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

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Single nuclei isolation

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We use this protocol and it's working

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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

Attachments

PDF

Single nuclei isolat...

383KB

Materials

MATERIALS

- DNase I (RNase-free) - 1,000 units **New England Biolabs** **Catalog #**M0303S
- Bovine Serum Albumin (BSA) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**A7906
- Magnesium chloride hexahydrate **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**M2670
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific** **Catalog #**10977023
- DAPI **Invitrogen - Thermo Fisher** **Catalog #**D3571
- Sucrose **Fisher Scientific** **Catalog #**S25590B
- Triton™ X-100 **Fisher Scientific** **Catalog #**AC215680010
- Potassium chloride solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**60142
- UltraPure™ 1M Tris-HCl pH 8.0 **Thermo Fisher Scientific** **Catalog #**15568025
- DL-Dithiothreitol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D9779-1G
- cOmplete™ Protease Inhibitor Cocktail **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**11697498001
- NxGen® RNAse Inhibitor **Lucigen** **Catalog #**30281-2
- Anti-NeuN Antibody **Merck Millipore (EMD Millipore)** **Catalog #**MAB377
- Goat anti-Mouse IgG (H L) Alexa Fluor 647 **Thermo Fisher Scientific** **Catalog #**A-21235
- OptiPrep™ Density Gradient Medium **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D1556)

STEP MATERIALS

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

Equipment

Dounce All-Glass Tissue Grinders	NAME
Tissue Grinder	TYPE
KIMBLE	BRAND
885300-0007	SKU
https://www.kimble-chase.com/advancedwebpage.aspx?cg=886&cd=4&SKUTYPE=202&SKUFLD=SKU&DM=1250&WEBID=6855	LINK

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	2 U/ml

		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 %

Protocol materials

- cOmplete™ Protease Inhibitor Cocktail **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**11697498001
- Anti-NeuN Antibody **Merck Millipore (EMD Millipore)** **Catalog #**MAB377
- DL-Dithiothreitol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D9779-1G
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- Anti-NeuN Antibody, clone A60 **Merck Millipore (EMD Millipore)** **Catalog #**MAB377

Safety warnings

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

Before start

- 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.

Note

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 .

Note

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	2 U/ml

		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 **Merck Millipore (EMD Millipore) Catalog #**MAB377

Note

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.

Note

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.

Note

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).

Note

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