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Adult mouse lung cell dissociation (on ice) V.3

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Human Cell Atlas Metho...

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Protocol status: Working
We use this protocol and it's working

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Abstract

This protocol was used to dissociate adult (8-10 wk) mouse lung tissue. The entire procedure is carried out on ice (to reduce artifact gene expression changes) and takes about half an hour. The yield was 16,240 non-RBC/mg tissue with 87% viability.

Guidelines

Enzyme Mixes

Coll. A/Elastase/Dispase Enzyme Mix (1.5 mL → *save 0.5 mL of coll./elastase/dispase mix in separate 1.5 mL tube.*)
90 µL Collagenase A 100 mg/mL – 6 mg/mL final (Sigma, 10103578001)
150 µL elastase 43 u/mL - 4.3 u/mL final (Worthington, LS002292)
150 µL Dispase 90 u/mL – 9 u/mL final (Worthington, LS02100)
7.5 µL 1 M CaCl2 – 5 mM final
7.5 µL DNAse (125 U/mL) (Applichem, A3778)
1095 µL PBS

B. Lich enzyme mix (1 mL)
899 µL DPBS (no Ca, no Mg) (Thermo Fisher, 14190144)
1 µL 0.5 M EDTA - 0.5 mM final conc. (Sigma, A8806)
100 µL *Bacillus Licheniformis* 100 mg/mL - 10 mg/mL final conc. (Sigma, P5380)

Preparing stock aliquots of reagents:
Enzyme stocks are made up in DPBS. I make up 100 mg/mL stock of Collagenase A and *Bacillus Licheniformis*. For elastase, I prepare 43 u/mL stock, and for dispase a 90 u/mL stock. Enzymes mixes are aliquoted in 150 µL (elastase, depase) or 100 µL (Collagenase A, Bacillus Licheniformis) and stored at -80 ºC. For the CaCl2, I prepare a 1 M stock and autoclave. The DNAse is diluted in DPBS, aliquoted and stored at -80 ºC.

Required reagents:
Red Blood Cell Lysis Buffer (Sigma, R7757) - store at RT
Trypan blue solution, 0.4% (ThermoFisher, 15250061)

(Optional) Dead Cell Removal Kit:
EasySep dead cell removal (Annexin V) kit (cat. #17899)
EasySep Magnet (cat. #18000)

Required Equipment & Consumables:
Centrifuges for 1.5 mL and 15 mL conicals (MLS)
Pipettes and pipet tips (MLS)
15, 50 ml Conicals (MLS)
1.5 mL tubes (MLS)
30 µM filters - Miltenyi (130-098-458)
Petri dishes (MLS)
Razor blades (MLS)
Ice bucket w/ice (MLS)
Hemocytometers - InCyto Neubauer Improved (DHC-NO1-5)

The protocol workflow is as follows:
A. Isolate lung
B. First layer
C. Second layer
D. Preparing cells for Chromium

Materials

MATERIALS

RBC Lysis Buffer **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**R7757

DNAse **AppliChem Catalog #**A3778

BSA

Troubleshooting

Before start

-Prepare enzyme mixes and leave on ice.
-Cool centrifuges to 4 °C.

Isolate tissue

1 Isolate lung (optional: perfuse lung with DPBS to reduce RBC). Place lung in ice-cold DPBS and transport on ice.

2 Using sterile forceps, transfer lung tissue to petri dish on ice. Remove excess DPBS using pipet. Mince lung tissue on petri dish on ice for 2 min until fine paste. Vigorously mince tissue using forceps to manipulate the tissue with one hand while using a grinding motion with the razor blade in the other hand.

00:02:00 mince tissue on ice

1st layer

3 Weigh out 25 mg of tissue on petri dish. Using a sterile razor blade or forceps place 25 mg tissue in 1 mL enzyme mix in 1.5 mL eppendorf tube, incubating on ice.

25 mg minced lung tissue

4 Incubate on ice. Shake tube vigorously every 30 secs. Begin triturating at 2 mins. Triturate 10X every 1.5 minute (first w/tip cut).

00:00:30 shake 00:01:30 triturate 10x

5 After 5 min pipet tissue + enzyme mix into new sterile petri dish on ice. Mince 2 min using razor blade to further break up residual chunks of tissue.

00:05:00 After 5 min. digesting pipet digest mix into petri dish

00:02:00 mince in petri dish

6 Pipet digest mix back into 1.5 mL tube. Rinse petri dish with 0.5 mL coll. A/elastase/dispase enzyme mix and pipet into same tube.

0.5 mL rinse petri dish

7 Continue triturating and shaking on ice for 2 additional minutes until you reach 9 minutes total digestion time

00:02:00 triturate on ice

8 At 9 min total digest time let tube settle for one min on ice. The chunks of tissue will settle to the bottom of the tube, leaving released cells in the supernatant. Pipet 80% of supernatant onto 30 µM filter on sterile 15 mL conical.

00:01:00 settle on ice

9 Rinse filter w/6 mL ice-cold PBS/BSA 0.04%. Leave filter on 15 mL conical for next steps.

6 mL ice-cold PBS/BSA 0.04%

2nd layer

10 Add additional 1 mL of 10 mg/mL b. lich enzyme mix to residual clumps of tissue in enzyme in the 1.5 mL tube.
1 mL B. lich enzyme mix

11 Continue triturating on ice 10x every 1.5 minute for 10 additional minutes (20 min total time). Shake every 30 sec.
00:10:00 digest on ice | 00:01:30 triturate 10x | 00:00:30 shake

12 Pipet entire volume onto same 30 µM filter on 15 mL conical - rinse w/6 mL ice-cold PBS/BSA 0.04%.
6 mL ice-cold PBS/BSA 0.04%

13 Spin 300 g for five minutes at 4 ºC. Remove all but 100 µL of supernatant - being careful not to disturb pellet.
4 °C spin at 300 g

RBC Lysis

14 Add 900 µL RBC lysis buffer to pellet. Triturate 20X using 1 mL pipet set to 700 µL and incubate for two min on ice.
900 µL RBC lysis buffer | 00:02:00 incubate on ice

15 Add 12 mL ice-cold PBS/BSA 0.04% to 15 mL conical to dilute RBC lysis buffer.
12 mL ice-cold PBS/BSA 0.04%

Preparing for Single-Cell Sequencing

16 Spin 15 mL conical 200 g (low-g spin) for 5 min at 4 ºC to pellet cells and leave small debris and platelets in supernatant.
00:05:00 spin at 200 g | 4 °C

17 Remove supernatant and re-suspend in 200 µL ice-cold PBS/BSA 0.04%.
200 µL ice-cold PBS-BSA 0.04%

18 Optional: to increase the % of viable cells, at this point in the procedure you can perform dead cell removal using the EasySep dead cell removal kit according to the manufacturer's instructions.

19 Examine cells using hemocytometer w/trypan blue. Adjust concentration to 1000 cells / µL for 10X Chromium or 100 cells / µL for DropSeq.