Single nuclei isolation

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Isolation of single nuclei from postmortem fresh frozen human brain and immunostaining for NeuN

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1 Works for me dx.doi.org/10.17504/protocols.io.6tuhenw

Neurodegeneration Method Development Community
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ABSTRACT

- Protocol based on Krishnaswami *et al.*, Nat Protoc. 2016, 3:499-524
- Used routinely in our lab for the isolation of single nuclei from fresh frozen postmortem brains from subjects with neurodegenerative disorders and healthy controls for single-nucleus RNA-seq
- Works also for isolation of nuclei from fresh frozen mouse brains
- Used for isolation of all nuclei or antibody-enriched populations (i.e., NeuN$^+$ nuclei)
- We always perform and strongly recommend FACS if used for single-nucleus RNA-seq

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Manuscript under review

Single nuclei isolation from postmortem frozen frozen brain_CobosLab_10282019.pdf

MATERIALS

NAME	CATALOG #	VENDOR
DNase I (RNase-free) - 1,000 units	M0303S	New England Biolabs
Bovine Serum Albumin (BSA)	A7906	Sigma Aldrich
Magnesium chloride hexahydrate	M2670	Sigma Aldrich
UltraPure™ DNase/RNase-Free Distilled Water	10977023	Thermo Fisher Scientific
DAPI	D3571	Invitrogen - Thermo Fisher
Sucrose	S25590B	Fisher Scientific
Triton™ X-100	AC215680010	Fisher Scientific
Potassium chloride solution	60142	Millipore Sigma
UltraPure™ 1M Tris-HCl pH 8.0	15568025	Thermo Fisher Scientific
DL-Dithiothreitol	D9779-1G	Sigma Aldrich
cOmplete™ Protease Inhibitor Cocktail	11697498001	Sigma Aldrich
NxGen® RNAse Inhibitor	30281-2	Lucigen
Anti-NeuN Antibody	MAB377	Merck Millipore

NAME	CATALOG #	VENDOR
Goat anti-Mouse IgG (H L) Alexa Fluor 647	A-21235	Thermo Fisher Scientific
OptiPrep™ Density Gradient Medium	D1556)	Sigma Aldrich

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Anti-NeuN Antibody, clone A60	MAB377	Merck Millipore

MATERIALS TEXT

- Material and tools needed: Forceps, spatula, blades, dounce tissue grinder, cell strainers, petri dishes

Dounce All-Glass Tissue Grinders
Tissue Grinder
KIMBLE 885300-0007

Buffer preparation (All solutions should be RNase-free for single-soma RNAseq experiments):

Isolation Medium #1 (IM1), 45 ml (optional)

Prepare in a 50 ml Falcon tube and store at 4 °C up to 6 months.

Amount	*Reagent*	*Final concentration*
7500 µl	1.5 M Sucrose	250 mM
1125 µl	1 M KCl	25 mM
225 µl	1M MgCl2	5 mM
450 µl	1 M Tris (pH 8)	10 mM
35.7 ml	RNase-free H2O	

Homogenization Buffer (3 ml per sample)

Prepare FRESH and keep ICED or at 4 °C . Discard after use.

Amount	*Reagent*	*Final concentration*
2925 µl	IM1	
3 µl	DTT 1mM	1 µM
30 µl	50x Protease Inhibitor	0.5 x
15 µl	RNaseIN 40U/µl	0.2 U/µl
30 µl	TritonX100 10 %v/v	0.1 %

Iodixanol dilutions

Prepare in a 50 ml Falcon tubes and store at 4 °C up to 6 months. Accuracy with Iodixanol and sucrose concentrations is critical.

- **Iodixanol medium (IDM), 45 ml**

Amount	*Reagent*	*Final concentration*
7500 µl	1.5 M Sucrose	250 mM
6750 µl	1 M KCl	150 mM
1350 µl	1 M MgCl2	30 mM
2700 µl	1 M Tris (pH 8)	60 mM

26.7 ml	Nuclease-free H2O	

- **Iodixanol 50%(v/v), 20 ml**

16.7 ml Iodixanol 60 %(v/v) + 3.3 ml IDM

- **Iodixanol 29%(v/v), 30 ml**

14.5 ml Iodixanol 60 %(v/v) + 15.5 ml IDM

Freezing Storage Buffer (FSB), 15ml (optional)

Prepare in a 50 ml Falcon tube and store at 4 °C up to 6 months.

Amount	*Reagent*	*Final concentration*
1.665 ml	1.5M Sucrose	166.5 mM
75 µl	1M MgCl2	5 mM
150 µl	1M Tris (pH8)	10 mM
13.1 ml	Nuclease-free H2O	

Buffer for Immunostaining, 10ml

Amount	*Reagent*	*Final concentration*
9400 µl	RNase-free PBS (pH7.4)	
500 µl	RNase-free 10 % BSA	0.5 %
50 µl	1 M MgCl2	5 mM
10 µl	DNAse I (2000 U/ml)	2 U/ml

Buffer for Antibody Incubation

Add to 1 ml of Buffer for Immunostaining:

Amount	*Reagent*	*Final concentration*
5 µl	RNaseIN 40 U/µl	0.2 U/µl

Collection medium for FACS (1 ml; 0.2 ml/vial)

Amount	*Reagent*	*Final concentration*
950 µl	RNase-free PBS (pH7.4)	
25 µl	RNAse IN	
*	BSA 10% *After collecting!	1 %

SAFETY WARNINGS

Please see SDS (Safety Data Sheet) for hazards and safety warnings.
Uses fresh human brain tissue - Biosafety Level 2 lab work.

BEFORE STARTING

- All solutions and materials should be **RNase-free** and **kept iced or at 4 °C** at all times
- Glassware and metal tools are **sealed with aluminum foil** and **baked at 220 °C for 06:00:00 .**

1 

Prepare **Homogenization Buffer** and cool it on ice.

Amount	*Reagent*	*Final concentration*
2925 µl	IM1	
3 µl	DTT 1mM	1 µM
30 µl	50x Protease Inhibitor	0.5 x
15 µl	RNaseIN 40U/µl	0.2 U/µl
30 µl	TritonX100 10 %v/v	0.1 %

Homogenization Buffer (3 ml per sample)

Prepare FRESH and keep ICED or at **4 °C** . Discard after use.

1.1 Add **2925 µl IM1** .

1.2 Add **3 µl DTT 1mM** .

1.3 Add **30 µl 50x Protease Inhibitor** .

1.4 Add **15 µl RNaseIN 40U/µl** .

1.5 Add **30 µl TritonX100 10 %v/v** .

2 Pre-cool the dounce tissue grinder on ice (**Kimble Kontes all glass tissue grinder**, 7 mL tubes, 0.02 - 0.056 mm clearance space between pestle and tubes)

3 Add **2.4 ml** of **Homogenization buffer** to the dounce tissue grinder.

4 Collect the brain chunk (~ **100 mg**) and transfer to a Petri dish on ice.

5 Cut out into small pieces using a chilled scalpel or blade.

6 Transfer all pieces into the dounce tissue grinder.

7 Homogenize the tissue, on ice (**~30 firm strokes**).
Check on **hematocytometer** while homogenizing and adjust the number of strokes.

Too few strokes – you will see cell clumps; too many strokes – you will get damaged nuclei.

8 Filter homogenate using **40 µm** Corning cell strainer to remove clumps.
--> Take sample for hemocytometer.

9 Transfer the homogenate into two precooled 1.5 ml Eppendorf tubes.

10 

Centrifuge at **1000 x g** for **00:08:00** at **4 °C** .

11 Slowly aspirate the supernatant from the side of the tube.
Avoid disturbing the pellets, up to **50 µl** of supernatant can be left.

12 Gently **resuspend** each pellet in **225 µl** (final volume) of cold **Homogenization buffer** and pool both tubes (final volume **450 µl**).

13 

Add an **equal** volume of cold **[M]50 %v/v iodixanol** .

Critical! Be very exact with volumes. **450 µl** suspension + **450 µl** 50 % iod.
Final iodixanol concentration is **25 %**.

14 

Gently pipette mix.

15 Add an equal volume of **29 % iodixanol** (**900 µl**) into a 2 ml Eppendorf tube.

16 Slowly layer off the 25 % iodixanol suspension mix over the 25 % iodixanol, without mixing them.

17 

Centrifuge at **13500 x g** for **00:20:00** at **4 °C** .

18 

Prepare **buffer for immunostaining.**

Amount	*Reagent*	*Final concentration*
9400 µl	RNase-free PBS (pH7.4)	
500 µl	RNase-free 10 % BSA	0.5 %
50 µl	1 M MgCl2	5 mM
10 µl	DNAse I (2000 U/ml)	2 U/ml

18.1 Add **9400 µl RNase-free PBS (pH7.4)** .

18.2 Add **500 µl RNase-free 10 % BSA** .

18.3 Add **50 µl 1 M MgCl2** .

18.4 Add **10 µl DNAse I (2000 U/ml)** .

19 Prepare **buffer for Antibody incubation** (1.5 ml per sample) by adding **5 µl** of RNaseIN 40 U/µl to **1 ml** of **buffer for Immunostaining** (final concentration 0.2U/µl)

20 After centrifugation, remove and discard the top myelin-rich debris layer (you can use a 1 ml pipette with the tip cut, or cotton swabs).

21 

Then, remove and discard the aqueous supernatant, without disrupting the **nuclei pellet**. Avoid contaminating with the top layer.

22 Use a small amount of **buffer for Antibody Incubation** to resuspend the pellet and transfer the solution to a new tube.

23 Gently resuspend in **500 µl** of **buffer for Antibody Incubation**.

24

Incubate for **00:15:00** , at **4 °C** or iced, for **blocking** nonspecific staining.

25 Take sample for hemocytometer.

26 Take a sample for **unstained** control. Take sample for **2AB-only** (nonspecific binding control).

27 Add primary antibody (**Ms-a-NeuN, 1:1000**) and incubate on a rotator in cold room (**4 °C**) for **00:40:00** .

Anti-NeuN Antibody, clone A60
by Merck Millipore
Catalog #: MAB377

Use Eppendorfs coated with BSA for collection (to coat the Eppendorf tubes, fill them with 10 % BSA solution in PBS for **00:05:00** , rinse with PBS, and dry at **4 °C** overnight).

Washing

28

Add **700 µl** of **buffer for Immunostaining** and invert several times.

29

Centrifuge at **500 x g** for **00:05:00** at **4 °C** .

30

Carefully transfer all the supernatant (don't leave any supernatant! It's ok to drag some pellet) to a new Eppendorf tube and resuspend the pellet in **400 µl** of **buffer for Antibody Incubation**.

31 Centrifuge the supernatant at **500 x g** for **00:05:00** at **4 °C** to recover non-pelleted nuclei.

32 Resuspend them in **200 µl** of **buffer for Antibody Incubation** and pool for a final volume of 600 µl.

33 Take samples for single color staining.

34 Add secondary antibody (**G-a-Ms Alexa Fluor 647, 1:1000**) and **Hoechst 33258** (5 µg/ml) to the same tube. (**Hoechst 1:1000 of 2,5 mg/ml stock**).

35 Check the quality of the sample and on hemocytometer.

For 100 mg of tissue, should have ~ 2 Million nuclei in ~0.6 ml.

36 Transfer to 7 ml culture tubes.

37 Place on ice and bring them to the FACS facility for sorting.

FACS sorting controls:
Non-stained
2AB only
Single stainings

38 Collect in BSA coated Eppendorfs containing **collection medium** (containing ~1/5 of the expected final volume after collection).

39 Add BSA after collection for final 1% BSA final concentration (tested for 10x Chromium v2 and v3).

Different Single Cell assays tolerate different BSA concentrations, but lowering it may increase nuclei aggregation.