Single soma isolation

Oct 28, 2019

Isolation of single somas from postmortem fresh frozen human brain and immunostaining for AT8 and MAP2

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

Neurodegeneration Method Development Community
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ABSTRACT

- Protocol optimized to isolate single neuronal somas with cytoplasmic protein aggregates from postmortem fresh frozen brain
- Works for the isolation of neuronal somas with neurofibrillary tangles from Alzheimer disease's brains
- Note that cell membranes are highly disrupted in tissues that were previously frozen. Thus, cytoplasmic transcripts are largely lost. This protocol is therefore useful for the isolation of specific cell populations using antibodies against cytoplasmic antigens, but not for the profiling of cytoplasmic mRNA.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Manuscript under review

Single soma isolation from postmortem frozen human brain_CobosLab_10282019.pdf

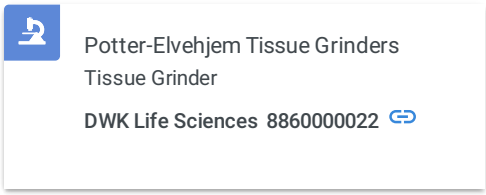
MATERIALS

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
Sucrose	S25590B	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-Microtubule-Associated Protein 2 (MAP2) Antibody	AB5622	Merck Millipore
Phospho-Tau (Ser202 Thr205) Monoclonal Antibody (AT8)	MN1020	Thermo Fisher Scientific
SYTOX™ Green Nucleic Acid Stain	S7020	Thermo Fisher Scientific
Goat anti-Rabbit IgG (H L) Alexa Fluor 647	A-21245	Thermo Fisher Scientific

NAME	CATALOG #	VENDOR
Goat anti-Mouse IgG (H L) Alexa Fluor 350	A-11045	Thermo Fisher Scientific
OptiPrep™ Density Gradient Medium	D1556	Sigma Aldrich

MATERIALS TEXT

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



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	1 M MgCl2	5 mM
450 µl	1 M Tris (pH 8)	10 mM
35.7 ml	H2O	

Homogenization Buffer (3 ml per sample)

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

Amount	*Reagent*	*Final concentration*
2955 µl	IM1	
3 µl	DTT 1mM	1 µM
30 µl	50x Protease Inhibitor	0.5 x
15 µl	RNaseIN 40 U/µl	0.2 U/µl
No Triton!		

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
3750 µl	1 M KCl	83 mM
750 µl	1 M MgCl2	17 mM
1500 µl	1 M Tris (pH 8)	33 mM
31.5 ml	H2O	

- **Iodixanol 42 %(v/v), 20 ml**
  14 ml Iodixanol 60 %(v/v) + 6 ml IDM

- **Iodixanol 25 %(v/v), 30 ml**
  12.5 ml Iodixanol 60 %(v/v) + 17.5 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	1.5 M Sucrose	166.5 mM
75 µl	1 M MgCl2	5 mM
150 µl	1 M Tris (pH8)	10 mM
13.1 ml	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 (pH 7.4)	
25 µl	RNAseIN	
*	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

- Aims for **minimal mechanical damage** (as little centrifugation and pipetting as possible)
- 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*
2955 µl	IM1	
3 µl	DTT 1mM	1 µM
30 µl	50x Protease Inhibitor	0.5 x
15 µl	RNaseIN 40 U/µl	0.2 U/µl
No Triton!		

Homogenization Buffer (3 ml per sample)

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

1.1 Add **2955 µl IM1** .

1.2 Add **3 µl DTT 1mM** .

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

1.4 Add **15 µl RNaseIN 40 U/µl** .

2 Pre-cool the dounce tissue grinder on ice (**Potter-Elvehjem tissue grinder**, 8 mL tubes, 0.1–0.15 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 (~ **200 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 Slowly homogenize the tissue, on ice (**8~15 strokes; slow**).
Check on **hematocytometer** while homogenizing and adjust the number of strokes.

Too few strokes – you will see clumps; too many strokes – you will get a higher proportion of naked nuclei.

8 Filter homogenate using a **100 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 **400 x g** for **00:05:00** 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 **42% v/v iodixanol**.

Critical! Be very precise with volumes. **450 µl** suspension + **450 µl** 50% iodixanol.
Final iodixanol concentration is **21%**.

14 

Gently pipette mix.

15 Add an equal volume of **25% iodixanol** (**900 µl**) into a 2 ml Eppendorf tube.

16 Slowly layer off the 21% iodixanol suspension mix over the 25% iodixanol, without mixing them.

17 

Centrifuge at **8000 x g** for **00:15:00** 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 

Remove and discard the aqueous supernatant, without disrupting the **somas 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 **200 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 antibodies (**Ms-a-AT8 1:150** and **Rb-a-MAP2 1:40**) and incubate on a shaker in cold room (**4 °C**) for **00:40:00** .

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 **500 µl** of **buffer for Immunostaining** and invert several times.

29 Centrifuge at **400 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 somas.

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 antibodies (**G-a-Ms Alexa350, 1:500** and **G-a-Rb Alexa647, 1:500**) and **Sytox Green** 1:400 (1:100 stock, final concentration **1:40.000**)

35 Check the quality of the sample on hemocytometer.

For 200 mg of tissue, we should have ~ 1-2 Million somas 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** (volume of collection medium should be ~1/5 of the expected final volume after collection).

39 Add BSA after collection for 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.