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Frozen tissue dissociation for single-nucleus RNA-Seq

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1 Works for me This protocol is published without a DOI.

Human Cell Atlas Method Development Community NCIHTAN

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ABSTRACT

The protocol described here relies on mechanical dissociation of frozen lung tissue (human or mouse), filtering and washing the nuclei suspension in SCC buffer followed by the FACS sorting. The resulting nuclei suspension can be processed on scRNA-Seq platform of choice, 10X Chromium, inDrops or other. The protocol has been validated on frozen human tissues of lung, breast pancreas and kidney as well as frozen mouse tissues of brain, lung, pancreas.

PROTOCOL CITATION

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KEYWORDS

nuc-seq, scRNA-Seq, snRNA-Seq, frozen tissue, lung

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25981

MATERIALS

NAME	CATALOG #	VENDOR
Nuclei EZ lysis buffer	EZ PREP NUC-101	Sigma
Dounce homogenizers	D8938-1SET	Sigma
Ambion RNase Inhibitior 40 U/µl	AM2682	Ambion
DEPC (Diethyl pyrocarbonate)	D5758-25ML	Sigma Aldrich
Hemocytometer	A25750	Thermo Fisher Scientific
CorningTM FalconTM Test Tube with Cell Strainer Snap Cap	352235	Thermo Fisher Scientific
Plastic petri dish 35 x 10 mm style	353001	Falcon
Protein LoBind tubes	022431081	Eppendorf
Disposable Scalpel	10148-882	Technocut
Ambion RNase Inhibitor 40U/uL	AM2682	Ambion
DEPC-treated water	AM9915G	Ambion
DNAse/RNAse free distilled water	10977023	Invitrogen
10% (w/v) BSA filtered through 0.2 µm membrane	A7906-100G	Sigma

NAME	CATALOG #	VENDOR
DAPI (46-Diamidino-2-Phenylindole Dihydrochloride) at 1 mg/1 mL	D1306	Invitrogen
Trypan Blue Solution 0.4%	15250061	
SSC (20X) RNase-free	AM9770	Invitrogen
DRNA Free Reagent Spray	UX-04397-24	
1M DTT	43816-10ML	Sigma
70% Ethanol spray		
Sucrose	S7903-250G	Sigma – Aldrich
Citric acid	251275-100G	Sigma – Aldrich

MATERIALS TEXT

Swinging bucket cooling centrifuge
Centrifuge
SorvallTM LegendTM X1 Centrifuge Series n/a

Countess II
Life Technologies AMQAX1000

10% (w/v) BSA: Prepare 10% BSA solution and filter through 0.22 µm membrane (PTFE). Store at -20 ºC.

Nuclei Lysis buffer:

Reagent	Volume, µl	Final concentration
2 M Sucrose	125	250 mM
250 mM Citric Acid	200	50 mM
DEPC treated water	675	- - -

Total volume 1.0 ml

Nuclei Wash buffer:

Reagent	Volume, µl	Final concentration
2 M Sucrose	125	250 mM
250 mM Citric Acid	200	50 mM
10% (w/v) BSA	10	1% (w/v)
1M DTT	20	20 mM
Ambion RNAse Inhibitor, 40U/ul	5	0.2 U/ul
DEPC treated water	640	- - -

Total volume 1.0 ml

Nuclei Resuspension buffer:

Reagent	Volume, µl	Final concentration
6X SCC	1500	3X

1M DTT	60	20 mM
10% (w/v) BSA	30	1% (w/v)
Ambion RNAse Inhibitor, 40U/ul	15	0.2
DEPC treated water	1395	- - -

Total volume 7.0 ml

EQUIPMENT

NAME	CATALOG #	VENDOR
Swinging bucket cooling centrifuge	n/a	Thermo Fisher Scientific
Countess II	AMQAX1000	Thermo Fisher Scientific

BEFORE STARTING

Evaluate RNA integrity

Before you start make sure that the frozen tissue has RIN value higher than 6.0

Tissue grinding 15m

1 **Prepare the laminar hood and the grinding glassware:** 3m

- Clean the surface and glassware thoroughly with DRNAse Free Reagent Spray followed by 70% ethanol.
- Bring the ice bucket in the cell culture hood and spray it with DRNAse Free Reagent.
- Place fully assembled Dounce homogenizer on ice and to let it cool.
- Bring sterile blade in the hood.

2 **Prepare a frozen tissue:** 3m

- On a dry ice, place a frozen tissue on a Petri dish and cut it into two or three smaller pieces (e.g. the size of which corresponds to a half-rice grain).

3 **Prepare Lysis buffer:** 1m

- Transfer **600 µl Lysis Buffer** in 1.5 mL tube, add **1.0 µl** of DEPC and vortex vigorously until solution becomes cloudy and homogenous.

> DEPC is not soluble in water and it will form layer of droplets on top. Therefore, mix vigorously to disperse DEPC droplets.

- Transfer ready-to-use lysis solution in 2 mL grinding glass tube.

4 **Grind the tissue:** 5m

- Place the small piece(s) of frozen tissue in 2 mL grinding glass tube.
- Using a large clearance pestle (Tube A) for the initial sample grinding gently move pestle up and down 10-15 times.

> Do not remove pestle out of the liquid while grinding the tissue, avoid creating bubbles, which may form if pestle is being moved too quickly.

- Next, use a small clearance pestle (Tube B) and grind tissue further by movinig up and down for 10-15 times.
- Tissue should be completely minced and homogenized.

Certain tissue types might be elastic and hard to break down completely. In such case move to next step.

- Keep the sample in a glass tube on ice.

5 **Record experimental details:** 2m

- Write down if something unusual happened, tissue did not dissociate, etc.

Straining/washing of single-nuclei suspension 25m

6 **IMPORTANT**: keep nuclei suspension cool at all times.

NOTE: For centrifugation use a swinging bucket centrifugure and 1.5 ml (or 2.0 ml) Protein LoBind Eppendorf tubes placed inside the 50 mL Falcon tube.

7 **Straining:** 2m

- Place 5 mL Falcon Round-bottom tube (typically used for FACS) with blue Snap Cap strainer on ice.
- Strain the nuclei suspension through 35 µm Cell Strainer Snap Cap and collect flow through fraction into the tube.

If it is difficult to aspirate the nuclei suspension by pipette another option is to pour all suspension out of 2 mL homogenizer tube directly onto the blue cap. However, care must be taken to not introduce any ice that might be stuck on the outside walls of a homogenizer tube.

- Transfer the filtered solution to 1.5 mL Protein LoBind Eppendorf tube, keep on ice.

8 **Centrifugation:** 7m

- Spin down the dissociated tissue suspension in a swinging bucket centrifuge at **500 x g, 4°C 00:05:00** .
- There should be a clearly visible pellet. If you don't see one, check the settings on your centrifuge.
- Remove and save supernatant from the centrifuged nuclei pellet. Do not disturb the pellet, leave ~ 10-20 uL of supernatant on top.

9 **Resuspension:** 2m

- Re-suspend the pellet in **1 mL Nuclei Wash Buffer** using 1000 µL pipette.

10 **Centrifugation:** 7m

- Spin down the tube in a swinging bucket centrifuge at **500 x g, 4°C 00:05:00** .
- Remove the supernatant without disrupting the pellet, the size of which should be smaller than in the previous step.

11 **Resuspension and straining:** 2m

- Re-suspend the nuclei pellet in **1 mL Nuclei Wash Buffer** , mix well using 1000 µL pipette until suspension becomes homogenious. The volume of buffer at this step can be adjusted as needed (e.g. 0.5 ml).
- Filter the nuclei suspension through 5 mL FACS Snap Cap strainer and collect into the tube.

12 **Nuclei evaluation under BF/FL microscope:** 5m

- Mix **10 µl nuclei suspension** with **0.2 µl 100X DAPI dye** and **10 µl 0.4% Trypan Blue dye** .
- Inspect nuclei suspension under the bright-field and fluorescence microscope. Record the images (**Figure 1**).
- Approximately 10^6- 10^7 nuclei per 1 ml should be expected.

When evaluating sample under the bright field there should be a lot of dark debris visible (Figure 1) making it hard to count the species. However, i the blue (DAPI) channel the nuclei should be clearly visible.

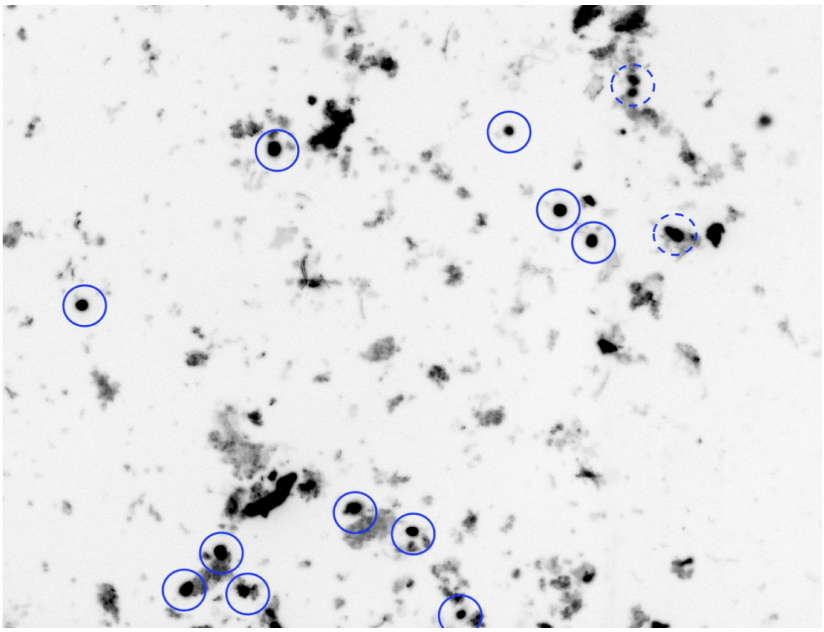

Figure 1. Bright field microscopy image of nuclei suspension before FACS (blue circles indicate individual nuclei).

FACS 25m

13 **Prepare the unstained nuclei sample:** 1m

- Take **50 µl nuclei suspension** and transfer to another 5 mL Falcon tube.
- Add **250 µl Nuclei Resuspension Buffer** .
- Label the tube as “reference” which will be used as a unstained population reference for FACS.
- Keep on ice

14 **Prepare the DAPI-stained nuclei sample:** 1m

- Take remaining, **950 µl nuclei suspension** and stain with **10 µl 100X DAPI dye** by mixing the suspension with 1 ml pipette.
- Keep on ice

15 **Prepare the FACS collection tube:** 1m

Add **0.2 mL Nuclei Resuspension Buffer** into 5 mL Falcon tube and swirl buffer all around to wet the inner walls.

16 **Sort DAPI positive nuclei on FACS** 20m

- Run the "reference" sample (from Step 13) and record the scatter plot.
- Run DAPI-positive nuclei suspenion (from Step 14), indetify the fluorescent population and record the scatter plot.
- Using the collection tube preapred in Step 15, sort the DAPI-postiive population into the collection tube

- The example of sorting gates (P4) is provided below:

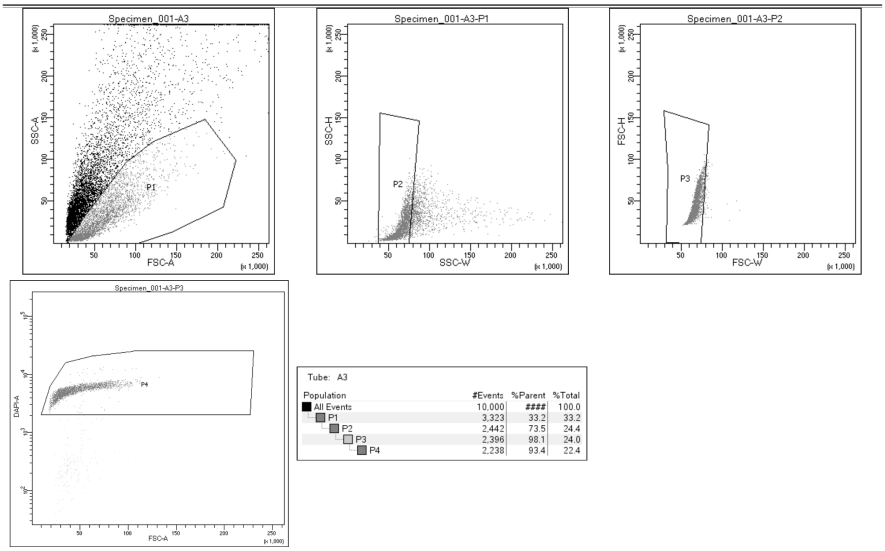

Figure 2. FACS plots and sorting-gates (P4) of DAPI stained nuclei suspension.

Post-FACS 10m

17 Concentrate the nuclei

- Concentrate nuclei by transferring FACS sorted suspension in 1.5 mL Protein LoBind tube and spinning in a swinging bucket centrifuge at **600 x g, 4°C 00:05:00** .

Using FACS counts aim to reach a dilution of 2000 nuclei/ul.

18 Count the nuclei

- After centrifugation carefully aspirate the supernatant leaving the desirable amount of suspension (e.g. 200 µl)
- Carefully disperse the nuclei by mixing suspension with a 200 µl pipette.
- Mixi **10 µl Nuclei Suspension** with **0.2 µl 100X DAPI dye** and **10 µl 0.4% Trypan Blue dye** and count the nuclei on Countess II instrument.
- Expected results are shown in Figure 3 and Figure 4.

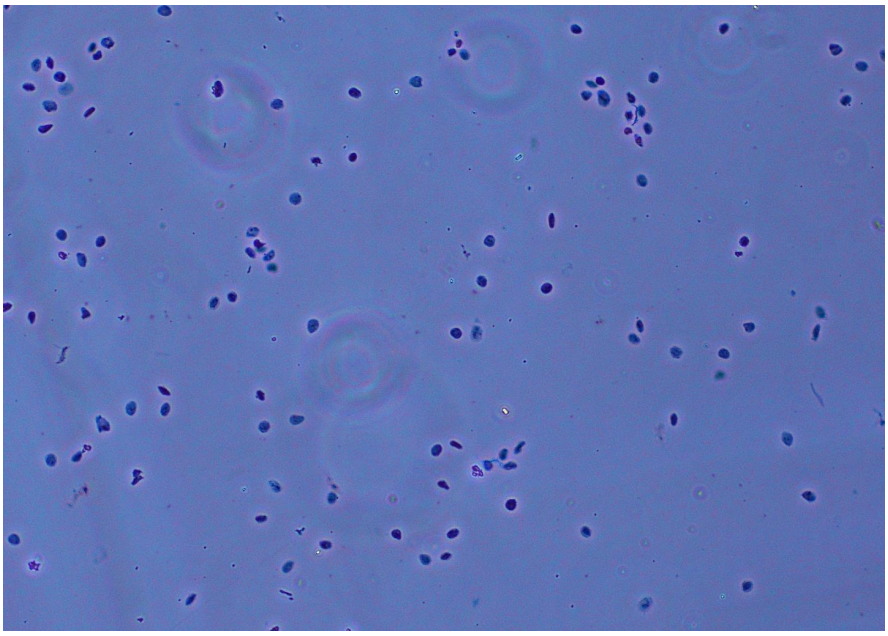

Figure 3. Bright field microscopy image of lung nuclei suspension obtained after frozen tissue dissociation and FACS.

Single nucleus RNA-Seq

19 Control experiment:

- If frozen tissue dissociation is being conducted for the first time perform bulk cDNA synthesis and evaluate the cDNA yields and profile.
- Otherwise, proceed to the reverse transcription reaction using the platform and method of your choice.

20 Barcoding, library preparation and sequencing

- Follow *10X genomics* protocol when using Chromium instrument 10X genomics protocol (v3.1 Chemistry)
- Follow *inDrops* protocol when using home-built inDrops plaform inDrops protocol

Sequencing

21 Sequence the DNA library

We use NovaSeq 6000 instrument to sequence the final DNA libraries using pair-end sequencing option, R1 read - 26 cycles, R2 read - 70 cycles, and index read - 8 cycles, aiming for ~100 million reads per ~5,000 single-nuclei.

FASQ files were processed using SEQC pipeline (Azizi et al., Cell, 2017) and mapped to reference genome with the default SEQC parameters to obtain the gene-cell count matrix.

Frozen Human Lung - SCLC

Small cell lung carcinoma (Human, Frozen)

Overall Statistics

# Reads:	159940090
% of uniquely mapped reads:	89.31%
% of multi-mapped reads:	5.31%
% of unmapped reads:	5.10%
% of filtered reads mapping to genome:	20.60%
Sequencing saturation rate:	93.21%
# Nuclei	2659
Median molecules per nucleus:	1966
Average reads per nucleus:	43221
Average reads per molecule:	14.73
% of cells filtered by high mt-RNA content:	4.55%

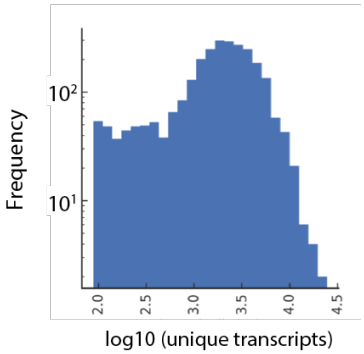

Figure 4. snRNA-Seq of previously frozen human small cells lung carcinoma sample.

Frozen Human Lung - cSCLC

Combined small cell lung carcinoma (Human, Primary Tissue, Frozen)

Overall Statistics

# Reads:	214898910
% of uniquely mapped reads:	83.20%
% of multi-mapped reads:	4.60%
% of unmapped reads:	11.85%
% of filtered reads mapping to genome:	27.45%
Sequencing saturation rate:	68.37%
# Nuclei	8598
Median molecules per nucleus:	3937
Average reads per nucleus:	15148
Average reads per molecule:	3.16
% of cells filtered by high mt-RNA content:	0.87%

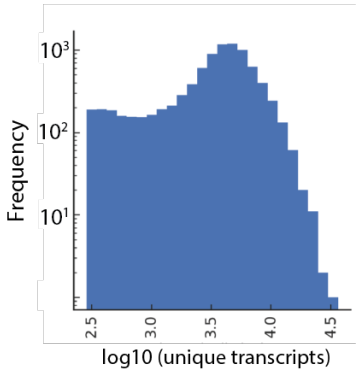

Figure 5. snRNA-Seq of previously frozen combined small cells lung carcinoma sample.