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

Single soma isolation

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We use this protocol and it's working

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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.

Attachments

PDF

Single soma isolatio...

246KB

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
- Sucrose **Fisher Scientific Catalog #**S25590B
- 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-Microtubule-Associated Protein 2 (MAP2) Antibody **Merck Millipore (EMD Millipore) Catalog #**AB5622
- Phospho-Tau (Ser202 Thr205) Monoclonal Antibody (AT8) **Thermo Fisher Scientific Catalog #**MN1020
- SYTOX™ Green Nucleic Acid Stain **Thermo Fisher Scientific Catalog #**S7020
- Goat anti-Rabbit IgG (H L) Alexa Fluor 647 **Thermo Fisher Scientific Catalog #**A-21245
- Goat anti-Mouse IgG (H L) Alexa Fluor 350 **Thermo Fisher Scientific Catalog #**A-11045
- OptiPrep™ Density Gradient Medium **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D1556

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

Equipment

Potter-Elvehjem Tissue Grinders	NAME
Tissue Grinder	TYPE
Kimble	BRAND
K886000-0022	SKU
https://www.kimble-chase.com/advancedwebpage.aspx?cg=625&cd=5&SBCatPage=	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	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 conc*

			entr ation
	5 µl	RNa seIN 40 U/µl	0.2 U/µl

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

	Amo unt	*Rea gent*	*Final conc entr ation*
	950 µl	RNa se-free PBS (pH 7.4)	
	25 µl	RNA seIN	
	*	BSA 10% *Aft er colle cting !	1 %

Troubleshooting

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

- 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.

Note

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**.

Note

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 .

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 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.

Note

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.

Note

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).

Note

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