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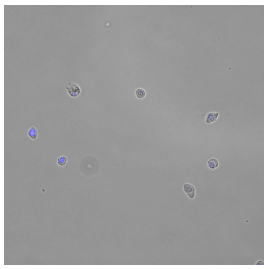
Cell dissociation from airway biopsies with cold-active protease for single-cell RNA-seq



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Laure-Emmanuelle Zaragosi[1], Pascal Barbry[1]

[1]Université Côte d'Azur, CNRS, IPMC, 06560 Valbonne, France

Human Cell Atlas Metho...

Laure-Emmanuelle Zaragosi
Institut de Pharmacologie Moléculaire et Cellulaire

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

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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.

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	Thermo	AMQAF10

	FL automated cell counter	Fisher Scientific	00

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

MATERIALS

- EDTA
- 23G Needles **Catalog #**4657667
- Protease from Bacillus Licheniformis **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P5380
- Quick-Read 10 Chamber Slide **Globe Scientific Catalog #**3805
- Countess™ Cell Counting Chamber Slides **Catalog #**C10314
- DPBS no calcium, no magnesium **Invitrogen - Thermo Fisher Catalog #**14190136
- 21G needle **VWR International (Avantor) Catalog #**BD-305165
- HBSS **Gibco - Thermo Fisher Scientific Catalog #**14060040

STEP MATERIALS

- Quick-Read 10 Chamber Slide **Globe Scientific Catalog #**3805
- Ammonium Chloride Solution 100 mL **STEMCELL Technologies Inc. Catalog #**7800
- Flowmi cell strainer **Catalog #**H13680-0040
- Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL Solution in Water **Thermo Fisher Scientific Catalog #**H3570
- NucGreen™ Dead 488 ReadyProbes™ Reagent **Thermo Fisher Scientific Catalog #**R37109

Protocol materials

- 23G Needles **Catalog #**4657667
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- Flowmi cell strainer **Catalog #**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 start

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	NAME
Medi-Globe	BRAND
GBF-21-18-120	SKU

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

Note

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

Note

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

Note

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 **STEMCELL Technologies Inc. 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

Note

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 **Thermo Fisher Scientific Catalog #**H3570

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

Note

Preparation of cell staining reagent:

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

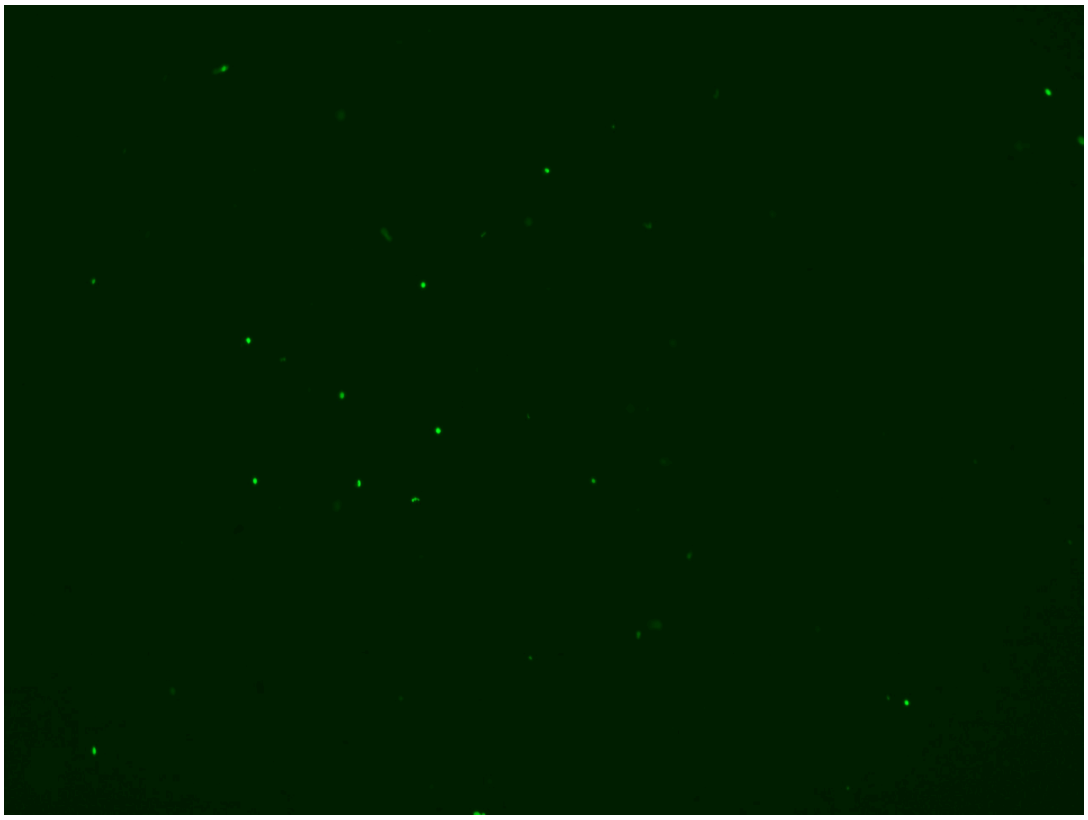
00:01:00

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

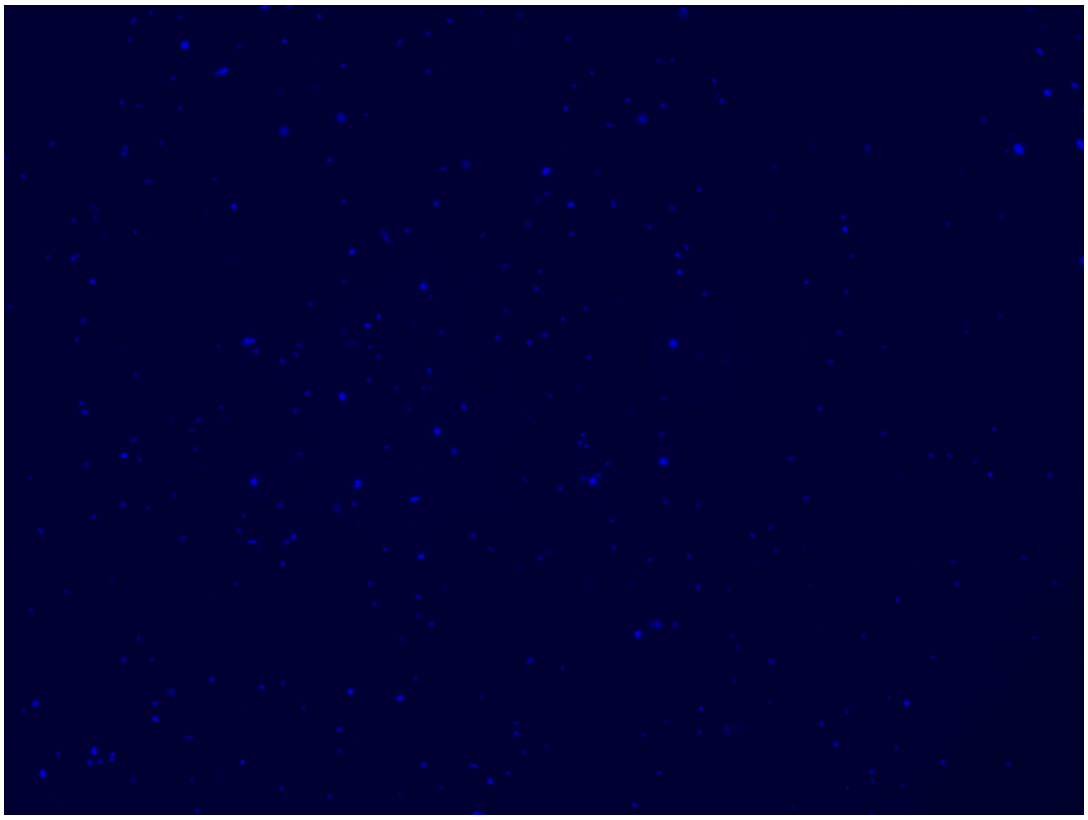
Equipment

new equipment	NAME
Thermo Fisher Scientific	BRAND
AMQAF1000	SKU
Countess™ II FL Automated Cell Counter with Dapi and GFP cubes	SPECIFICATIONS

Expected result

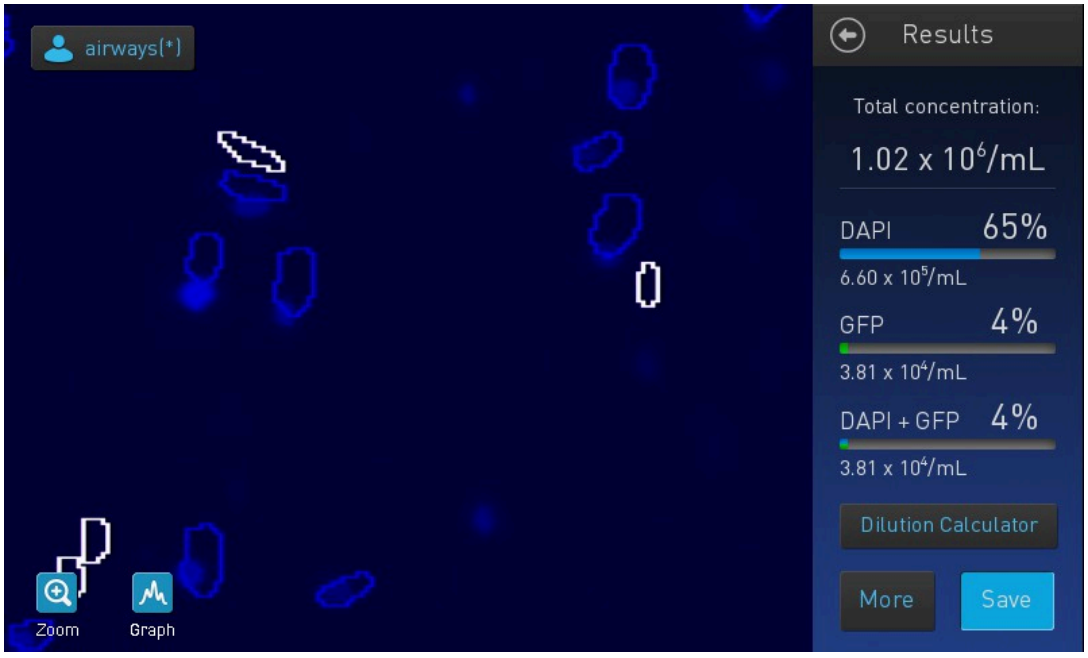


Countess GFP image after NucGreen and Hoescht33342 staining



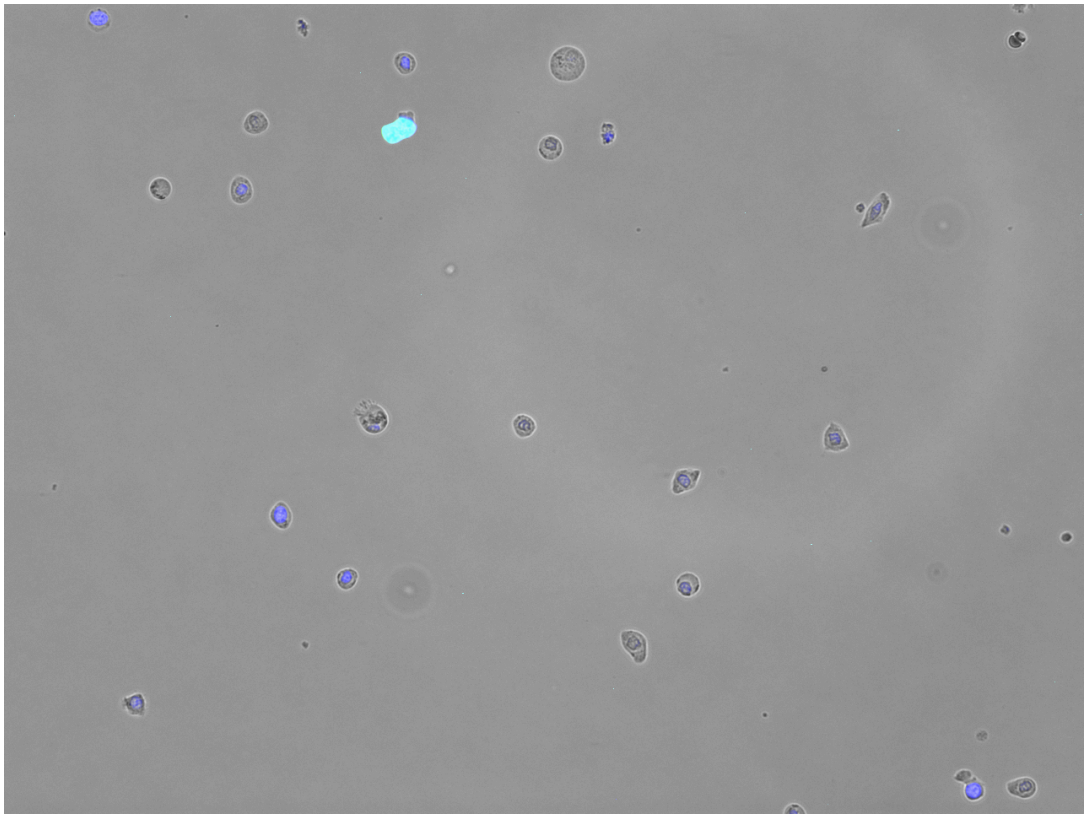
Countess Dapi image after NucGreen and Hoescht33342 staining

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.