Mouse Brain Flow Protocol

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Purpose:

To identify and characterize the number and activation state of microglia and infiltrating immune cells of the mouse brain. This protocol was created by combining aspects from all the references listed. Currently this protocol has only been used with a whole brain. Given the amount of recovery it may be possible to isolate sufficient cells from specific brain regions using this protocol.

Materials:

- 1x PBS – 10mls for perfusion/mouse
- 10cm petri dishes – 1 per mouse
- Razor blade
- Sterile transfer pipettes – Pasteur pipettes (glass)
- 15ml tube – 2 per mouse
- 50ml tubes – 3 per mouse
- P1000 tips with wide bore (pre-cut the ends of the tips to create wide bore tips, then autoclave)
- Normal P1000 Tips
- P1000 Pipette
- Enzymes
    - Make stocks in MilliQ water and filter sterilize
    - **Stock DNAase 1 20mg/ml** – add 100ul of stock to each brain for final concentration of 0.5mg/ml
    - **Stock liberase 5mg/ml** – add 80ul of stock to each brain for final concentration of 0.1mg/ml
- Serum free Media
    - RPMI +
- Serum Media
    - RPMI + 10% FBS and 1% Pen-strep
- Stock Isotonic Percoll (SIP) Stock media – this needs to be at room temperature before starting procedure
    - 10% of 10x PBS
    - 90% Percoll

- The below solutions are made from the SIP stock:

	9mls each (enough for 2 brains)	For 10 brains – 45mls each
70% SIP	7ml SIP 2mls 1x PBS	63 ml SIP 14ml 1x PBS
37% SIP	3.7mls SIP 5.3mls 1x PBS	33.3 ml SIP 26.5 mls 1x PBS
30% SIP	3mls SIP 6mls 1x PBS	15 mls SIP 30mls 1x PBS

Procedure:

1. Perfuse mouse with 10mls of PBS using a 10ml syringe and 26 gauge needle.
2. Extract brain and place in a small petri dish with 1 mL of serum-free media.
3. Using a razor blade, mince the brain.
4. Transfer the minced brain to a 15 mL tube, then rinse the petri dish with 1 mL of serum-free media.
5. Put the 15 mL tube on ice. Each tube should have 2 mls of media and minced brain.
6. In the lab, add 100 µL of DNase I (Final concentration of 0.5 mg/mL) and 80µL Liberase (final concentration of 0.1 mg/mL) to each sample.
7. Add 1.820 mL of serum-free media to each sample. (If you alter your stock enzyme concentrations you can change the volume here – make sure final volume added to each brain is 4mls)
8. Incubate tubes at 37C for at 40 minutes (This can be in incubator or in warm water bath) , inverting every 10 minutes.
9. Using a large gauge needle – 18 Gauge and 5ml syringes – mechanically disrupt the mixture by syringing up and down at least 5 times. See photo below. Figure from (Bordt et al, 2020).

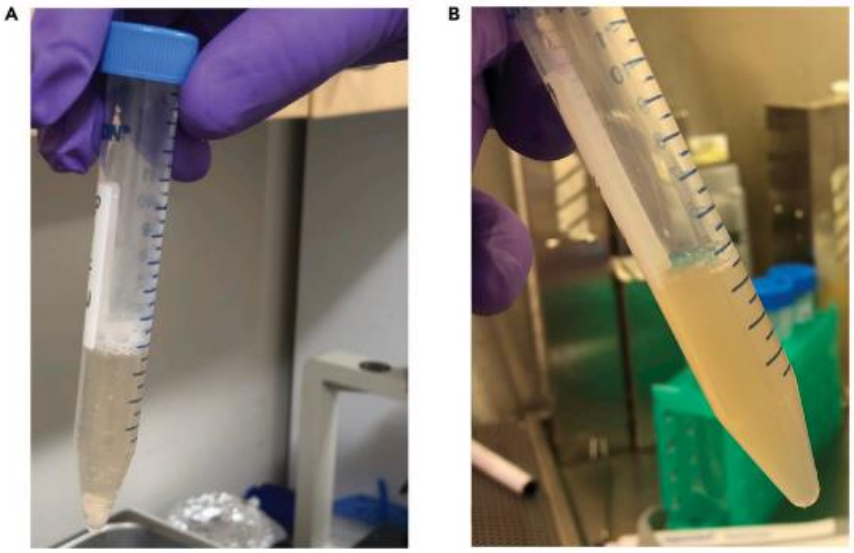
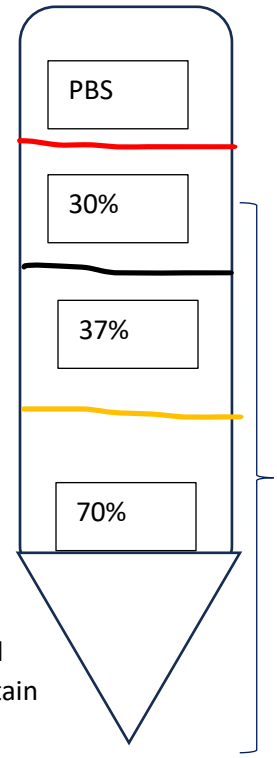


Figure 3. Dissociation of Tissue to a Single-Cell Suspension
Appearance of tissue (A) prior to and (B) after dissociation steps

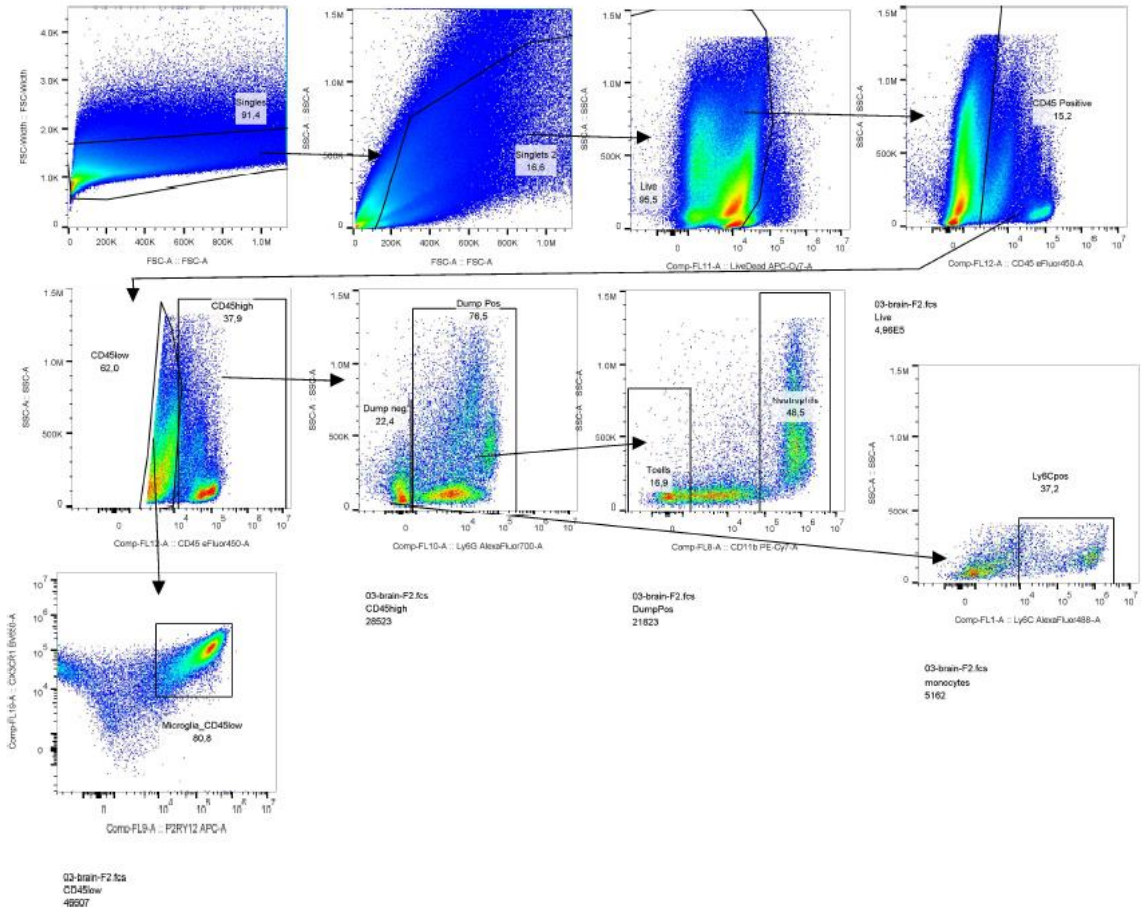
10. Neutralize enzymes by adding 5 mL of serum media to each tube.
11. Transfer sample to 50ml conical tubes.
12. Spin down tubes at 360 g for 5 minutes.
13. Filter the samples through a 70 µm cell strainer into a new 50ml tube. then centrifuge at 360 g for 5 minutes.
14. Resuspend pellet in 4 mL of 37% SIP and transfer to a new 15 mL tube.
15. Slowly add 4 mL of 70% SIP, 4 mL 30% SIP and 2 mL of 1x PBS. *This must be done very slowly and with control to get the best banding.*
16. Spin tubes at 300 g, brake set to 1, at **room temperature** for 40 minutes.
17. Using a transfer pipette, remove the myelin layer and discard. The layer indicated in red in the drawer.
18. Collect the layer of cells between the 70-37% interface into a clean 50 mL tube, collecting as much as possible from above and below. Ie. Collect everything below the myelin layer (see bracket in figure beside). Your cells should band by the yellow spot but the band can be hard to see so collect everything below the myelin layer.
19. Add 3x the interphase volume as PBS (dilute Percoll 3x).
20. Spin tubes at 365 g for 5 minutes at room temperature. Brake can be turned back on now.
21. **For flow:**
    - **a.** Resuspend cells in 200 µL PBS and transfer to V bottom plate.
        - **i.** For controls you can rinse out the tubes with PBS
        - **ii.** You should consider euthanizing an extra mouse to do all FMO controls and unstained.
    - **b.** Spin at 400 g for 5 minutes at 4°C to pellet cells.
    - **c.** Remove supernatant with pipette (cells are not very sticky so will fall out of plate if you flip upside down to remove the supernatant) and stain for 30 minutes in 50 uL stain.
    - **d.** Spin at 400 g for 5 minutes, then remove supernatant.
    - **e.** Fix cells for 8 minutes with 200 µL fixlyse.
    - **f.** Spin at 400 g for 5 minutes, then wash with 200 µL of PBS.
    - **g.** Spin at 400 g for 5 minutes and resuspend in 240 µL of facswash.



Stain Mix:

	Colour	uL/sample
CD45	eF450	0.5
CD11b	PE-Cy7	0.125
F4/80	PE-Dazzle 594	0.2
Ly6G	AF700	0.1
CD3	AF700	0.2
CD86	PerCp	1
MHC-II	PE	1
CCR2	BV785	0.8
Ly6C	AF488	**0.5**
P2RY12	APC	1
CX3CR1	BV650	0.1
LiveDead	Zombie NearIR	0.1
	PBS	44.375

Proposed Gating Strategy – This is loosely based on the gating and markers used in Spiteri AG, et al. 2021.



References:

1) Bordt. EA, et al. Isolation of Microglia from Mouse or Human Tissue. Star Protocols Cell Press June 19 2020, doi: 10.1016/j.xpro.2020.100035
2) Spiteri AG, et al. High-parameter cytometry unmasks microglial cell spatio-temporal response kinetics in severe neuroinflammatory disease. Journal of Neuroinflammation July 26 2021. doi: 10.1186/s12974-021-02214-y
3) Biolegend protocols: https://www.biolegend.com/en-us/protocols/whole-mouse-brain-processing-for-microglia-isolation-cell-separation-and-flow-cytometry
4) Lee JK and Tansey MG. Microglia isolation from Adult Mouse Brain. Methods Mol Biol. Aug 27 2014. Doi:10.1007/978-1-62703-520-0_3