Supplementary Material For:

Trehalose-enhanced isolation of neuronal sub-types from adult mouse brain

Protocol For:

RNA extraction from sorted neuronal subtypes

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Reagents

D-Trehalose (Sigma-Aldrich Catalog # 90208)
Phenol red free PBS
AP-V [DL-AP5, Cat # 0105 Tocris Bioscience]
Kynurenic acid [K-3375, Sigma-Aldrich]
Saline solution
D-MEM/F12 without phenol red [Gibco, 11039–021, Invitrogen]
FBS (Fetal Bovine Serum) [cat # 12103C-100ML, Nichirei co.]
HS (Horse Serum HI) [26050–070, 100 mL Gibco]
TRIZOL LS [10296010 100ML Invitrogen], Chloroform, Ethanol
Worthington papain dissociation kit (LK003150)
Purelink, RNA micro kit [12183016, Invitrogen]

Solution preparation

1. 50% w/v D-trehalose in dH2O, autoclave

Solutions from Worthington kit

2. EBSS (Earle's Balanced Salt Solution) with bicarbonate and phenol red (vial 1), equilibrate with sterile O_2:CO_2
3. Ovomucoid protease inhibitor with BSA (Ovomucoid inhibitor 10 mg with albumin 10 mg, vial 4)
Add 32 mL EBSS (vial 1) to Ovomucoid protease inhibitor with BSA (vial 4). Allow contents to dissolve

* ***HINT:*** *Reconstitute for the first use, then store at 4C and reuse. Refrigerate between uses and equilibrate with sterile O_2:CO_2 before each use.*

4. Mixed EBSS#1 solution

mixed EBSS #1 for 1 mouse	EBSS	25 mM AP-V	100 mM KA	50%(w/v) Trehalose
Total 10 mL	8.9 mL	20 µl (0.05 mM)	80 µL (0.8 mM)	One mL

5. Papain solution, (100 units of papain in 1 mM L-cysteine and 0.5 mM EDTA, vial 2)
Add 5 mL of EBSS #1 to Papain vial 4. Incubate 10 min at 37C or until the solution appears clear and equilibrate the solution with 95%O_2: 5%CO_2.

* ***HINT:*** *The solution should be used promptly but can be held at RT during the dissection. A separate papain vial is provided for each dissociation.5 single use vials per package.*

6. DNase-I solution, (1000 units of DNase-I, vial 3)
Add 500 µL of EBSS to DNase-I vial. Mix gently

* ***HINT:*** *Avoid vigorous mixing.*

7. Papain DNase I solution
Add 250 µL of DNase I solution to prepared papain solution in step 4 (vial 2).
Final concentration is 20units/mL Papain and 0.005% DNase I
Store the rest of DNase I solution on ice.

8. Medium solution without serum

Medium without serum	D-MEM/F12 without phenol red	50%(w/v) Trehalose	25 mM AP-V	100 mM KA
Total 21.1 mL	18.9 mL	2.1 mL	21 µl	84 µl

9. Medium solution with serum

Medium	D-MEM/ F12 without phenol red	FBS (Fetal bovine serum)	HS (Horse serum)	50%(w/v) Trehalose	25 mM AP-V	100 mM KA
Total 10ml	7.0 mL	1.0 mL	1.0 mL	1.0 mL	10 µl	40 µl

Procedure

I. Dissection

1. Dissect the whole brain, extract cortex. Place cortex in a Petri dish containing 5 mL equilibrated mixed EBSS#1 solution.
2. Cut the tissue into small pieces in 5 mL equilibrated mixed EBSS#1 solution.
3. Transfer tissue to the 50 mL tube that has been previously

covered with foil and discard EBSS#1 from the dish.
4. Add the equilibrated 5.250 mL Papain Dnase-I solution.
5. Equilibrate again by passing 95% O_2: 5% CO_2 in the space above the solution until the color change indicates pH 7.2–7.4 (pH color chart is in the kit).
6. Incubate at 37°C in water bath for 60 min with constant agitation (150 s/min).
7. After 5 min of incubation equilibrate again. Equilibrate by passing 95% O_2:5% CO_2 in the space above the solution until the color change indicates pH 7.2–7.4 (pH color chart is in the kit). Put tissue back for incubation at 37C.

Prepare mixed EBSS#2 solution 15 min before the end of tissue digestion.

Mixed EBSS#2 solution

mixed EBSS #2 for 1 sample	EBSS	25 mM AP-V	100 mM KA	Albumin-ovomucoid inhibitor (vial 4)	DNase I (vial 3)	50% (w/v) Trehalose
Total 6.0ml	4.73 mL	20 µl	48 µl	350 µl	250 µl	600 µl

II. Trituration (All steps in Falcon 50 mL covered in foil)

8. Add 5ml of mixed EBSS#2 solution and mix the tissue suspension avoiding air bubbles. Divide the solution into two 50 mL tubes

* *HINT: It is important to divide into 2 since this trituration remains incomplete if whole cortex is processed in one tube.*

9. Centrifuge the suspension at 1500 rpm (100 g) for 10 min.
10. Discard the supernatant and resuspend the pellet with 150 µL of mixed EBSS#2.
11. Mechanically dissociate the digested tissue using a plastic disposable tip attached to a P200 micropipettor, which has been set to 0.18 mL (180 µL), by pipetting up and down gently 20 - 30 times until a smooth and "creamy" suspension is obtained.

* *HINT: The suspension should be able to pass through the plastic tip bore without getting lodged in the tip. Amount of pipetting required is proportional to the age of mouse and pipetting must be performed until a creamy suspension is obtained.*

12. Add 0.1 mL (100 µL) of mixed EBSS#2 solution to bring the volume to approximately 300 µL.
13. Pipette the suspension approximately 15 more times with a disposable plastic tip attached to a P1000 micropipettor (set to 280 µL) to achieve a homogenous cell suspension without any remaining chunks of tissue.
14. Add 5 mL Medium without serum to the tube and mix with the cells.
15. Wash the cell suspension by centrifugation at 1500 rpm for 10 min.
16. Discard the supernatant and resuspend the pellet by adding 0.2 mL (200 µL) of mixed EBSS#2 solution and pipetting up and down 15 times using a plastic disposable tip attached to a P200 micropipettor.
17. Add 5 mL Medium without serum, mix the cells and wash the cell suspension by centrifugation at 1500 rpm for 10 min.
18. Resuspend the final pellet in 1 mL in Medium with serum for each tube.
Gather everything in one tube and mix. Cover with foil.
19. Take a 50 µL aliquot of cells and dilute in equal volume of trypan blue. Mount on slides and put coverslip. Take these to FACS unit to check under microscope.

III. Before sorting

20. Confirm that the flow cytometer tubing was rinsed with one run of RNase free dH2O, then bleach followed by RNase free dH2O.
21. Confirm that the nozzle is changed to a larger size to ensure smooth passage of neurons
22. Confirm the sorting will be conducted at slow speed to prevent damage to neurons
23. Observe under microscope to check if cells are well dissociated and viable.
Take images before sorting.
24. Prepare 1.5 mL eppendorf tubes containing 750 µL Trizol LS. Cells must be sorted directly in Trizol LS for RNA preparation. Since only 25% sample v/v is recommended, don't collect more than 250 µL per tube containing 750 µL Trizol LS. For one whole cortex, in our hands, 250 µL is equivalent to 20,000 cells.

* *HINT You may need to dilute the 2 ml cell solution for efficient sorting. Use the medium solution with serum for dilution.*

IV. After sorting

25. Collect cells on slide after first few cells are sorted to take pictures and:
* check if cells are fluorescence positive.
* Look out for false positives (debris) and oddly fluorescent cells
* check the intensity of the fluorescence and report it.
Gating can be adjusted at this stage to ensure that fluorescence positive cells alone are collected.
26. Set up the timer when starting to sort and collect 20,000 cells/tube in 750ul Trizol LS. From 1 whole cortex, we usually collect 7–8 tubes of 20,000 pyramidal cells in 1–1.5 h
Restrict sorting time to 1–2 h to prevent RNA degradation.

V. RNA extraction and purification

RNA can be extracted using method of choice. We use invitrogen purelink kit with modifications
Modification: Invitrogen recommends that equal volume of 100% Ethanol be added to aqueous phase to get a final concentration of 50% Ethanol. We find that increasing final concentration to 70% increases the short RNA and therefore the total yield of the sample.

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