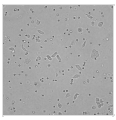

Cell dissociation from nasal and bronchial brushings with cold-active protease for single-cell RNA-seq

Version 2

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Human Cell Atlas Method Development Community

Laure-Emmanuelle Zaragosi

ABSTRACT

This protocol provides details on the cell dissociation that should be performed to obtain single-cell suspensions from nasal epithelium brushings.
Cell dissociation is performed at 4°C to avoid gene expression alterations and maximize viability.
The typical cell number recovery is 200 000 - 300 000 for one brushing.
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
Hypothermosol	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 brushing of the epithelium of the nasal cavity
2. Dissociation: triturate on ice or store on ice without trituration
3. Remove red blood cells if necessary
4. Prepare cells for Chromium/DropSeq

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

MATERIALS

NAME	CATALOG #	VENDOR
EDTA		
23G Needles	4657667	
Protease from Bacillus Licheniformis	P5380	Sigma
HypoThermosol® FRS 100 mL	7935	Stemcell Technologies
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
Quick-Read 10 Chamber Slide	3805	Globe Scientific
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	**Hypothermosol**	**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 nasal brush biopsy in the nasal cavity in the inferior nasal conca zone (UBERON_0005922) (to be performed by a medical doctor)

EQUIPMENT

Medi-Globe GCB-02-18-120

2.0 mm cytology brush

2 Cut the cytology brush and place it in a 5 mL eppendorf tube containing 1 mL of ice-cold dissociation buffer.

1 ml

PREPARATION OF DISSOCIATION MIX (Fresh at each experiment)

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

For 1 mL of dissociation mix add:
- 850 microlitres of Hypothermosol
- 100 microlitres of protease (Final concentration:10 mg/mL)
- 50 microlitres of EDTA (Final concentration: 0.5 mM)

4 °C

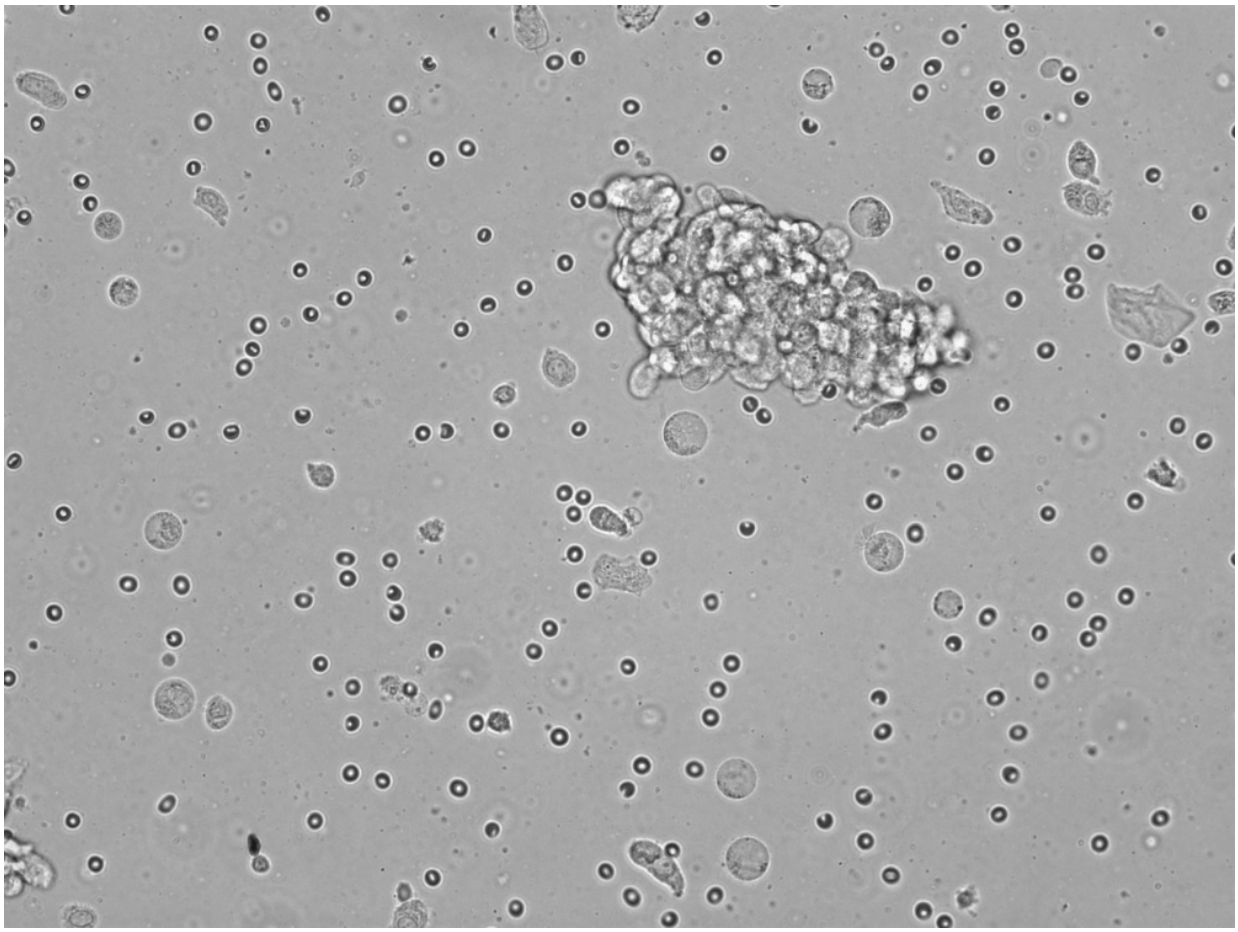
3 If cells are processed directly after brushing, use DPBS instead of hypothermosol and go directly to step 4.
If transportation or storage is necessary: place tube on ice or in polystyrene box containing ice packs. Brushings can be stored for 60 min in dissociation buffer.

4 Shake the brush into the buffer to put cells in suspension then spin the tube for 2 min at 150g to remove residual cells from the brush.

5 Discard the cytology brush and observe cells under an inverted microscope.

Quick-Read 10 Chamber Slide
by Globe Scientific
Catalog #: 3805

EXPECTED RESULT

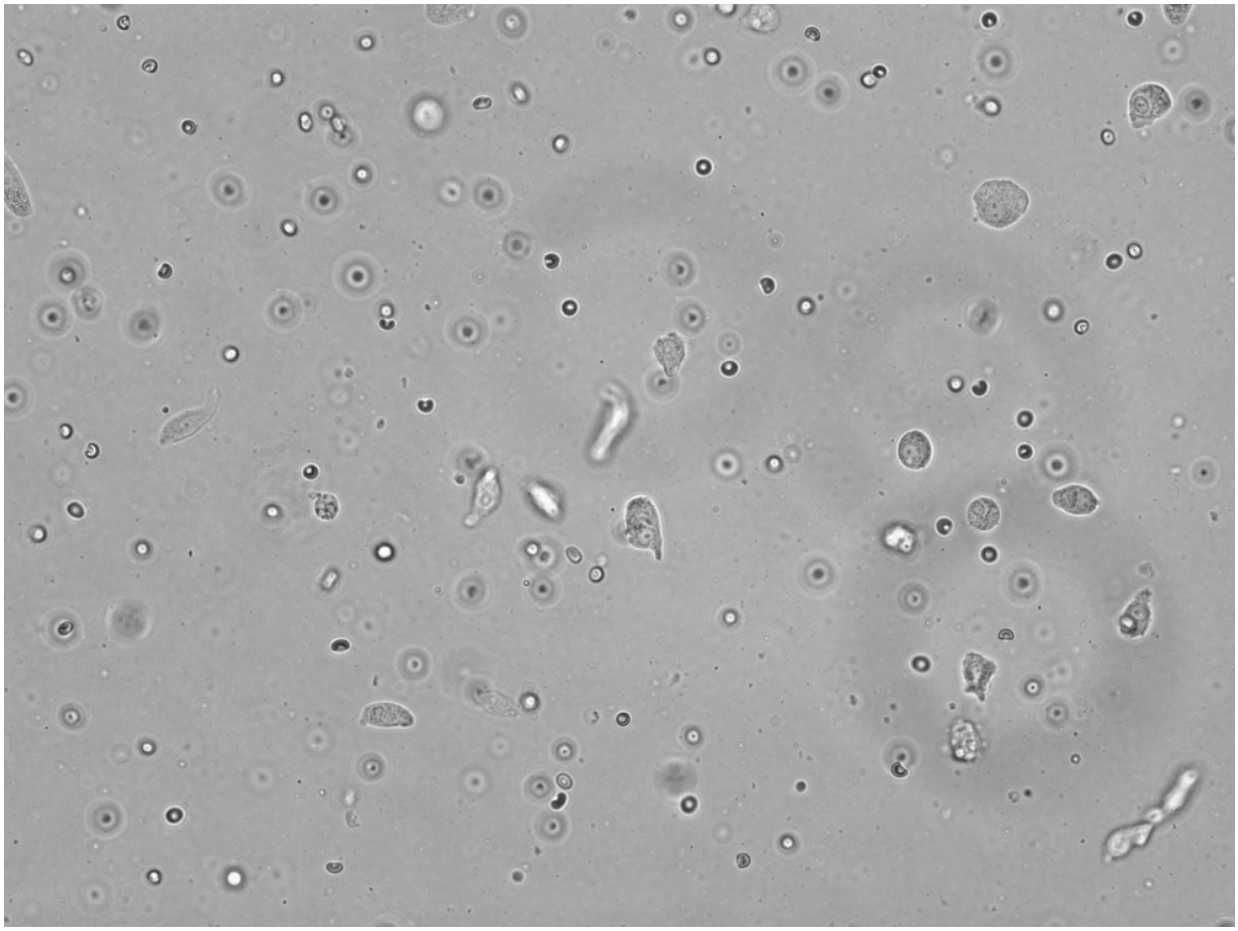


6 Incubate cells on ice for 30 min, with gentle trituration with needles 5 times every 5 min. Use needle with decreasing sizes from 21G to 23G. If storage and/or transportation has been performed for at least 60 min, skip the incubation step, only perform trituration steps.

00:30:00 Incubation

00:05:00 Trituration

EXPECTED RESULT



4 °C

7 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

8 Spin at 400g for 5 min at 4°C

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

10 Resuspend in 200 µL of wash buffer (HBSS + 1% BSA)

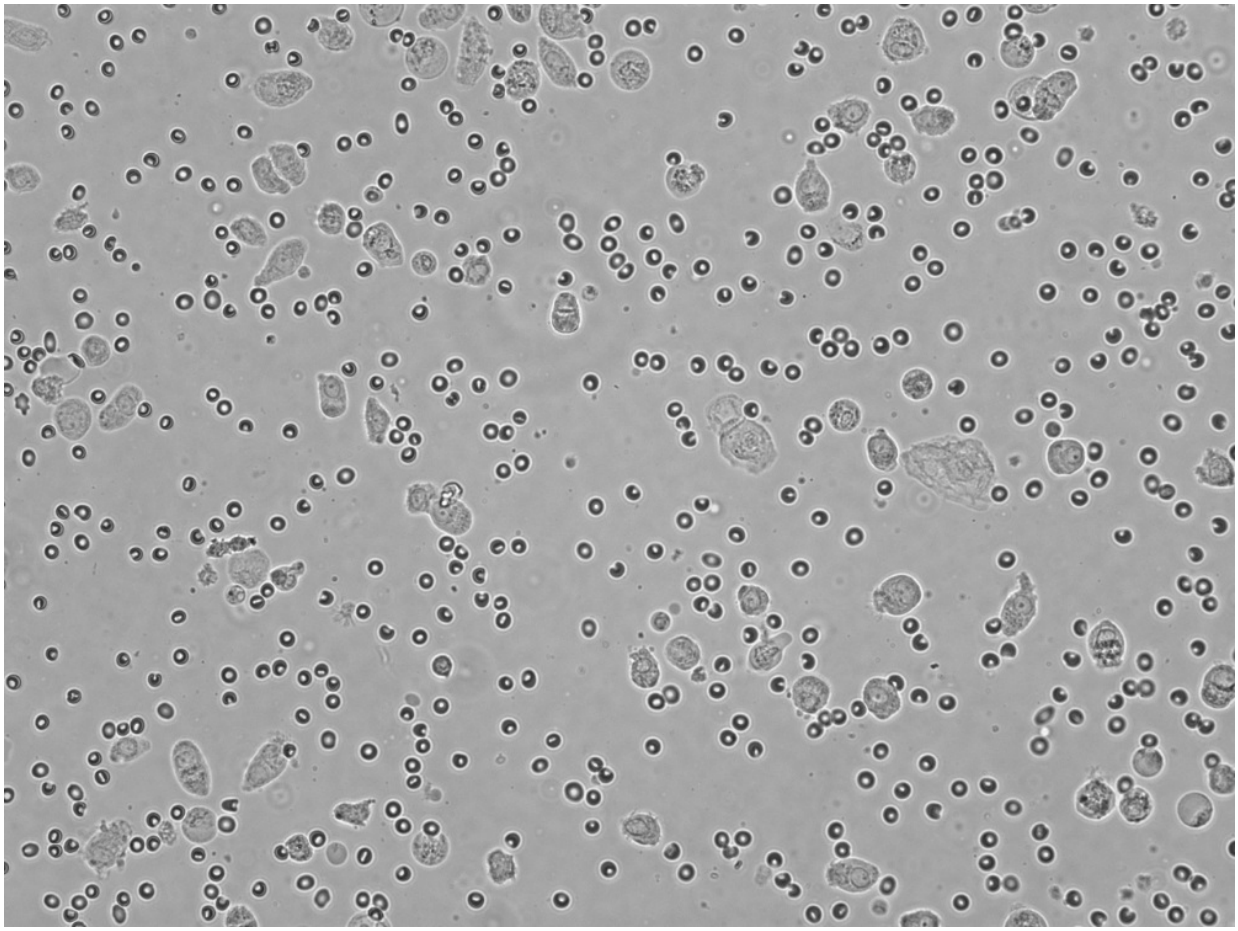
Prepare Wash buffer:

HBSS : 20 mL

HBSS/BSA 2%: 20 mL

11 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.

EXPECTED RESULT



12 If RBC content iis lower than 50%, go directly to step 18.
Perform RBC lysis: add 1.8 mL of Ammonium Chloride 0.8% to 200 µL of cell suspension (9 volumes), transfer in a 5 mL eppendorf.

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

2.25 ml Ammonium Chloride 9 vol. for 1 cell vol.

13 Incubate on ice for 10 min.

00:10:00 RBC lysis

4 °C

Add 400 µL of Inactivation buffer

14 2 ml **Inactivation buffer**

15 Spin at 400g for 5 min at 4°C

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

17 Resuspend in 250 µL of wash buffer and monitor correct RBC lysis

250 µl **wash buffer**

18 Add 1 mL of wash buffer

1 ml **wash buffer**

19 Spin at 400g for 5 min at 4°C

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

21 Resuspend in 500 µL of wash buffer

500 µl **wash buffer**

22 Filter cell suspension through Flowmi cell strainer

Flowmi cell strainer
Catalog #: H13680-0040

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

23 Add 500 µL of wash buffer to filtered cells.

500 µl **wash buffer**

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

25 Resuspend in 50 µL of resuspension buffer (HBSS + 0.05% BSA).

50 µl **Resuspension buffer**

Prepare Resuspension buffer:

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

26 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

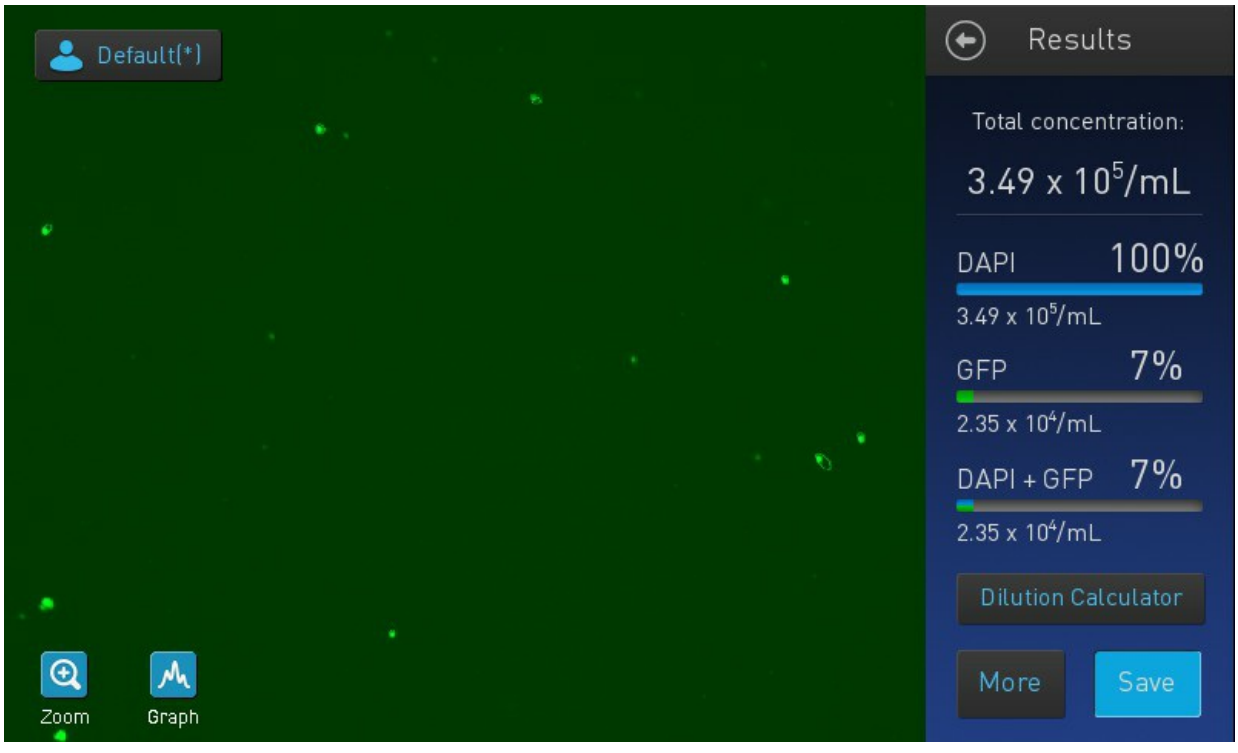
27 Count with Countess automated cell counter using both sides of chambers. Monitor the percentage of nucleated cells (Hoechst +) and dead cells (GFP+).

EQUIPMENT

Thermo Fisher Scientific AMQAF1000

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

EXPECTED RESULT

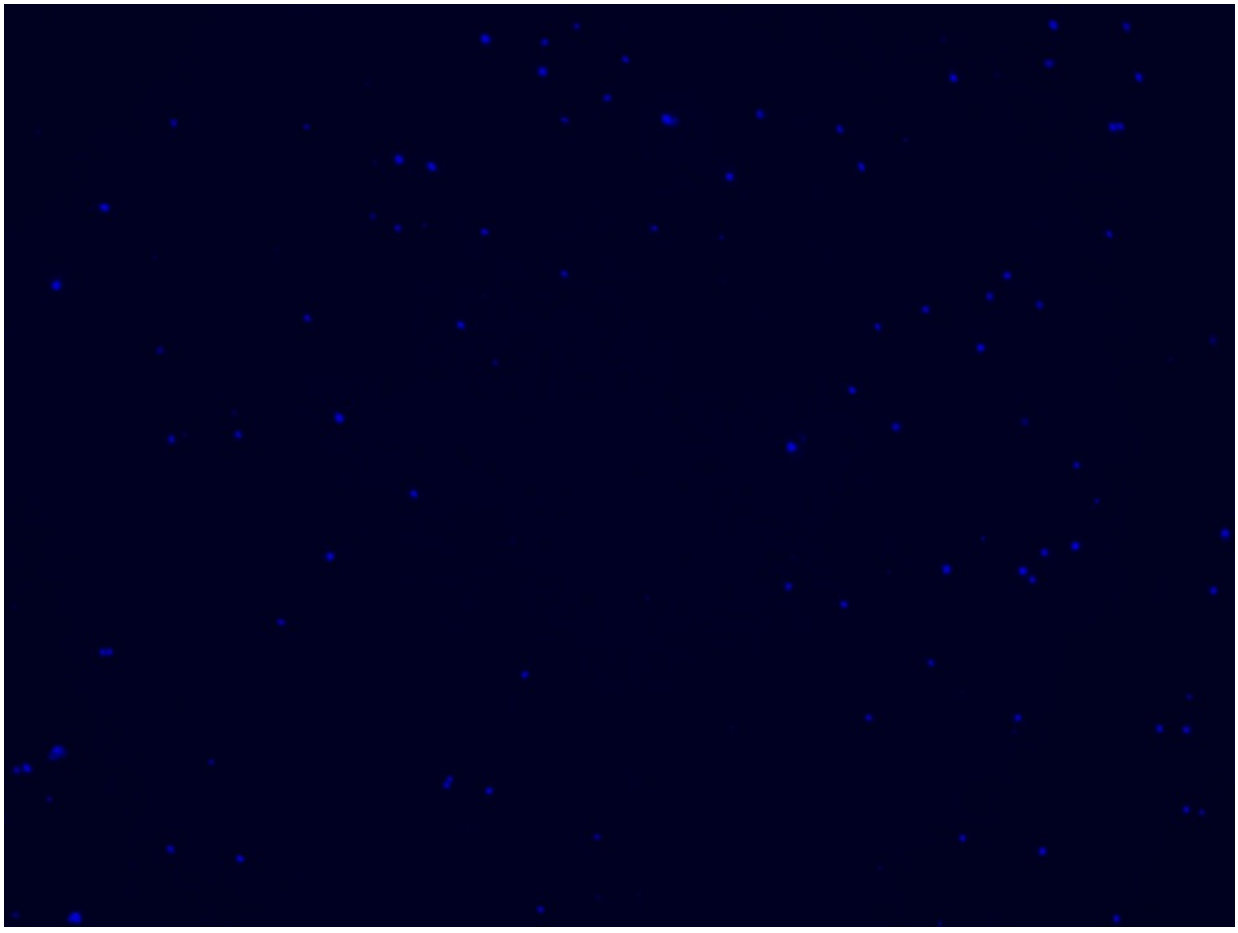


Countess report after NucGreen and Hoescht33342 staining

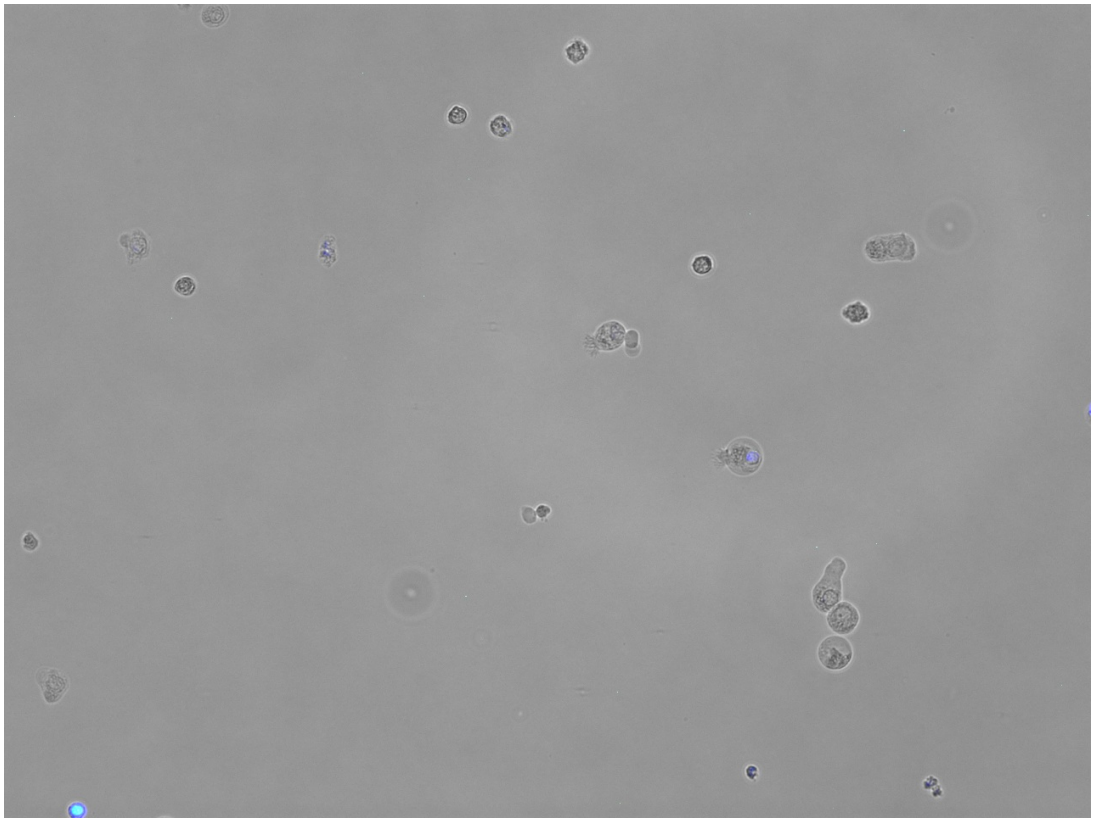
EXPECTED RESULT

Countess bright field image after NucGreen and Hoescht33342 staining

EXPECTED RESULT



Countess Dapi image after NucGreen and Hoescht33342 staining



Floid microscope image after NucGreen and Hoescht33342 staining

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