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Production of High-Purity Cell Suspensions from Human Precision Cut Lung Slices for Single-Cell RNA Sequencing

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We use this protocol and it's working

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Abstract

Background:
In vitro models reduce the risk to humans caused by medical research but are often limited to a single or few cell types. Precision Cut Lung Slices (PCLS), which model the human lung tissue, contain major cell types in situ allowing the preservation of intra cell type interactions that occurs during both homeostasis and disease states. Here we demonstrate a procedure to produce high-quality single-cell RNA sequencing data from cultured human Precision Cut Lung Slices.
Methods: Cryogenically frozen PCLS slices were thawed and cultured on Transwell membranes. Slices were put into contact with media, but not submerged. Slices were fed every other day for 8 days and put into single cell suspension by enzymatic dissociation. Mechanical separation using cell strainers and density gradient centrifugation was used to deplete residual agarose. Single cell suspensions were captured and sequenced.
Results: Single-cell suspensions from PCLS slices yielded greater than 80% viable cells. Mechanical and density gradient centrifugation reduced both the amount and the size of agarose in the single-cell suspensions. A total of 34,069 cells were captured for single-cell RNA sequencing, 97.4% of Barcodes were valid, and 97.4% of Reads were Mapped to Genome. 16 cell types, including all major cell types in the lung (endothelial, epithelial, mesenchymal, and immune), were identified.
Conclusion:
The described method produced single-cell suspensions from Precision Cut Lung Slices that resulted in high-quality single-cell RNA sequencing data of all major cell types.

Materials

- DMEM High Glucose + L-Glutamine **Gibco - Thermo Fischer Catalog #**11-965-118
- Heat-Inactivated FBS **Gibco - Thermo Fischer Catalog #**10-082-147
- 1X Non-Essential Amino Acids **Gibco - Thermo Fischer Catalog #**11-140-050
- Sodium Pyruvate **Gibco - Thermo Fischer Catalog #**11-360-070
- 1M HEPES Buffer **Gibco - Thermo Fischer Catalog #**15-630-080
- Penicillin/Streptavidin **Gibco - Thermo Fischer Catalog #**15-140-122
- Amphotericin-B **Gibco - Thermo Fischer Catalog #**15-290-018
- Gentamicin **Gibco - Thermo Fischer Catalog #**15-750-060
- Divalent Cation Free DPBS **Lonza Catalog #**BW17-512F
- Magnesium Chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**7791-18-6
- Potassium Chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**793590-500G
- Sodium Chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**746398-500G
- Calcium Chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**21115-1ML

PPE:

1 Lab coat, gloves and face shield should be worn at all times. All consumables (e.g. tips) should be disinfected in 10% bleach after use. Surfaces should be cleaned prior and after use with Virex followed by 70% ethanol. Experimenter should not open door to hall while working with tissue. No one is allowed into the tissue culture room while the tissue is being actively worked on. All work should be done at a BSL2 level.

PCLS-TW Media Prep

2 Combine the following to the top chamber of a bottle-top vacuum filter:

- 500 mL DMEM high glucose (4.5g/L) + L-Glutamine
- 50 mL Heat-inactivated FBS
- 5 mL of 100x Non-Essential Amino Acids
- 5 mL Sodium Pyruvate 100nM
- 7.5 mL 1M HEPES Buffer
- 5 mL of 10,000u/mL Penicillin/Streptavidin
- 500 µL (125ug) Amphotericin B
- 800 µL (40ug) Gentamicin

Using vacuum, filter media into bottom chamber. Aliquot and store at 4°C until needed.

Tissue Growth (Day 1 & 2)

3 **Prep:**
Warm water bath containing autoclaved diH2O to 37°C

4 **Day One:**
Obtain vial(s) of PCLS and place vial into warm water bath. Making sure not to submerge the cap and gently swirl vial until only small ice crystals remain. Do not thaw completely.

5 In biosafety cabinet, decant the vial swiftly over a transwell membrane of a 6-well transwell membrane letting the slice wash out. If the slice does not easily fall out, rinse the vial with PBS, repeat as needed.
If necessary, sterile flat head forceps may be used to gently seize the slice and place atop the transwell membrane. Be sure to uncurl the slice so it lies flat.

6 Gently dispense 500 µL of DPBS with Ca2+/Mg2+ onto the slice. Gently swirl plate and then remove DPBS, making sure not the disturb the slice. Repeat.

7 Dispense 800 µL of PLCS-TW media into the bottom chamber of the transwell. Media should come into contact with PCLS, but not submerge PCLS.
Label each well of transwell plate accordingly.

8 To ensure wells do not dry out during incubation, dispense 800 µL of PLCS-TW media to any unused wells.

9 Place plate in incubator at 37°C with 5% CO_2.

10 **Day Two:**
Remove transwell plate from the incubator and move to biosafety cabinet.

11 Aspirate the media from bottom chamber of each well and discard.

12 Dispense 800 µL of fresh PCLS-TW media into the bottom chamber of each well.

13 Return transwell plate to incubator at 37°C with 5% CO_2.

Single Cell Dissociation

14 **Prep:**
- Set incubator to 37°C
- Turn on centrifuge and set the temperature to 4°C
- Place the following into the biosafety cabinet:
  - Pasteurs
  - p1000
  - 1000uL tips
  - Tube holder
  - Forceps
  - Chlorox spray bottle
  - 15mL tube holder
  - Bleach beaker
  - Dissociation cocktail

15 **Dissociation Cocktail:**
1. *Digestion Buffer* (may be made ahead, store at 4°C):
a) Divalent cation free DPBS (1 L)
+ 10mM HEPES-NaOH (pH 7.4) (use 10mL of 1M stock)
+ 150mM NaCl (8.77g)

+ 5mM KCl (0.37g)
+ 1mM MgCl2 (0.1g)
+ 1.8mM CaCl2 (0.2g)

2. *Make dissociation cocktail then add the following enzymes:*

a) *Collagenase Type A from Clostridium*; 2mg/ml final (powder) (0.15units/mg and 0.30 units/mL). Stored as lyophilized dry powder at 2-8°C, dry enyzme is stable for 6-12 months.

b) *Dispase II*; 1mg/ml final (liquid), 1:5 dilution of total digestion cocktail volume (50 units/mL stock, 10 units/mL).

c) *Elastase*: Twice crystallized from Porcine Pancreas (0.5mg/mL final) (3 units/mg and 1.5 units/mL). Elastase in unstable at pH 3.5. Elastase product codes: ES and ESL have poor solubility at neutral pH and at concentrations greater than 0.25%. It is suggested that primary solutions be made in KCl or alkaline buffers and diluted into the reaction mixtures or media, compensating for ionic strength of pH changes.

3. *DNase-Deoxyribonuclease-1 from Bovine pancreas; 2mg/mL final (powder).*

a) 10mg/mL solution of DNase 1 in 0.15 M NaCl may lose <10% of its activity when stored for a week in aliquots at -20°C. The remains active for up to five hours at 60°C between pH 5 and 7, and loses activity in <10 minutes at 68°C. Activity of 1 mg/mL solution is lost at the rate of 6%/hour in acetate buffer (pH 5) or tris buffer (pH 7.2)

For 10 mL of dissociation cocktail (in a 15 mL conical):

+ 10 mL of Digestion Buffer
+ 20 mg Collagenase type A
+ 200 µL Dipase II (1 mg/ mL)
+ 5 mg Elastase
+ 20 mg DNase

16 Measure and dispense 500 µL of dissociation cocktail into appropriately labeled 5mL culture tubes, one tube per slice.

17 Place each PCLS into separate 5mL culture tubes containing dissociation cocktail.
Label the tubes accordingly.

18 Place tubes on orbital shaker located in a 37°C incubator.

19 Incubate and shake 1000 rpm, 37°C, 00:40:00 .

20 Place the following items inside the biosafety cabinet:
- Tray full of wet ice
- 70uM cell strainers

- 6 well place on ice
- DPBS with Ca2+/Mg2+ (DPBS++) on ice
- Labeled 15mL tubes
- 3mL syringes

21 After the 40 minute incubation, add 500 µL DPBS++ to each tube and pour into cell strainer on top of a well.

22 Rinse each set of tubes with an additional 500 µL of DPBS ++ to maximize cell recovery.

23 Push tissue through the cell strainer with the plunger end of the syringe. Gently swirl in circular motions, repeat 5-10 times.
Note: Samples can be pooled at this time if needed.

24 Add 500 µL of DPBS++ and push remaining tissue through the cell strainer with the plunger end of the syringe.
Note: Large agarose pieces and fibrous tissue may remain.

25 Collect strained cells using pipette and place in 15mL tube.

26 Centrifuge 15mL tube 1000 x g, 4°C, 00:10:00 10m

Debris Removal (Performed on 0.5-1 g estimated pooled tissue) 20m

27 Aspirate supernatant completely.

28 Resuspend cell suspension carefully with 6200 µL of cold buffer (DPBS++) and transfer cell suspension to a 15mL tube. Do not vortex.

29 Add 1800 µL of cold Debris Removal Solution.

30 Mix well by pipetting 10 times slowly up and down using a 2 mL pipette.

31 Overlay very gently with 4 mL of cold buffer (DPBS++) making sure to tilt tube and pipette very slowly to ensure that the PBS/D-PBS phase overlays the cell suspension and phases are not mixed.

32 Centrifuge 3000 x g, 4°C, 00:10:00 with full acceleration and full brake. Three phases are formed. 10m
Note: The acceleration and brake can be reduced to increase separation of phases.

33 Vacuum aspirate the top two phases completely and discard (approximately 4mL).

34 Add cold DPBS++ to a final volume of 15 mL

35 Gently invert the tube three times to mix. Do not vortex.

36 Centrifuge 1000 x g, 4°C, 00:10:00 with full acceleration and full brakes. 10m

37 Aspirate supernatant completely making sure not to disturb pellet.

38 Carefully resuspend cells by adding 175 µL of cold DPBS++ by pipetting slowly up and down. Do not vortex. Place on ice.

39 Count the cells.