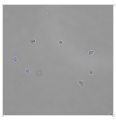

Cell dissociation from airway biopsies with cold-active protease for single-cell RNA-seq

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Human Cell Atlas Method Development Community

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ABSTRACT

This protocol provides details on the cell dissociation that should be performed to obtain single-cell suspensions from airway biopsies.
Biopsies may come from tracheal, bronchial or nasal epithelium.
Cell dissociation is performed at 4°C to avoid gene expression alterations and maximize viability.
The typical cell number recovery is 40 000 cells for one biopsy.
Cell suspensions are suitable for single-cell RNA-sequencing protocols.

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

Storage Conditions of Reagents

Reagent	Storage Condition
HBSS	4°C
20 mM EDTA	room temperature
BSA (Sigma, A8806)	4°C
Protease from *Bacillus Licheniformis* (Sigma, P5380)	Store 100 µL aliquots (100 mg/mL) in DPBS at -80°C
Hoechst 33342 (10 mg/mL)	4°C
NucGreen™ Dead 488 ReadyProbes™ Reagent	room temperature

Required Equipment

Equipment	Supplier	Catalog no.
Countess II FL automated cell counter	Thermo Fisher Scientific	AMQAF1000

The protocol workflow is as follows:

1. Perform airway biopsies in the desired zone
2. Dissociation: mince with scalpel then triturate on ice in dissociation buffer
3. Remove red blood cells if necessary
4. Prepare cells for Chromium/DropSeq

All steps should be performed on ice or at 4°C

Use wide-bore 1 mL pipet tip for all biopsy transfers.

MATERIALS

NAME	CATALOG #	VENDOR
EDTA		
23G Needles	4657667	
Protease from Bacillus Licheniformis	P5380	Sigma
Quick-Read 10 Chamber Slide	3805	Globe Scientific
Countess™ Cell Counting Chamber Slides	C10314	
DPBS no calcium, no magnesium	14190136	Invitrogen - Thermo Fisher
21G needle	BD-305165	VWR international Ltd
HBSS	14060040	Gibco - Thermo Fischer

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Ammonium Chloride Solution 100 mL	7800	Stemcell Technologies
Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL Solution in Water	H3570	Thermo Fisher Scientific
NucGreen™ Dead 488 ReadyProbes™ Reagent	R37109	Thermo Fisher Scientific
Flowmi cell strainer	H13680-0040	

SAFETY WARNINGS

Samples coming from patients with undetermined viral status should be process in cell culture rooms with the appropriate safety level.

BEFORE STARTING

Prepare *Bacillus Licheniformis* enzyme mix just prior to starting dissociation:

Volume (µl)	Reagent	Final concentration
850	DPBS	**1X**
50	**20 mM EDTA**	**0.5 mM**
100	**Protease from *B. Licheniformis* (100 mg/mL)**	**10 mg/mL**

Prepare Inactivation buffer*:*

Make stock of 10% BSA in HBSS and store at -20 °C.
To make HBSS/BSA 2% aliquot 40 mL of HBSS in 50 mL conical and pipet in 10 mL of 10% BSA stock.

Prepare Wash buffer*:*

To make HBSS/BSA 1% aliquot 20 mL of HBSS in 50 mL conical and pipet in 20 mL of HBSS/BSA 2%.

Prepare Resuspension buffer*:*

To make HBSS/BSA 0.05% aliquot 1 mL of HBSS/BSA 2% in 50 mL conical and pipet in 39 mL of HBSS.

Prepare cell staining reagent:

- HBSS: 500 µL
- Hoechst 33342 (10 mg/mL): 1 µL
- NucGreen™ Dead 488 ReadyProbes™ Reagent: 1 drop

1 Perform bronchial biopsy at the desired level of the airways (to be performed by a medical doctor)

EQUIPMENT

Biopsy forceps

Medi-Globe GBF-21-18-120

2 Put the biopsy in 1 mL DPBS in a well of a 6-well plate, observe aspect, and then transfer into 1 mL of ice-cold dissociation buffer in a 1.5 mL eppendorf tube. Use wide-bore 1 mL pipet tip for all biopsy transfers.

1 ml

PREPARATION OF DISSOCIATION MIX (Fresh at each experiment)

Ingredients:

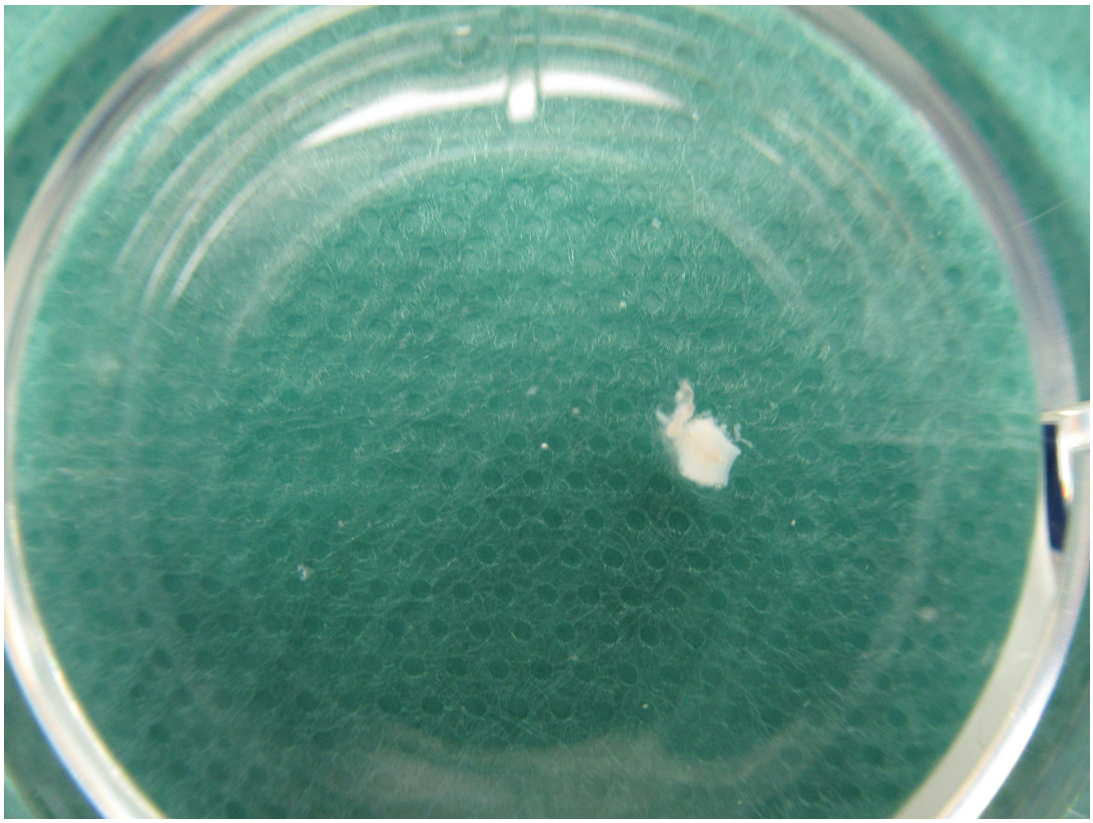
- PBS
- Protease from Bacillus Licheniformis (100 mg/mL stock solution in PBS)
- EDTA 10 mM

For 1 mL of dissociation mix add:

- 850 microlitres of PBS
- 100 microlitres of protease (Final concentration:10 mg/mL)
- 50 microlitres of EDTA (Final concentration: 0.5 mM)

4 °C

EXPECTED RESULT



3 If transportation or storage is necessary: place tube on ice or in polystyrene box containing ice packs. Biopsies can be stored for 60 min in dissociation buffer.

4 Carefully remove biopsy from the dissociation buffer, with a wide-bore 1 mL pipet tip and place in a 100 mm petri dish, taking as little dissociation buffer as possible. Mince with a scalpel equipped of a 10 blade. Drag the biopsy out of the liquid and mince very carefully into the smallest possible pieces. With a wide-bore pipet tip, transfer back the minced biopsy with a small volume of dissociation buffer. Rince the petri dish with dissociation buffer, at the location of biopsy mincing to recover as many cells as possible.

5 Incubate cells on ice for 90 to 120 min after mincing, with gentle trituration with needles 5 times every 5 min. Use needle with decreasing sizes from 21G to 23G.

01:30:00 **Incubation**
00:05:00 **Trituration**
4 °C

6 Inactivate protease by adding 200 µL of Inactivation buffer (HBSS containing 2% BSA)

200 µl **Inactivation buffer**

Prepare Inactivation buffer:

HBSS : 40 mL
10% BSA stock: 10 mL

7 Spin at 400g for 5 min at 4°C

8 Discard supernatant leaving 10 µL of residual liquid on the pellet.

9 Resuspend in 100 µL of wash buffer (HBSS + 1% BSA)

100 µl wash buffer

Prepare Wash buffer:

HBSS : 20 mL
HBSS/BSA 2%: 20 mL

10 Observe cells under an inverted microscope to evaluate red blood cells (RBC) content.
RBC content is better evaluated using an automated cell counter such as Countess, after addition of Hoechst 33342 to an aliquot of the cell suspension to discriminate nucleated cells from non-nucleated cells.

11 If RBC content iis lower than 50%, go directly to step 18.
Perform RBC lysis: add 900 µL of Ammonium Chloride 0.8% to 100 µL of cell suspension (9 volumes).

Ammonium Chloride Solution 100 mL
by Stemcell Technologies
Catalog #: 7800

900 µl Ammonium Chloride 9 vol. for 1 cell vol.

12 Incubate on ice for 10 min.

00:10:00 RBC lysis

4 °C

13 Add 200 µL of Inactivation buffer

200 µl Inactivation buffer

14 Spin at 400g for 5 min at 4°C

15 Discard supernatant leaving 10 µL of residual liquid on the pellet.

16 Resuspend in 100 µL of wash buffer and monitor correct RBC lysis under microscope

100 µl wash buffer

17 Add 1 mL of wash buffer

1 ml wash buffer

18 Spin at 400g for 5 min at 4°C

19 Discard supernatant leaving 10 µL of residual liquid on the pellet.

20 Resuspend in 500 µL of wash buffer

500 µl wash buffer

21 Filter cell suspension through Flowmi cell strainer

Flowmi cell strainer
Catalog #: H13680-0040

https://www.youtube.com/watch?v=taS1BuTnvds

22 Add 500 µL of wash buffer to filtered cells.

500 µl wash buffer

23 Discard supernatant leaving 10 µL of residual liquid on the pellet.

24 Resuspend in 40 µL of resuspension buffer (HBSS + 0.05% BSA).

40 µl Resuspension buffer

Prepare Resuspension buffer:

HBSS : 39 mL
HBSS/BSA 2%: 1 mL

25 Mix 10 µL of cells with 10 µL of cell counting solution (HBSS with 20 µg/mL Hoechst 33342 and NucGreen). Incubate for 1 min at room temperature.

Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL Solution in Water
by Thermo Fisher Scientific
Catalog #: H3570

NucGreen™ Dead 488 ReadyProbes™ Reagent
by Thermo Fisher Scientific
Catalog #: R37109

Preparation of cell staining reagent:

- HBSS: 500 µL
- Hoechst 33342 (10 mg/mL): 1 µL
- NucGreen™ Dead 488 ReadyProbes™ Reagent: 1 drop

00:01:00

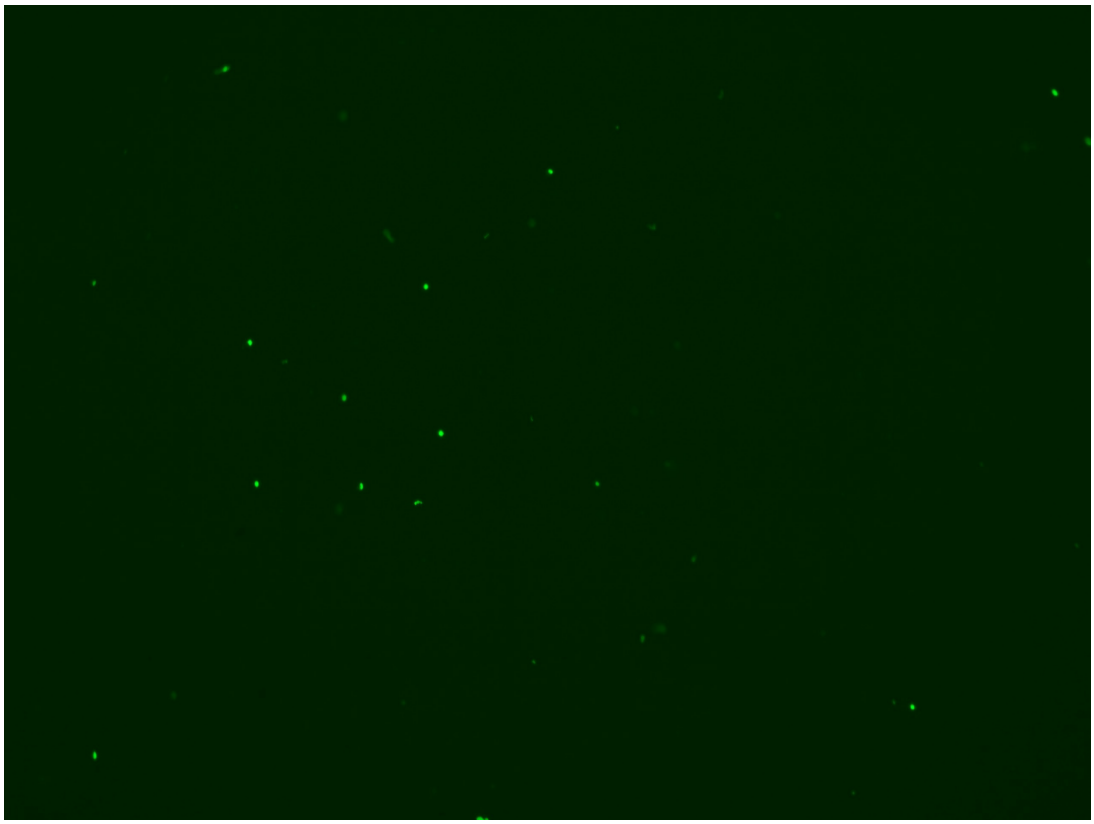
26 Count with Countess automated cell counter using both sides of chambers. Monitor the percentage of nucleated cells (Hoechst +) and dead cells (GFP+).

EQUIPMENT

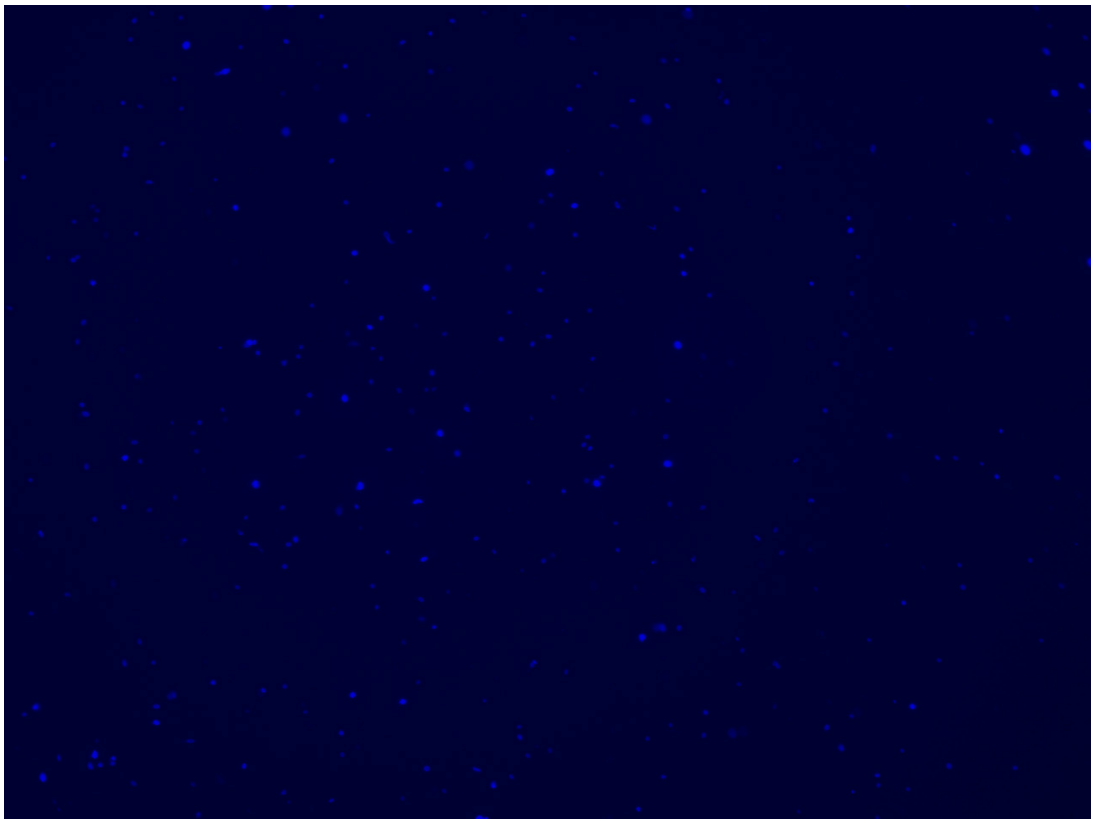
Thermo Fisher Scientific AMQAF1000

Countess™ II FL Automated Cell Counter with Dapi and GFP cubes

EXPECTED RESULT

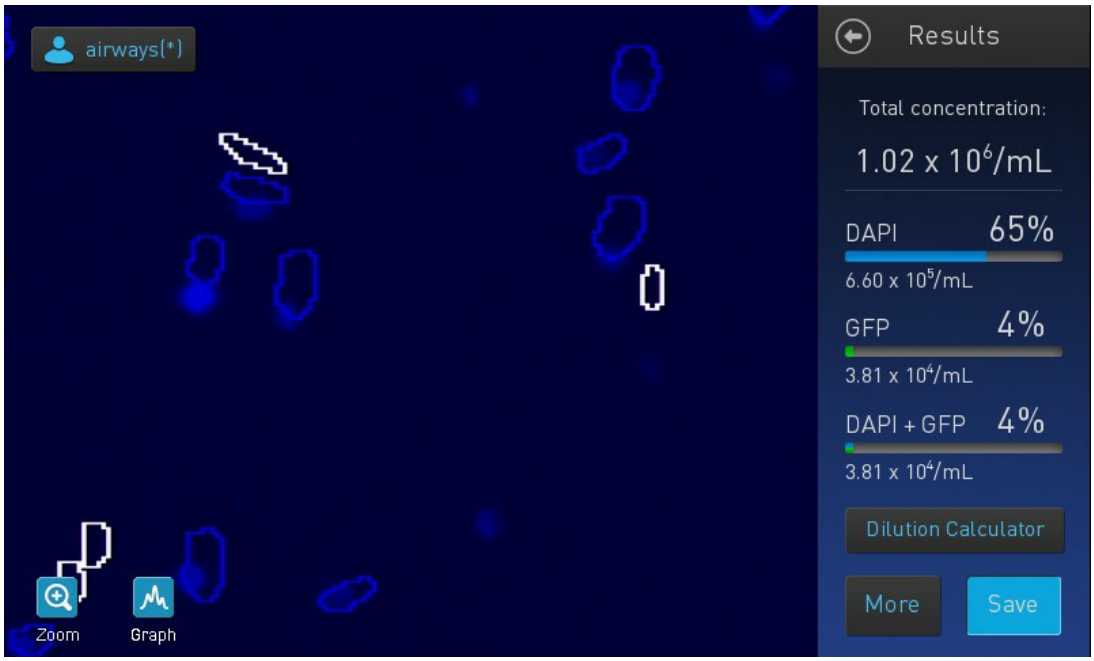


Countess GFP image after NucGreen and Hoescht33342 staining



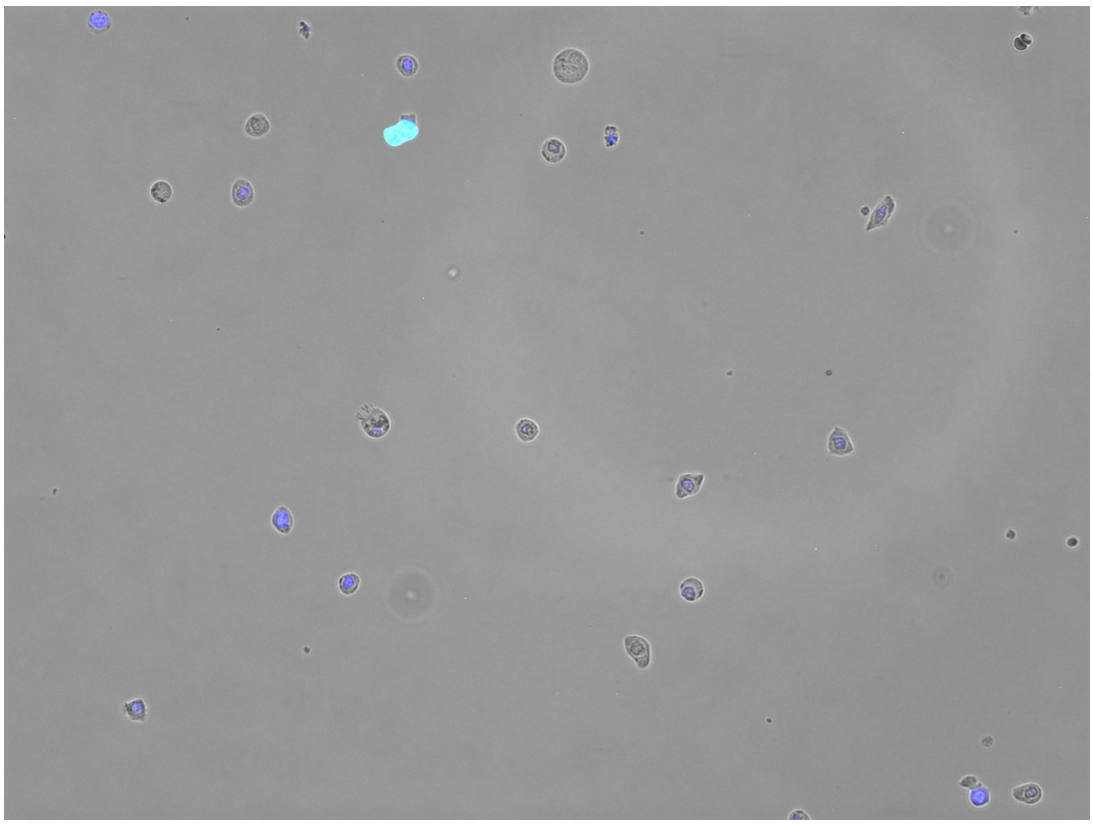
Countess Dapi image after NucGreen and Hoescht33342 staining

Countess report after NucGreen and Hoescht33342 staining



Countess report after NucGreen and Hoescht33342 staining

EXPECTED RESULT



Floid image after NucGreen and Hoescht33342 staining

27 Adjust concentration to a range of 700 to 1000 cells/µL (with wash buffer) for 10X Chromium. Monitor final cell concentration.

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