Protocol: Single-cell suspensions from acute adult mouse brain

By Arpy Saunders. Version 1. 4/12/18

Saunders et al. 2018. A Single-Cell Atlas of Cell Types, States, and Other Transcriptional Patterns from Nine Regions of the Adult Mouse Brain

Disclaimer

Creating healthy and representative single-cell suspensions from adult brain tissue is a challenge. There are no systems that will guarantee success, no magic bullets/buffers; Indeed, there are likely many experimental solutions (one of which I present here) that give you the *opportunity* to create high-quality suspensions. Ask any brain slice electrophysiologist and they'll tell you how variable slice quality can be from prep to prep, even on the same day, with same buffers and littermate animals. Anyone can learn to make adult cell suspensions, but practice is required. From my experience, there are four universal keys to a successful experiment:

1. **Reagent quality**. Make sure your buffers/reagents are *fresh* and at the *correct* osmolarity/pH
2. **Speed**. You need to be *fast* in your dissections
3. **Touch**. You need to be gentle, avoiding rolling or shearing the tissue
4. **Temperature**: You need to keep the brain as *cold* as possible, through the perfusion, dissection, and slicing. I recommend ALWAYS chilling the brain from the inside out with a cardiac perfusion and dissecting the brain from the skull while it's submerged in ice-cold buffer.

I hope this protocol allows any experimenter – regardless of experience level – to learn to produce healthy brain suspensions.

Overview

Here I describe the step-by-step protocol I used to generate single-cell suspensions from P60–70 C57Blk6/N mice. The protocol is based on two different buffers. Mice are perfused and brains cut in an ice-cold liquid slushy of high-sucrose "Cutting Buffer" (CB). Acute slices are then washed and dissected in "Dissociation Buffer" (DB). Dissected tissue volumes are then digested in "Dissociation Buffer + Enzymes." Digested tissue is then titrated into single-cells using "Dissociation Buffer + Enzyme Inhibitors." Cells are then pelleted, cleaned of debris and processed in "Dissociation Buffer + BSA."

The DB buffer is Ca^{2+}-free, helping limit prevalent Ca-dependent downstream events like immediate early gene expression and excitotoxicity during protease digestion. DB Ionic concentrations are estimated to maintain cells at V_m = -30.5, a membrane potential that should inhibit action potential firing (by inactivating voltage-gate sodium channels).

Acknowledgements

I'd like to thank Dr. John Campbell from Brad Lowell's group at the Beth Israel Deaconess Medical Center for showing me his system for making single-cell suspensions from the hypothalamus. The protocol workflow follows his general design.

Improvements

This protocol is a starting point and many improvements can be madeI'm especially excited about the possibility of using cold-activated proteases – as described in this paper – which achieve digests while not subjecting RNA contents to elevated temperatures which drive degradation. Adding Actinomyosin as

described here and here or other transcriptional inhibitors can help limit active transcription during the prep.

Supplies

Reagents

- Buffers & Proteases/Inhibitor Stocks (see “Appendix” for recipes)
    - “Dissociation Buffer (DB)”
    - “Cutting Buffer (CB)”
    - 10x stocks of the Protease 23 and Papain proteases (“10X DB + P23” & “10X DB + PN”) and their respective inhibitor solutions (“10X DB + TI” & “10X DB + OI”)

Equipment

- Glass Pasteur pipets, fire-polished to successively smaller bore sizes
- Ice buckets
- Vibratome (eg Leica VT1000 S) for cutting acute brain slices
- Immersion blender
- Sylgard-coated dissection dish
- Freezable dissection tray (eg Tissue-Tek)
- Centrifuge, refrigerated to 4ºC. 15 ml falcon tube bucket adaptors (Spins are at 300g).
- Water bath (34ºC) & holders that snuggly hold 15 ml falcon tubes
- Perfusion system. We use a peristaltic pump (Watson-Marlow 120 Series).
- 40 µm cell strainer (Falcon Corning, 352340)
- Haemocytometer (eg Propper)
- Stereoscope for dissection (eg Leica MZ10)
- Inverted microscope for examining cell suspensions. If using our “LDH” cell viability system, epifluorescence also required.

Requirements per Replicate

- Each cell suspension replicate will require:
    - 15 mL falcon tubes (n=3)
    - 5 mL eppendorf tube (n=1)
    - 40 µm cell strainer

Step-by-Step Protocol

1. Set-up for digest. Prepare DB falcon tubes with protease, inhibitor and BSA cocktails (n=3 separate 15 mL falcon tubes). These buffers are used for protease digestion, digestion inhibition, and re-suspending cells for sequencing, respectively. Place on ice.
    a. Prepare 5 ml of 1x DB containing P23 and Papain ("DB P23 + PN") [4 mL of 1x DB, with 0.5 mL each of "10X DB + P23" and "10X DB + PN"]
    b. Prepare 10 mL of 1x DB containing Trypsin and Ovomucoid inhibitors ("DB TI + OI") [8 mL of 1x DB, with 1 mL each of "10X DB + TI" and "10X DB + OI"]
    c. Prepare 10 mL of DB + 0.01% BSA

2. Set-up dissection dish and tools. Fill sylgard-coated dissection dish with chilled DB (no additives). Place on ice. Prepare tools for blocking cuts and micro-dissection of brain regions.

3. Pre-warm 5 mL aliquot of "DB P23 + PN" buffer in 15 mL falcon tube. Transfer the tube to the water bath (set to 34°C).

4. Set-up for trans-cardiac perfusion and brain slicing
    a. Partially freeze >300 mL of CB, then blend with an immersion blender to make a liquid-ish slushy that is smooth (ie no ice crystals that could damage the brain)
    b. Fill a small weigh boat (or other container) with the CB slushy. Place on ice. After perfusion, the brain will be immediately placed into this slushy.

5. Perform a trans-cardiac perfusion of the mouse with ice-cold CB, following the guidelines of your animal protocol. Decapitate directly into weigh boat with CB slushy. Carefully dissect out the brain, keeping it as submerged (and cold) as possible. Avoid torqueing the brain or piercing it pieces of skull. Evaluate the quality of the perfusion (successfully perfusions will remove all blood and the brain will appear brightly white; if the perfusion is poor, start again).

6. Perform blocking cuts on brain. Remove brain from CB slushy with spatula. Quickly and carefully perform blocking cuts appropriate for the region and orientation of your planned acute slices.

7. Transfer the blocked brain to the vibratome. Gently cover the brain with ice-cold CB slushy and cut acute slices. (For our study, I cut slices that were 400 μm thick. The logic being that 400 μm slabs are thick enough to preserve the structure of the tissue while thin enough to allow 0_2 penetration and light-based imaging of cell somata.) [*troubleshooting tip: examine your slices under the microscope to check for the presence of healthy intact-looking soma, using the DB buffer to cover the slices. If you don't see intact and healthy looking cells in your slices, your suspensions will (unsurprisingly) be cell-poor. Swelling or shriveling of the cells in slices indicates that your DB is either hypo- or hyper-osmotic. If you don't know what you're looking at, ask a slice electrophysiologist friend to examine your slices on their rig. They can help explain what healthy and unhealthy cells look like and the overall quality of your slice.*]

8. Gently transfer slices containing regions of interest to your dissection dish containing chilled DB. Keep the DB buffer in the dissection dish extra-cold by placing it on top of a frozen-tray. Position the dish under the microscope and proceed to gently dissect out regions of interest. Use straight, sharp cuts and avoiding pulling or torqueing the tissue. Image the slices before and after your dissections so you know what exact tissue areas went into your single-cell suspensions. [*pro-tip: less is often more, tissue-wise. It's better to set up parallel digests of the optimal amounts of tissue then to overload any single tube with too much tissue (<30 mg), which will be produce a lot of debris that's difficult to remove.*]

9. Gently transfer the tissue volumes containing regions of interest into the pre-warmed "DB P23 + PN" using fine forceps. Avoid torqueing or tearing the tissue. Return to the tube to the water bath and allow tissue to digest for the empirically-determined optimal time, occasionally gently disturbing the slices to avoid sticking. [*troubleshooting tip: to determine optimal digest times for each region (Table 1), I did many paired experiments, splitting tissue from the two hemispheres into two digest conditions and comparing the resulting number of recovered single cells. This experimental strategy makes digest-time comparisons "paired," helping to control for variability in experimental factors (like tissue quality) upstream of digest time. In practice, I found digest times varied between 1 – 3 hrs in adult mice, with the longer times for regions with higher white matter content]. Also note that there may be a trade-off between higher cell yield and RNA quality – you may find that longer digest times yield higher cell numbers, but this can come at a cost of more RNA degradation.*

10. Remove tube containing the digested tissue from the water bath and place on ice. Gently remove the "DB P23 + PN" buffer and gently replace with 5 mL of ice-cold inhibitor solution "DB TI + OI". Return to ice.

11. Titrate the digested tissue into single cells using the fire-polished glass Pasteur pipets. The goal here is to use successively smaller-bore pipets to break up the tissue into successively smaller pieces, using as few passes as possible. If tissue is over-digested, it will immediately dissipate into a cloud of debris; if under-digested, it will take many passes to break up. There is a sweet-spot, a middle path. Avoid introducing bubbles at all costs, as they will annihilate your cells. I start with a P1000 and tip to break up any large chunks, and then work my way through several passes with each of the glass pipets. Target tissue chunks, do not just pass the entire volume. Return to ice.

12. Centrifuge cells into pellet (Pelleting #1). Place titrated suspension in 15 mL falcon tube into counter-balanced centrifuge, spin for 10 minutes at 300g.

13. Remove the supernatant. The supernatant contains debris, while the dissociated somata should make up the pellet. I use a P1000 to remove ~4.5 mL of the supernatant. For the last bit that's close to the pellet, I used a P200 and *VERY* carefully remove as much of the suspension as possible. (Even small amounts of the residual supernatant can contribute large amounts of debris). Do not disturb the pellet.

14. Gently resuspend the pellet in 5 mL DB + BSA.

15. Centrifuge cells into pellet (Pelleting #2). Spin for 10 minutes at 300g.

16. Remove the supernatant as before. Resuspend the pellet in DB + BSA. This resuspension volume will determine your cell concentration for downstream processing, so choose wisely.

17. Filter the suspension. Pre-wet the 40 μm cell strainer with DB + BSA. Place over the collection tube. (In my case, I used 5 mL Eppendorf tubes, in spite of the improper strainer fit. I avoided 50 mL falcons because I was afraid of dropping/pouring the cells from that height.) Pass the

suspension through the filter. Use a P1000 to pull the suspension that is stuck to the bottom of the filter. Add to the total suspension.

18. Measure cell concentrations and/or cell viability. After gently pipetting the suspension up and down to unsettle the cells, take a suspension sample, add to haemocytometer (typically 10 µl). Repeat, gathering a second replicate. Examine the suspensions under the microscope and calculate cell concentrations. What type of somatic morphologies do you see? You should see neuron, astrocytes, oligodendrocytes and a host of smaller cells without obvious processes.
    - **a.** To assay the membrane integrity of individual cells in your suspension, you can use our modified "LIVE/DEAD/HOECHST" fluorescence assay ("LDH Assay", Appendix).
    - **b.** Add 20 µl of suspension to 20 µl of 2x LDH assay. Allow cells to sit at room temperature for 10 minutes in the dye-cocktail. Gently pipet to un-pellet, then load sample into haemocytometer, image the three fluorescent channels and calculate the fraction of "healthy" cells (Hoechst/Calcein-AM double positive) vs "injured" cells with leaky nuclear membranes (EtBR+)
    - **c.**

Appendix

A. Buffer Recipes

HEPES-Sucrose "Cutting Buffer"

	MW	final concentration (mM)	for 1L (in g or ml)	for 2L (in g or ml)
NaCl	58.44	110	6.43	12.86
HEPES	238.3	10	2.383	4.766
glucose	180.2	25	4.505	9.01
sucrose	342.3	75	25.6725	51.345
$MgCl_2$ $6H_20$	203.31	7.5	7.5 ml (1M stock)	15 ml (1M stock)
KCl	74.56	2.5	2.5 ml (1M stock)	5 ml (1M stock)

- adjust to pH 7.4 with NaOH
- acceptable osmolarity range: 350-360 mOsm

"Dissociation Buffer"

	MW	final concentration (mM)	for 1L (in g or ml)	for 2L (in g or ml)
Na_2SO_4	142.04	82	11.65	23.3
K_2SO_4	174.26	30	5.23	10.46
glucose	180.2	10	1.81	3.62
HEPES	238.3	10	2.39	4.78
$MgCl_2$ $6H_20$	203.31	5	5 ml (1M stock)	10 ml (1M stock)

- adjust to pH 7.4 with NaOH
- acceptable osmolarity range: 290 – 300 mOsm

Storage: Buffers should be stored at 4°C and discarded < 2 weeks.

B. Protease and Protease Inhibitor Cocktails

Prepare 10x stocks of the Protease 23 and Papain:

- **Protease 23** [Sigma, P4032]. Prepare a 10x stock of (30 mg/ml) in Dissociation Buffer. I prepare 50 mL at a time in falcon tube, vortex into solution, then store frozen (-20°C) in 550 ul aliquots. This stock is called “10X DB + P23” in the protocol.

- **Papain** [Worthington, LK003178]. Prepare a 10x stock (100 units/ml Papain, 5 mM L-Cysteine and 2.5 mM EDTA) by adding 1 ml of Dissociation buffer into each small vial, warm at 37°C for 10 minutes to dissolve, then store frozen (-20°C) in 550 ul aliquots. This stock is called “10X DB + PN”.

Prepare 10x stocks of the Trypsin and Ovomucoid Inhibitors:

- **Trypsin Inhibitor** [Sigma, T6522]. Prepare a 10x stock (10 mg/ml) in Dissociation Buffer. I prepare 50 mL at a time in falcon tube, vortex into solution, then store frozen (-20°C) in 1.1 mL aliquots. This stock is called “10X DB + TI”.

- **Ovomucoid protease inhibitor and BSA** [Worthington, OI-BSA]. Prepare a 10x stock (10 mg/ml) in Dissociation Buffer. I add 32 mL of Dissociation Buffer to the large Worthington vial and gently vortex into solution. Store frozen (-20°C) in 1.1 mL aliquots. This stock is called “10X DB + OI”.

C. Fluorescence-based Assay of Cell Health

To evaluate the quality of suspensions, it can be helpful to measure how intact/healthy the cells are (in addition to simply total cell numbers).

We modified the LIVE/DEAD viability/cytotoxicity kit from Thermo with Hoechst dye ("LDH Assay"). This cocktail of 3 dyes can be mixed with the suspension and imaged to reveal different qualities of cell health:

a. *Calcein-AM* ("Live" Marker) – reports intactness of plasma membrane through esterase-based accumulation of the Calcein dye. Green channel.

b. *Hoechst* ("Live" Marker) – indicates relatively unperturbed nuclei by reporting double-stranded DNA through minor-groove intercalculation. Blue channel.

c. *EthD-1* ("Dead" Marker) – indicates permeable nuclear membranes. Dye binds to major-groove DNA but under normal circumstances is prevented from entering the nuclei by the intact nuclear membrane

Make a 2x working stock of the dyes at the following concentrations (Calcein-AM, 20 µM; EthD-1, 20 µM; Hoechst 33342, 40 µM). Freeze 25 µl aliquots at -20ºC.

D. Adult Mouse Brain Region Cell-Suspension Table

Region	Digestion Time (hrs)	Slice Orientation	Resuspension Volume (ml)	Mice / Replicate
Frontal Cortex (FC)	2	Coronal	3 or 4	1
Posterior Cortex (PC)	2	Coronal	3 or 4	1
Hippocampus (HC)	1.5	Horizontal (10-15 pitch forward)	3	1
Thalamus (TH)	2.5	Coronal & Sagittal	3	1
Cerebellum (CB)	3	Sagittal	4	1
Striatum (STR)	1	Coronal	3	1
Globus Pallidus externus & Nucleus Basalis (GP/NB)	2.5	Sagittal	3	2
Entopeduncular nucleus and Subthalamic nucleus (EP/STN)	3	Sagittal	3	4
Substantia Nigra and Ventral Tagmental Area (SN/VTA)	2.5	Coronal	3	2 or 3