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**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)+rubber bulb
* 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 SIP2mls 1x PBS | 63 ml SIP14ml 1x PBS |
| 37% SIP | 3.7mls SIP5.3mls 1x PBS | 33.3 ml SIP26.5 mls 1x PBS |
| 30% SIP | 3mls SIP6mls 1x PBS | 15 mls SIP30mls 1x PBS |

**Procedure:**

1. Perfuse the mouse with 10mls of PBS using a 10ml syringe and 26 gauge needle.
2. Extract the brain and place it 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 (910 μL × 2) of serum-free media to each sample. (If you alter your stock enzyme concentrations, you can change the volume here – make sure the final volume added to each brain is 4mls)
8. Incubate tubes at 37C for 40 minutes (This can be in an incubator or in a 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 the photo below. Figure from (Bordt et al, 2020).

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1. Neutralize enzymes by adding 5 mL of serum media (37 C) to each tube.
2. Transfer the sample to 50ml conical tubes.
3. Spin down tubes at 360 g for 5 minutes. Remove supernatant and resuspend in 10 mL of serum media. Filter the samples through a 70 μm cell strainer into a new 50ml tube. Wash the old 50mL conical tube with 10 mL of serum media, and wash the filter with 5 mL of serum media.
4. Centrifuge at 360 g for 5 minutes.
5. Take out supernatant and resuspend pellet in 4 mL of 37% SIP and transfer to a new 15 mL tube.
6. Slowly add 4 mL of 70% SIP, 4 mL of 30% SIP, and 2 mL of 1x PBS (RT). This must be done very slowly and with control to get the best banding. Use a *p1000 pipet to control speed.*
7. Spin tubes at 300 g, brake set to 1, at **room temperature** for 40 minutes.

1. Using a transfer pipette, remove the myelin layer and discard. The layer indicated in red in the drawer.

PBS

1. 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. *Firstly, suck up the myelin layer with a pasteur pipet, then collect interphase with a new Pasteur pipet tip. Collect as much as possible, but do not touch red blood cells at the bottom of the tube.*

30%

1. Add 3x the interphase volume as PBS (dilute Percoll 3x).

37%

1. Spin tubes at 365 g for 5 minutes at room temperature. The brake can be turned back on now.
2. **For flow:**
	1. Resuspend cells in 200 μL PBS and transfer to V bottom plate.
		1. For controls you can rinse out the tubes with PBS

70%

* + 1. You should consider euthanizing an extra mouse to do all FMO controls and unstained.
	1. Spin at 400 g for 5 minutes at 4°C to pellet cells.
	2. Remove the supernatant with a pipette (the cells are not very sticky, so they will fall out of the plate if you flip it upside down to remove the supernatant) and stain for 30 minutes in 100 uL of LiveDead Blue [1 in 1000].
	3. Fash twice with FacsWash
	4. Resuspend in Facs+ Fc block (1%) and incubate for 5 mins
	5. Add 50 uL of stain and incubate for 5min.
	6. Spin at 400g for 5 min 4 C to pellet the cells
	7. Fix cells for 8 minutes with 200 μL fixlyse.
	8. Spin at 400 g for 5 minutes, then wash with 200 μL of PBS.
	9. 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](https://doi.org/10.1007/978-1-62703-520-0_3)