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Nuclei Isolation for 10x Chromium single-nuclei RNA sequencing

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We use this protocol and it's working

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Abstract

Single-nucleus RNA sequencing (sn-RNA seq) enables the profiling of nuclear gene expression in isolated cells. Herein, we present a step-wise protocol for single nuclei isolation from a fresh-frozen small biopsy of rat brain containing a human xenograft. The described method includes human-neuronal nuclei isolation and debris removal using fluorescent-activated
nuclei sorting. The isolated nuclei were processed through the 10x Chromium Controller platform for snRNA-seq. Compared to single-cell RNA-seq (sc-RNA seq), the use of nuclei avoids dissociation-associated transcriptional artefacts and is
compatible with frozen tissue.

Guidelines

General notes

Input nuclei suspension should contain >90% dead cells with intact nuclei. The presence of a high fraction of dying cells with damaged nuclei per ambient RNA and cellular debris may influence sequencing performance

Lysis

There are various types of lysis buffer that can be used to lyse the cells and release the nuclei. This very much depends on the tissue type. The basic buffer can either be hypotonic (which might help lyse the cells and release the nuclei) or isotonic (which may be more intrinsically gentle for the preparation). The detergent that is used to lyse the cells can be varied (as well as the concentration). Commonly used buffers alternative to the one we used are:

Lysis buffer NST (isotonic+NP40)

- 10mM Tris HCl pH 7.4
- 146mM NaCl
- 3mM MgCl2
- 0.1% NP40
- 40U/ml of RNase inhibitor

Lysis buffer IST (hypotonic + NP40)

- 10mM Tris HCl pH 7.4
- 10mM NaCl
- 3mM MgCl2
- 0.1% NP40
- 40U/ml of RNase inhibitor

For every type of tissue, the number of strokes with the dounce homogenizer needs to be calibrated. The incubation time after the dounce homogenization can also be varied. For each tissue an optimization needs to be performed.

Tubes

Nuclei as opposed to cells are problematic since they are smaller and they are more sticky because of DNA leakage and other factors. This means that there is much more significant loss after each centrifugation step. To try to minimize the loss, we have tried various methods to prevent the nuclei sticking to the sides of the tubes. One that work and we recommend is the coating of the tubes for about one hour before use with PBS+1%BSA. Recently, we have tried using polystyrene 5ml FACS tubes but we cannot comment if they are any better at reducing loss.

Materials

Material input

- Adult athymic (CBHrnu) nude rats
- Stem cells-derived neural progenitors (differentiated as in 10.1016/j.xpro.2020.100065)

Reagents

Lysis Buffer

- PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Thermo Fisher Scientific** **Catalog #**AM9625
- Nuclei Isolation Kit: Nuclei EZ Prep **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**NUC-101
- Recombinant RNAse Inhibitor **Takara Bio Inc.** **Catalog #**2313A

Wash Buffer

- PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Thermo Fisher Scientific** **Catalog #**AM9625
- Ultrapure BSA **Ambion** **Catalog #**AM2616
- Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**03335399001

Staining

- DAPI **Thermo Fisher Scientific** **Catalog #**D1306
- PE Anti-Human Nuclear Antigen antibody **Abcam** **Catalog #**Ab215755
- Rabbit anti-NeuN antibody **Abcam** **Catalog #**Ab104225
- Alexa Fluor 647 AffiniPure Goat Anti-Rabbit IgG **Jackson ImmunoResearch Laboratories, Inc.** **Catalog #**111-605-144
- Trypan Blue Stain (0.4%) for use with the Countess™ Automated Cell Counter **Thermo Fisher** **Catalog #**T10282

Consumables

- DNA LoBind Tubes, 1.5 ml (Eppendorf, cat. no. 22431021)
- 15ml Falcon tubes (Corning , cat. no. 430052)
- MACS Smart Strainers, 30 µm (Miltenyi Biotech, cat. no. 130-098-458)
- Gloves (nitrile/latex, assorted manufacturers/sizes)

Equipment

Equipment	
NucleoCounter®	NAME
NC-200™	TYPE
Chemometec	BRAND
NC-200	SKU
https://chemometec.com/products/nucleocounter-nc-200-automated-cell-counter/	LINK

- Centrifuge with swinging bucket rotor for 1.5/2.0 mL microcentrifuge tubes and for 15ml Falcon tubes
- FACS sorter (i.e. FACS Aria III) equipped with lasers to detect Alexa Fluor 647, Phycoerythrin and DAPI
- 10ul, 20ul, 200ul and 1000ml Pipettes and filtered tips
- Disposable serological pipettes (5ml and 10ml) and pipette guns

Troubleshooting

Safety warnings

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

Preparation of reagents and materials

1

1. Prepare ~ 50 mL of ice-cold PBS and store it on ice
2. Prepare 8 mL of ice-cold lysis buffer containing 0.2U/ul of RNase inhibitor by adding 40 µL of RNase inhibitor to 8 mL of Nuclei EZ-prep
3. Prepare 10 mL of ice-cold wash buffer containing 1%BSA and 0.2U/ul of RNase inhibitor by adding 2 mL of 5%BSA and 50 µL of RNase inhibitor to 8 mL of 1xPBS

Transcardial perfusion

2

1. Perfuse the animal with ice-cold PBS
2. Rapidly and On ice, extract the brain and using fine forceps, dissect the rat striatum containing the human xenograft
3. Immediately transfer the tissue in an ice-cold 1.5 mL tube and snap-freeze in in liquid nitrogen or dry ice
4. Store the sample to -80 °C until use

Nuclei isolation

1h 53m

3

1. On ice Transfer the tissue from the -80 °C to the dounce homogenizer containing 1.5 mL of cold lysis buffer On ice 1h 53m
2. Homogenize with 24 strokes On ice
3. Transfer the homogenized sample to a 15ml Corning tube on ice
4. Wash the dounce with 1.5 mL of lysis buffer
5. Allow the tube to stand for 00:05:00 on On ice
6. Centrifuge at 500 x g for 00:05:00 at 4 °C
7. Remove the supernatant
8. Wash with 1 mL of lysis buffer on ice
9. Wait 00:05:00 on On ice
10. Centrifuge at 500 x g for 00:05:00 at 4 °C
11. Remove the supernatant

12. Resuspend in 1 mL wash buffer (gently, without creating bubbles) On ice
13. Filter with a 30um cell strainer (MACS SmartStrainers) On ice
14. Wash the strainer with 1 mL wash buffer On ice
15. Centrifuge at 500 x g for 00:05:00 at 4 °C
16. Remove the supernatant
17. Resuspend in 300 µL wash buffer On ice
18. Count the nuclei. We recommend using both Trypan Blue to assess nuclei integrity and a fluorescent cell counter When looking at the nuclei in the counter, look at the integrity of them – how round they are with clear border and how much they are single and dispersed and not in clumps or in small groups of cells
19. Stain using HNA-PE and NeuN-Rb (1:100 diluted in wash buffer)
20. Incubate for 00:30:00 at 4 °C in the dark
21. Add secondary antibody (Rb-647, 1:200)
22. Incubate for 00:20:00 at 4 °C in the dark
23. Wash with 1 mL of wash buffer On ice
24. Centrifuge at 500 x g for 00:05:00 at 4 °C
25. Wash with 1ml of wash buffer 00:20:00
26. Centrifuge at 500 x g for 00:05:00 at 4 °C
27. Resuspend in DAPI solution (dilute the stock of 1mg/ml in 1:1000, 1ul in 1ml)
28. FACS sort the nuclei in 35 µL of wash buffer using a 70 µm nozzle, 21– 22 p.s.i.

    The sort should be done for HNA^+ $NeuN^+$ $DAPI^+$ single nuclei
29. After FACS sorting, centrifuge the nuclei 600 x g for 00:08:00
30. Carefully remove as much supernatant as possible and count in a cell counter. Again, look at the integrity of the nuclei. The nuclei should be counted twice and the average concentration calculated.
31. The nuclei are now ready for a 10x run. In our case sample processing was performed using the Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (10X Genomic, California, # PN-1000121) and
    the Chromium Controller (10X Genomics, California) per manufacturer's instructions as published in User Guide CG000204 Rev D (10X Genomics,California)

Protocol references

Staining

In the described protocol, we were interested in human neuronal nuclei isolation, thereby we decided to stain the nuclei for a human (HNA) and a neuronal (NeuN) marker. This step can be avoided if the cell types of interest are different i.e. use of GFAP if the cells of interest are astrocytes. DAPI counter-staining and sorting for single nuclei is not necessary and other methods exist to remove cell debris. In our hands, the use of DAPI and sorting allowed to achieve the cleanest sample and the performance was superior compared to the use of myelin removal kit (Miltenyi) or sucrose gradient (as recommended by 10x in https://cdn.10xgenomics.com/image/upload/v1660261285/support-documents/CG000124_Demonstrated_Protocol_Nuclei_isolation_RevF.pdf