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Version 1

Multi-Seq & Antibody-based Negative selection for the 'Frankenstein' protocol for nuclei isolation from fresh and frozen (brain) tissue for snRNAseq V.1



Forked from ['Frankenstein' protocol for nuclei isolation from fresh and frozen tissue for snRNAseq](#)



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

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We use this protocol and it's working

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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.



Attachments



Frankenstein.p...

379KB

Guidelines

Note

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 to 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](#))

Note

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

MATERIALS

⊗ Nuclei EZ lysis buffer Merck MilliporeSigma (Sigma-Aldrich) Catalog #EZ PREP NUC-101

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 Buffer¹ for Single Cell Gene Expression 3' reagents (DO NOT add RT enzyme)

RT Reagent Mix	50 μL
RT primer	3.8 μL
Additive A	2.4 μL
H2O	(31.7 - X - Y) μL

10x RT Buffer¹ 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



	H ₂ O	(31.7 - X - Y) μL
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¹RT 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 H₂O 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.

Ethics statement

Tissue collection for this protocol requires prior approval by the users' Institutional Ethics Board or equivalent ethics committee.

Before start

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.

Note

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. For fibrous brain-associated tissues, we additionally mince the tissue with a small scissors.
- 3 Homogenize the sample using a douncer (stroking 10-20 times).
- 4 Transfer the homogenate (~  500 μL) into a 2 mL tube.

Nuclei Isolation

25m

- 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.
- 7 Centrifuge the nuclei in a swing-bucket centrifuge at  500 x g for  00:05:00 at  4 $^{\circ}\text{C}$ 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 in a swing-bucket centrifuge at  500 x g for  00:05:00 at  4 $^{\circ}\text{C}$, remove supernatant as much as possible without disturbing pellet (if pellet looks loose leave ~  50 μL behind).

- 10 Add  500 μL PBS without Ca and Mg [PBS (-)] and incubate  00:05:00 **without resuspending** to allow buffer interchange.

Note

It is crucial that the PBS does **not** contain BSA or FBS since this would quench the anchor/co-anchor.



- 11 After incubation, add  1 mL of PBS (-) and resuspend the nuclei.
- 12 Centrifuge the nuclei in a swing-bucket centrifuge at  500 x g for  00:05:00 at  4 °C , remove supernatant leaving behind ~  50 μL .

Nuclei Barcoding with Multi-Seq

25m

- 13 During centrifugation, prepare the multiplexing anchor solution.

The Multi-seq assay was conducted in line with the Sigma protocol:

<https://www.sigmaaldrich.com/DE/de/technical-documents/technical-article/genomics/sequencing/multi-seq-sample-multiplexing-single-cell-analysis-sequencing>

For each sample the following solution was generated:

A	B
Anchor Oligo (50 μM)	1 μl
Unique sample barcode oligo (50 μM)	1 μl
PBS (-)	23 μl

Multiplexing anchor solution. Make sure to record the unique barcodes used.



14 Resuspend cells in  180 μL PBS (-) and add  20 μL multiplexing anchor solution to each sample and incubate  00:05:00 on ice.

5m

15 During anchor-incubation, prepare the co-anchor solution.

For each sample the following solution was generated. If you have more than 1 sample the solution for all samples can be prepared in the same tube.

	A	B
	Co-anchor Oligo (50 μM)	1 μl
	PBS (-)	24 μl

Multiplexing anchor solution. Make sure to record the unique barcodes used.

16 Add  20 μL multiplexing co-anchor solution to each sample and incubate for another  00:05:00 on ice.

5m

17 Quench the unbound anchors and co-anchors by adding  1 mL Nuclei Wash and Resuspension Buffer.

18 Centrifuge the nuclei in a swing-bucket centrifuge at  500 x g for  00:05:00 at  4 $^{\circ}\text{C}$, remove supernatant leaving behind ~  50 μL .

5m

19 Gently resuspend nuclei in  1.4 mL Nuclei Wash and Resuspension Buffer.

20 Centrifuge the nuclei in a swing-bucket centrifuge at  500 x g for  00:05:00 at  4 $^{\circ}\text{C}$, remove supernatant leaving behind ~  50 μL .

5m

Nuclei Staining

25m

21 During centrifugation, prepare the FACS antibody master mix for negative selection of nuclei. The volume is  50 μL per sample. For brain derived nuclei in our hands, the master mix consists of the following components:



A	B
Anti-NeuN Antibody	1 μ l
Anti-Olig2 Antibody	1 μ l
Nuclei Wash and Resuspension Buffer	48 μ l

FACS master mix for 1 sample.

22 Gently resuspend nuclei in  50 μ L FACS master mix.

23 Incubate at  4 $^{\circ}$ C in the dark for 20 min.

Note

IMPORTANT: Protect from light from here forward.

24 Gently resuspend nuclei in  1.4 mL Nuclei Wash and Resuspension Buffer.

25 Centrifuge the nuclei in a swing-bucket centrifuge at  500 x g for  00:05:00 at  4 $^{\circ}$ C , remove supernatant leaving behind ~  50 μ L .

5m

26 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).

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

Cytometry and Single-cell analysis

28 Filter nuclei (at least once) with a 35- μ m cell strainer. Visually inspect nuclei integrity under a microscope.

**Note**

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

- 29 Perform cytometric analysis. Identify single nuclei and sub-populations based on DNA content, gate and sort directly into 10x RT Buffer prepared with RNase inhibitor and **without the RT Enzyme Mix**.
- 30 Proceed immediately with the 10x Genomics Single Cell Protocol and minimize the time between nuclei preparation/sorting and chip loading.
- 31 Add  10 μ L RT Enzyme Mix to the sorted nuclei in RT buffer, mix well but gently and load chip as per the 10x, Parse or Seekgene protocols.