMEM/F12. Concentration varies by lot.
- 27-gauge Hypodermic Needle (Fisher 50-209-2577) and 1mL syringe (BD309659)
- Mr. Frosty Cryopreservation container (Fisher 1535050)
- Lung Cell Culture Media

MICHIGAN MEDICINE

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Procedures

Thawing Bud Tip Progenitor Organoids (BTOs)

Note: BTOs are frozen as intact organoids in matrigel. 3x 40 µL droplets of BTOs were frozen in 1.5 mL 10% serum, 10% DMSO and 80% culture media at p1.5. They will be p2 at thaw.

1. Thaw rapidly in a 37°C water bath until partly thawed
2. In a 15mL tube, add 8.5 mL Serum Free Basal Media to thaw/transfer the cryovial contents
3. Spin 300g for 5 minutes at 4°C, BTOs should pellet and remain in Matrigel.
4. Aspirate Serum Free Basal Media and resuspend in 1 mL of 3F media, triturate gently to knock off residual Matrigel and transfer to 1.5 mL tube.
5. Spin 300g for 5 minutes at 4°C, BTOs should pellet with residual Matrigel on top.
6. Remove residual Matrigel layer and 3F media, leaving BTOs. Resuspend in 260 µL of Matrigel.
7. Make 6x 40 µL wells in 24 well plate. Invert plate to avoid BTOs falling to the bottom of the well and incubate for 10 minutes at 37°C.
8. Add 750 µL of 3F + 10 µM Y-27632. Add DBPS + primocin/gentamycin in the empty wells.
9. Next day replace with 3F (no Y-27632).
10. Change the media every 2 - 3 days (e.g. M/W/F).

Passaging Bud Tip Organoids (BTOs)

Needle passage every 7 to 10 days. Morphology should be cystic and the media should not turn yellow.

1. Collect the Matrigel drop in media and triturate with a P1000 10x.
2. Spin suspension at 300g at 4°C for 5 minutes. BTOs should pellet with residual Matrigel on top.
3. Remove 3F Media and residual Matrigel, leaving the BTOs. Resuspend in 1 mL of 3F media.
4. Using a 27-gauge needle and 1 mL syringe briskly passage intact BTOs through the needle 3 times (up and down = 1 time). BTOs should fragment into multiple pieces.
5. Spin down fragmented BTOs at 300xG at 4°C for 5 minutes. Remove 3F media.
6. Resuspend BTOs in appropriate amount of Matrigel and make new 40 µl droplets in wells of a 24-well plate.
7. Invert plate to avoid BTOs falling to the bottom of the well and incubate for 10 minutes at 37°C.
8. Add 750 µl of 3F + 10 µM Y-27632. Add DBPS + primocin/gentamycin in the empty wells.
9. The next day replace with 3F (no Y-27632).
10. Change the media every 2 - 3 days (e.g. M/W/F).

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Passaging AT2 Lung Organoids

Passage cells when they reach ~80% of the Matrigel (AT2s do not become confluent)

1. Harvest cells in warm TrypLE (~0.5 mL/drop) and triturate 30x with a cut tip of a P1000
2. Incubate for 10 mins at 37 degrees Celsius, then Triturate 30x; repeat
3. Spin cells at 300g for 3 mins
4. Remove supernatant
5. Resuspend cells in 5 mL DPBS
6. Spin cells at 300g for 3 mins
7. Remove supernatant, resuspend cells in 1 mL DPBS wash and transfer to a 1.5 mL tube
8. Count cells in a hemocytometer with Trypan blue
9. Resuspend cells to 1,000 cells/µL Matrigel
10. Plate cells in 20 µL Matrigel drops
11. Invert plate to avoid BTOs falling to the bottom of the well and incubate for 10 minutes at 37°C.
12. Add AT2 media + Y27632 to wells. Add DBPS + primocin/gentamycin in the empty wells. Remove Y27632 on the next media change.
13. Change the media every 2 - 3 days (e.g. M/W/F).

Thawing Trachea Organoids

1. Thaw rapidly in a 37°C water bath until partly thawed
2. In a 15-mL tube add 8mL Advanced DMEM or DMEM/F-12 plus 1mL FBS/CBS plus Y27632 to thaw/transfer the cryovial contents
3. Centrifuge at 500g for 5 min
4. Add 1 mL cold PBS, resuspend cells and transfer to a 1.5 mL tube

Cell Seeding

5. Check the cell density, then transfer the cells into a new tube (10 - 50k cells, usually 20k per well)
6. Centrifuge new tube at 300g for 3 min
7. Discard the supernatant and resuspend in cell culture media and then mix with Matrigel for a final concentration of 8mg/mL and a volume of 20uL per well
8. Invert plate to avoid BTOs falling to the bottom of the well and incubate for 10 minutes at 37°C.
9. Add media. Add DBPS + primocin/gentamycin to the empty wells.

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Passaging Trachea Organoids in Matrigel

Passage organoids when there are several large organoids, the cells are too dense/sparse, or after 2-4 weeks

1. Remove media from the wells and add 5-10mL total DPBS + Y27632 to the wells
2. Cut the p1000 barrier tip to collect organoids/Matrigel in a 15mL tube
3. Triturate to separate cells from the Matrigel
4. Centrifuge at 500g for 5 min
5. Discard supernatant

Single-cell suspension preparation

6. Add 1-2 mL TrypLE and triturate vigorously, leave in the 37 °C incubator
7. Triturate every 10-15 mins until mostly single cells can be observed in the tube under the microscope
8. Deactivate 2x volume with 10% FBS with Y27632
9. Centrifuge at 500g for 5 min
10. Discard supernatant
11. Add 1 mL cold PBS, resuspend cells and transfer to a 1.5 mL tube
    a. *Alternatively, add 1mL CryoStor per vial ~6w/24w thaw for cell preservation or flash freeze 1-2w/24w per vial for RNA preservation

Cell Seeding

12. Check the cell density using the hemocytometer with Trypan blue
13. Transfer appropriate number of cells to a new tube
14. Centrifuge new tube at 500g for 5 min
15. Discard the supernatant and resuspend in cell culture media and then mix with Matrigel for a final concentration of 8mg/mL Matrigel and a volume of 20uL per well.
16. Place in the incubator of 10 min upside down (hanging drops)
17. Add media after 10 min. Add DBPS + primocin/gentamycin in the empty wells.

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Passaging Trachea Organoids on 3T3-J2i cells

Passage cells every 1-2 weeks or when ~80% confluent. High density can irreversibly differentiate esophagus cells and stop growth. 3T3-J2i cells lose viability after 1-2 weeks.

A. Plate Preparation

a) Add 100,000 cells per well of a 6-well plate, at 24hrs (at least 4hrs) before passaging. Other sized plates may be used.

B. Passaging cells

a) Add 500 µl of trypsin EDTA to each well of culture cells.
b) Incubate at 37°C for 5-10 minutes.
    a. Cells should be able to be loosened with GENTLE scraping or trituration.
c) Inactivate trypsin by adding FANY and transferring to a centrifuge tube.
d) Spin cells at 500g for 5 minutes.
e) Resuspend cells in 1000 µL of FANY per well of a 6 well and triturate gently cells with a P1000 pipette.

C. Freezing cells

a) Add 500 µl of trypsin EDTA to each well of culture cells.
b) Incubate at 37°C for 5-10 minutes.
    a. Cells should be able to be loosened with GENTLE scraping or trituration.
c) Inactivate trypsin by adding FANY and transferring to a centrifuge tube.
d) Spin cells at 500g for 5 minutes.
a) Resuspend cells into CryoStor or FANY with 10% DMSO and 10% serum.
b) Add 1 mL of cell suspension per cryovial.
c) Transfer to a Mr. Frosty freezing container.
d) Place in -80C. Transfer vials to liquid nitrogen within 24 hrs of cryopreservation.

Harvesting primary lung tissue

Materials

1. **<u>Lung digestion solution</u>** in serum free MEM containing a final concentration of
    a. Collagenase A (1mg/ml) (Roche, 10103578001)
    b. Elastase (2-4U/ml) (Worthington, LS002274); dilution is LOT dependent! Check activity and amount of protein on each tube!
    c. DNASE (0.1mg/ml) to prevent stickiness and clumping of cells from DNA of dead cells. (Roche cat#10104159001; 100mg vial. Dilute 1:100 from a 10mg/mL stock solution)
2. **<u>Miscellaneous</u>**
    a. Red Blood Cell Lysis Buffer: Roche, 11814389001
    b. 100um cell strainer: Fisher, 22363549

Methods

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1. Cut lung tissue into small 1-2cm^2 sized cubes. Chop the lung cubes into a paste like consistency with scissors and place it into 20mL of digestion solution in a 50mL conical tube.
    a. Note: More than 2cm^2 of tissue per 20mL of digestion solution can get too sticky. Multiple tubes can be merged into 1 tube after isolation of single cell suspension
2. Rotate at 37°C for 1 hour
3. In a cell culture hood, triturate with a 25mL or 10mL pipette
4. Rotate at 37°C for 1 hour
5. Pipette tissue up and down again multiple times to disperse cells from the tissue.
6. Prepare 100µm filter over a fresh 50mL conical tube and pour digested tissue through the filter. Multiple filters may have to be used if sticky.
7. Dilute each 20ml of digestion solution into a total volume of 100ml with PBS split between two 50ml conical tubes. (For removal of excess fat in the single cell suspension)
3. Spin for 10 minutes at 500g.
4. Remove supernatant. You may rewash if the supernatant is too cloudy.
5. Add 1-2 ml red blood cell lysis buffer to the pellet and incubate for 3 minutes.
6. Add 20ml of PBS to stop the reaction and filter through a 100um filter into a fresh 50ml conical tube.
7. Centrifuge for 5-7 minutes at 500g and remove the supernatant. The pellet should have few red blood cells.
8. Begin with cell seeding procedures for the appropriate lung section or freezing in appropriate freezing media in a Mr. Frosty freezing container.