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Sample preparation for single nuclei sequencing of brain

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Protocol status: Working
We use this protocol in our group and it worked.

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Abstract

Sample preparation for single nuclei sequencing using fresh frozen tissue from human postmortem brain. The protocol is a combination of an already existing and published protocol in Nature protocols *Using single nuclei for RNA-seq to capture the transcriptome of postmortem neurons* and the 10x Genomics handbook *Sample preparation, isolation of nuclei for single nuclei RNA sequencing*.

Materials

MATERIALS

- Optiprep (Iodixanol) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D1556-250ML
- Sucrose **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S0389
- RNaseZap® RNAse Decontamination Solution **Life Technologies Catalog #**AM9780
- Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T8787
- Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**3335402001
- LS Columns **Miltenyi Biotec Catalog #**130-042-401
- Buffer Kit RNase-free **Thermo Fisher Scientific Catalog #** AM9010
- Nuclease-Free Water **Thermo Fisher Scientific Catalog #** AM9939
- Dithiothreitol (DTT) **Thermo Fisher Scientific Catalog #**707265ML
- cOmplete™ EDTA-free Protease Inhibitor Cocktail **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**11873580001
- Myelin Removal Beads II human mouse rat **Miltenyi Biotec Catalog #**130-096-733
- Ultra pure BSA (50 mg/mL) **Thermo Fisher Scientific Catalog #**cat# AM2616
- SUPERase• In™ RNase Inhibitor (20 U/µL) **Thermo Fisher Scientific Catalog #**cat# AM2694
- PBS 1x without calcium & magnesium **VWR International (Avantor) Catalog #**Cat# 21-040-CVR

STEP MATERIALS

Buffers:

Nuclei isolation medium 1 (NIM1)

	Component	Volume (µl)	Final concentration (mM)
	1.5 M sucrose	2500	250
	1 M KCl	375	25
	1 M MgCl2	75	5
	1 M Tris buffer	150	10
	Nuclease-free water	11900	-
	Total volume	15000	-

Important! This buffer should be prepared in advance, it can be stored in 50-ml tube at 4C for up to 6 months.

Nuclei isolation medium 2 (NIM2)

	Component	Volume (µl)	Final concentration (mM)
	NIM1	4895	
	1 mM DTT	5	1 µM
	50x protease inhibitor	100	1x
	Total volume	5000	

Important! This buffer should be prepared fresh and placed at 4C or on ice for use. Afterwards, it should be discarded

Homogenization buffer

	Component	Volume (µl)	Final concentration (mM)
	NIM2	1455	1 x
	RNaseIn 40 U µl−1	15	0.4 U µl−1
	Superasin 20 U µl−1	15	0.2 U µl−1
	Triton X-100 10% (v/v)	15	0.1% (v/v)
	Total volume	1500	

Important! This buffer should be prepared fresh, protected from the light and placed at 4C or on ice for use. Afterwards, it should be discarded.

Nuclei Wash and Resuspension Buffer

1x PBS
1% BSA
0.2 U ul-1 Rnase inhibitor

LS Column Calibration Buffer

1x PBS

0.5% BSA

Iodixanol medium (IDM)

	Component	1× volume (µl)	Final concentration (mM)
	1.5 M sucrose	2500	250
	1 M KCl	2250	150
	1 M MgCl2	450	30
	1 M Tris buffer, pH 8.0	900	60
	Nuclease-free water	8900	-
	Total volume	15000	

The medium can be stored at 4C for up to 6 months.

Iodixanol dilutions

	Component	1× volume (µl)	Final concentration
	Iodixanol 60% (vol/vol)	12500	50% vol/vol
	IDM	2500	-
	Total volume	15000	
	Component	**1× volume (µl)**	**Final concentration**
	Iodixanol 60%	7250	29% vol/vol
	IDM	7750	-
	Total volume	15000	

The reagents can be stored at 4C for up to 6 months.

Safety warnings

Regulations about working with tissue samples may vary between institutions, it is important to be aware about the guidelines before to start any experiment.

Before start

- Run Bioanalyser to check RNA integrity number (RIN) and evaluate the quality of the tissue.
- Clean workstation, pipettes and tools with RNaseZap solution before to start the experiment.
- Precool the Dounce homogenizer and pestles on ice. Once they are cooled, fill the homogenizer with 1ml of cold homogenization buffer and keep it on ice.

Nuclei isolation

1 Place a Petri dish on dry ice and transfer the tissue there, cut it using a chilled scalpel. Transfer the tissue into the precooled Dounce homogenizer and keep the homogenizer on ice during the rest of the protocol (to reduce heat caused by friction).

Equipment

Dounce Tissue Grinder Set with Two Glass Pestles	NAME
Wheaton	BRAND
cat# 357538	SKU
https://us.vwr.com/store/product/4832641/dounce-tissue-grinders-wheaton	LINK

2 Homogenize the tissue with five strokes of the loose pestle, followed by 10–15 strokes of the tight pestle.

3 Filter the homogenate through a BD Falcon tube with a cell strainer cap.

Equipment

Falcon™ Test Tube with Cell Strainer Snap Cap	NAME
Corning	BRAND
cat# 352235	SKU
https://www.fishersci.com/shop/products/falcon-tube-cell-strainer-cap-mesh-size-35um/0877123	LINK

4 Transfer the volume to a chilled 1.5 ml eppendorf and centrifuge at 1000 rcf for 00:08:00 at 4 °C .

5 Remove the supernatant without disrupting the nuclei pellet.

Myelin removal

6 Add 1800 µL nuclei wash and resuspension buffer to the pelleted nuclei and, using a regular-bore pipette tip, gently pipette mix 8-10 times.

7 Add 200 µL Myelin Removal Beads II to the resuspended nuclei. Mix with a wide-bore pipette tip (no vortex). Incubate for 00:15:00 at 4 °C .

8 Meanwhile, place LS columns in the magnetic field of a MACS separator each with 3 mL LS Column Calibration Buffer to rinse the columns.

Equipment

QuadroMACS Separator	NAME
MACS Separator - magnetic field	TYPE
Miltenyi biotec	BRAND
cat# 130-090-976	SKU
https://www.miltenyibiotec.com/SE-en/products/macs-cell-separation/separators/quadromacstm-separator-and-starting-kits.html	LINK

Equipment

NAME	BRAND	SKU	LINK
MACS MultiStand	Miltenyi Biotec	cat# 130-042-303	https://www.miltenyibiotec.com/SE-en/products/macs-cell-separation/separators/macs-multistand.html

9 Using a 10 ml serological pipette, dilute the nuclei suspension with 10 mL Nuclei Wash and Resuspension buffer, gently pipette mix 5 times.

10 Centrifuge the nuclei at 500 rcf for 00:10:00 at 4 °C .

11 Remove the supernatant without disrupting the nuclei pellet and add 2 mL Nuclei Wash and Resuspension buffer.

12 Apply 1 mL nuclei suspension in each column.

13 Wash each of the columns with 1 mL Nuclei Wash and Resuspension buffer

go to step #13 once more.

14 Collect the effluent into two 5ml Eppendorf tubes.

15 Centrifuge the two vials with nuclei at 500 rcf for 00:05:00 at 4 °C .

16 Remove the supernatant from the two vials without disrupting the nuclei pellet and add 500 µL Nuclei Wash and Resuspension buffer.

17 Pool both resuspended nuclei in a new pre-chilled 1.5 ml Eppendorf.

Density gradient centrifugation

18 Centrifuge at 1000 rcf for 00:08:00 at 4 °C .

19 Meanwhile, prepare the iodixanol dilution mix (IDM) and iodixanol dilutions to place them on ice.

20 Aspirate the supernatant (~1,000 µl) and gently resuspend the pellet in 250 µl of homogenization buffer.

21 Strain the mixture through a BD Falcon tube with a cell strainer cap to remove any remaining aggregates, and place it on ice.

22 Gently mix the nuclei with 250 µl of 50% (vol/vol) iodixanol, and, to a new Eppendorf tube, add 500 µl of 29% iodixanol. Slowly layer 500 µl of the nuclei mixture over the 29% iodixanol.

23 Centrifuge at 13.500 rcf for 00:20:00 at 4 °C .

24 Remove and discard the supernatant without disrupting the nuclei pellet.

25 Resuspend the final pellet in 60 µL Nuclei Wash and Resuspension buffer. The sample is ready for QC-nuclei counting.