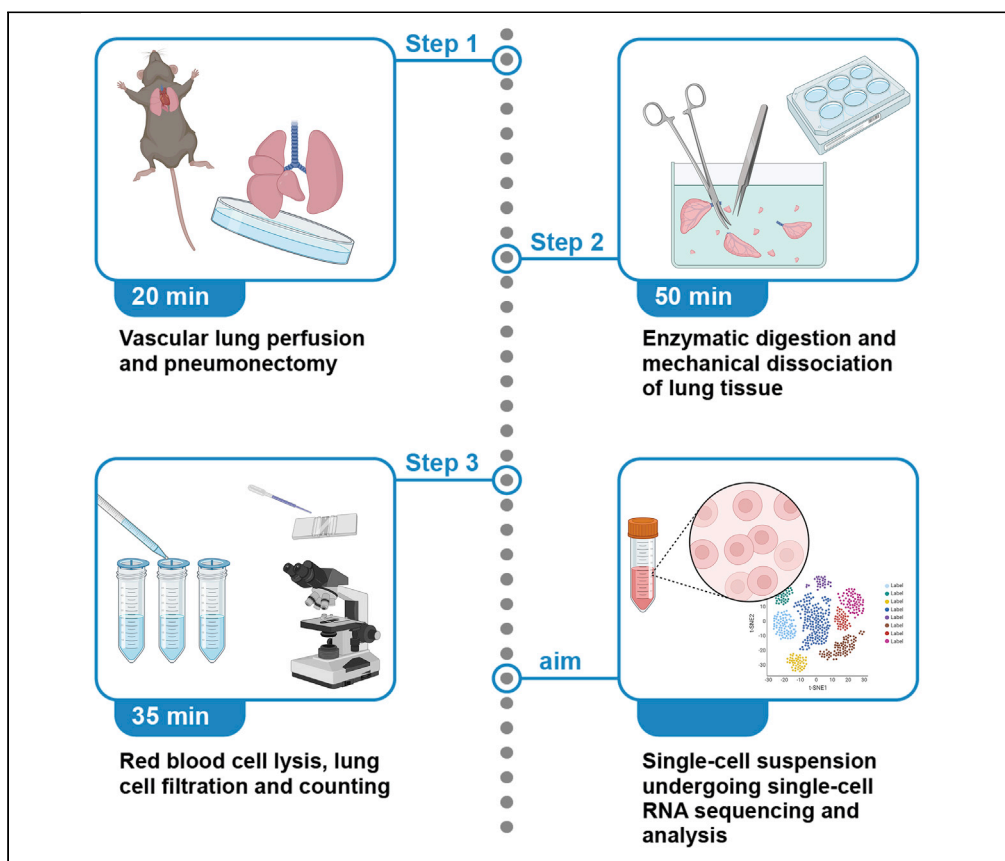# STAR Protocols

Protocol

Protocol to dissociate healthy and infected murine- and hamster-derived lung tissue for single-cell transcriptome analysis

Peter Pennitz,
Cengiz Goekeri,
Jakob Trimpert, ...,
Michael D.
Mühlebach, Martin
Witzenrath,
Geraldine Nouailles

peter.pennitz@charite.de
(P.P.)
cengiz.goekeri@charite.
de (C.G.)
geraldine.nouailles@
charite.de (G.N.)

Highlights

Protocol to isolate murine and hamster lungs with and without prior perfusion

Enzymatic digestion and dissociation to obtain highly viable single-cell suspension

Protocol suitable for infected animals in biosafety laboratory conditions 1–3

In infectious disease research, single-cell RNA sequencing allows dissection of host-pathogen interactions. As a prerequisite, we provide a protocol to transform solid and complex organs such as lungs into representative diverse, viable single-cell suspensions. Our protocol describes performance of vascular perfusion, pneumonectomy, enzymatic digestion, and mechanical dissociation of lung tissue, as well as red blood cell lysis and counting of isolated cells. A challenge remains, however, to further increase the proportion of pulmonary endothelial cells without compromising on viability.

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

Protocol

Protocol to dissociate healthy and infected murine- and hamster-derived lung tissue for single-cell transcriptome analysis

Peter Pennitz,[1,8,9,*] Cengiz Goekeri,[1,2,8,9,*] Jakob Trimpert,[3] Emanuel Wyler,[4] Aileen Ebenig,[5] Chantal Weissfuss,[1] Michael D. Mühlebach,[5,6] Martin Witzenrath,[1,7] and Geraldine Nouailles[1,10,*]

[1]Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Department of Infectious Diseases and Respiratory Medicine, Berlin, Germany

[2]Cyprus International University, Faculty of Medicine, Nicosia, Cyprus

[3]Freie Universität Berlin, Institute of Virology, Berlin, Germany

[4]Max Delbrück Center for Molecular Medicine in the Helmholtz Association (MDC), Berlin Institute for Medical Systems Biology (BIMSB), Berlin, Germany

[5]Division of Veterinary Medicine, Paul-Ehrlich-Institut, Langen, Germany

[6]German Center for Infection Research (DZIF), Gießen-Marburg-Langen, Germany

[7]German Center for Lung Research (DZL), Berlin, Germany

[8]These authors contributed equally

[9]Technical contact

[10]Lead contact

*Correspondence: peter.pennitz@charite.de (P.P.), cengiz.goekeri@charite.de (C.G.), geraldine.nouailles@charite.de (G.N.)
https://doi.org/10.1016/j.xpro.2022.101957

SUMMARY

In infectious disease research, single-cell RNA sequencing allows dissection of host-pathogen interactions. As a prerequisite, we provide a protocol to transform solid and complex organs such as lungs into representative diverse, viable single-cell suspensions. Our protocol describes performance of vascular perfusion, pneumonectomy, enzymatic digestion, and mechanical dissociation of lung tissue, as well as red blood cell lysis and counting of isolated cells. A challenge remains, however, to further increase the proportion of pulmonary endothelial cells without compromising on viability.

For complete details on the use and execution of this protocol, please refer to Nouailles et al. (2021),[1] Wyler et al. (2022),[2] and Ebenig et al. (2022).[3]

BEFORE YOU BEGIN

The protocol below describes how to prepare a single-cell suspension from infected murine lung tissue that were challenged with human pathogens and required biosafety level 2 (BSL-2) working conditions. However, a very similar protocol can be used to isolate pulmonary cells from SARS-CoV-2 infected Syrian hamsters with just minor adaptations to implement specific BSL-3 safety regulations. The protocol was likewise suitable for corresponding uninfected mouse and hamster controls.

The lungs harbor a variety of structural and immune cells, which collectively facilitate crucial physiological processes including gaseous exchange and pulmonary secretions (surfactant, mucus), while also enabling antimicrobial functions such as bacterial sensing and phagocytosis.[5]

Institutional permissions

All procedures affecting animals were approved by the institutional and local governmental authorities (Landesamt für Gesundheit und Soziales Berlin, Germany or Regierungspräsidium Darmstadt,

Germany). Users of this protocol should note that permissions for experiments on live vertebrates must be acquired in advance from the relevant institutions. Local and governmental regulations concerning handling of (genetically modified) infectious agents must also be obeyed and the respective procedures authorized and carried out under the appropriate biosafety conditions until authorized inactivation of all biological material takes place.

Preparation of reagents and buffers

⏱ Timing: 2–7 days before experiment

1. Calculate and prepare the required amounts of stock solutions and buffers ahead of the experiment (see materials and equipment).
   a. Prepare bovine serum albumin (BSA)-containing buffers in 1× phosphate-buffered saline (PBS). For convenience, prepare a 4% BSA/PBS stock solution by dissolving BSA in 1× PBS, filter through a 0.22 μm membrane for sterilization and store at 4°C until required.
   b. Two – 7 days before experiment dilute stock solution 1:4, 1:8 and 1:100 in sterile 1× PBS to prepare 1%, 0.5% and 0.04% BSA/PBS buffers, respectively.
   c. Prepare sterile 1× red blood cell (RBC) lysis buffer by diluting 10× RBC lysis solution 1:10 with distilled water, followed by filtration through a 0.22 μm membrane.
   d. Prepare sterile 2 mg/mL actinomycin D stock solution by dissolving actinomycin D powder in methanol followed by filtration. Aliquot and store solution in liquid nitrogen or −150°C until usage.

Note: Actinomycin D stock solution should be protected from light, stored frozen and added to corresponding buffers on the day of the experiment. Please be aware that actinomycin D adheres to plastic and glass and therefore solutions must be prepared fresh from frozen stock every time.

Note: As an extensive stress gene signature could overlay biological gene expression patterns, it is beneficial to add transcriptional inhibitors like actinomycin D[6] (used in this protocol) to buffers and digestion media to reduce procedure-induced stress gene transcription. Since these inhibitors reduce the rate of *de novo* RNA transcription, the impact of the procedure on live cells can be minimized.

Note: Even though sterility is not required for this protocol, we recommend sterile filtration of buffers (e.g., by vacuum filtration through 0.22 μm membranes), as it prolongs storage times and enables use of buffers with other protocols.

2. Calculate and aliquot required quantities of enzymes and store aliquots at appropriate temperature (collagenase CLS II at 4°C, DNase I at −20°C, dispase at −20°C; see materials and equipment for enzyme quantities per whole lung).

Get reagents and buffers ready for the experiment

⏱ Timing: 2–5 h before experiment (for step 3)

The quality of results critically depends on fast processing of lung cells after explantation. Thus, all time-consuming steps that can be performed prior to pneumonectomy should be prepared and/or executed in advance.

3. Prepare fresh lung digestion medium by dissolving appropriate amounts of aliquoted enzymes in thawed dispase solution (see materials and equipment).

4. Thaw the appropriate amount of Dispase solution for lung perfusion medium (see materials and equipment).

5. Thaw aliquoted actinomycin D stock solution and add to 1% BSA/PBS buffer, lung perfusion buffer and lung digestion medium to a final concentration of 2 μg/mL.

6. Place lung digestion medium, lung perfusion medium, buffers, 1× PBS and RBC lysis buffer on ice until required.

Note: Do not use any buffers containing EDTA, as it interferes with dispase and with reverse transcriptase for cDNA conversion in subsequent single-cell RNA-sequencing (scRNA-seq) workflows.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD16/32 (clone: 2.4G2); use 1:200	BD Biosciences	Cat# 553142; RRID: AB_394657
FITC rat anti-mouse CD45 (clone: 30-F11), use 1:400	BD Biosciences	Cat# 553080; RRID: AB_394610
PE anti-mouse CD326 EpCAM (clone: G8.8), use 1:100	BioLegend	Cat# 118205; RRID: AB_1134176
PerCP/Cyanine5.5 anti-mouse CD31 (clone: MEC13.3), use 1:333	BioLegend	Cat# 102522; RRID: AB_2566761
Chemicals, peptides, and recombinant proteins		
RBC lysis buffer, 10×	Santa Cruz Biotechnology	Cat# sc-296258
Gibco™ DPBS, no calcium, no magnesium	Thermo Fisher Scientific	Cat# 14190094
Actinomycin D	Sigma-Aldrich /(Merck)	Cat# A9415-5MG
Albumin, from bovine serum	Sigma-Aldrich /(Merck)	Cat# A7030-100G
Dispase	Corning	Cat# 354235
DNase I	Panreac AppliChem	Cat# A3778,0500
Collagenase type II, Cls II	Sigma-Aldrich /(Merck)	Cat# C2-22-1G
Collagenase B	Roche	Cat#11088815001
Fixable Viability Dye eFluor™ 780, use 1:333	eBioscience	Cat# 65-0865-14
Trypan blue	Sigma-Aldrich /(Merck)	Cat# 111732
Methanol >99,5%,	Carl Roth GmbH	Cat# CP43.2
Critical commercial assays		
Chromium Next GEM Single Cell 3′ Kit v3.1	10x Genomics, Inc.	Cat# 1000268
Chromium i7 Multiplex Kit	10x Genomics, Inc	Cat# 120262
Chromium Next GEM Chip G Single Cell Kit	10x Genomics, Inc	Cat# 1000120
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific	Cat# Q32851
KAPA Library Quantification Kits - Complete Kit (Universal)	Roche Sequencing	Cat# 07960140001
Bioanalyzer High Sensitivity DNA Analysis	Agilent	Cat# 5067-4626
NextSeq 500/550 High Output Kit v2.5 (150 Cycles)	Illumina	Cat# 20024907
Deposited data		
Raw Data Mouse	Pennitz et al.[4]	https://www.health-atlas.de/data_files/563?graph_view=tree
scRNA-seq Hamster	Ebenig et al.[3]	GEO: GSE196938
Experimental models: Organisms/strains		
Mouse: C57BL/6JRj, female / male, adult (6–12 weeks)	Janvier Labs	RRID: MGI:2670020
Hamster: RjHan:AURA, female / male, adult (6–12 weeks) HsdHan:AURA, female / male, adult (6–12 weeks)	Janvier Labs Envigo	N/A Art# 8903
Software and algorithms		
R	R Core Team	https://www.r-project.org/
R Studio	R Studio Inc.	https://www.rstudio.com/
R Package: Seurat	Satija Lab	https://satijalab.org/seurat/
R Package: ggplot	Tidyverse	https://ggplot2.tidyverse.org/

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cell Ranger Software	10x Genomics, Inc.	https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/installation
GraphPad Prism, Version 9	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
FlowJo	FlowJo	https://www.flowjo.com/
FACS Diva	BD Biosciences	https://www.bdbiosciences.com/
Inkscape	Inkscape Community	https://inkscape.org/de/
Other		
6-well plate	Corning	Cat# 353046
5 mL polystyrene tubes	Corning	Cat# 352052
50 mL conical tubes	Corning	Cat# 352070
70 μm cell strainer	Greiner Bio-One	Cat# 542070
40 μm cell strainer	Greiner Bio-One	Cat# 542040
Syringe filters ROTILABO®	Carl Roth GmbH	Cat# P666.1
Flowmi® Cell Strainers	Sigma-Aldrich /(Merck)	Cat# BAH136800040-50EA
500 mL Vacuum Filter/Storage Bottle System	Corning	Cat# 431097
Qubit 4 Fluorimeter	Thermo Fisher	Cat#Q33238
2100 Bioanalyzer Instrument	Agilent	Part#G2939BA
Novaseq 6000	Illumina	Cat#20013850
NextSeq 550	Illumina	Cat#SY-415-1002
Chromium Controller	10x Genomics	Prod# 1000204

MATERIALS AND EQUIPMENT

The buffer recipes refer to whole murine lungs and right caudal lobes of Syrian hamsters. If whole Syrian hamster lungs shall be analyzed a triplication of the amount is advised.

2 mg/mL actinomycin D stock solution

Reagent	Final concentration	~ Amount per murine lungs / hamster lung lobe
Methanol	–	40 μL
Actinomycin D	2 mg/mL	80 μg
Total		40 μL

Prepare in advance, perform sterile filtration and store in liquid nitrogen* for up to 6 months. Protect from light. Prepare in excess. * Storage at - 80°C is not an alternative due to the freezing point of methanol.

4% BSA/PBS stock solution

Reagent	Final concentration	~ Amount per murine lungs / hamster lung lobe
1× PBS	–	10 mL
BSA	40 mg/mL	0.4 g
Total		10 mL

Prepare in advance, perform sterile filtration and store at 4°C for up to two months. Prepare in excess.

1% BSA/PBS buffer*

Reagent	Final concentration	~ Amount per murine lungs / hamster lung lobe
1× PBS	–	18.75 mL
4% BSA/PBS stock solution	10 mg/mL	6.25 mL
2 mg/mL actinomycin D stock solution	2 μg/mL	25 μL
Total		25 mL

Prepare 1% BSA/PBS in advance and store at 4°C. Actinomycin D needs to be protected from light and added fresh on the day of the experiment. Unused solution should be discarded. *instead of 1% BSA/PBS, 0.5% BSA/PBS buffer has successfully been used.

0.04% BSA/PBS buffer

Reagent	Final concentration	∼ Amount per murine lungs / hamster lung lobe
1× PBS	–	24.75 mL
4% BSA/PBS stock solution	0.4 mg/mL	0.25 mL
Total		25 mL

0.04% BSA/PBS is the concentration of BSA recommended for mRNA barcoding steps. Prepare in advance and store at 4°C for up to two months. Ingredients should be sterile. Can be prepared in excess.

Lung perfusion medium (optional)

Reagent	Final concentration	Amount per murine lungs / hamster lung lobe
Dispase	5.000 IU/mL	5 mL
2 mg/mL actinomycin D stock solution	2 μg/mL	5 μL
Total		5 mL

Prepare fresh on the day of the experiment as per experimental need, store at 4°C. Unused solution should be discarded. Ingredients should be sterile.

Lung digestion medium

Reagent	Final concentration	Amount per murine lungs / hamster lung lobe
DNase I	1 mg/mL	2 mg
Collagenase	750 IU/mL	1.500 IU (Collagenase CLS II)
	alternative: 0.4395 U/mL	*alternative: 0.879 U (Collagenase B)*
Dispase	5.000 U/mL	2 mL
2 mg/mL actinomycin D stock solution	2 μg/mL	2 μL
Total		2 mL

Prepare fresh on the day of the experiment as per experimental need, store at 4°C prior to the experiment. Unused solution should be discarded. Ingredients should be sterile. Collagenase B has successfully been used instead of Collagenase CLS II.

RBC lysis buffer

Reagent	Final concentration	∼ Amount per murine lungs / hamster lung lobe
10× RBC lysis buffer	1×	1 mL
ddH$_2$O	–	9 mL
Total		10 mL*

Prepare in advance as per experimental need, store at 4°C and use at 20°C. ddH$_2$O: Double-distilled water. * Non-perfused lungs may require up to 10 mL due to the residual blood. For perfused mouse lungs 2 mL of 1× RBC lysis buffer is sufficient, while hamster lungs required 5 mL. Prepare in excess in case first lysis attempt fail.

⚠ CRITICAL: The following reagents are harmful and precautions should be taken in order to avoid the indicated hazards.

- Dispase may cause allergy, asthma symptoms or breathing difficulties if inhaled.
- Actinomycin D is fatal if swallowed, may cause cancer, damage fertility and harm the unborn child.
- Methanol is a highly flammable liquid and vapor, toxic if swallowed, and may cause irritation in case of contact with skin or inhalation and may also cause ocular damage.
- Collagenase CLS II may cause allergy, asthma symptoms or breathing difficulties if inhaled.
- Trypan blue may cause cancer.

Alternatives: Published alternatives for the transcription inhibitor actinomycin D, which blocks *de novo* RNA transcription during cell isolation, include DSP (dithiobis succinimidyl propionate)[7] and CellCover[8] reagent.

Alternatives: Published alternatives for enzymes used to isolate viable lung cells include Liberase[9] (commercially available purified enzyme blends containing collagenase and neutral

protease), collagenase B or elastase.[10] For kidney tissue cold protease digestion was success-fully tested to significantly reduce the expression of stress genes and artifacts.[11,12]

STEP-BY-STEP METHOD DETAILS
Vascular lung perfusion and pneumonectomy

⏱ Timing: 20 min per lung (plus 10 min for each additional experimental animal) (for step 1)

This step details handling of experimental animals and lung excision with or without prior perfusion (optional). Animals are sacrificed either by bleeding out via heat puncture (after step 1) or by bleeding out through transection of the abdominal vena cava (step 5).

1. Subject laboratory animals to deep anesthesia according to approved protocols e.g., with 3-fold overdosed anesthetics. Confirm depth of anesthesia by loss of pedal withdrawal reflex.

   Note: If working under BSL-3 conditions, make sure to wear appropriate PPE including chain gloves when handling animals while they are still awake and to use appropriate safety needles and sharps.

   Note: If protocol without perfusion is performed animals can be sacrificed at this step by bleeding out via heart puncture. If analysis of blood or serum is to be performed in parallel, it can be collected here and placed in an EDTA or clotting tube, respectively. EDTA blood should be kept on ice, whilst clotting should be allowed to occur at 20°C.

   Note: Protocols with and without perfusion generated valid results. Past reasons to follow a protocol without perfusion were in our case e.g., i) necessity to increase speed of experimental procedures, ii) regulatory limitations restricting use of sharps in biosafety level 3 laboratory and iii) approved animal experimental protocols for sacrifice by bleeding out via heart puncture instead of vena cava.

2. Place anesthetized animals in a secured supine position.
3. Soak abdominal and thoracic areas with 70% ethanol to disinfect the fur and keep the surgical instruments sterile.

   ⚠ CRITICAL: Ensure that the laboratory animal does not aspirate ethanol as it could potentially damage cells in upper and lower airways.

4. Open the abdominal cavity by incising the abdominal skin and peritoneum from the sternum to the pubis using surgical scissors and forceps.
5. Sacrifice the animal by bleeding out through transection of the abdominal vena cava (in case bleeding out via heart puncture has not been performed after step 1).

   Note: If analysis of blood or serum is to be performed in parallel, it can be collected here from the abdominal vena cava in a 1 mL syringe using a 27G needle and placed in an EDTA or clotting tube, respectively. EDTA blood should be kept on ice, whilst clotting should be allowed to occur at 20°C.

6. Open the thoracic cavity and expose the heart and lungs.
7. **Without perfusion** continue with step 9, **with perfusion** proceed with step 8.
8. Perfuse the pulmonary vessels through the animals' right heart ventricle by slowly flushing with 5 mL lung perfusion medium using a 5 mL syringe capped with a 26G needle (Figure 1).

   Note: The perfusion step allows for removal of blood cells within the pulmonary vessels.
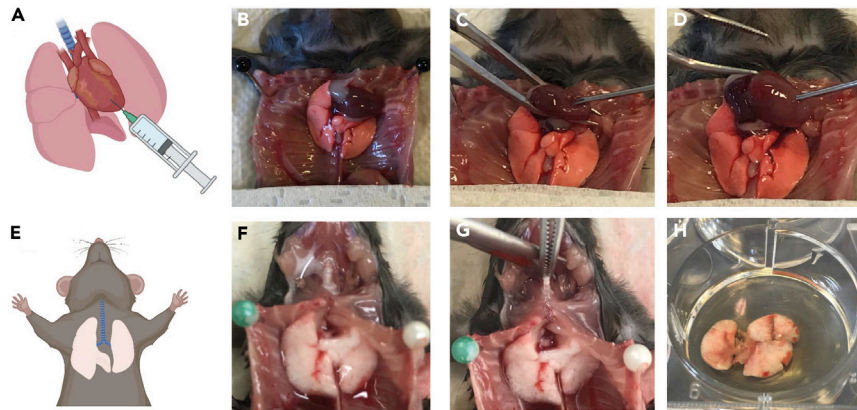
Figure 1. Lung perfusion and pneumonectomy
(A) Illustration displaying perfusion of pulmonary vessels through the right heart ventricle.
(B) Medial thoracotomy was performed to enable access to the heart.
(C and D) (C) The heart is fixed in position with tweezers to inject perfusion needle and (D) ejection of lung perfusion media.
(E) Illustration displaying prepared trachea and perfused lungs.
(F) Heart and thymus are removed facilitating access to the lungs.
(G) Pneumonectomy is performed without tissue contact by lifting the trachea.
(H) Once transferred to cell culture dish, remaining parts of the trachea are removed and dissected lungs (without trachea) are stored in digestion media.

9. Lift up the lungs by pulling the trachea, dissect them free and dip them twice into ice-cold PBS to remove residual fluid or blood.
10. Remove the trachea.
11. Place the lungs (mice) or right caudal lobe (hamsters) in an individual well of a 6-well plate (Figure 1H) or in an individual petri dish and add 2 mL lung digestion medium.

   Note: If dissecting multiple animals one after another, place lungs into 3 mL RPMI medium or PBS containing 1% BSA and 2 μg/mL actinomycin D on ice until all animals are dissected, then proceed with step 11.

Enzymatic digestion and mechanical dissociation of lung tissue

   ⏱ Timing: 50 min per lung (plus 10 min for each additional lung)

This step details how enzymatic digestion in combination with gentle mechanical force allows for dissociation of solid lung tissue into single cell suspension.

   Note: For processing multiple lungs, the following steps are carried out in parallel for each well. Clean forceps with 1× PBS before moving on to the next well.

   ⚠ CRITICAL: Rapid, yet gentle cell isolation reduces cell stress and ensures high quality transcriptome data.

12. Within the digestion medium, mechanically dissociate the tissue by securing it with straight anatomical forceps in one hand and repeatedly hitting it with a second curved anatomical set of forceps held in the other hand for approximately 2–3 min. Alternatively, lungs can be cut into <1 mm pieces using pointed scissors (Figure 2).

   Note: We have experienced that dissociating lung tissue with clapping forceps results in higher numbers of structural lung cells.
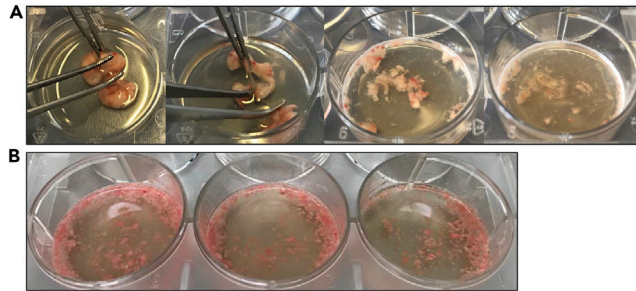
Figure 2. Mincing of murine lung tissue in digestion medium
(A) Mechanical dissociation of lung tissue by repeatedly clapping with forceps.
(B) Result when of lung tissue was minced using pointed scissors.

13. Incubate at 37°C (with occasional shaking, e.g., using an orbital shaker at 0.26 × g) for 30 min.
14. Add 8 mL ice-cold 1% BSA/PBS buffer to stop enzymatic digestion.
15. Pipette the tissue and liquid up and down 3–10 times using a 5 mL serological pipette to further disrupt the lung tissue. Avoid introducing air bubbles.
16. Collect the complete suspension in a 10 mL serological pipette and discharge onto a 70 μm cell strainer placed on top of a 50 mL conical tube.
17. Use the plunger of a 5 mL syringe to gently swipe remaining lung tissue through the cell strainer.
18. Rinse the well with 10 mL cold 1% BSA/PBS or 0.5% BSA/PBS buffer and pass it through the cell strainer to retrieve the maximal number of cells.
19. Centrifuge the cell suspension at 300 × g for 5 min at 4°C and discard the supernatant.

Red blood cell lysis, cell filtration and counting

⏲ Timing: 35 min per lung (plus 20 min for each additional lung) (for step 20)

This step details red blood cell lysis, and washing and counting of lung cells.

20. Add RBC lysis buffer (2–10 mL) to the cell pellet and carefully resuspend. Incubate the suspension for 2–5 min at 20°C.

Note: The required volume and incubation time is dependent on the rodent species and prior vascular perfusion of lungs. We recommend testing appropriate volume and incubation times for individual experimental settings. In our hands, 2 mL RBC lysis buffer and 2 min incubation time was sufficient for perfused mouse lungs while 10 mL RBC lysis buffer and 5 min incubation time was sufficient for non-perfused hamster lungs.

21. Stop RBC lysis by adding at least 10 mL 0.04% BSA/PBS buffer.
22. Centrifuge cell suspension at 300 × g for 6 min at 4°C and discard supernatant.
23. To ensure that RBC are lysed, check if pellet has turned colorless (Figure 3). If not repeat steps 18–21.
24. Resuspend the cell pellet in 4–10 mL of 0.04% BSA/PBS buffer.
25. Filter the single cell suspension by discharge onto a 40 μm cell strainer placed on top of a 15 mL conical tube.
26. Determine the cell concentration by counting cells using a Neubauer hemocytometer:
    a. Dilute the single cell suspension 1:10 in trypan blue solution by adding 20 μL cell suspension to 180 μL trypan blue solution and gently pipette to allow mixing.
    b. Count cells in the 4 square subdivisions placed at the corners of a Neubauer hemocytometer (S1 – S4).
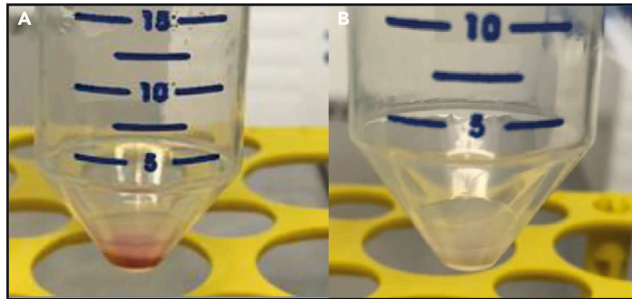   c. Calculate the cell concentration for each sample.

Figure 3. Cell pellets prior and after red blood cell lysis
(A and B) (A) Cell pellet containing RBC (as indicated by the red color) prior to adding RBC lysis buffer (left) and (B) after successful RBC lysis (right).

$$cell\ count\ =\ \frac{S1 + S2 + S3 + S4}{4}\ x\ dilution\ factor\ x\ 10\ 000\ x\ sample\ volume\ [mL]$$

27. Visually check cell viability and singularity on the hemocytometer.

   Note: Viability should be at least 80%. A high proportion of dead cells indicates problematic handling of tissue, e.g., due to enzyme concentrations, incubation time and other processing steps being too harsh. See troubleshooting section (problem 1).

   Note: To increase the fraction of viable cells in the single cell suspension, depletion of dead cells using magnetic cell separation or fluorescence-activated cell sorting can be performed.

28. Adjust the single cell suspension to approximately 500 μL to 1 mL suspension with an appropriate concentration for single-cell RNA-sequencing by diluting with 0.04% BSA/PBS buffer.
29. Filter 500 μL to 1 mL of the diluted suspension with Flowmi® cell strainers (40 μm) to ultimately remove cell clumps (Figure 4).

   Note: The optimal cell density for the demonstrated single-cell sequencing approach using the Chromium Next GEM Single Cell 3′ Kit v3.1 (10x Genomics) was $4 \times 10^5 - 2 \times 10^6$ cells/mL. Low concentrations of BSA in buffers are recommended for mRNA barcoding steps by the manufacturer.
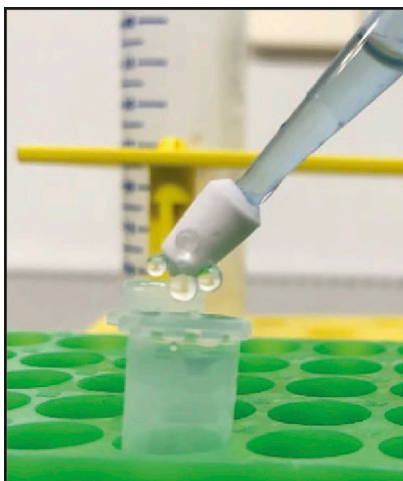

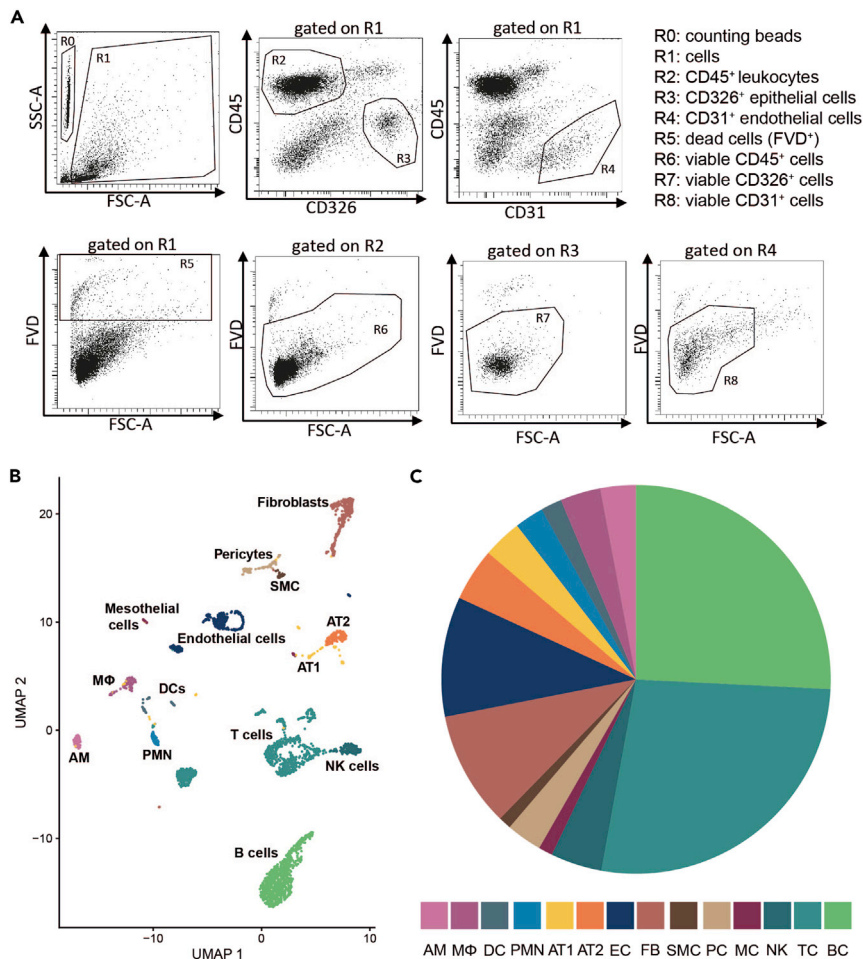**Figure 4. Removal of cell clumps**
Cell filtering via Flowmi.

Figure 5. Cell viability control by flow cytometry and anticipated cell population identified by single-cell analysis
(A) Dot plots of isolated single-cells blocked with CD16/32 (FcR block, anti-mouse CD16/32, use 1:200) and stained with monoclonal antibodies (mAbs) against CD45 (leukocyte marker, FITC anti-mouse CD45, use 1:400), CD326 (epithelial cell marker, PE anti-mouse CD326, use 1:100), CD31 (endothelial cell marker, PerCP/Cyanine5.5 anti-CD31, use 1:333) and fixable viability dye (FVD; dead cell marker, use 1:333). Forward scatter (FSC) and side scatter (SSC) plots represent size and granularity of cells, respectively. R0: represents counting beads, R1 represents all cells minus debris. Cells gated on R1 are displayed for CD45, CD326 and CD31 positive populations to identify leukocytes (R2), epithelial cells (R3) and endothelial cells (R4), respectively. FVD was used to stain dead cells (R5) and to allow discrimination of viable leukocytes, epithelial and endothelial cells (R6 to R8) from dead cells.
(B and C) (B) UMAP plot displaying clustering and annotation of sequenced and bioinformatically evaluated naive murine lung cells and (C) pie chart indicating proportions of sequenced and annotated lung cell types. AM: Alveolar macrophages, Mφ: Interstitial macrophages/monocytes, DC: Dendritic cells, PMN: Polymorphonuclear leukocytes, AT1: Alveolar epithelial cells type 1, AT2: Alveolar epithelial cells type 2, EC: Endothelial cells, FB: Fibroblasts, SMC: Smooth muscle cells, PC: Pericytes, MC: Mesothelial cells, NK: Natural killer cells, TC: T cells, BC: B cells, UMAP: Uniform manifold approximation and projection.

Note: Labeling or barcoding of mRNA needs to be performed as soon as possible after processing of the final single cell suspension. Please refer to the desired manufacturer's protocol for step-by-step instructions.

In the demonstrated approach that generated the data shown in Figure 5, the Chromium Next GEM Single Cell 3′ Kit v3.1 (10x Genomics) was used for mRNA barcoding, reverse transcription, and single-cell transcriptome library preparation (https://assets.ctfassets.net/an68im79xiti/1eX2FPdpeCgnCJ tw4fj9Hx/7cb84edaa9eca04b607f9193162994de/CG000204_ChromiumNextGEMSingleCell3_v3.1_

Code Box 1. Creation of Seurat Object using SCTransform in R

```
>library(ggplot2)

>library(dplyr)

>library(Seurat)

>library(patchwork)

>library(sctransform)

>library(hdf5r)

>library(glmGamPoi)

>MLung_data <- Read10✕_h5("…/outs/filtered_feature_bc_matrix.h5", use.names = TRUE) #In
this case H5 Files are publically available to start the Pipeline. If filtered_feature_bc_
matrix Objects are available start Workflow with:

# >MLung_data <- Read10✕(data.dir = ".../outs/filtered_feature_bc_matrix")

>MLung <- CreateSeuratObject(MLung_data, project = "Mouse_lung")

>MLung <- PercentageFeatureSet(MLung, pattern = "^mt-", col.name = "percent.mt")

>MLung <- SCTransform(MLung, vars.to.regress = "percent.mt")

>if (!requireNamespace("BiocManager", quietly = TRUE)) install.packages("BiocManager")

>MLung <- SCTransform(MLung, method = "glmGamPoi", vars.to.regress = "percent.mt")

>MLung <- RunPCA(MLung)

>ElbowPlot(MLung, ndims = 50)

#credits to: Harvard Chan Bioinformatics Core[16]:https://hbctraining.github.io/scRNA-seq/lessons/
elbow_plot_metric.html

>pct <- MLung[["pca"]]@stdev / sum(MLung[["pca"]]@stdev) * 100

>cumu <- cumsum(pct)

>co1 <- which(cumu > 90 & pct < 5)[1]

>co1

>co2 <- sort(which((pct[1:length(pct) - 1] - pct[2:length(pct)]) > 0.1), decreasing = T)
[1] + 1

>co2

>pcs <- min(co1, co2)

>pcs

>plot_df <- data.frame(pct = pct,

>           cumu = cumu,

>           rank = 1:length(pct))

>ggplot(plot_df, aes(cumu, pct, label = rank, color = rank > pcs)) +

>  geom_text() +

>  geom_vline(xintercept = 90, color = "grey") +

>  geom_hline(yintercept = min(pct[pct > 5]), color = "grey") +

>  theme_bw()

>MLung <- RunUMAP(MLung, dims = 1:39)

#  seems to work better if maximal information according to line 27 is considered
```

Continued

```
>MLung <- FindNeighbors(MLung, dims = 1:39)

>MLung <- FindClusters(MLung, resolution = 0.8)

#  resolution = 0.8 is default setting, increase to get more clusters, decrease for less

>DimPlot(MLung, label = TRUE)

>#saveRDS(MLung, file = ".../MLung_seurat_from_H5.rds")
```

Rev_D.pdf). Next generation sequencing was performed using a NextSeq 500/550 High Output Kit v2.5 (150 Cycles) on a NextSeq500 device (both Illumina).

EXPECTED OUTCOMES

The aim of this protocol is to provide an easy-to-handle lung cell isolation and processing workflow that has been performed under high containment biological laboratories and is thus suitable for infectious disease research. Processing naïve lung tissue according to steps 1 to 29 should provide a single cell suspension containing approximately 1×10^7 cells (murine C57BL/6J lungs and Syrian hamsters' caudal lung lobe). Cell numbers can vary, depending on age, disease, infectious agent and time point of analysis. Cell suspensions should include alveolar, interstitial, and inflammatory macrophages, dendritic cells, granulocytes, alveolar epithelial cells type 1 and type 2, ciliated cells, endothelial cells, lymphatic endothelial cells, fibroblasts, smooth muscle cells, pericytes, mesothelial cells, natural killer cells, T cells and B cells with singularity and viability rates of above 90%, thereby fulfilling the necessary technical quality criteria for subsequent single-cell RNA-sequencing. The expected unsorted (not enriched or depleted for any specific cell type) physiological frequencies of pulmonary cell types after barcoding, library preparation and sequencing are displayed in Figure 5.

Generated sequencing data can be processed in a straightforward way using the demonstrated Vignette of R Package Seurat.[13–15] The adapted code for generating a murine lung Seurat Object from a single sequencing file is displayed in Code Boxes 1 and 2. Integrational approaches (including different species data) and more complex analyses have been described previously.[4]

Flow cytometry (optional)

After the isolation of single cells, major quality control criteria (cell singularity, viability and diversity) for subsequent scRNA-seq can be evaluated via flow cytometry. If a particular population of cells is of specific interest, we highly recommend carefully practicing the protocol and analyzing the samples via flow cytometry prior to proceeding with single-cell barcoding experiments. For instance, staining for major cell populations of the lung (e.g., CD45+ leukocytes, CD326+ epithelial cells and CD31+ endothelial cells) and for cell viability (via measuring 7AAD- or FVD- cells) can be performed, which allows the frequencies of viable singlets amongst the parent population (or total numbers of cells) to be determined.

Bioanalyzer and Qubit

Before sequencing the final single-cell gene expression libraries, quality control measures should be performed to determine the concentration (Qubit) and cDNA fragment sizes (Bioanalyzer) (Figure 6).

> *Note:* Instead of using a Bioanalyzer, the fragment sizes can also be measured using a fragment analyzer or tape station.

KAPA PCR (optional)

It is recommended to perform qPCR on final single-cell gene expression libraries using the KAPA Library Quantification Kit (Roche Sequencing) for specific quantification of cDNA fragments of interest, since Bioanalyzer and Qubit also detect cDNA fragments which cannot be sequenced.

Code Box 2. Visualization of the generated Seurat Object

```
>MLung <- readRDS(".../ MLung_seurat_from_H5.rds ")

#Marker genes in Dotplot

>features <- c("Marco","Adgre1", "Flt3", "Cxcr2", "Siglecf", "Ncr1", "Cd3e", "Cd79a", "Epcam",
"Akap5", "Lamp3", "Foxj1", "Pecam1", "Cdh5", "Mmrn1", "Inmt", "Acta2", "Cox4i2", "Msln")

>DotPlot(MLung, features = features) + RotatedAxis()

>MLung$populations <- paste("C", MLung$seurat_clusters)

>MLung@active.ident= factor(MLung$populations)

>MLung <- RenameIdents (MLung, "C 0" = "B cells", 'C 1' = "Endothelial cells", 'C 2' = "T cells",
'C 3' = "B cells", 'C 4' = "T cells", 'C 5' = "Fibroblasts", 'C 6' = "T cells", 'C 7' = "AT2", 'C 8' =
"NK cells", 'C 9' = "Fibroblasts", 'C 10' = "T cells", 'C 11' = "AT1", 'C 12' = "AM", 'C 13' =
"Pericytes", 'C 14' = "Endothelial cells", 'C 15' = "PMN", 'C 16' = "Macrophages", 'C 17' = "T
cells", 'C 18' = "DC", 'C 19' = "T cells", "C 20" = "T cells", "C 21" = "Mesothelial cells", "C
22" = "SMC", "C 23" = "Macrophages")

>MLung$populations <- paste(MLung@active.ident)

>UMAPPlot(MLung, label = T) + NoLegend()

>Cellorder = c('AM', 'Macrophages', 'DC', 'PMN', 'AT1', 'AT2', 'Endothelial cells',
'Fibroblasts', 'SMC', 'Pericytes', 'Mesothelial cells' , 'NK cells', 'T cells', 'B cells')

>Nogpalette <- c("#CB769E", "#A85C85", "#4F6D7A", "#0081AF", "#F7C548", "#F97E44",
"#0D3B66", "#B2675E", "#644536", "#C4A381", "#832D52", "#246A73", "#368F8B", "#62C370")

>my_levels <- c(Cellorder)

>MLung@active.ident<- factor(x =MLung@active.ident, levels = my_levels)

>UMAPPlot(MLung, label = T, cols = c(Nogpalette)) + NoLegend() + coord_fixed(ratio=1)

>ggsave(".../Umap_Mlung_SCT_H5.pdf", width = 7, height = 7)

#Piechart

>tbl_ct <- table(MLung$populations, MLung$orig.ident)

>sums = colSums(tbl_ct) # sum across columns of the object = colSums(..

>tbl_ct[,1] <- tbl_ct[,1]/sums[1]

>tbl_ct

>

>freq_ct <- data.frame(tbl_ct)

>colnames(freq_ct) <- c("celltype", "ident", "percentage")

>freq_ct$celltype <- factor(freq_ct$celltype, levels = c(Cellorder))

>freq_ct <- freq_ct[order(freq_ct$celltype),]

>ggplot(freq_ct, aes(x="", y=percentage, fill=celltype)) +

>  geom_bar(stat="identity", width=1) + scale_fill_manual("legend", values = Nogpalette) +

>  coord_polar("y", start=0) + theme_void()

>ggsave(".../Piechart_MLung.pdf", width = 7, height = 5)
```

LIMITATIONS

Although the demonstrated protocol was established to represent all major lung cell types, we observe that the distribution of isolated cell types might not necessarily represent cellular
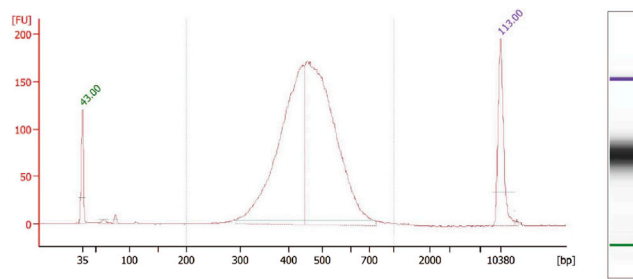
Figure 6. Quality control of generated gene expression libraries
Example of a final gene expression library with fragment size distribution suitable for next generation sequencing.

frequencies of physiological lung tissue. Estimates based on morphological identification and counting of cells in normal lung tissue sections across species suggest that alveolar type 1 epithelial cells represent between 8%–12%, alveolar type 2 epithelial cells 8%–16%, endothelial cells 30%–46% and alveolar macrophages 3%–10% of total lung cells.[5] Over- or underrepresentation of cell types of interest depend on the type and amount of enzymes used during digestion and processing of the tissue.

TROUBLESHOOTING

Problem 1
Laboratory animals do not exhibit the depth of anesthesia (evaluated by loss of pedal withdrawal reflex) necessary for dissection, despite receiving the correct amount per body weight (step 1).

Potential solution
The anesthetics might take longer to be effective in individual animals. The injection might have been faulty, although injection into the peritoneal cavity minimizes this risk. Consult your laboratory veterinarian if a second dose might need to be applied. Alternatively, perform a cervical dislocation to sacrifice the animal. All procedures performed on animals need prior approval of the institutional and local governmental authorities.

Problem 2
Vascular lung perfusion was unsuccessful, lungs remain pinkish (optional step 8).

Potential solution
Identifying and hitting the right ventricle can be challenging at first (Figure 1). If the lungs do not start turning white upon the initiation of vascular perfusion, remove the needle from the heart and try flushing the right ventricle a second time or try to inject into the pulmonary artery. Before ejecting the perfusion medium out of the syringe, make sure to obstruct the first injection site with tweezers. Lungs turning whitish indicate a successful perfusion.

Problem 3
Low viability of lung single cell suspension (step 27).

Potential solution
In order to prevent cell death, preparation and processing time should be kept as short as possible. Ensure that cell suspensions are not exposed to fluctuating temperatures during and in between centrifugation steps. Ideally, cell suspensions should be kept on ice and centrifuged at cold temperatures during processing unless the protocol requires it otherwise.

Do not place cell pellets on ice, instead resuspend immediately after centrifugation and place cell suspension on ice.

Problem 4
Low number of desired lung cell populations (step 27).

Potential solution
Consider enrichment or depletion of specific cell populations via magnetic or flow cytometry-based cell sorting. If hard to dissociate cells, like structural lung cells are of specific interest, combining or replacing single-cell RNA-sequencing with single-nucleus RNA-sequencing could be beneficial as the nuclei isolation procedures reduce the dissociation bias.

Problem 5
Unusual Bioanalyzer measurements (expected outcomes – bioanalyzer and qubit).

Potential solution
In case of unexpected cDNA fragment size distribution or measurements, try to run the samples again with a freshly prepared gel-dye mix. Make sure the sample is diluted according to the manufacturer's instructions in order to prevent overloading of the Bioanalyzer chip due to excessive concentrations. On the other hand, if cDNA fragment size distribution curves are too small or hardly detectable, running a pure or less-diluted sample could be attempted.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Geraldine Nouailles (geraldine.nouailles@charite.de).

Materials availability
This study did not generate new unique reagents.

Data and code availability
The data chosen for exemplary naïve murine lung cell analysis was previously published[4] and raw data files deposited under "Health Atlas: https://www.health-atlas.de/data_files/563?graph_view= tree." Code for shown example data can be found in Code Boxes 1 and 2.

The raw data for exemplary scRNA-seq analysis of infected hamster lung cells was deposited in the under "Gene Expression Omnibus: GSE196938." As a reference, raw data of bulk RNA-seq analysis of similarly infected hamster lung cells was deposited under "Gene Expression Omnibus: GSE195939."

REFERENCES

1. Nouailles, G., Wyler, E., Pennitz, P., Postmus, D., Vladimirova, D., Kazmierski, J., Pott, F., Dietert, K., Muelleder, M., Farztdinov, V., et al. (2021). Temporal omics analysis in Syrian hamsters unravel cellular effector responses to moderate COVID-19. Nat. Commun. *12*, 4869. https://doi.org/10.1038/s41467-021-25030-7.

2. Wyler, E., Adler, J.M., Eschke, K., Teixeira Alves, G., Peidli, S., Pott, F., Kazmierski, J., Michalick, L., Kershaw, O., Bushe, J., et al. (2022). Key benefits of dexamethasone and antibody treatment in COVID-19 hamster models revealed by single-cell transcriptomics. Mol. Ther. *30*, 1952–1965. https://doi.org/10.1016/j.ymthe.2022.03.014.

3. Ebenig, A., Muraleedharan, S., Kazmierski, J., Todt, D., Auste, A., Anzaghe, M., Gömer, A., Postmus, D., Gogesch, P., Niles, M., et al. (2022). Vaccine-associated enhanced respiratory pathology in COVID-19 hamsters after TH2-biased immunization. Cell Rep. *40*, 111214. https://doi.org/10.1016/j.celrep.2022.111214.

4. Pennitz, P., Kirsten, H., Friedrich, V.D., Wyler, E., Goekeri, C., Obermayer, B., Heinz, G.A., Mashreghi, M.F., Büttner, M., Trimpert, J., et al. (2022). A pulmonologist's guide to perform and analyse cross-species single lung cell transcriptomics. Eur. Respir. Rev. *31*, 220056. https://doi.org/10.1183/16000617.0056-2022.

5. Crapo, J.D., Barry, B.E., Gehr, P., Bachofen, M., and Weibel, E.R. (1982). Cell number and cell characteristics of the normal human lung. Am. Rev. Respir. Dis. *125*, 740–745. https://doi.org/10.1164/arrd.1982.125.6.740.

6. Sunkara, V., Heinz, G.A., Heinrich, F.F., Durek, P., Mobasheri, A., Mashreghi, M.F., and Lang, A. (2021). Combining segmental bulk- and single-cell RNA-sequencing to define the chondrocyte gene expression signature in the murine knee joint. Osteoarthritis Cartilage *29*, 905–914. https://doi.org/10.1016/j.joca.2021.03.007.

7. Attar, M., Sharma, E., Li, S., Bryer, C., Cubitt, L., Broxholme, J., Lockstone, H., Kinchen, J., Simmons, A., Piazza, P., et al. (2018). A practical solution for preserving single cells for RNA sequencing. Sci. Rep. *8*, 2151. https://doi.org/10.1038/s41598-018-20372-7.

8. Wohnhaas, C.T., Leparc, G.G., Fernandez-Albert, F., Kind, D., Gantner, F., Viollet, C., Hildebrandt, T., and Baum, P. (2019). DMSO cryopreservation is the method of choice to preserve cells for droplet-based single-cell RNA sequencing. Sci. Rep. *9*, 10699. https://doi.org/10.1038/s41598-019-46932-z.

9. Nakano, H., Nakano, K., and Cook, D.N. (2018). Isolation and purification of epithelial and endothelial cells from mouse lung. Methods Mol. Biol. *1799*, 59–69. https://doi.org/10.1007/978-1-4939-7896-0_6.

10. Angelidis, I., Simon, L.M., Fernandez, I.E., Strunz, M., Mayr, C.H., Greiffo, F.R., Tsitsiridis, G., Ansari, M., Graf, E., Strom, T.M., et al. (2019). An atlas of the aging lung mapped by single cell transcriptomics and deep tissue proteomics. Nat. Commun. *10*, 963. https://doi.org/10.1038/s41467-019-08831-9.

11. Denisenko, E., Guo, B.B., Jones, M., Hou, R., de Kock, L., Lassmann, T., Poppe, D., Clément, O., Simmons, R.K., Lister, R., and Forrest, A.R.R. (2020). Systematic assessment of tissue dissociation and storage biases in single-cell and single-nucleus RNA-seq workflows. Genome Biol. *21*, 130. https://doi.org/10.1186/s13059-020-02048-6.

12. Adam, M., Potter, A.S., and Potter, S.S. (2017). Psychrophilic proteases dramatically reduce single-cell RNA-seq artifacts: a molecular atlas of kidney development. Development *144*, 3625–3632. https://doi.org/10.1242/dev.151142.

13. Hafemeister, C., and Satija, R. (2019). Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression. Genome Biol. *20*, 296. https://doi.org/10.1186/s13059-019-1874-1.

14. Hao, Y., Hao, S., Andersen-Nissen, E., Mauck, W.M., 3rd, Zheng, S., Butler, A., Lee, M.J., Wilk, A.J., Darby, C., Zager, M., et al. (2021). Integrated analysis of multimodal single-cell data. Cell *184*, 3573–3587.e29. https://doi.org/10.1016/j.cell.2021.04.048.

15. Hafemeister, C., and Satija, R.. Using sctransform in Seurat. https://satijalab.org/seurat/articles/sctransform_vignette.html.

16. HarvardChanBioinformatics. Introduction to single-cell RNA-seq. https://hbctraining.github.io/scRNA-seq/lessons/elbow_plot_metric.html.