Establishing Murine Lung Organoids Mimicking Cell Plasticity and Regeneration

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Summary

Organoids are widely used for disease modeling due to their faithful recapitulation of tissue homeostasis, regeneration, and disease processes. While organoids are typically cultured under stemness-promoting conditions with several growth factors and chemicals, these stimulated stem cell niches may not accurately represent the regenerative environment. Herein, we present a detailed, efficient protocol for generating and culturing murine lung organoids (LOs) that mimic regeneration. We also describe how to establish genetically engineered LOs using viral transduction.

For complete details on the use and execution of this protocol, please refer to Kim et al. [1,2]

Before you begin

Given that mouse LOs are established from mouse lung tissue, animal experiments should be performed with an appropriate protocol approved by the Institutional Animal Care and Use Committee (see Institutional Permissions). Viral transduction-related experiments should be performed with adequate personal protective equipment under institutional guidelines and an approved biosafety protocol.

Institutional Permissions

All animal procedures performed here were based on the Association for the Assessment and Accreditation of Laboratory Animal Care guidelines and institutionally approved protocol (00001377; the University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee). The study was compliant with all relevant ethical regulations regarding animal research.

Key resources table

REAGENT or RESOURCE	Source	Identifier
Antibodies		
Anti-Ter-119 MicroBeads, mouse	Milteny Biotec	Cat# 130-049-901 RRID: AB_2936424
CD45 MicroBeads, mouse	Milteny Biotec	Cat# 130-052-301 RRID: AB_2877061
CD326 (EpCAM) MicroBeads, mouse	Milteny Biotec	Cat# 130-105-958 RRID: AB_2936423
CD31 MicroBeads, mouse	Milteny Biotec	Cat# 130-097-418 RRID: AB_2814657
Hop (HOPX) Antibody (E-1)	Santacruz	Cat# sc-398703 RRID: AB_2687966
Anti-Prosurfactant Protein C (SPC) antibody	Abcam	Cat# ab40879 RRID: AB_777473
Anti-Uteroglobin (SCGB1A1) antibody	Abcam	Cat# ab40873 RRID: AB_778766
Anti-Tubulin, Acetylated (Ac-TUB) antibody	Sigma	Cat# T6793 RRID: AB_477585
Chemicals, peptides, and recombinant proteins		
Leibovitz's L-15 Medium	Gibco	Cat# 11415064
DMEM/F-12	Gibco	Cat# 11320033
Penicillin-Streptomycin	Gibco	Cat# 15140122
Cell Dissociation Buffer, enzyme-free, PBS	Gibco	Cat# 13151014
ACK (Ammonium-Chloride-Potassium) Lysing Buffer	Gibco	Cat# A1049201
Trypsin-EDTA	Gibco	Cat# 25200056
GlutaMAX	Gibco	Cat# 35050061

HEPES (1 M)	Gibco	Cat# 15630080
Collagenase Type I	Worthington	Cat# CLS-1 LS004197
Elastase	Worthington	Cat# ESL LS002294
DNase I	Worthington	Cat# D LS002007
Bovine Gelatin	Sigma	Cat# G1393
EDTA solution	Sigma	Cat# E7889
Heparin	Sigma	Cat# H3149
Endothelial cell growth supplement (ECGS) from bovine neural tissue	Sigma	Cat# E2759
Dimethyl sulfoxide (DMSO)	Sigma	Cat# D2650
Hexamethrine bromide (Polybrene)	Sigma	Cat# TR-1003-G
Polyethylene glycol (PEG) 8,000	Thermo Scientific	Cat# 043443
Cultrex Basement Membrane Extract, Type 2, Pathclear	R&D	Cat# 3532-005-02
Experimental models: Cell lines		
HEK 293T	ATCC	Cat# CRL-3216
Experimental models: Organisms/strains		
Mouse: 2- to 10- week-old male or female C57BL/6	The Jackson Laboratory	JAX:000664; RRID:IMSR_JAX:000664
Recombinant DNA		
Plasmid: pMD2.G	Addgene	Cat# 12259
Plasmid: psPAX2	Addgene	Cat# 12260
Plasmid: pLenti-CMV-MSC-RFP-SV-Puro	Addgene	Cat# 109377
Other		
Clean bech cabinet	n/a	n/a
CO2 Incubator	n/a	n/a
Refrigerator centrifuge	n/a	n/a
LS Columns	Milteny Biotec	Cat# 130-042-401
MACS® (magnetic-activated cell sorting) MultiStand	Milteny Biotec	Cat# 130-042-303
QuadroMACS™ Separator	Milteny Biotec	Cat# 130-090-976
Stereomicroscope	KRUSS	n/a
Inverted microscope	ZEISS	n/a
15 mL Conical tube	Fisher scientific	Cat# 14-959-49B
50 mL Conical tube	Fisher scientific	Cat# 05-539-6
Cell Lifters	Fisher scientific	Cat# 08-100-240
0.2 μm Disposable PES Filter Units	Fisher scientific	Cat# FB12566502
Sterile Syringe Filter PES (0.45 μm)	Fisher scientific	Cat# 09-720-514
40 μm Cell Strainer	Corning	Cat# 352340
70 μm Cell Strainer	Corning	Cat# 352350
Cryogenic Vials	Corning	Cat# 430488
Transwell-Clear insert, PET membrane, diameter 6.5 mm, pore size 0.4 μm	Corning	Cat# 3470
1.5 mL tubes	Genesee Scientific	Cat# 24-282

10 μL filter tips	Genesee Scientific	Cat# 24-401
200 μL filter tips	Genesee Scientific	Cat# 23-412
1000 μL filter tips	Genesee Scientific	Cat# 23-430
BD Luer-Lok Syringe (30 ml)	Becton Dickinson	Cat# 302832
Freezing container	Thermo Scientific	Cat# 5100-0001

Materials and equipment

Lung dissociation buffer		
Reagent	**Final concentration**	**Amount**
Leibovitz's L-15 medium		1.2 ml
Collagenase type I (20 mg/ml)	2 mg/ml	150 μl
Elastase (20 mg/ml)	2 mg/ml	150 μl
DNase I (20 mg/ml)	0.4 mg/ml	30 μl

MACS buffer		
Reagent	**Final concentration**	**Amount**
Leibovitz's L-15 medium		89 ml
FBS	10 %	10 ml
Penicillin-streptomycin	1 %	1 ml
EDTA (0.5 M)	1 mM	200 μl

LuEC growth media		
Reagent	**Final concentration**	**Amount**
DMEM		77.6 ml
FBS	20 %	20 ml
Penicillin-streptomycin	1 %	1 ml
Heparin (25 mg/ml)	100 μg/ml	0.4 ml
ECGS (10 mg/ml)	100 μg/ml	1 ml

LO growth media		
Reagent	**Final concentration**	**Amount**
DMEM/F-12		44 ml
Insulin/Transferrin/Selenium		500 μl
FBS	10 %	5 ml
Penicillin-streptomycin	0.5 %	250 ul
Glutamax	0.5 ×	250 ul
HEPES (1 M)	1 mM	50 ul

PEG solution

Reagent	Final concentration	Amount
Water		Up to 200 ml
10 × PBS (pH 7.4)	1 ×	20 ml
PEG-8000	40 % (W/V)	80 g
NaCl	1.2 M	14 g
Sterile by filtering through 0.2 um vacuum filter		

[Storage condition]
Stocks of Collagenase type I (20 mg/ml), Elastase (20 mg/ml), and DNase I (20 mg/ml) are stored at -20 °C for up to 6 months without repeated freeze-thaw cycles.
Lung dissociation buffer is prepared freshly.
MACS buffer is stored at 4 °C for up to a month.
LuEC growth media is stored at 4 °C for up to a week.
LO growth media is stored at 4 °C for up to a week.
PEG solution is stored at 4 °C for up to 6 months.

Step-by-step method details

Murine lung endothelial cell isolation and culture

This section describes how to extract lung endothelial cells (LuECs) from the mouse lung, culture them on the gelatine-coated plate, and cryopreservation them for LO culture (Figure 1). LuECs will be used as feeder cells for LO culture, as previously described [1-3]. The cryopreservation of LuECs is available, and every batch of LuECs may show biological variances. Therefore, we recommend stocking enough LuECs at once to minimize biological variances of LO culture.

Day 1. Timing: [4 hours]
Prepare ice, ice-cold ammonium–chloride–potassium (ACK) lysing buffer, and ice-cold magnetic activated cell sorting (MACS) buffer. Prepare LuEC growth media at 37 °C.

1. Before isolating LuECs, prepare a 0.2 % gelatin solution in phosphate-buffered saline (PBS) filtered through a 0.2 μm filter and store it at 37°C.

2. Prepare lung dissociation buffer and put it at 37 °C.

3. Euthanize the 2-3 weeks-old mice by CO_2 inhalation, followed by cervical dislocation.

4. Excise the lung lobes and transfer them to cold Leibovitz's L-15 Medium containing 3 % penicillin-streptomycin.
    a. Before excising the lungs to remove immune cells and red blood cells (RBCs) in the lung, flush PBS to the right ventricle slowly until the lung becomes white using a syringe with a 22 G needle. Typically, 5-10 ml of PBS is used for one mouse.

       Note: The lung lobes can be stayed on ice for up to 1 hour to transfer lung lobes from the euthanized place to the tissue processing room.

5. Place tissues on 10 cm Petri dishes on ice to process.
    a. Remove the esophagus, trachea, and extra connective tissues using forceps.
    b. Mince the tissues into 1 mm3 pieces with surgical blades or surgical scissors.
    c. Collect minced tissues in a 2 ml Eppendorf tube (or 15 ml conical tube).

6. Dissociate the lung with 1.7 ml of the lung dissociation buffer at 37 °C for 30 minutes.
    a. After 10 minutes of digestion, pipet tissues with 1 ml pipet tips until all the pieces are passed through the tip. Stop pipetting when all the pieces can pass through the tip.

       Note: The lung dissociation time can be increased up to 45 minutes.

       Note: When processing the multiple lungs, use each tube containing 1.7 ml of the lung dissociation buffer

7. Stop the digestion by adding 300 μl of FBS, followed by pipetting.

8. Perform RBC lysis using ACK lysing buffer.

    CRITICAL: Perform this procedure at 4 °C.

    a. Filter the digested cells through a 70 μm sterile strainer and collect cells in a 2 ml Eppendorf tube.
    b. Centrifuge the cells (4,500 g, 1 minute) at 4 °C and discard the supernatant by aspiration.
    c. Gently pipet the pellet with 1 ml of ACK lysing buffer and incubate it for 3 minutes at 4 °C.

    CRITICAL: Avoid vigorous pipetting.

    d. Centrifuge the cells (4,500 g, 1 minute) at 4 °C and discard the supernatant by aspiration.

9. Resuspend cells in 400 μl of MACS buffer, filter the cells through a 30 μm sterile strainer, and collect cells in a 1.5 ml Eppendorf tube.

    Note: When processing the multiple lungs, resuspend cells in 400 μl of MACS buffer per lung and aliquot 400 μl in each tube.

10. Add 40 μl of anti-mouse CD45 magnetic beads, 40 μl of anti-mouse Ter119 magnetic beads, and 40 μl of anti-mouse CD326 (EpCAM) magnetic beads to cell suspension and incubate for 45 minutes at 4 °C.

11. Perform the negative magnetic sorting with an LS column.

    Note: When processing the multiple lungs, use one LS column per lung.

    a. Collect cells 3 times with 5 ml of MACS buffer into a 15 ml conical tube while the magnetic stand holds the column.
    b. Centrifuge the cells (1,000 g, 5 minutes) at 4 °C and discard the supernatant by aspiration.

12. Resuspend cells in 400 μl of MACS buffer and collect cells in a 1.5 ml Eppendorf tube.

    Note: When processing the multiple lungs, resuspend cells in 400 μl of MACS buffer per lung and aliquot 400 μl in each tube.

13. Add 40 μl of anti-mouse CD31 magnetic beads to the cell suspension and incubate for 45 minutes at 4 °C.

14. During the incubation, prepare the gelatin-coated cell culture plate.

    Note: When processing the multiple lungs, prepare one dish per lung.

a. Spread the 37 °C incubated 0.2 % gelatin solution into a 10 cm culture dish. Typically, 4 ml of solution can cover one 10 cm culture dish.
b. Incubate the gelatin-covered dish for 15 minutes at 37 °C.

Note: The incubation time can be increased up to 4 hours.

c. Aspirate the gelatin solution and wash the dish with PBS 2 times.
d. Dry the dish at room temperature (RT) for 15 minutes.

15. Perform the positive magnetic sorting with an LS column.

Note: When processing the multiple lungs, use one LS column per lung.

a. Wash the column 3 times with 5 ml of MACS buffer while the magnetic stand holds the column.
b. Separate the column from the MACS magnetic stand and collect cells 2 times with 5 ml of MACS buffer into a 15 ml conical tube.
c. Centrifuge the cells (1,000 g, 5 minutes) at 4 °C and discard the supernatant by aspiration.

16. Resuspend the cell pellet with 12 ml of LuEC growth media and culture them on the gelatin-coated plate until cells are 80-90 % confluent.
    a. Culture the cells in one dish per mouse.
    b. Typically, cells reach 80-90 % confluent in 6-7 days.
    c. Replace the LuEC growth media every other day.

Day 7. Timing: [2 hours]

17. Detach the cells from the plate for secondary sorting.

CRITICAL: Secondary sorting is a vital step to obtain high purity of LuECs.

a. Aspirate the culture media and wash the dish with PBS 2 times.
b. Add 2 ml of enzyme-free cell dissociation buffer to the dish.

CRITICAL: Cell dissociation enzymes such as trypsin should be avoided for secondary sorting.

c. Incubate the dish for 5 minutes at 37 °C.
d. Detach the cells using a cell lifter.
e. Stop the dissociation with 2 ml of DMEM (10 % FBS and 1 % penicillin-streptomycin) and collect cells into a 15 ml conical tube.
f. Centrifuge the cells (1,000 g, 5 minutes) at 4 °C and discard the supernatant by aspiration.

18. Resuspend cells in 400 μl of MACS buffer per dish and aliquot 400 μl into each Eppendorf tube.

19. Add 40 µl of anti-mouse CD31 magnetic beads per tube to the cell suspension and incubate for 45 minutes at 4 °C.

20. During the incubation, prepare the gelatin-coated cell culture plate.
    a. Spread the 37 °C incubated 0.2 % gelatin solution into a 10 cm culture dish. Typically, 4 ml of solution can cover one 10 cm culture dish.
    b. Incubate the gelatin-covered dish for 15 minutes at 37 °C.

       Note: The incubation time can be increased up to 4 hours.

    c. Aspirate the gelatin solution and wash the dish with PBS 2 times.
    d. Dry the dish at RT for 15 minutes.

21. Perform the positive magnetic sorting with an LS column.

       Note: When processing the multiple lungs, use one LS column per lung.

    a. Wash the column 3 times with 5 ml of MACS buffer while the magnetic stand holds the column.
    b. Separate the column from the MACS magnetic stand and collect cells 2 times with 5 ml of MACS buffer into a 15 ml conical tube.
    c. Centrifuge the cells (1,000 g, 5 minutes) at 4 °C and discard the supernatant by aspiration.

22. Resuspend the cell pellet with 12 ml of LuEC growth media and culture them on the gelatin-coated plate until cells are 80-90 % confluent.
    a. Culture the cells in one dish per mouse.
    b. Typically, cells reach 80-90 % confluent in 3-4 days.
    c. Replace the LuEC growth media every other day.

Day 11. LuEC passaging: [1 hour]

Prepare 0.2 % gelatin solution, trypsin-EDTA, and LuEC growth media at 37 °C.

23. Before passaging the LuECs, prepare the gelatin-coated cell culture plate.

       Note: Passage LuECs into a 1:5 ratio.

    a. Spread the 37 °C incubated 0.2 % gelatin solution into a 10 cm culture dish. Typically, 4 ml of solution can cover one 10 cm culture dish.
    b. Incubate the gelatin-covered dish for 15 minutes at 37 °C.
    c. Aspirate the gelatin solution and wash the dish with PBS 2 times.
    d. Dry the dish at RT for 15 minutes.

24. Passage LuECs
    a. Wash cells with PBS 2 times.
    b. Add 2 ml trypsin-EDTA into the plate and incubate at 37 °C until cells are detached.
    c. Inactivate trypsin by adding 0.5 ml of FBS.

d. Centrifuge the cells (1,000 g, 5 minutes) at 4 °C and discard the supernatant by aspiration.
e. Resuspend the cell 12 ml per dish of LuEC growth media and culture them on the gelatin-coated plate until cells are 80-90 % confluent.
f. Passage the cells into a 1:5 ratio.
g. Typically, cells reach 80-90 % confluent in 3-4 days.
h. Replace the LuEC growth media every other day.

Day 14. LuEC cryopreservation: [1 hour]
Prepare trypsin-EDTA at 37 °C. Prepare ice-cold LuEC growth media and ice-cold FBS.

25. LuECs cryopreservation
    a. Wash cells with PBS 2 times.
    b. Add 2 ml trypsin-EDTA into the plate and incubate at 37 °C until cells are detached.
    c. Inactivate trypsin by adding 0.5 ml of FBS.
    d. Centrifuge the cells (1,000 g, 5 minutes) at 4 °C and discard the supernatant by aspiration.
    e. Resuspend the cells with LuEC growth media and count them.
    f. Add LuEC growth media to reach 1.1×10^6 cells/ml, followed by adding 10 % DMSO.
    g. Aliquot cells into cryogenic vials at 1 ml/vial and store them at -80 °C using a freezing container.
    h. Next day, move the vials into a liquid nitrogen tank.

    Note: Storing LuECs at -80 °C decreases cell viability and growth rate

Lung organoid culture

This section describes how to culture LOs with LuECs in a liquid-air interface (Figure 2). Typically, one lung is enough to culture more than 50 wells. The LOs are observed by a light microscope on day 4 and gradually grow (Figure 3A). Mature LOs generate alveolar, bronchiolar, and bronchioalveolar organoids (Figure 3B). The size of LOs increases until maximum size, followed by cell death, which depends on the cultured epithelial cell numbers. Therefore, the optimization of LO growth is recommended. In our hands, LOs reached the maximum size at around day 16. Thus, we cultured LOs for up to 14 days, followed by harvesting or passaging them.

Day -1 Timing: [45 minutes]
Prepare 0.2 % gelatin solution, DMEM (10 % FBS and 1 % penicillin-streptomycin), and LuEC growth media at 37 °C.

> **Note:** If LuECs were stored at -80 °C, culture them 7 days before LO culture. It takes more time to grow compared to the cells stored in a liquid nitrogen tank.
> **Note:** Typically, one plate of LuECs can culture more than 20 wells of LOs when it confluent to 70-80 %.

26. Before culturing the LuECs, prepare the gelatin-coated cell culture plate.

a. Spread the 37 °C incubated 0.2 % gelatin solution into a 10 cm culture dish. Typically, 4 ml of solution can cover one 10 cm culture dish.
b. Incubate the gelatin-covered dish for 15 minutes at 37 °C.
c. Aspirate the gelatin solution and wash the dish with PBS 2 times.
d. Dry the dish at RT for 15 minutes.

27. Culture LuECs
    a. Put cryovial containing LuECs at 37 °C until they are thawed.
    b. Add 9 ml of DMEM complete media to the cells.
    c. Centrifuge the cells (1,000 g, 5 minutes) at 4 °C and discard the supernatant by aspiration.
    d. Resuspend the cell 12 ml per dish of LuEC growth media and culture them on the gelatin-coated plate until cells are 70-90 % confluent.
    e. They are ready to use for LO culture.

Day 4. Timing: [4 hours]
Prepare ice, ice-cold ACK buffer, ice-cold MACS buffer, and ice-cold LO growth media.

28. Thaw aliquoted basement membrane at 4°C.

29. Prepare lung dissociation buffer and put it at 37°C.

30. Euthanize the mice by CO_2 inhalation, followed by cervical dislocation.

31. Excise the lung lobes and transfer them to cold Leibovitz's L-15 Medium containing 3 % penicillin-streptomycin.
    a. Before excising the lungs to remove immune cells and red blood cells (RBCs) in the lung, flush PBS to the right ventricle slowly until the lung becomes white using a syringe with a 22 G needle. Typically, 5-10 ml of PBS is used for one mouse.

       Note: The lung lobes can be stayed on ice for up to 1 hour to transfer lung lobes from the euthanized place to the tissue processing room.

32. Place tissues on 10 cm Petri dishes on ice to process.
    a. Remove the esophagus, trachea, and extra connective tissues using forceps.
    b. Mince the tissues into 1 mm^3 pieces with surgical blades or surgical scissors.
    c. Collect minced tissues in a 2 ml Eppendorf tube (or 15 ml conical tube).

33. Dissociate the lung with 1.7 ml of the lung dissociation buffer at 37 °C for 30 minutes.
    a. After 10 minutes of digestion, pipet tissues with 1 ml pipet tips until all the pieces are passed through the tip. Stop pipetting when all the pieces can pass through the tip.

       Note: The lung dissociation time can be increased up to 45 minutes.

       Note: When processing the multiple lungs, use each tube containing 1.7 ml of the lung dissociation buffer

34. Stop the digestion by adding 300 μl of FBS, followed by pipetting.

35. Perform RBC lysis using ACK lysing buffer.

    CRITICAL: Perform this procedure at 4 °C.

    a. Filter the digested cells through a 70 μm sterile strainer and collect cells in a 2 ml Eppendorf tube.
    b. Centrifuge the cells (4,500 g, 1 minute) at 4 °C and discard the supernatant by aspiration.
    c. Gently pipet the pellet with 1 ml of ACK lysing buffer and incubate it for 3 minutes at 4 °C.

    CRITICAL: Avoid vigorous pipetting.

    d. Centrifuge the cells (4,500 g, 1 minute) at 4 °C and discard the supernatant by aspiration.

36. Resuspend cells in 400 μl of MACS buffer, filter the cells through a 30 μm sterile strainer, and collect cells in a 1.5 ml Eppendorf tube.

    Note: When processing the multiple lungs, resuspend cells in 400 μl of MACS buffer per lung and aliquot them 400 μl in each tube.

37. Add 40 μl of anti-mouse CD45 magnetic beads, 40 μl of anti-mouse Ter119 magnetic beads, and 40 μl of anti-mouse CD31 magnetic beads to cell suspension and incubate for 45 minutess at 4 °C.

38. Perform the negative magnetic sorting with an LS column.

    Note: When processing the multiple lungs, use one LS column per lung.

    a. Collect cells 3 times with 5 ml of MACS buffer into a 15 ml conical tube while the column is held by the magnetic stand.
    b. Centrifuge the cells (1,000 g, 5 minutes) at 4 °C and discard the supernatant by aspiration.

39. Resuspend cells in 400 μl of MACS buffer and collect cells in a 1.5 ml Eppendorf tube.

    Note: When processing the multiple lungs, resuspend cells in 400 μl of MACS buffer per lung and aliquot them 400 μl in each tube.

40. Add 40 μl of anti-mouse CD326 (EpCAM) magnetic beads to the cell suspension and incubate for 45 minutes at 4 °C.

41. During the incubation, prepare the LuECs.
    a. Wash cells with PBS 2 times.

b. Add 2 ml trypsin-EDTA into the plate and incubate at 37 °C until cells are detached.
c. Inactivate trypsin by adding 0.5 ml of FBS.
d. Centrifuge the cells (1,000 g, 5 minutes) at 4 °C and discard the supernatant by aspiration.
e. Resuspend the cell 1 ml per dish of LO growth media, followed by counting cells.
f. Centrifuge the cells (1,000 g, 5 minutes) at 4 °C and discard the supernatant by aspiration.
g. Resuspend the cell with an ice-cold basement membrane at 1×10^6 cells/ml and store in ice.

42. Perform the positive magnetic sorting with an LS column.

Note: When processing the multiple lungs, use one LS column per lung.

a. Wash the column 3 times with 5 ml of MACS buffer while the column is held by the magnetic stand.
b. Separate the column from the MACS magnetic stand and collect cells 2 times with 5 ml of MACS buffer into a 15 ml conical tube.
c. Centrifuge the cells (1,000 g, 5 min) at 4 °C and discard the supernatant by aspiration.
d. Resuspend the cell pellet with 1 ml of LO growth media, followed by counting cells.
e. Add LO growth media for cell density of 2×10^5 cells/ml.

43. Culture LOs

a. Mix 50 μl/well of LuECs in the basement membrane and 50 μl/well of lung epithelial cells in the LO growth media and mix pipet gently.

Note: One well of LO culture contains 5×10^4 LuECs and 1×10^4 lung epithelial cells.

b. Add 100 μl/well of the LuEC and lung epithelial cells mixture into the top of the transwell with 0.4 μm pore.

CRITICAL: Pipette gently to avoid generating bubbles.

c. Incubate the transwell plate at 37 °C for 30 minutes to solidify basement membranes.
d. During the incubation, place LO culture media at 37 °C.
e. Add 500 μl of LO culture media at the bottom of the transwell.
f. Replace LO culture media every other day.
g. Small LOs can be seen under the microscope at 2-3 days of culture. LOs can be cultured for up to 14 days.

Organoid passaging

This section describes how to passage LOs. Unlike typical organoid culture using many growth factors and chemicals, LOs cultured with feeder cells lose their stemness and ability to form organoids after several passaging. We observed a severe decrease in organoid-forming efficiency from passage 4 (Figure 3D). Therefore, we recommend culturing LOs until passage 3.

Day 1. Timing: [45 minutes]
Prepare 0.2 % gelatin solution, DMEM (10 % FBS and 1 % penicillin-streptomycin), and LuEC growth media at 37 °C.

> **Note:** If LuECs were stored at -80 °C, culture them 7 days before LO culture. It takes more time to grow compared to the cells stored in a liquid nitrogen tank.
> **Note:** Typically, one plate of LuECs can culture more than 20 wells of LOs when it confluent to 70-80 %.

44. Before culturing the LuECs, prepare the gelatin-coated cell culture plate.
    a. Spread the 37 °C incubated 0.2 % gelatin solution into a 10 cm culture dish. Typically, 4 ml of solution can cover one 10 cm culture dish.
    b. Incubate the gelatin-covered dish for 15 minutes at 37 °C.
    c. Aspirate the gelatin solution and wash the dish with PBS 2 times.
    d. Dry the dish at RT for 15 minutes.

45. Culture LuECs
    a. Put cryovial containing LuECs at 37 °C until they are thawed.
    b. Add 9 ml of DMEM complete media to the cells.
    c. Centrifuge the cells (1,000 g, 5 min) at 4 °C and discard the supernatant by aspiration.
    d. Resuspend the cell 12 ml per dish of LuEC growth media and culture them on the gelatin-coated plate until cells are 70-90 % confluent.
    e. They are ready to use for LO culture.

Day 4. Timing: [1 hour]
Prepare ice, ice-cold LO culture media, ice-cold FBS, and ice-cold PBS. Prepare trypsin-EDTA at 37 °C.

46. At least 2 hours before passaging LOs, thaw the aliquoted basement membrane at 4°C.

47. Discard culture media at the bottom of the transwell and put the plate on the ice.

48. Add 500 μl of ice-cold PBS at the bottom of the transwell.

49. Add 200 μl of ice-cold PBS at the top of the transwell and gently pipet to dissolve the basement membrane.

50. Collect dissolved basement membrane containing LOs into a 1.5 ml Eppendorf tube.

51. Centrifuge the cells (6,000 g, 1 min) at 4 °C and discard the supernatant by pipetting carefully.

52. Add 1 ml of ice-cold PBS and gently pipet.

53. Centrifuge the cells (6,000 g, 1 min) at 4 °C and discard the supernatant by pipetting carefully.

54. Add 500 μl of trypsin-EDTA, gently pipet, and incubate at 37 °C for 30 minutes.

55. During the incubation, prepare LuECs.

a. Wash cells with PBS 2 times.
b. Add 2 ml trypsin-EDTA into the plate and incubate at 37 °C until cells are detached.
c. Inactivate trypsin by adding 0.5 ml of FBS.
d. Centrifuge the cells (1,000 g, 5 min) at 4 °C and discard the supernatant by aspiration.
e. Resuspend the cell 1 ml per dish of LO growth media, followed by counting cells.
f. Centrifuge the cells (1,000 g, 5 min) at 4 °C and discard the supernatant by aspiration.
g. Resuspend the cell with an ice-cold basement membrane at 1×10^6 cells/ml and store in ice.

56. Inactivate trypsin by adding 500 μl of FBS into LOs.

57. Centrifuge the cells (6,000 g, 1 min) at 4 °C and discard the supernatant by pipetting carefully.

58. Add 1 ml of LO culture media, followed by counting cells.

59. Add LO growth media for cell density of 2×10^5 cells/ml.

60. Culture LOs
    a. Mix 50 μl/well of LuECs in the basement membrane and 50 μl/well of lung epithelial cells in the LO growth media and mix pipet gently.

       Note: One well of LO culture contains 5×10^4 LuECs and 1×10^4 lung epithelial cells.

    b. Add 100 μl/well of the LuEC and lung epithelial cells mixture into the top of the transwell with 0.4 μm pore.

       CRITICAL: Pipette gently to avoid generating bubbles.

    c. Incubate the transwell plate at 37 °C for 30 minutes to solidify basement membranes.
    d. During the incubation, place LO culture media at 37 °C.
    e. Add 500 μl of LO culture media at the bottom of the transwell.
    f. Replace LO culture media every other day.

Lentivirus preparation

Lentivirus can be produced by using packaging plasmids, Lentivirus plasmid with PEI, and Opti-MEM as described elsewhere [4-6]. To increase transfection efficiency, we selected 2nd generation lentivirus packaging system for high titers [4], followed by polyethylene glycol (PEG)-based virus concentration [7]. We transfected 293T cells with packaging and lentiviral plasmids on day 1 and changed the medium on day 2. We then collected the media two times on day 3 and day 4. Collected media on day 3 is stored at 4 °C, followed by combining media collected on day 4. We used vector-expressing fluorescent proteins such as GFP and mCherry to titrate the virus.

Note: Using a vector expressing fluorescent proteins allows us to titrate the virus with a fluorescent microscope. Otherwise, a qPCR-based virus titration kit is also available for using a vector without expressing fluorescent protein.

Day 4 Timing : [30 minutes]
Prepare ice-cold PEG solution.

61. Filter the virus with a 0.45 μm syringe filter.

62. Add PEG solution to media in a 1:3 ratio (PEG solution 1: virus-containing media 3).

63. Mix the virus-PEG mixture well by vigorous shaking and incubate it at 4 °C overnight.

    Note: Incubate the virus-PEG mixture for at least 4 hours up to 24 hours.

64. Culture 5×10^4 cells/well of 293T cells into 48-well cell culture plates.

    Note: These cells will be used for virus titration. We titrated the virus using six serially diluted viruses and one negative control by duplicate (7 wells × duplicate = 14 wells).

Day 5 Timing : [1.5 hours]
Prepare ice-cold LO culture media.

65. Centrifuge the mixture (1,600 g, 60 min) at 4 °C and discard the supernatant carefully.

66. Reconstitute the virus with 1 ml of LO culture media and incubate at 4 °C for 15 minutes.

67. Centrifuge the virus (20,000 g, 3 min) at 4 °C and collect the supernatant to remove protein debris.

68. Take 35 μl of the virus and serially dilute 5-6 times.

    Note: This serial dilution of the virus will be used for virus titration. Use 10 μl of virus as 1 ×. The serially diluted virus will be 1-0.00001 ×.

69. Aliquot the rest of the virus and store them at -80 °C.

70. Add serially diluted virus into 293T cells with 10 μg/ml of polybrene.

Day 7 Timing : [1 hour]

71. Measure the virus transduction units (TU) using fluorescence microscopy.

$$Titer\ (TU) = \frac{N \times F}{V}$$

N = Number of cells transduced, F = Fraction of cells with fluorescence, V = Virus volume
(5×10^4 cells $\times$ 0.25 [25 % expressing fluorescence]) / (0.1 μl of virus (0.01 X)) = 2×10^6 TU/μl

Lentiviral transduction

This section describes how to transduce lentivirus into mouse LOs. In this protocol, we describe how to introduce lentiviruses into the LOs. This protocol will be useful to introduce genes of interest or CRISPR-Cas9-based gene editing by lentivirus. Co-expression of fluorescent proteins will be helpful to visualize infection efficiency (Figure 3E). Since the basement membrane has strong autofluorescence, a strong promoter for fluorescent proteins is required. Additionally, tag-proteins such as FLAG, HA, or MYC can be used to address infection efficiency by immunostaining using formalin-fixed paraffin embeddings (FFPEs) of LOs.

LO culture

We described the LO culture method above. The virus infection of LOs can be performed during the passaging of LOs.

LO infection

Day 1. Timing: [45 minutes]

Prepare 0.2 % gelatin solution, DMEM (10 % FBS and 1 % penicillin-streptomycin), and LuEC growth media at 37 °C.

> **Note:** If LuECs were stored at -80 °C, culture them 7 days before LO culture. It takes more time to grow compared to the cells stored in a liquid nitrogen tank.
> **Note:** Typically, one plate of LuECs can culture more than 20 wells of LOs when it confluent to 70-80 %.

72. Before culturing the LuECs, prepare the gelatin-coated cell culture plate.
    a. Spread the 37 °C incubated 0.2 % gelatin solution into a 10 cm culture dish. Typically, 4 ml of solution can cover one 10 cm culture dish.
    b. Incubate the gelatin-covered dish for 15 minutes at 37 °C.
    c. Aspirate the gelatin solution and wash the dish with PBS 2 times.
    d. Dry the dish at RT for 15 minutes.

73. Culture LuECs
    a. Put cryovial containing LuECs at 37 °C until they are thawed.
    b. Add 9 ml of DMEM complete media to the cells.
    c. Centrifuge the cells (1,000 g, 5 minutes) at 4 °C and discard the supernatant by aspiration.
    d. Resuspend the cell 12 ml per dish of LuEC growth media and culture them on the gelatin-coated plate until cells are 70-90 % confluent.
    e. They are ready to use for LO culture.

Day 4. Timing : [6 hours]

74. Thaw the stored virus and basement membrane at 4 °C.

75. Harvest cells from LOs and count the single cells as described in the Organoid passaging section (Organoid passaging section, Steps 3-11, 12-14).

76. Prepare virus-containing medium with polybrene (7 μg/ml in 500 μl) in LO growth media.

77. Add polybrene-containing medium to the cell pellet and transfer the cell suspension.

78. Centrifuge the tube for 1 hr with 600 g at ambient temperature (set the temperature at 32°C if possible).

    Note: Centrifuge at 20°C-25°C is also applicable. We confirmed that the infection efficiency is comparable to that of the cells infected at 32°C.

79. Incubate the tube in the 37°C incubator for 4 hours.

80. Before 30 minutes of incubation, prepare LuECs
    a. Wash cells with PBS 2 times.
    b. Add 2 ml trypsin-EDTA into the plate and incubate at 37 °C until cells are detached.
    c. Inactivate trypsin by adding 0.5 ml of FBS.
    d. Centrifuge the cells (1,000 g, 5 minutes) at 4 °C and discard the supernatant by aspiration.
    e. Resuspend the cell 1 ml per dish of LO growth media, followed by counting cells.
    f. Centrifuge the cells (1,000 g, 5 minutes) at 4 °C and discard the supernatant by aspiration.
    g. Resuspend the cell with an ice-cold basement membrane at 1×10^6 cells/ml and store in ice.

81. Centrifuge the cells (6,000 g, 1 minute) at 4 °C and discard the supernatant by pipetting carefully.

    Note: The fluid waste containing adenovirus should be discarded properly. Bleaching (with 10% final bleach volume) for 20 minutes is recommended.

82. Wash the cells with 1 ml of LO growth media, centrifuge the cells (6,000 g, 1 minute) at 4 °C, and discard the supernatant by pipetting carefully.

83. Add 1 ml of LO culture media, followed by counting cells.

84. Add LO growth media for a cell density of 2×10^5 cells/ml.

    Note: The antibiotics selection during lung organoid culture is available. If you put antibiotics into the LOs, lung epithelial cell numbers can be increased up to 5X depending on the infection efficiency.

85. Culture LOs
    a. Mix 50 μl/well of LuECs in the basement membrane and 50 μl/well of lung epithelial cells in the LO growth media and mix pipet gently.

       Note: One well of LO culture contains 5×10^4 LuECs and 1×10^4 lung epithelial cells.

    b. Add 100 μl/well of the LuEC and lung epithelial cells mixture into the top of the transwell with 0.4 μm pore.

       CRITICAL: Pipette gently to avoid generating bubbles.

    c. Incubate the transwell plate at 37 °C for 30 minutes to solidify basement membranes.
    d. During the incubation, place LO culture media at 37 °C.
    e. Add 500 μl of LO culture media at the bottom of the transwell.
    f. Replace LO culture media every other day.

Expected outcomes

This protocol aims to establish a standard method for culturing mouse LOs mimicking regeneration. Although many culture methods for lung organoids have been used, they used various growth factors and chemicals, or cultured specific-isolating cells aimed at particular lung structures [8]. Here we used broad lung epithelial cells with LuECs, using minimized external stimulus to culture lung organoids mimicking regeneration. We observed that LOs generated all lung structures (alveolar, bronchiolar, and bronchioalveolar).

Limitations

This protocol mimics regeneration using LuECs as feeder cells. Not only endothelial cells but also various other cell types such as immune and mesenchymal cells [9]. Therefore, further optimization of LO is required using various cells including immune and mesenchymal cells, to precisely recapitulate regeneration.

This protocol is optimized for C57BL/6 mouse-derived LO experiments. All the timelines and scales have been verified in C57BL/6 but not in other species or strains. Therefore, further testing is required to apply this protocol to different strains or species.

Troubleshooting

Problem 1:

LuECs do not attach to the plate well. (Related to steps 1-25).

Potential solution:

Gelatin coating is important to culture LuECs for attaching cells on culture plates. Perform gelatin coating at least for 15 minutes at 37 °C.

Problem 2:

LOs are not growing well. (Related to steps 26-43).

Potential solution:

The quality of lung epithelial cells is important to culture LOs. Avoid vigorous pipetting during all the steps of lung digestion, RBC lysis, cell sorting, and cell seeding steps. After lung digestion, we recommend performing all steps at 4 °C except the magnetic sorting steps.

Problem 3:

The basement membrane of LOs looks dark and cloudy. (Related to steps 26-43).

Potential solution:

Possible contamination of one of lung epithelial cells or lung endothelial cells. LOs will stop growing and start apoptosis. After collecting the tissues, proceed with all the steps under the clean bench. The Leibovitz's L-15 Medium is easy to contaminate. Throw out the old Medium and use the new one. When any white particles are seen in Leibovitz's L-15 Medium, it should not be used.

Problem 4:

LO size and the growth rate vary in each LO culture or passaging. (Related to steps 26-43).

Potential solution:

The number of lung epithelial cells affects LO growth. After digesting lungs or organoids, make sure the cells are digested or are trypsinized well, into the single-cell suspension. Accurate cell counting is required for consistent LO culture. With various cell numbers, LO culture needs to be optimized.

Problem 5:

There are too few lentivirus-infected cells. (Related to steps 65-80).

Potential solution:

Optimization of infection is recommended. The virus titers influence cell viability and infection efficiency. Too high a virus titer increases cell death of LOs and too low a virus titer shows a low

infection rate. After concentrating the virus, calculate the MOI using 293T cells, and optimization of MOIs for LO infection is recommended.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jae-Il Park (jaeil@mdanderson.org), or technical contact, Bongjun Kim (bkim6@mdanderson.org).

Material availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets or codes.

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Author contributions

B.K. performed the experiments. B.K. and J.-I.P. analyzed the data. B.K. wrote the manuscript. J.-I.P. reviewed and edited the manuscript.

Declaration of interests

The authors declare no competing interests.

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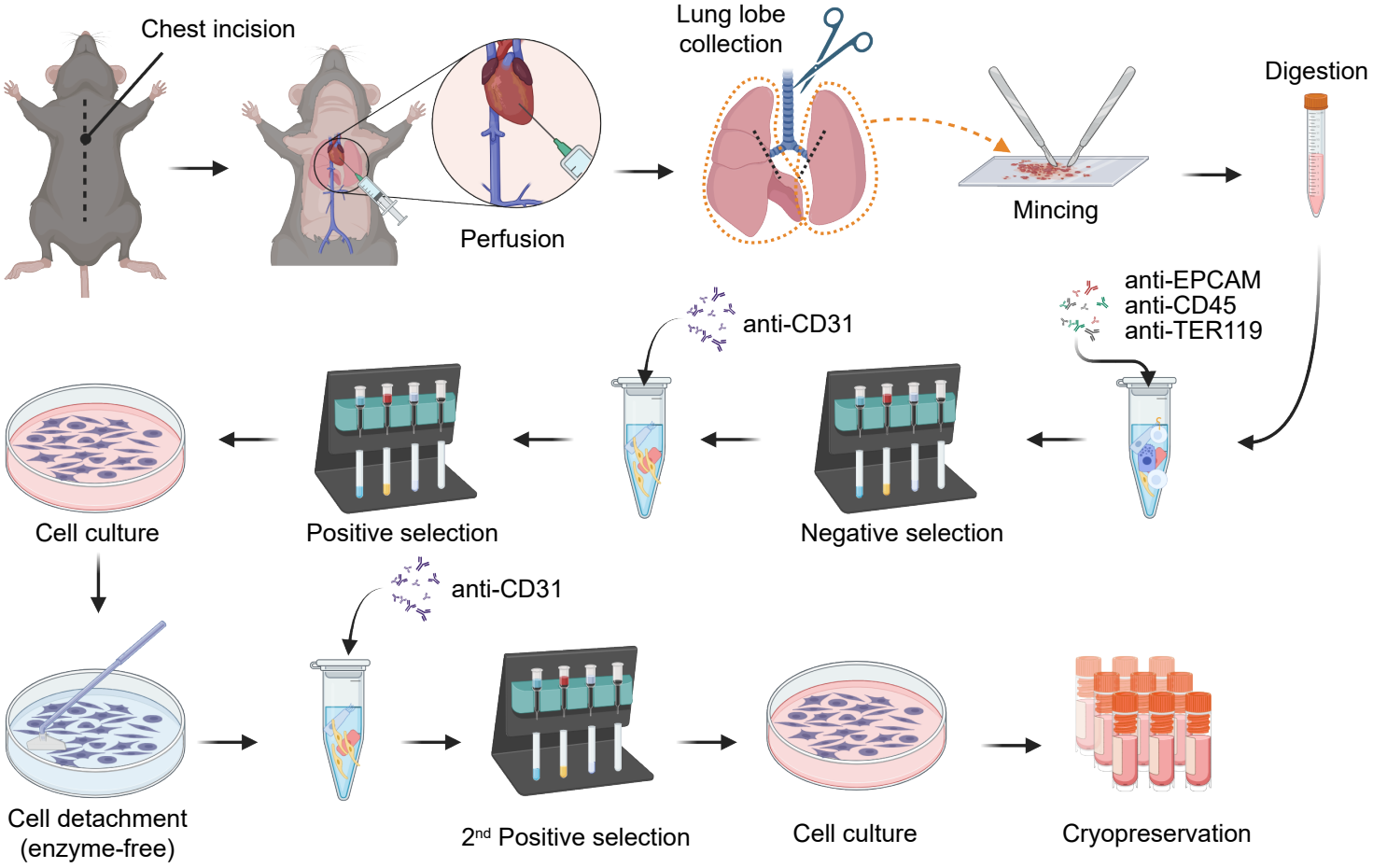
Figure legends

Figure 1: Schematic process of murine lung endothelial cell culture. After performing the perfusion, mouse lung tissues are collected and digested. LuECs are sorted out by serial negative and positive selection, and the sorted cells are seeded on the gelatin-coated culture plates. Cultured cells are sored another round of positive selection to improve the cell purity followed by cell expansion and cryopreservation.

Figure 2: Schematic process of murine lung organoid culture. After performing the perfusion, mouse lung tissues are collected and digested. Lung epithelial cells are sorted out by serial negative and positive selection, and the sorted cells are seeded into a liquid-airway interface after combining with the cultured LuECs and basement membrane.

Figure 3: Lung organoid culture and viral infection process. (A) Representative z-stacked light microscope images of LO culture at the indicated time point. Scale bar, 500 μm; d: day. (B, C) Serial sections of LOs were applied for immunostaining. (B) Representative images of LOs fluorescently immunostained for alveolar cell markers (HOPX [alveolar type I cell] and SPC [alveolar type II cell]). Scale bar, 200 μm. (C) Representative images of LOs fluorescently immunostained for bronchiolar cell markers (SCGB1A1 [club cell] and Ac-TUB [ciliated cell]). Scale bar, 200 μm. (D) Representative z-stacked light microscope images of LO culture on day 14 of indicated passages. Scale bar, 500 μm; p: the number of passages. (E) LOs were infected with the lentivirus containing pLenti-CMV-MSC-RFP-SV-Puro vector during the passaging. Representative light microscope and fluorescence microscope images of LOs on day 14. Scale bar, 200 μm.

Figure1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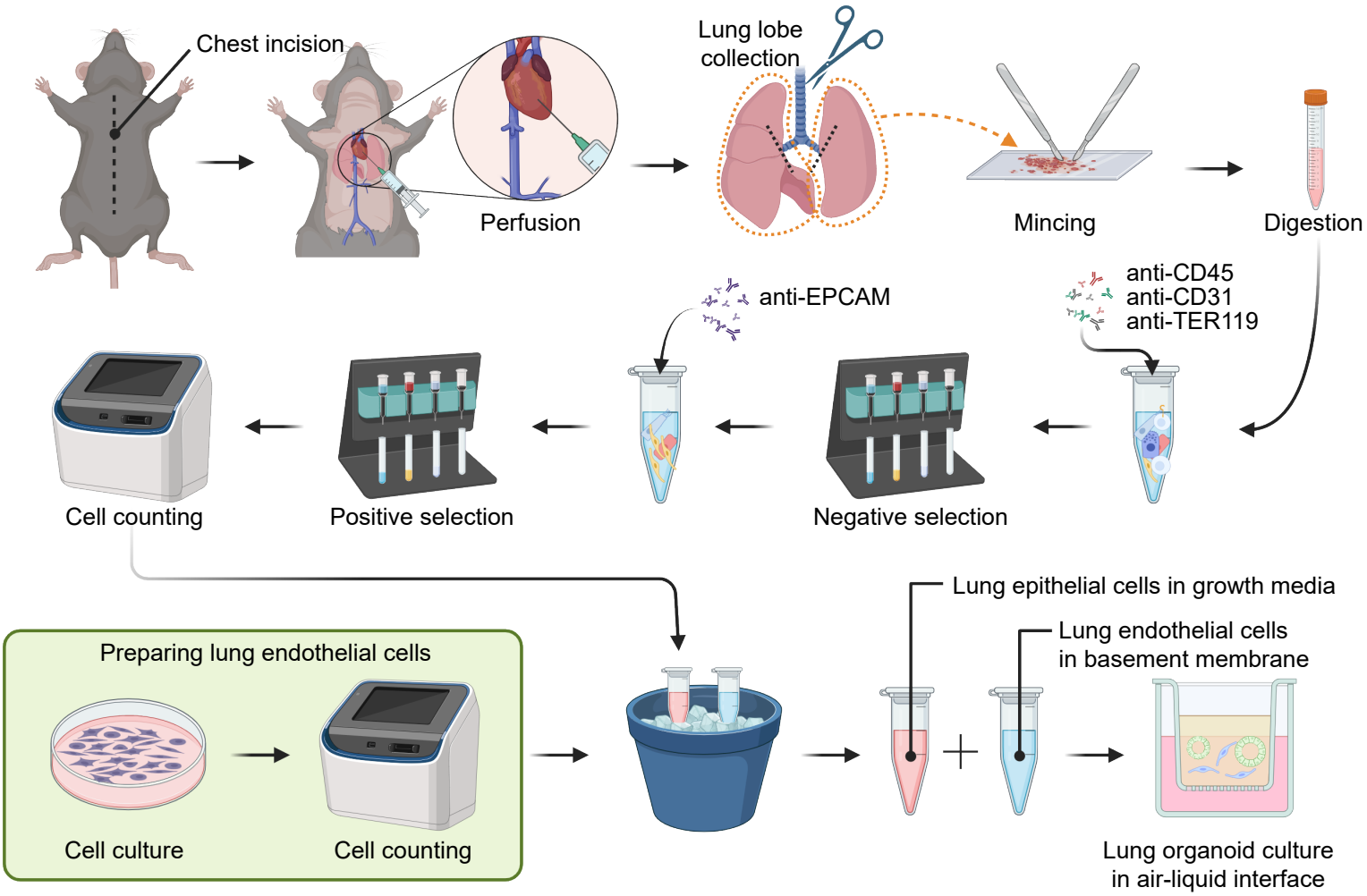
Perfusion
Lung lobe collection
Mincing
Digestion
anti-EPCAM
anti-CD45
anti-TER119
anti-CD31
Negative selection
Positive selection
Cell culture
Cell detachment (enzyme-free)
anti-CD31
2nd Positive selection
Cell culture
Cryopreservation

Figure2


Chest incision
Perfusion
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Digestion
anti-CD45
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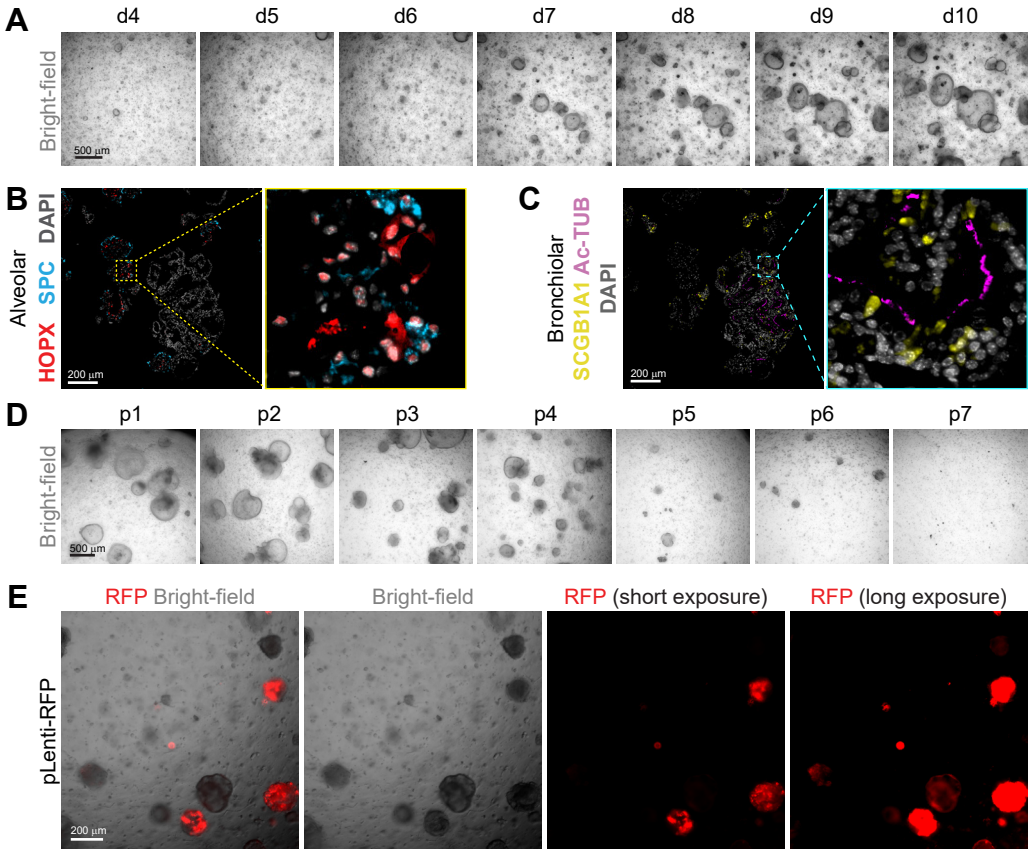
anti-TER119
anti-EPCAM
Cell counting
Positive selection
Negative selection
Preparing lung endothelial cells
Cell culture
Cell counting
Lung epithelial cells in growth media
Lung endothelial cells in basement membrane
Lung organoid culture in air-liquid interface

Figure3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d4
d5
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Bright-field
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B
Alveolar
DAPI
SPC
HOPX
200 μm
C
Bronchiolar
SCGB1A1 Ac-TUB
DAPI
200 μm
D
p1
p2
p3
p4
p5
p6
p7
Bright-field
500 μm
E
RFP Bright-field
Bright-field
RFP (short exposure)
RFP (long exposure)
pLenti-RFP
200 μm