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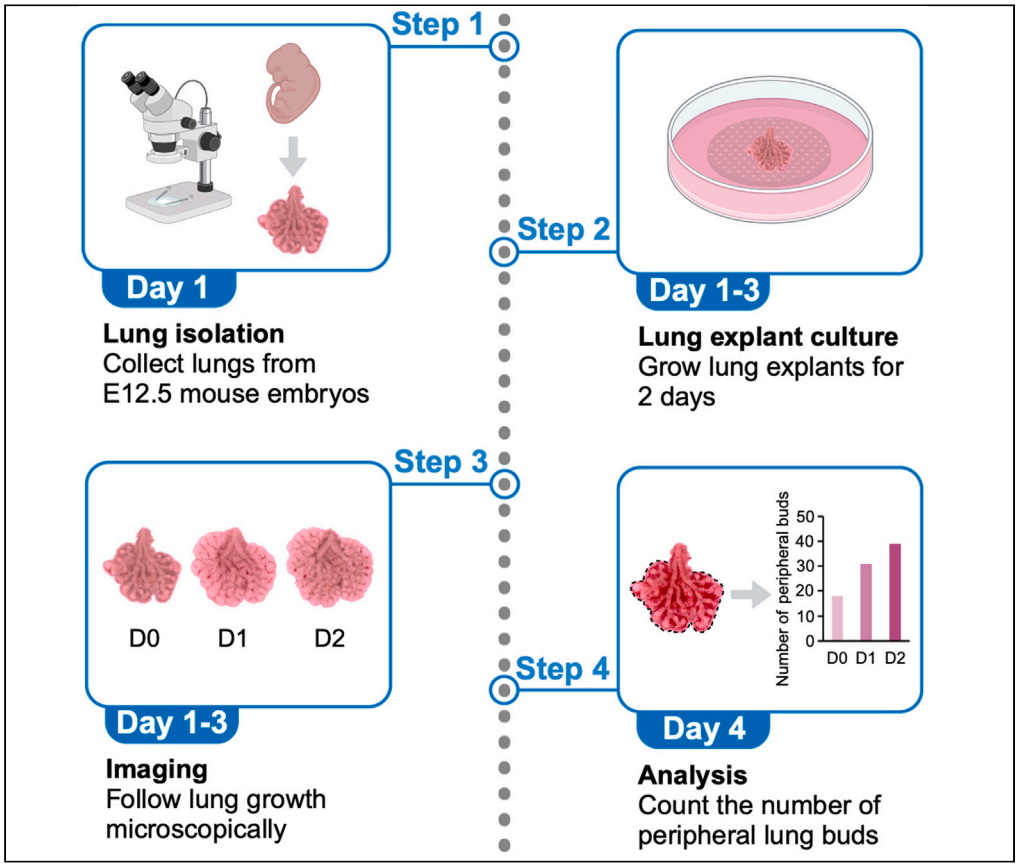

Mouse lung branching morphogenesis creates epithelial tree structures required for respiration. Here, we present a protocol for studying mouse lung developmental branching using lung explant cultures. We describe steps for isolating lungs with a video at embryonic day 12.5 (E12.5) and culturing as an explant for 2 days. We also detail procedures for microscopic imaging on days 0–2 and analysis of peripheral lung buds. This technique has the potential to investigate lung development in various conditions.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for studying mouse lung developmental branching using explant cultures

Steps for isolating embryonic lungs at E12.5 and culturing as an explant for 2 days

Guidance on analyzing the number of peripheral lung buds by imaging lungs on days 0–2

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Protocol to study early lung developmental branching in mouse embryos using explant culture

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SUMMARY

Mouse lung branching morphogenesis creates epithelial tree structures required for respiration. Here, we present a protocol for studying mouse lung developmental branching using lung explant cultures. We describe steps for isolating lungs with a video at embryonic day 12.5 (E12.5) and culturing as an explant for 2 days. We also detail procedures for microscopic imaging on days 0–2 and analysis of peripheral lung buds. This technique has the potential to investigate lung development in various conditions.

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

BEFORE YOU BEGIN

Mouse lung development initiates at embryonic day 9.5 (E9.5). During the pseudoglandular stage (E10.5–E16.5), a highly regulated and repetitive combination of branching and elongation leads to a complex airway network formation, known as branching morphogenesis. This generates epithelial trees that facilitate gas exchange, filtering, and secretion processes.[2] Because *ex vivo* cultured lungs closely resemble the *in vivo* lungs in structure and function, the explant culture model enables studying various processes crucial to lung development, function, and pathologies.[3,4]

Here, the protocol describes an approach for studying mouse lung developmental branching using lung explant cultures. The process involves the harvesting of the mouse embryonic lung by dissection at stage E12.5 using a stereo microscope, careful placement on membranes in a correct orientation, and culture as an explant for two days. During the culture, samples are microscopically imaged on days 0, 1, and 2. Finally, the number of peripheral lung buds is analyzed.

Institutional permissions

All animal experiments were formally reviewed and approved by the Ethical Committee for Animal Experimentation in Finland.

> *Note:* The experiments on animals must be performed in accordance with relevant institutional and national guidelines and regulations.

Timed mating of the mice

Timing: 14–17 days (if the vaginal plug formation is followed for 4 days)

1. Generate pregnant female C57BL/6N mice for collection of lungs at embryonic day 12.5 (E12.5).
   a. For each mating, put one male and one virgin female C57BL/6N mouse (approximately 2–6 months old) together to the same cage on the afternoon.
   b. Check for vaginal plugs in the following mornings (by 9 a.m.) for the next 4 days.
      i. Examine the vaginal opening for a whitish plug by lifting the female by the base of the tail.
   c. A pregnant female is identified based on the presence of a vaginal plug. The day of the vaginal plug appearance is considered embryonic day 0.5 (E0.5).
   d. When the vaginal plug is present, separate the female mouse from the male mouse.
   e. Wait for an additional 12 days until the embryos reach a gestational age of E12.5.

Note: Some female mice may not get pregnant even with a vaginal plug; therefore, using at least 2–3 mice pairs is recommended for matings.

Preparation of reagents and buffers

Timing: 0.5 h

2. Prepare the lung explant culture medium.
3. Store the culture medium and 1× PBS solution at +4°C. During the dissection, keep these solutions on ice until needed.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
1× PBS solution, pH 7.4	Gibco, Thermo Fisher Scientific	Cat#10010-015
DMEM/F12 medium (1:1)	Gibco, Thermo Fisher Scientific	Cat#21331-020
ES screened fetal bovine serum, heat-inactivated	Biowest	Cat#S181B
Penicillin-Streptomycin (10,000 U/mL)	Gibco, Thermo Fisher Scientific	Cat#15140122
Experimental models: Organisms/strains		
Mouse: C57BL/6N, wild-type, 2–6 months old, male and female	Charles River Laboratories, Wilmington, MA	RRID:MGI:2159965
Software and algorithms		
ImageJ/Fiji	Schindelin et al.[5]	RRID:SCR_002285; http://fiji.sc
Other		
M60 stereo microscope	Leica	N/A
Axio Zoom.V16 stereo microscope with AxioCam 105 color camera and 1.0× PlanApo Z objective	Zeiss	N/A
O_2/CO_2 incubator	Sanyo	Cat#MCO-18M
Iris scissors	World Precision Instruments	Cat#501758-G
Forceps 115 mm, sharp/curved	LLG	Cat#9171124
Dumont #55 forceps	World Precision Instruments	Cat#14099
30G needle	BD Microlance, BD	Cat#304000
1 mL syringe	BD Plastipak, BD	Cat#300013
3 mL graduated Pasteur pipette	Pastette	Cat#LW4111
200 µL Optifit pipette tip	Sartorius	Cat#790200
50 mL Falcon tube	Corning	Cat#352070
⌀ 10 cm polystyrene plastic dish	Corning	Cat#430591
Polystyrene 12-well plate	Corning	Cat#3737
Polystyrene 6-well plate	Corning	Cat#3736
8.0 µm pore size Whatman Nuclepore track-etched membranes	Merck	Cat#WHA10417506

MATERIALS AND EQUIPMENT

Lung explant culture medium		
Reagent	Final concentration	Amount
DMEM/F12 medium	98.5%	98.5 mL
ES screened fetal bovine serum, heat-inactivated	0.5%	0.5 mL
Penicillin-Streptomycin	200 U/mL	1 mL
Total	N/A	100 mL
Storage: Keep at 4°C for up to two months.		

⚠ CRITICAL: To avoid microbial contamination, prepare the medium in a laminar flow hood.

The lung isolation from the embryos should be performed in a clean environment, ideally in a clean dissecting room with minimal air movement and a disinfected working area.

The lung explants are grown on a 6-well plate including 1.5 mL culture medium and an 8.0 μm pore size Whatman Nuclepore Track-Etched membrane.

A humidified 37°C incubator with a carbon dioxide concentration of 5% is required for the lung explant culture.

STEP-BY-STEP METHOD DETAILS

Isolation of the mouse embryonic lung

Timing: 1–3 h (depending on how many lungs are collected)

Lungs are collected from E12.5 mouse embryos for explant culture.

1. Euthanize a pregnant female mouse with CO_2 and ensure death by cervical dislocation.
2. Remove the uterus from the animal using scissors and 115 mm forceps.
   a. Place the mouse on its back and spray 70% ethanol on the abdominal part to prevent contamination.
   b. Carefully make a vertical incision to the abdominal area and open the abdominal wall and the peritoneum.
   c. Remove the whole uterus from the mouse.
3. Immediately, place the uterus in a 50 mL falcon tube filled with cold PBS.
4. Keep the uterus on ice for a minimum of 30 min to ensure the euthanasia of the embryos.
5. Place the uterus on a ø 10 cm polystyrene plastic dish filled with cold PBS (Figure 2A) and detach embryos from the uterus under a stereo microscope (Figure 1A) using fine Dumont #55 forceps (Figure 1B).

   Note: A pregnant mouse may have up to 12 embryos.

6. Place the dissected embryos on a 12-well plate containing cold PBS (Figure 2B).
   a. Transfer the embryos: e.g., with a 3 mL plastic Pasteur pipette with a tip cut larger to fit the embryo (Figure 1C).
   b. Keep the embryos on ice until their lungs are harvested.
7. Under a stereo microscope, harvest the embryonic lungs one by one in 30 mL of cold PBS on the lid of a ø 10 cm polystyrene plastic dish with the fine forceps (Figures 2C–2J and Video S1).
   a. Transfer the embryo to the dish (Figure 2C).

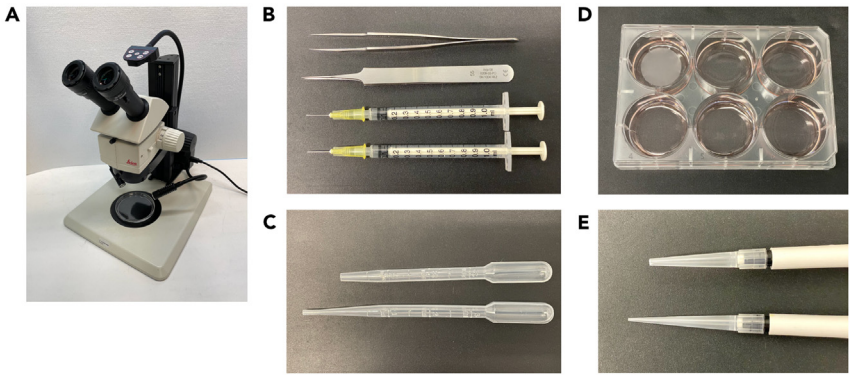

Figure 1. Useful equipment for the protocol

(A) The dissection is performed under a stereo microscope such as Leica M60. The embryos can be dissected on a ø 10 cm polystyrene plastic dish lid filled with cold PBS.

(B) Fine forceps (such as Dumont #55 forceps) are needed for precise lung isolation. Needles might be useful for cleaning the lungs or placing the lung lobes in the correct orientation (e.g., 30G needles with 1 mL syringes).

(C) The embryos can be transferred e.g., with a 3 mL plastic Pasteur pipette with a tip cut larger to fit the embryo.

(D) The lung explants are grown on a 6-well plate including 1.5 mL culture medium and an 8.0 μm pore size Whatman Nuclepore Track-Etched membrane. Here, the membrane is placed on the first well.

(E) The embryonic lungs can be transferred with a 200 μL pipette with a tip cut larger.

b. Keep the embryo on its left side and take a good holding position of the embryo with the forceps.

Note: The orientation of the dissection can be reversed if preferred.

c. First, remove the tail and the limbs from the right side of the embryo (Figure 2D).

Note: If genetically modified mice are used, collect the tail for genotyping.

d. Carefully remove skin and organs around the lungs.

Note: The lungs are located behind the heart. The lower part of the lungs is close to the upper part of the liver, close to the spine.

i. Remove skin and tissue from the right side of the embryo where the right front limb used to be (Figure 2E).
ii. Detach the liver and connecting tissue below the heart (Figure 2F). Next, remove the heart (Figure 2G).
iii. Just below the lungs, cut any tissue holding them in place, such as the esophagus (Figure 2H).
iv. Detach the lungs by pulling cautiously from the upper part of the trachea (Figures 2I and 2J).

8. Clean the lungs with the fine forceps or needles under the stereo microscope (Figures 1B and 2K and Video S1).
   a. Pay attention to delicately removing the part of the esophagus located vertically behind the lungs (Figure 2L).

⚠ CRITICAL: Avoid microbial contamination by using clean equipment and surroundings: e.g., by disinfecting all the scissors and forceps before use.

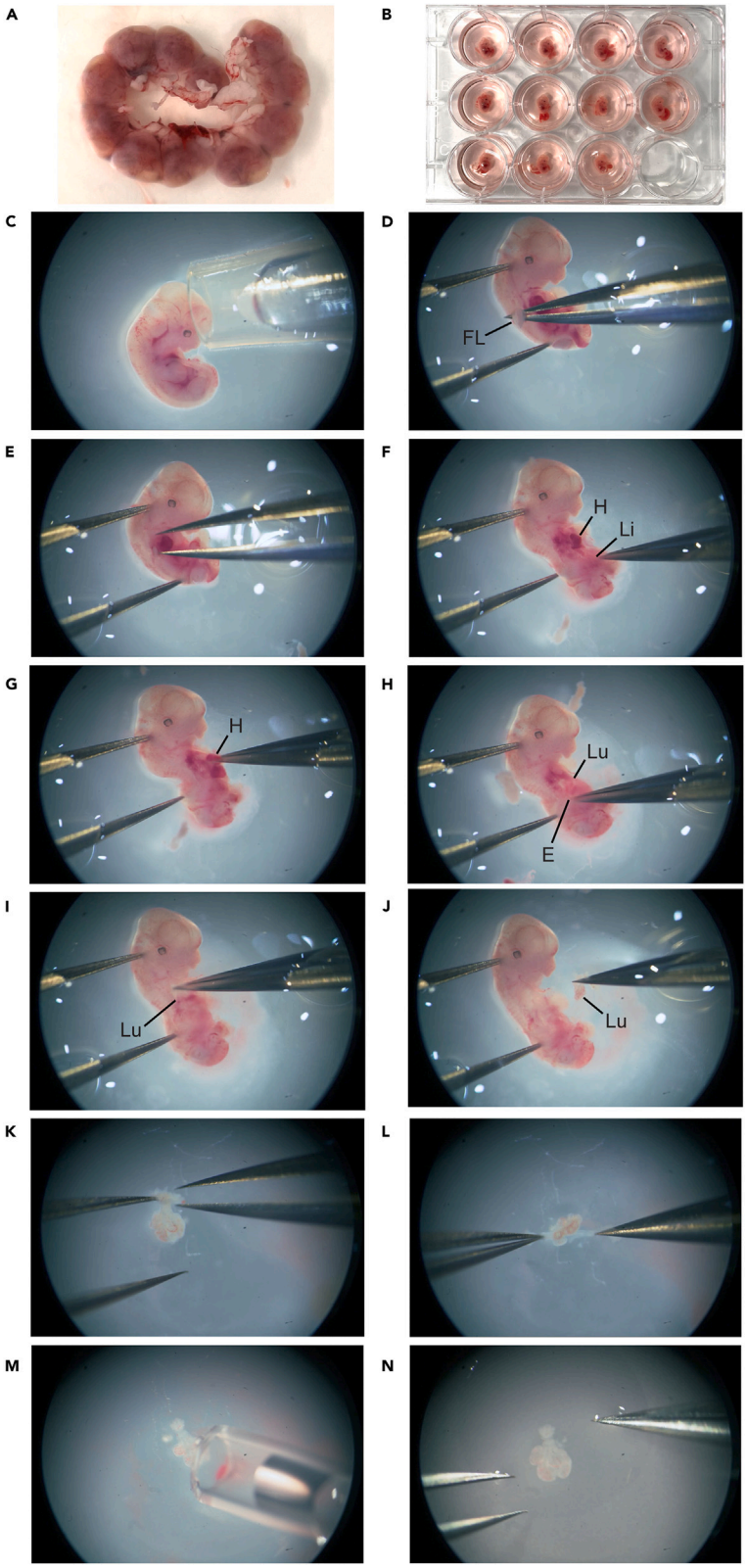
A
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Figure 2. Steps for lung isolation

The embryonic lungs at E12.5 are harvested under a stereo microscope. (C)–(N) are still images captured from Video S1.

(A and B) An example of the uterus (A) and dissected embryos (B) at E12.5. A pregnant mouse may have up to 12 embryos. Here, the litter size is 11.

(C) The embryo is transferred to the dish containing cold PBS.

(D) The embryo is kept on its left side (the orientation of the dissection can be reversed if preferred), and the tail and the limbs are removed from the right side of the embryo.

(E–G) Skin and organs are carefully removed around the lungs. First, skin and tissue are taken away from the right side of the embryo where the right front limb used to be (E). Then, the liver and connecting tissue below the heart are detached (F). Next, the heart is removed (G).

(H–J) Any tissue holding the lungs in place, such as the esophagus, is cut just below the lungs (H). Finally, the lungs are detached by pulling cautiously from the upper part of the trachea (I–J).

(K and L) The lungs are cleaned with fine forceps or needles under the stereo microscope (K). The part of the esophagus located vertically behind the lungs is carefully removed (L).

(M) The lungs are transferred on the membrane with a 200 μL pipette with a tip cut larger.

(N) The lung lobes are carefully placed in a correct orientation on the membrane: the four right lobes should be on the left and the left lobe on the right on the membrane. FL, front limb; Li, liver; H, heart; Lu, lungs; E, esophagus. See also Video S1.

⚠ CRITICAL: The embryonic lungs fall apart easily. Therefore, very careful handling of the lungs is needed.

Lung explant culture

Timing: 3 days

The lung explants are placed on a membrane on day 1 and cultured for 2 days.

9. Add 1.5 mL culture medium to the wells of a 6-well plate in a laminar flow hood.
10. Place an 8.0 μm pore size Whatman Nuclepore Track-Etched membrane carefully on top of the medium in a laminar flow hood (first well in Figure 1D).
    a. Make sure the matte side is up and the shiny side is facing the medium.
11. Transfer the lung explant on the membrane with a 200 μL pipette with a tip cut larger (Figures 1E and 2M and Video S1).

    Note: The lungs can easily get stuck to the pipette tip, so we recommend cutting the tip larger.

12. Place the lung lobes in a correct orientation on the membrane: the four right lobes should be on the left and the left lobe on the right (Figures 2N and 3A).

    Note: Make sure that the lung lobes are intact and not twisted or on top of each other.

13. Culture the lung explants at 37°C in a humidified atmosphere with 5% CO_2 for two days.

Imaging

Timing: 3 days

The lung growth is followed microscopically on days 0–2.

14. Image the samples on time points D0 (0 h), D1 (24 h), and D2 (48 h) after the start of the culture (Figure 3A).
    a. The microscope setup can be following or similar: AxioZoom.V16 stereo microscope (Zeiss) with AxioCam 105 Color camera and 1.0× PlanApo Z objective.

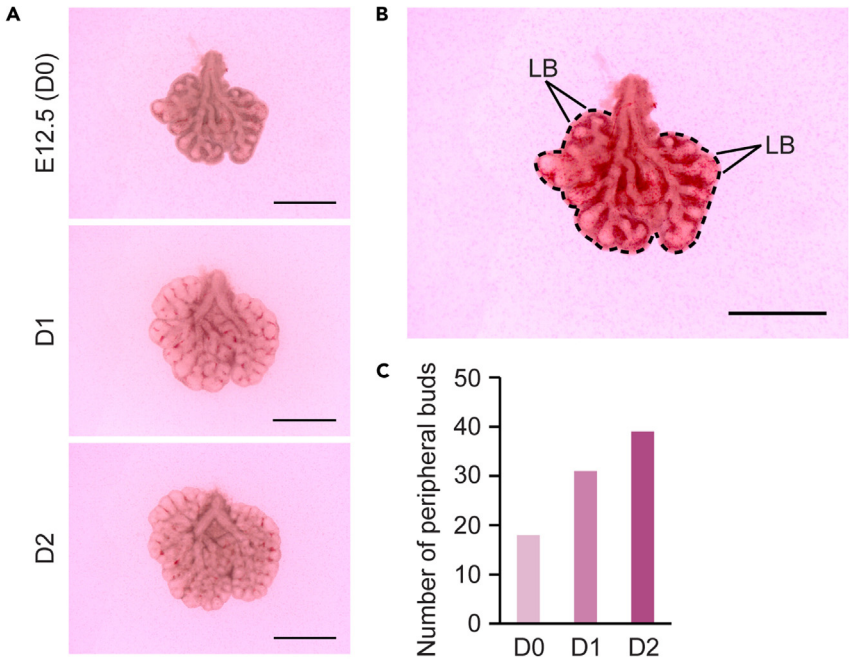

Figure 3. Lung explant culture, imaging, and analysis

(A) A lung explant from the E12.5 embryo is cultured for two days and imaged on days 0, 1, and 2 (D0–D2), such as with the following microscope: AxioZoom.V16 stereo microscope (Zeiss) with AxioCam 105 Color camera and 1.0× PlanApo Z objective. Scale bars: 1,000 μm.

(B) An example of peripheral lung bud analysis at D0. To count the peripheral buds by eye, contrast has been added to the image using ImageJ/Fiji software. In the image, the peripheral lung buds located on the outer parts of the lung are shown with a dashed line. Here, the number of peripheral buds is 18. Scale bar: 1,000 μm. LB, lung bud; each line points to one bud.

(C) The number of peripheral buds of the lungs explant is shown (D0, 18; D1, 31; D2, 39).

Note: After two days, the analysis of the lung buds gets challenging.

⚠ CRITICAL: Aim to take images at the same position each day so that the lung growth is easy to analyze.

EXPECTED OUTCOMES

The lobes of the harvested lungs at E12.5 should be intact and in a correct orientation (Figure 3A). A successful lung explant culture should show an increase in the peripheral lung buds when cultured for 24 h (D1) and 48 h (D2) compared to the starting point (D0), as seen in Figures 3A and 3C. Additionally, the cultures should show no signs of microbial contamination.

QUANTIFICATION AND STATISTICAL ANALYSIS

Count the number of peripheral lung buds.

Next, the number of peripheral lung buds on the outer parts of lung explants is counted by eye from the figures obtained (Figure 3A). The peripheral buds lie on the terminal tips of the tree-like structure of the lung. For better visualization of the buds, add light and contrast to the image with, e.g., ImageJ/Fiji software,[5] and zoom in to the picture. The peripheral buds located on the outer parts of the lung at D0 are shown with a dashed line in Figure 3B. The progression of branching morphogenesis from D0 to D2 is compared in the lung explants (Figure 3C). The comparison between the lung explants can be based on, e.g., different genotypes, as shown in Talvi et al.[1]

Note: Normalization can be done to remove the size variation between different litters from different pregnant dams. Normalization method for each litter: the number of peripheral buds in a lung at D0, D1, or D2 is divided by the biggest number of peripheral buds of the litter at D0.

LIMITATIONS

After day 2, the analysis of peripheral lung buds becomes difficult as the buds continue to divide into smaller compartments. Therefore, we do not recommend continuing the experiment after day 2. For similar reasons, we do not recommend experimenting with lungs from older than E12.5 mouse embryos. However, at an earlier stage E11.5, the experiment might be possible to execute, albeit the lungs are more fragile, and more careful handling is needed.

TROUBLESHOOTING

Problem 1

Microbial contamination in the lung explant cultures.

Potential solution

Avoid microbial contamination by cleaning the equipment and surroundings.

Problem 2

Peripheral lung bud analysis of the lung explants is challenging due to extra material from surrounding tissues (related to Step 8).

Potential solution

Aim to clean the lungs as much as possible during the isolation without breaking the lung structure. Fine forceps or needles are needed for the lung cleaning process.

Problem 3

The lungs are not in the correct orientation (related to Step 12).

Potential solution

Aim to place the lung lobes in a correct orientation: the four right lobes should be on the left and the left lobe on the right on the membrane. Additionally, make sure that the lung lobes are not twisted or on top of each other (see Figure 3A). Fine needles might be useful for placing the lung lobes in the correct orientation; however, caution is required not to break the lungs.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jyrki Heino (jyrki.heino@utu.fi).

Technical contact

Technical questions about executing this protocol should be directed to and will be answered by the technical contact, Salli Talvi (sakakei@utu.fi).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103198.

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AUTHOR CONTRIBUTIONS

S.T.: conceptualization, data curation, formal analysis, funding acquisition, investigation, validation, visualization, writing – original draft, and writing – review and editing. J.J.: conceptualization, data curation, formal analysis, funding acquisition, investigation, validation, visualization, writing – original draft, and writing – review and editing. P.R.: conceptualization, funding acquisition, resources, writing – original draft, and writing – review and editing. J.H.: conceptualization, funding acquisition, project administration, supervision, resources, writing – original draft, and writing – review and editing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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