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Working

'Frankenstein' protocol for nuclei isolation from fresh and frozen tissue for snRNAseq

Version 2

Luciano Martelotto[1]

[1]University of Melbourne

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Human Cell Atlas Method Development Community

Luciano Martelotto
University of Melbourne

ABSTRACT

This protocol is the result of the combination of various nuclei isolation protocols for single cell RNA-seq experiments using droplet-based methods, hence the name Frankenstein. Developed to prepare nuclei isolates from small sample sizes (as little as a grain of rice), this protocol uses FACS to identify cell subpopulations based on ploidy (e.g. tumor versus stroma), to ensure that nuclei suspensions are not clumped, and to remove any debris, especially ambient RNA, to help reduce background. The reference protocols can be found in the following papers: Hu, et al., Habib, et al. (2016), Habib, et al. (2017), Lake, et al., and Lacar, et al.

This protocol is routinely used in the single-cell innovation lab for single nuclei experiments using 10x Genomics technologies.

The protocol has been demonstrated to work successfully with fresh, snap/flash frozen, cryopreserved cells, and cell lines, as well as various solid cancers: pancreas, pheochromocytomas, paragangliomas, breast cancer, lymphoma, xenografts and tumors.

EXTERNAL LINK

https://research.unimelb.edu.au/centre-for-cancer-research/our-research/single-cell-innovation-lab

PDF

___Frankenstein____protocol_for_nuclei_isolation_from_fresh_and_frozen_tissue_FINAL.pdf

GUIDELINES

This protocol requires access to a cell sorter and familiarity with sorting cells/nuclei into 96-well plates.

1. Use a plastic pestle to mechanically homogenize tissue and release nuclei
2. Separate the nuclei from debris using a cell sorter
3. Collect a specific number of nuclei in a 96-well plate containing 10x RT Buffer*
4. Immediately load the sample into a Single Cell Chip for processing according the Single Cell 3' Reagents User Guide or Single Cell V(D)J 5' Reagents User Guide

*Assume that nuclei recovery is 57%, and use this to determine the number of nuclei to collect for each of your samples. (This value is derived from the Cell Suspension Volume Calculator Table in the Single Cell 3' Reagents User Guide or Single Cell V(D)J 5' Reagents User Guide)

The protocol did not work with cardiomyocytes due to the high level of myosin heavy chain (MHC).

10x Genomics Products

Chromium Single Cell Immune Profiling Solution - https://www.10xgenomics.com/solutions/vdj/

Chromium Single Cell Gene Expression Solution - https://www.10xgenomics.com/single-cell/

MATERIALS

NAME	CATALOG #	VENDOR
Nuclei EZ lysis buffer	EZ PREP NUC-101	Sigma

MATERIALS TEXT

Nuclei wash and resuspension buffer (prepare chilled, 4°C)

1x PBS
1.0% BSA
0.2 U/µl RNase Inhibitor

Nuclei wash and resuspension buffer with DAPI (prepare chilled, 4°C)

1x PBS
1.0% BSA
0.2 U/µl RNase Inhibitor
10 µg/mL DAPI

10x RT Buffer[1] for Single Cell Gene Expression 3' reagents (DO NOT add RT enzyme)

RT Reagent Mix	50 µL
RT primer	3.8 µL
Additive A	2.4µL
H20	(31.7 - X - Y) µL

10x RT Buffer[1] for Single Cell Immune Profiling 5' reagents (DO NOT add RT enzyme)

RT Reagent Mix	50 µL
RT primer	5.9 µL
Additive A	2.4 µL
H20	(31.7 - X - Y) µL

[1]RT Buffer Notes

- X ('sorting volume'): In the cytometric analysis setup described in this protocol, each droplet is 1 nL. Example: 10,000 nuclei = 10,000 nL = 10 µL 'sorting volume'.
- Y ('additional volume'): This accounts for any additional volume deposited by the flow cytometer nozzle. In the cytometric analysis setup described in this protocol (i.e. 75 µm nozzle) there is no additional volume deposited by the nozzle, so Y = 0 If in doubt, or to be on the safe side, just make Y= 5-10 µL.
- The 1 nuclei/nL assumption was corroborated empirically by sorting 10,000 nuclei in ten wells containing 70 µL PBS and then measuring the final volume post sorting. It is highly recommended to determine X empirically as value may vary depending on different sorters/nozzle combinations. It is recommended to determine it at least once.
- Always measure the volume after sorting and top up to 90 µL with PBS or H_2O if required.

- After adding the RT Enzyme Mix the final volume will be 100 µL.
- It is crucial to work as fast as possible. Do not leave nuclei sitting on ice for too long (e.g. 30' is too long).
- Reduce as much as possible the time from sorting-to-controller run, ideally keep it under 40 minutes. The longer the time the higher the background will be.

SAFETY WARNINGS

See SDS (Safety Data Sheet) for safety warnings and hazards.

BEFORE STARTING

All samples and reagents are kept on ice or at 4 °C (wet ice).

Prepare all buffers and reagents as described in the "Materials" section.

Tissue Homogenization

1 Mince/chop tissue with a razor blade to small pieces. The tissue may be as small as a grain of rice.

For mincing the tissue, you may take the tube out of ice, however, be quick and return to ice.

2 Add **500 µl** chilled Nuclei EZ Lysis Buffer to the tissue in 1.5 mL tube.

3 Homogenize the sample using a douncer (stroking 10-20 times).

4 Transfer the homogenate (~ **500 µl**) into a 2 mL tube.

Nuclei Isolation and Staining

5 Add **1 ml** of chilled Nuclei EZ Lysis Buffer, mix gently and incubate on ice for **00:05:00** . Gently mix with a wide bore tip. Repeat 1-2 times during the incubation.

6 Filter homogenate using a 70 µm-strainer mesh. Collect flow through in a polystyrene round-bottom FACS tube and transfer volume back into a new 2 mL tube.

7 Centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C** and remove supernatant leaving behind ~ **50 µl**.

8 Gently resuspend nuclei in another **1.5 ml** of EZ Lysis buffer, incubate for **00:05:00** on ice.

9 Centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C**, remove supernatant as much as possible without disturbing pellet (if pellet looks loose leave ~ **50 µl** behind).

10 Add 500 µl Nuclei Wash and Resuspension Buffer and incubate 00:05:00 **without resuspending** to allow buffer interchange.

11 After incubation, add 1 ml of Nuclei Wash and Resuspension Buffer and resuspend the nuclei.

12 Centrifuge the nuclei at 500 rpm for 00:05:00 at 4 °C, remove supernatant leaving behind ~ 50 µl.

13 Gently resuspend nuclei in 1.4 ml Nuclei Wash and Resuspension Buffer and transfer to a 1.5 mL tube (easier to see small pellets).

14 Repeat Step 13 and resuspend in 500 µl **Nuclei Wash and Resuspension Buffer supplemented with DAPI**. Collect all nuclei by washing off nuclei from the wall of centrifuge tube.

IMPORTANT: Protect from light from here forward.

go to step #13

Cytometry and 10x

15 Filter nuclei (at least once) with a 35-µm cell strainer. Visually inspect nuclei integrity under a microscope.

Count the number of nuclei with a cell counter or hematocytometer if required.

16 Perform cytometric analysis. Identify single nuclei and sub-populations based on DNA content, gate and sort directly into 10x RT Buffer prepared **without the RT Enzyme Mix**.

17 Proceed immediately with the 10x Genomics Single Cell Protocol and minimize the time between nuclei preparation/sorting and chip loading.

18 Add 10 µl RT Enzyme Mix to the sorted nuclei in RT buffer, mix well but gently and load chip as per the Single Cell 3' Reagents User Guide or Single Cell V(D)J 5' Reagents User Guide.