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Brain Single Cell Isolation for Flow Cytometry

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Protocol status: Working

We use this protocol and it's working



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Abstract

This protocol is used to isolate single cells from mouse brains and process these samples for flow cytometry analysis. The protocol can be optimized/altered to account for staining of intracellular cellular markers.

SETUP

- 1 Make reagents needed:
 - Flow media: RPMI 1640 + 10%FBS + 1% Penicillin/Streptomycin + 1% L-Glutamine
 - 10X Stock Enzyme: 1g Collagenase IV in 100mL serum-free media (RPMI 1640).
Aliquot into 5mL and store at  -20 °C until use. To use (working concentration) thaw on ice, QS to 38mL using serum-free media. Final concentration with 2mL sample is ~1mg/mL Collagenase IV.
 - 90% Percoll: 18mL percoll + 2mL 10X PBS
 - 70% Percoll: 7mL 90% + 3mL 1X PBS
 - Staining buffer: 2% FBS in 1X DPBS without calcium and magnesium
- 2 Pre-heat water bath to 37C and pre-cool centrifuge to 4C. Label and set-up 6-wel plates, 50mL and 15mL conical tubes.

PROCEDURE

- 3 Isolate brain tissue using standard perfusion protocols and dissection techniques based on desired brain regions.
- 4 Place tissue in flat-bottom-6-well cell culture plate with 2mL flow media until tissue collection is complete.
- 5 Prepare enzyme and add 6mL working concentration to labeled 15mL conical tubes.
- 6 Mince isolated tissue with small scissors and transfer using pre-cut 1000µL pipette tips to conical tubes with enzyme.
- 7 Shake in water bath at 37C for 20 minutes: at 10 minutes, vortex and pipette up and down with Pasteur pipette to break up tissue then continue timer.
- 8 Transfer to 70µm cell strainers placed in 50mL tubes and smash tissue using the plunger of a 5mL syringe, rinsing with DPBS or flow media.
- 9 Spin at 1800RPM for 8min at 4C (brake on high)
- 10 Decant supernatant and re-suspend pellet in 7mL of flow media in 15mL conical tube.



- 11 Vortex cell suspension with 3mL 90% Percoll.
- 12 Inject 1.5mL of 70% Percoll under the 90% mixture SLOWLY. It is recommended to take-up 2mL of Percoll to inject, in order to prevent bubbles or disturbing the layer.
- 13 Spin at 1500RPM for 30min at 4C (no brake). DO NOT DISTURB GRADIENT.
- 14 Check for cells - should be suspended between pink and clear layer. Vacuum supernatant and fat to about 7mL line.
- 15 Transfer cells to a clean 15mL conical tube and fill rest of the way with PBS. DO NOT TAKE PELLET!
- 16 Vortex and spin at 1800RPM for 8min at 4C (brake on high).
- 17 Aspirate and leave 200uL liquid with pellet. Pipette up and down to break up pellet and transfer to round-bottom 96-well plate for staining. Label plate appropriately.
 - Splenocytes: wells for single color control and unstained control (and optional: well for mastermind + splenocytes) - see steps below for splenocyte isolation
 - Brain samples: mastermix only
- 18 Spin plate at 1500RPM for 5 min. Decant supernatant.
- 19 Re-suspend cells (only brain samples) in 100μL Fc Block (1:100 in staining buffer (FBS/PBS)) OR 100uL of staining buffer to splenocytes. Incubate on ice for 15 minutes, then add 100uL staining buffer on top for volume.
- 20 Spin plate at 1500RPM for 5min. Decant supernatant.
- 21 Make up antibody cocktail (mastermix) and single colour controls:

To make mastermix: add antibodies at their specific concentrations to at least 500uL FBS/PBS (or based on how many samples you have)



To make single colour controls: use volume of 2% FBS/PBS that will allow for 1 μ L of each antibody. (i.e if CD45 is 1:200, use 200 μ L of FBS/PBS)

- 22 Re-suspend cells in 50 μ L antibody cocktail in staining buffer. Incubate 20-30 minutes on ice or at 4C.
- 23 Add 150 μ L staining buffer on top for 200 μ L total volume.
- 24 Spin 1500RPM for 5min. Decant supernatant.
- 25 Re-suspend cells in 200 μ L 2%PFA. Incubate on ice or at 4C.
- 26 Spin 1500RPM for 5min. Decant supernatant. Re-suspend in 200 μ L FBS/PBS.
- 27 For storage: foil and parafilm and place plate in 4C. Before running samples on flow cytometer, filter samples with a strainer cap into 5mL FACS tubes.

SPLENOCYTE ISOLATION

- 28 Isolate spleen from perfused mouse and place in 70 μ m strainer in 50mL conical tube.
- 29 Smash spleen with media and spin down at 1500RPM for 5min.
- 30 Decant media and add 5mL ACK lysis buffer into tube for 3-5 minutes. Neutralize with 10mL 1X DPBS.
- 31 Spin 1500RPM for 5min. Decant and resuspend in 10mL DPBS.
- 32 Spin 1500RPM for 5min. Aspirate and resuspend in 10mL flow media.