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Flow-cytometry approach to characterize phagocytic properties of acutely-isolated adult microglia and brain macrophages in-vitro

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Cover Letter

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The Editor
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Dear Dr Myers,

I am pleased to submit our article entitled “Flow-cytometry approach to characterize phagocytic properties of acutely-isolated adult microglia and brain macrophages“ for consideration for publication in The Journal of Visualized Experiments *(JoVE)*.

Microglia and infiltrating brain macrophages, together called the central nervous system mononuclear phagocytes (CNS-MPs), form the majority of the brain phagocytic population. CNS-MPS adopt a distinct disease-associated phenotype and can play complex pathological roles in neurological diseases. Their phagocytic roles can contribute to amyloid beta and debris clearance as well as direct synaptic and neuronal injury in Alzheimer’s disease. Characterizing these phagocytic properties can provide a functional readout that compliments molecular profiling of microglia using traditional flow cytometry, transcriptomics and proteomics approaches. Phagocytic profiling of microglia has relied on microscopic visualization and *in-vitro* cultures of mouse neonatal microglia. The former approach suffers from limited sampling while the latter approach is inherently poorly reflective of the true *in-vivo* state of adult CNS-MPs.

We describe optimized protocols to phenotype phagocytic properties of acutely-isolated mouse CNS-MPs by flow cytometry. CNS-MPs are acutely isolated from adult mouse brain using mechanical dissociation followed by percoll density centrifugation, incubated with fluorescent microspheres or fluorescent Aβ fibrils, washed, and then labeled with panels of antibodies against surface markers (CD11b, CD45). Using this approach, we can compare phagocytic properties of brain-resident microglia with CNS-infiltrating macrophages and then assess the effect of aging and disease pathology on these phagocytic phenotypes. This rapid method also holds potential to functionally phenotype acutely-isolated human CNS-MPs from post-mortem or surgical brain specimens. Additionally, specific mechanisms of phagocytosis by CNS-MP subsets by inhibiting select phagocytic pathways can be investigated.

We believe that this detailed video-based methods paper will provide the neuroscience community with a well-characterized and easily-implemented functional assay of adult acutely-isolated CNS-MPs which can be easily incorporated into existing flow cytometry protocols to provide a functional dimension to traditional immuno-phenotyping.

We have no conflicts of interest to disclose.

Thank you for your consideration

Sincerely,

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TITLE:

Flow Cytometry Approach to Characterize Phagocytic Properties of Acutely-Isolated Adult Microglia and Brain Macrophages In Vitro

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KEYWORDS:

Alzheimer's disease, amyloid beta, flow cytometry, microglia, neuroinflammation, neurodegeneration, phagocytosis, stroke

SUMMARY:

Assessment of phagocytic properties of microglia and brain macrophages can provide a valuable functional dimension to molecular profiling studies. We describe a validated protocol that uses flow cytometry to rapidly and reliably quantify phagocytosis of fluorescent microspheres and amyloid beta fibrils by acutely-isolated microglia and brain macrophages from mouse models.

ABSTRACT:

Microglia and central nervous system (CNS)-infiltrating macrophages, collectively called CNS mononuclear phagocytes (CNS-MPs), play central roles in neurological diseases including neurodegeneration and stroke. CNS-MPs are involved in phagocytic clearance of pathological proteins, debris and neuronal synapses, each with distinct underlying molecular pathways. Characterizing these phagocytic properties can provide a functional readout that compliments molecular profiling of microglia using traditional flow cytometry, transcriptomics and proteomics approaches. Phagocytic profiling of microglia has relied on microscopic visualization and in vitro cultures of mouse neonatal microglia. The former approach suffers from limited sampling while the latter approach is inherently poorly reflective of the true in vivo state of adult CNS-MPs. This paper describes optimized protocols to phenotype phagocytic properties of acutely-isolated mouse CNS-MPs by flow cytometry. CNS-MPs are acutely isolated from adult mouse brain using mechanical dissociation followed by density gradient centrifugation, incubated with fluorescent microspheres or fluorescent Aβ fibrils, washed, and then labeled with panels of antibodies against surface markers (CD11b, CD45). Using this approach, it is possible to compare phagocytic properties of brain-resident microglia with CNS-infiltrating macrophages and then assess the effect of aging and disease pathology on these phagocytic phenotypes. This rapid method also holds potential to functionally phenotype acutely-isolated human CNS-MPs from post-mortem

or surgical brain specimens. Additionally, specific mechanisms of phagocytosis by CNS-MP subsets can be investigated by inhibiting select phagocytic pathways.

INTRODUCTION:

The innate immune cells of the central nervous system (CNS) are predominantly comprised of microglia and infiltrating monocytes/macrophages, together called as the CNS-mononuclear phagocytes (CNS-MPs)[1]. CNS-MPs are implicated in neurodegenerative diseases such as Alzheimer's disease (AD), neuroinflammatory disorders, and stroke[2-4]. CNS-MPs, along with astrocytes, pericytes and ependymal cells, have phagocytic functions[5,6]. In their homeostatic state, CNS-MPs are involved in constant surveillance of the local microenvironment, along with phagocytic clearance of apoptotic cell debris and proteins, and synaptic pruning to remodel neuronal connections[7-10]. In neurodegenerative conditions such as AD, CNS-MPs adopt distinct disease-associated molecular and functional phenotypes which can play pathological roles including clearance of aggregated amyloid-beta (Aβ) and neuronal elements, as well as inflammatory cytokine and factor release, resulting in complex pro-inflammatory, detrimental as well as anti-inflammatory, protective roles[11-14]. Phagocytosis of macroparticles, cellular debris, proteins and other infectious particles by CNS-MPs are mediated by distinct receptors expressed on their surface[9]. Disruption in these phagocytic pathways can lead to defective clearance and progressive accumulation of Aβ ultimately leading to progressive neuronal damage in AD[4,9]. While advances in molecular profiling of CNS-MPs using transcriptomic and proteomic approaches have provided invaluable insights into the molecular heterogeneity within CNS-MPs in neurological diseases[15,16], functional characterization of the phagocytic properties of CNS-MPs and their subsets is currently lacking. Functional characterization of the phagocytic properties of CNS-MPs can complement molecular profiling strategies, facilitate better functional phenotyping, and assist in assessing the efficacy of therapeutics that can rectify defective phagocytosis in disease models[17].

Traditional phagocytosis assays for CNS-MPs include incubating primary microglia along with fluorescent substrates such as Aβ or latex/polystyrene particles. Phagocytosis is then studied as a function of uptake of the fluorescent substrate using immunofluorescence microscopy[18-20]. It is well-established that CNS-MPs, when maintained in long cultures, can dramatically change their morphology and transcriptional profiles[21,22]. This hinders studying the phagocytic properties of CNS-MPs in their representative state in the CNS. A functional flow cytometry assay to profile acutely-isolated live CNS-MPs can provide a rapid assessment of phagocytic properties and can sample a much larger pool of CNS-MPs than microscopy approaches[9,23]. Furthermore, flow cytometry eliminates the need for maintaining the CNS-MPs in culture as well as provides a platform to study phagocytotic properties in different sub-populations of CNS-MPs. This manuscript describes optimized protocols to phenotype phagocytic properties of acutely-isolated mouse CNS-MPs by flow cytometry. Adapting phagocytosis assays using flow cytometry allows rapid multiplexing of the phagocytic phenotype of CNS-MPs coupled with immune phenotyping, thereby providing insights into heterogeneity of phagocytic properties within CNS-MPs at the single cell resolution.

PROTOCOL:

All mouse studies were conducted after obtaining approval from the Emory University's Institutional Animal Care and Use Committee (IACUC), and in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

1. **Preparation on the day of isolation**

1.1. Prepare an ice bucket to keep all reagents, buffers, and cell suspensions cold throughout the isolation procedure.

1.2. Place the bottle of 1× phosphate-buffered saline (PBS) (at least 1 L) at 4 °C for 30 min to 1 h prior to perfusion and keep 1× PBS on ice during the perfusion and isolation procedure.

1.3. Prepare working concentration of the density gradient medium (referred to as 35% SIP, see **Table of Materials**).

1.4. Label all the conical and flow cytometry tubes used in the experiment prior to cell isolation to avoid delays in processing times. Additionally, gather all other supplies necessary for the isolation prior to perfusion, such as 40 µm cell strainers, syringes, and needles.

2. Acute isolation of adult mouse microglia

2.1. Anesthetize an adult mouse by placing the mouse in a glass induction chamber with isoflurane (5 mL) until the mouse stops breathing and fails to respond to hindlimb paw pinch.

2.2. Use a 26 G needle and 10 mL syringe to perform cardiac perfusion with 30 mL of ice-cold 1× PBS until there is no visual blood.

2.3. Decapitate the mouse with surgical scissors. Use small scissors to cut the skin, exposing the skull. Remove the skull without damaging the brain tissue as described[24]. Remove the brain immediately and transfer to a sterile 40 µm cell strainer placed on top of a 50 mL conical tube.

2.4. Use the backside of the plunger of a 3 mL syringe to push the brain through the strainer and mechanically dissociate the entire brain tissue. Wash the cell strainer and plunger intermittently during the dissociation with 2 mL of ice-cold 1× PBS each time to ensure all the tissue passes through the strainer.

2.5. Scrape the bottom of the cell strainer with the plunger to collect any residual suspension into the conical. Thoroughly flush the cell strainer with ice-cold 1× PBS and pour the suspension into the 50 mL conical tube.

NOTE: When processing multiple animals in series, place the cell suspensions on ice until further processing.

2.6. Add ice-cold 1× PBS to bring the cell-suspension volume to 30 mL and centrifuge at 800 × *g* for 5 min at 4 °C.

2.7. Pour off the supernatant without disturbing the pellet and re-suspend the pellet in 6 mL of 35% SIP with a 10 mL serological pipette. Transfer the cell suspension + 35% SIP mixture to a new 15 mL conical tube and centrifuge at 800 × *g* for 25 min at 15 °C with no brake.

CAUTION: Take special care to avoid introduction of bubbles during re-suspension. Excessive bubbles can be detrimental to cell yield.

2.8. Carefully remove the 15 mL conical tube from the centrifuge without disturbing the layers and aspirate the top floating myelin layer.

2.9. Transfer the cell suspension + 35% SIP mixture using a 10 mL serological pipette to a new 50 mL conical tube containing 30 mL of ice-cold 1× PBS and centrifuge at 800 × *g* for 5 min at 4 °C. Carefully pour the supernatant off, leaving behind the cell pellet along with 500–800 µL of 1× PBS.

2.10. Re-suspend the cell pellet by gently pipetting up and down with a p1000 pipette and transfer to a 5 mL round bottom tube. Add 2 mL of ice-cold 1× PBS to the round bottom tube and centrifuge at 1,400 × *g* for 2 min at 4 °C. Quickly pour off the supernatant, leaving the cell pellet containing CNS-MPs in about 100 µL of 1× PBS (**Figure 1**).

3. **Phagocytosis set-up and flow cytometric staining**

3.1. Transfer 20 µL (≈200,000 cells) of the re-suspended CNS-MPs to a fresh 5 mL round bottom tube and add 80 µL of ice-cold 1× PBS. Do not add any fluorescent substrates or flow cytometry antibodies to this tube. These cells serve as a negative control to determine background fluorescence of CNS-MPs for flow cytometry analyses.

3.2. To establish the viability of the isolated CNS-MPs, use amine-based live/dead dyes (e.g., Fixable Blue UV).

3.2.1. Resuspend the rest of the cell pellet in 500 µL of 1× PBS and add 1 µL of the reconstituted dye to each sample tube except the negative control.

3.2.2. Gently vortex, cover the tubes with foil, and incubate them at room temperature for 30 min. Add 1 mL of 1× PBS to each tube and gently vortex.

3.2.3. Centrifuge the cells at 1,400 × *g* for 2 min at 4 °C. Quickly pour off the supernatant, and re-suspend the cell pellet in 100 µL of 1× PBS.

NOTE: Amine-based dyes will bind to the extracellular Aβ fibrils confounding the results of phagocytosis. Other live/dead indicators might interfere with the fluorescence of Aβ

fibrils/microspheres. Hence it is advised to used Live/dead indicator only in preliminary experiments to establish the viability of the isolated CNS-MPs.

3.3. Thoroughly vortex the tube containing PE-polystyrene microspheres and add 2 µL (≈200 microspheres/cell) to 100 µL of cell suspension in flow tubes except for the negative control. Gently vortex each tube to ensure equal distribution of the substrate with the cells and place the round bottom tubes in a sterile humidified incubator for 30 min at 37 °C with 5% CO_2.

3.3.1. Gently vortex and incubate the negative control tube to ensure similar experimental conditions between positive and negative samples.

NOTE: If using Aβ fibrils instead of PE-microspheres, prepare fluorescent HiLyte488 Aβ fibrils as described previously[9]. The final concentration of Aβ fibrils is 200 µM which is then diluted to prepare fresh 20 µM stock in PBS of fluorescent Aβ monomers. Add 12.5 µL of the 20 µM stock Aβ monomers to each tube containing isolated cells.

3.4. Remove the cells from the incubator and add 1 mL of 1× PBS to each tube and gently vortex. Centrifuge the cells at 1,400 × g for 2 min at 4 °C and quickly pour off the supernatant. Repeat the wash with 1 mL 1× PBS, centrifuge at 1,400 × *g* for 2 min, pour off the supernatant, and re-suspend the cell pellet in 100 µL of 1× PBS.

NOTE: After each spin, it is important to keep the cells on ice to inhibit ongoing phagocytic uptake.

3.5. Vortex the flow-cytometry antibodies and centrifuge them in a microcentrifuge for 10 s. To each cell suspension tube add 1 µL each of CD11b-APC/Cy7 and CD45-PE/Cy7 for Aβ phagocytosis assay, or CD45-FITC for PE-microsphere phagocytosis. Gently vortex each tube and incubate in the dark for 30 min at room temperature.

NOTE: Users can also include a separate tube containing potent phagocytic cells such as cultured BV2 microglia or peritoneal macrophages as positive control and treat them with PE-microspheres, and flow cytometric antibodies as described above. Data from this sample can be used to define the positive control denoting true phagocytosis in preliminary experiments.

3.6. While the cells are in incubation, set up compensation tubes using four round bottom 5 mL flow tubes. Thoroughly vortex the compensation beads and add 1 drop to each tube. Add 1 µL of CD11b-APC/Cy7 to the first tube and CD45-FITC to second the tube. Add 1 µL of PE-polystyrene to the third tube and keep unstained compensation beads in fourth tube. Use this fourth tube as a negative control to set up a compensation panel on the flow cytometer.

NOTE: When Aβ fibrils are used to study phagocytosis, add CD45-PECy7 to second tube instead of CD45-FITC. Add 1 µL of separate FITC tagged flow cytometry antibody or FITC-secondary antibody (any species) to the third compensation tube. This is important since the beads cannot bind to Aβ fibrils and will get washed away upon washing of the compensation tubes.

3.7. Allow the compensation beads to incubate with their respective antibodies for at least 10 min. After incubation, add 1 mL of 1× PBS to each tube, centrifuge at 1,400 x *g* for 2 min at 4 °C, and quickly pour off the supernatant. Place the compensation tubes on ice and cover them with the foil until further processing.

3.8. Following incubation with flow-cytometry antibodies, wash the stained cells with 1 mL of 1× PBS. Gently vortex and centrifuge the cells at 1,400 × *g* for 2 min at 4 °C. Quickly pour off the supernatant and repeat the wash with 1ml 1× PBS. Pour off the supernatant and re-suspend the cell pellet in 100 µL of 1× PBS.

3.9. Place the sample tubes on ice and cover them with the aluminum foil until flow cytometry.

NOTE: Use 1× PBS (pH 7.0) without Ca^{2+}/Mg^{2+} as flow buffer throughout the experiment. Ensure to exclude fetal bovine serum (FBS) in the flow buffer to avoid possible microglial activation.

4. Flow cytometry and analysis

4.1 Instrument set-up and compensation

NOTE: The experiments in this manuscript were performed on a flow cytometer with ultra-violet, violet, blue, yellow-green and red lasers. This experiment can be also adapted to three-laser flow-cytometers with blue, yellow-green and red lasers only. Users need to undergo formal training before handling the instrument.

4.1.1. Refill the flow sheath buffer tank and turn on the cytometer. “Prime” the cytometer with a 5 mL round bottom flow tube containing ddH_2O. Log into the analysis software on the computer connected to the flow-cytometer. Create an “experiment” and select FSC, SSC, FITC, PECy7 and APC-Cy7 as the parameters. If using PE-microspheres, select PE instead of PECy7 in the parameter panel.

4.1.2. Create an appropriate compensation panel.

NOTE: In our experiments, typically the FSC/SSC voltages for compensation beads is around 350/280, whereas FSC/SSC voltages for CNS-MPs is around 320/260. These values are highly variable between any two different cytometers and needs to be calibrated prior to the actual experiment.

4.1.3. Reconstitute each compensation and cell suspension tube with 500 µL of 1× PBS, briefly vortex, and place on ice. Run the negative control/unstained bead tube to set appropriate voltages such that the peak on each fluorophore histogram is below 10^2. Run the other single-color compensation tubes to identify their distinct positive peaks.

NOTE: There should be at least half log (base 10) difference between the positive and negative peaks of each fluorophore.

4.1.4. Record 5000 events from each compensation tube to allow the computer to auto-calculate the compensation set-up. Link the compensation set-up to the current experiment[25].

4.1.5. Create a new "tube" under the sample tab. Gently vortex and run the negative control tube containing unstained CNS-MPs. Adjust the forward and side scatter voltages to capture the population of interest. Record the events and sub-gate on the live CNS-MPs as mentioned below.

4.1.6. Capture and record events from all subsequent tubes as separate files keeping the parameters and voltages consistent. Gently vortex each tube before running them on the flow-cytometer.

4.2. Gating strategy and measurement of phagocytosis

4.2.1. Draw an initial mononuclear gate to capture all CNS-MPs. These live mononuclear cells must be gated in the following graph to include only single cells, excluding couplets and triplets.

4.2.2. Project the gated single, live CNS-MPs population on a subsequent graph displaying CD11b and CD45 fluorophores.

NOTE: Brain Researchident microglia are typically $CD11b^{+}CD45^{intermediate}$, whereas infiltrating macrophages are $CD11b^{+}CD45^{high}$. Additional markers such as Ly6c may be included in the panel to further confidently separate inflammatory monocytes ($Ly6c^{high}$ and $CD45^{high}$) from microglia ($Ly6c^{low}$ $CD45^{intermediate}$).

4.2.3. Apply appropriate gates to further study phagocytosis in respective populations as shown in **Figure 2F**.

NOTE: Phagocytosis in CNS-MPs is measured as a function of their Hilyte488/Alexa488 (Aβ) or PE (microsphere) fluorescence uptake. Distinct positive peak on their respective histograms indicate that the CNS-MPs have phagocytosed the substrate. Quantity of material phagocytosed is directly proportional to the intensity of the fluorescence. Hence, presence of multiple peaks must be interpreted as higher phagocytosis. These can be subsequently confirmed by immunohistochemistry.

4.2.4. After recording all the sample tubes on the flow-cytometer, export the data files in.fcs format for further analysis.

4.3. Statistical data analysis

4.3.1 Use any flow-cytometry analysis software along with spreadsheets and statistical analysis software for all data analyses.

4.3.2 Add.fcs files to the analysis software and double click on the first file. A graph displaying all the recorded events pops up. Follow similar gating strategy as shown in **Figure 2** to subsequently gate single, live mononuclear cells and study phagocytosis on specific population such as $CD11b^{+}CD45^{intermediate}$ microglial cells or $CD11b+CD45^{high}$ infiltrating macrophages (**Figure 2H**).

NOTE: Phagocytic index is assessed based on the proportion of cells internalizing fluorescent Aβ fibrils or ≥1 microsphere(s). As defined previously, low-level phagocytosis is uptake of at least 1 microsphere and high-level phagocytosis is >1 bead uptake[8,9]. Based on prior experiments, inhibition of actin-dependent processes by cytochalasin D completely inhibits >1 bead uptake and partly inhibits 1 bead uptake[9] as well as completely inhibits Aβ uptake, confirming actin-dependent phagocytic uptake rather than passive binding to the cell surface. Immunocytochemistry also confirms that each sequential peak in the microsphere assay is representative of a unitary increase in the numbers of beads phagocytosed[8,9].

REPRESENTATIVE RESULTS:

To exemplify typical results of phagocytic uptake of microspheres and Aβ42 fibrils by acutely-isolated CNS-MPs, acutely-isolated CNS-MPs were obtained from the ipsilateral hemisphere following transient middle cerebral arterial occlusion (MCAO)[8]. For details regarding phagocytic properties of CNS-MPs in the MCAO model, please refer to prior publications[8]. Briefly, after a 72 h recovery, CNS-MPs were acutely isolated from fresh brain using mechanical dissociation followed by density gradient centrifugation, and incubation with either fluorescent microspheres or Aβ fibrils (**Figure 1**). Subsequently, CNS-MPS were labeled with panels of fluorescent-conjugated antibodies against two surface markers, CD11b and CD45, and analyzed on a flow cytometer for phagocytic uptake of microspheres or Aβ fibrils (**Figure 1**). This protocol has been applied to determine changes in phagocytic properties with aging, progressive neuropathology, and following ischemic brain[8,9,26] injury as well as assess the effects of neuro-immunomodulatory therapies in vivo[9].

The gating strategy used for data analysis is presented in **Figure 2A**. An initial scatter plot showing forward, and side scatter is used to draw a gate for live CNS mononuclear cells which includes CNS-MPs as well as some lymphocytes, and neutrophils. The mononuclear cells using this strategy are 96% viable using this current protocol and can then be further sub-gated to include single cells and exclude doublets and triplets which could induce potential bias in the analysis (**Figure 2B**). Following selection of single cells, CNS-MPs are identified based on their surface expression of CD11b and CD45 (**Figure 2C**). Microglia using this strategy are $CD11b^{+}CD45^{intermediate}$ while infiltrating macrophages are $CD11b^{+}CD45^{high}$. Lymphocytes are $CD11b^{-}CD45^{high}$. This protocol ensures high viability of isolated CNS-MPs. Live/dead amine dyes such as Fixable-blue UV can be used as mentioned above to confidently sub-gate on live CNS-MPs (**Figure 2D**) prior to studying phagocytosis on the populations of interest.

Phagocytosis in these single, live CNS-MPs is studied using histograms or scatter plots for respective fluorophores (PE-microspheres; Hilyte488-Aβ fibrils). As described above, the sample with no fluorophores added serves as a negative control to identify the positive peaks for microspheres (**Figure 2D,** grey histogram) and Aβ fibril uptake (**Figure 2E,** grey histogram).

Phagocytic uptake of PE microspheres is indicated by multiple peaks of PE fluorescence, and each peak observed represents unitary increase in numbers of microspheres phagocytosed by CNS-MPs. By sorting individual peaks of fluorescence intensity following uptake, it was possible to confirm by fluorescence microscopy that cells in the first positive peak have phagocytosed one PE-microsphere, cells in the second peak have taken up two microspheres, while higher-order peaks have higher phagocytic properties (**Figure 2D**). In contrast to the PE-microsphere assay, only one positive peak of green fluorescence is observed in the Aβ phagocytosis assay (**Figure 2E**). Based on negative unstained controls and cytochalasin D mediated inhibition experiments, it has also been previously shown that Aβ fluorescence is indicative of an actin-dependent process[9].

Since flow cytometry allows to capture several dimensions of data on a given cell, phagocytic properties can be coupled with surface marker expression, such as CD11b and CD45 and others. Based on this advantage of flow cytometry over traditional microscopic approaches, the differences in phagocytic uptake by CNS-MP subsets can be analyzed in a post-hoc manner, after completion of the assay and acquisition of all raw data. Using CD11b and CD45 expression, CNS-MPs can be subdivided into microglia ($CD11b^{+}CD45^{intermediate}$) and infiltrating macrophages ($CD11b^{+}CD45^{high}$) and additional markers such as CD11c, Ly6c, and Ly6g can be simultaneously used to further subdivide CNS-MP populations. Sub-gating single mononuclear cells and a comparison of PE-microsphere and Aβ phagocytic uptake by microglia, infiltrating macrophages and lymphocytes, are shown in **Figure 2F–H**. Unlike microglia and macrophages, lymphocytes ($CD11b^{-}CD45^{high}$) which account for <5% of all isolated cells have minimal phagocytic activity. Within CNS-MPs, microglia show slightly lower phagocytic activity for PE-microspheres compared to macrophages (**Figure 2G**), which Aβ uptake is markedly higher for $CD45^{high}$ infiltrating macrophages compared to microglia (**Figure 2H**). Readers are additionally referred to publications utilizing this assay to phenotype phagocytic properties of acutely-isolated CNS-MPs and splenocytes in aging mice and in mouse models of AD and ischemic stroke[8,9,26]. Lastly, the underlying molecular mechanisms for PE-microsphere uptake and Aβ fibril uptake by microglia appear to be distinct[9].

FIGURE LEGENDS:

Figure 1: Overview of acute isolation of CNS-MPs and phagocytosis assay. Workflow summarizing isolation and purification of $CD11b^{+}/CD45^{+}$ central nervous system (CNS)-infiltrating macrophages (MPs) from an adult mouse brain. Following mechanical dissociation of fresh, whole mouse brain and density gradient centrifugation, CNS mononuclear cells (CNS-MCs) are incubated with either PE-conjugated microspheres or Hiltye488-conjugated Aβ fibrils. CNS-MCs are stained with fluorescent-conjugated antibodies for flow cytometry.

Figure 2: Gating strategy and flow cytometry fluorescent microsphere and Aβ fibril phagocytosis assay to characterize acutely-isolated CNS-MPs. (**A**) Representative forward scatter (FSC) and side scatter (SSC) profile of CNS mononuclear cells. (**B**) Representative single cell gating strategy of CNS-MCs based on FSC and SSC. (**C**) Antibody separation of mononuclear, single cells with CD11b and CD45 surface markers. (**D**) Representative graph showing proportion

of live cells of the $CD11b^{+}CD45^{+}$ CNS-MPs. (**E**) Representative flow cytometric histograms displaying baseline negative peak of phycoerythrin (PE) microsphere phagocytosis (grey) by unstained CNS-MPs and positive peaks indicative of microsphere phagocytosis (red) by CNS-MPs. Multiple peaks represent varying quantity of beads phagocytosed by the cells. (**F**) Representative flow cytometric histograms displaying baseline negative peak of Hiltye488 Aβ fibrils phagocytosis (grey) by unstained CNS-MPs and positive peaks indicative of Hiltye488 Aβ fibrils phagocytosis (red) by CNS-MPs. (**G**) Antibody separation of mononuclear, single cells with CD11b and displaying three different cell populations: $CD11b^{+}CD45^{intermediate}$ ($CD11b^{+}CD45^{int}$) $CD11b^{+}CD45^{high}$, and $CD45^{only}$. (**H**) Representative flow cytometric histograms displaying PE microsphere phagocytosis by different CNS-MP cell populations. (**I**) Representative flow cytometric histograms displaying Hilyte488 Aβ fibrils phagocytosis by different CNS-MP cell populations.

DISCUSSION:

Flow-cytometry is a technique that can detect the expression of proteins or markers of interest on the cell surface or in intracellular compartments using fluorescently labeled probes (typically antibodies) to label these markers of interest. Cell-surface or intracellular antibodies are typically conjugated with laser-excitable fluorochromes that have unique emission spectra. Cells incubated with these antibodies can be categorized into multiple sub-populations based on patterns of expression of these markers. CNS-MPs contain primarily microglia and CNS-infiltrating monocytes/macrophages. Microglia and CNS monocytes/macrophages have distinct functional and molecular characteristics which additionally can be altered in disease conditions such as Alzheimer's disease. Characterizing these differences with minimal modulation from their representative state in CNS can be facilitated using flow-cytometric approaches. This manuscript describes a protocol to rapidly assess the phagocytic properties of acutely isolated CNS-MPs and forgoes maintaining them in long-term cultures as is typically required in traditional methods.

The method described here can characterize the ability of CNS-MPs to phagocytose larger particles such as microspheres, as well as protein aggregates such as Aβ fibrils. Both are mediated by distinct groups of receptors, as shown previously[9]. Previous work has also confirmed actin-dependence of uptake of both substrates, based on inability of cells to demonstrate fluorescent uptake when treated with Cytochalasin D. Unlike CNS-MPs which are phagocytes, non-phagocytic cells such as lymphocytes show minimal uptake of microspheres or Aβ using this assay. This flow cytometric assay provides a much more unbiased sampling of cells and provides subpopulation-level heterogeneity in phagocytic properties than traditional microscopic approaches. For example, it is possible to compare phagocytic properties of microglia, brain-infiltrating macrophages/monocytes and lymphocytes, as well as other brain cell types (endothelial cells or astrocytes) simultaneously from a single sample. By coupling single-cell level phagocytic activity with surface epitope expression, the assay can be extended to sub-phenotype different populations of microglia and macrophages, and further to compare homeostatic microglia to disease-associated microglia that are seen in advanced aging and neurodegenerative disease states. It has also been previously shown that $CD11b^{+}CD45^{high}$ macrophages have higher phagocytic properties compared to $CD11b^{+}CD45^{int}$ microglia[9]. This assay also allows the study of age-dependent changes in phagocytic properties in microglia and brain macrophages in models

of neurodegeneration[9], ischemic stroke[8] or for that matter, any other neurological disease model that impacts the brain or spinal cord. The assay can be extended to other particles as well, including synaptosomes, neurons, protein aggregates and bacteria[25]. As previously demonstrated, specific phagocytic mechanisms can also be investigated. For example, previous data show that bulk-phase uptake of microspheres requires receptors such as MARCO and TLR2 while Aβ42 fibril uptake required CD148, CD36 and MSR1 receptors, highlighting the utility of flow cytometric assays to better understand specific phagocytic mechanisms[9].

Our protocol is specifically designed to study phagocytosis in CNS-MPs including microglia and infiltrating macrophages/monocytes, and thus has certain caveats. CNS-MPs are extremely sensitive to their microenvironment. Minimizing the total time from isolation to the functional assay by rapid tissue processing greatly improves the viability of these populations. Secondly, by adapting mechanical dissociation of cells to obtain a single-cell suspension as opposed to enzymatic digestion, ensures preservation of surface receptor integrity, in turn, improving the sensitivity of the assay. Our protocol adapts exclusion of serum in the flow cytometry buffer to avoid possible cell-activation of CNS-MPs. Finally, it is critical to stop phagocytotic activity by placing the samples on ice after the specified duration of incubation with microspheres or Aβ fibrils to ensure consistency and reproducibility. The limitation of the assay is that the shear stress of isolation procedures themselves can impact the functional assay, necessitating the use of well-matched controls for the studies, and validating key findings with other orthogonal approaches, such as immunohistochemistry and in vitro assays.

Flow-cytometric assays for functional phagocytic phenotyping of CNS-MPs has the added advantage of coupling experiments with fluorescent activated cell-sorting approaches to collect individual populations for down-stream transcriptomics or proteomics analyses, including contrasting highly-phagocytic with less phagocytic CNS-MPs. This rapid method also holds potential to functionally phenotype acutely-isolated human microglia and brain macrophages from fresh post-mortem or surgical brain specimens, as well as to investigate specific mechanisms of phagocytosis by brain macrophage subsets by inhibiting select phagocytic pathways.

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DISCLOSURES:

The authors have nothing to disclose

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Figure 1

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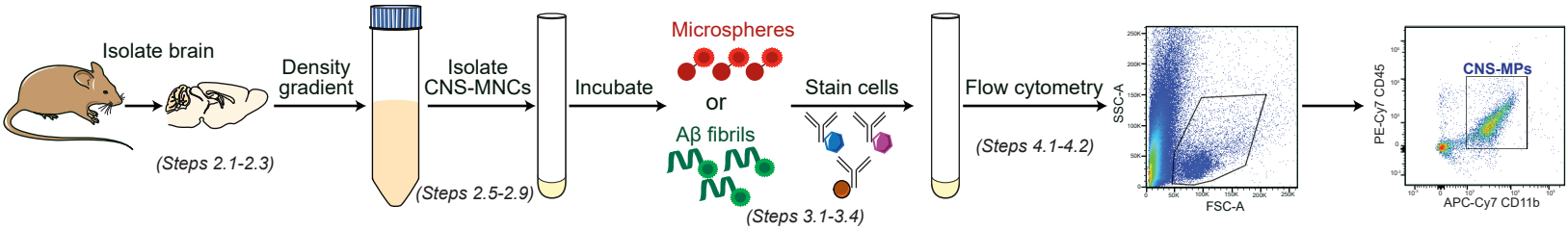

Figure 2

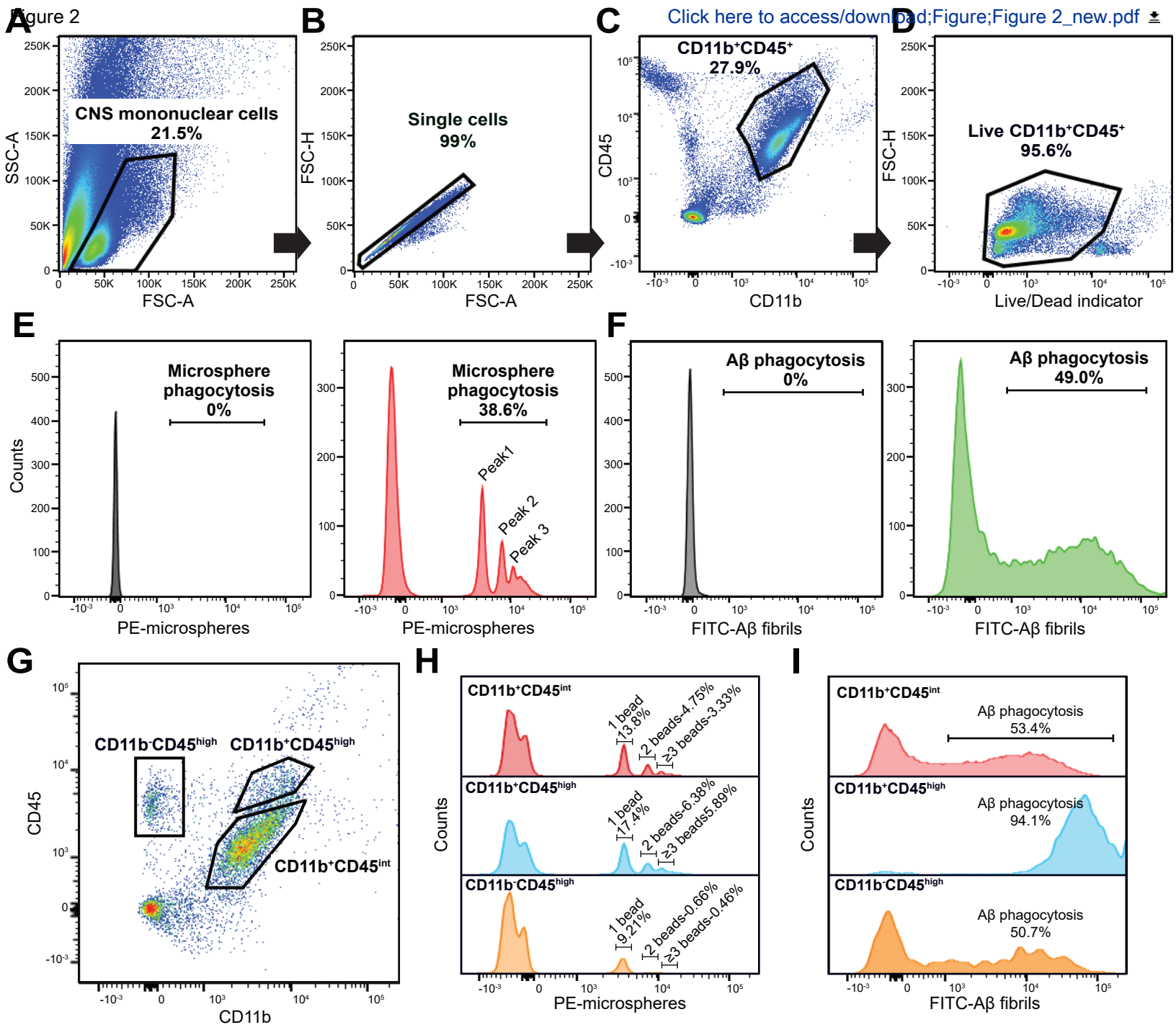

Figure1.ai

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Figure 1_updated_SR.ai

Figure2.ai

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Figure 2_new.ai

Name of Material/Equipment	Company	Catalog Number
10x Hank's balanced salt solution(10x HBSS)	ThermoFisher	14065056
1x Hank's balanced salt solution(1x HBSS)	ThermoFisher	14175095
1x Phosphate Buffered Saline (PBS)		
35% SIP		
APC-Cy7 rat anti-CD11b	BD Pharmingen	557657
FITC rat anti mouse CD145	BD Pharmingen	553080
LIVE/DEAD Fixable Blue Dead Cell Stain Kit	ThermoFisher	L34961
OneComp eBeads compensation beads	ThermoFisher	01-1111-42
PE-Cy7 rat anti mouse CD145	BD Pharmingen	552848
Percoll pH8.5-9.5	Sigma	P1644
Phycoerythrin-conjugated microspheres	ThermoFisher	F13083
β-Amyloid (1 - 42), HiLyte Fluor 488 - labeled, Human	AnaSpec	AS-60479-01

Comments/Description

Store at 4 °C. Used to make SIP as described in the protocol

Store at 4 °C. Used to make 35% SIP as described in the protocol

https://www.sigmaaldrich.com/technical-documents/protocols/biology/western-blotting/buffers-recipes/10x-phosphate-buffered-saline.html Store 10xPBS at Room temperature. Prepare fresh 1xPBS by diluting one part 10xPBS to nine parts of MilliQ dH20 and store it at 4 °C for an hour before use. Make sure the pH of the 1xPBS is 7.0 before refridgeration.

To make 20 mL of 35% SIP, use 7 mL of SIP and 13 mL of ice-cold 1× HBSS. Keep on ice until further processing

Store at 4 °C or keep on ice when in use. Sheild from light. Flow cytometry dilution - 1:100

Store at 4 °C or keep on ice when in use. Sheild from light. Flow cytometry dilution - 1:100

Store at -20 °C. Add 50ul of DMSO to one tube of the dye, vortex throughly and centrifuge at maximun speed for 1 min. Make 10µL aliquots and store them at -20 °C. Use a fresh aliquot for each experiment and add to the samples at 1:500 dilution.

Store at 4 °C, throughly vortex the tube before adding to the sample tube(s), keep on ice when in use.

Store at 4 °C or keep on ice when in use. Sheild from light. Flow cytometry dilution - 1:100

Store at 4 °C. To make 10 mL of Standard Isotonic Percoll (SIP): Use 9 mL of cold Percoll and 1 mL of cold 10× HBSS.

Store at 4 °C or keep on ice when in use.

The final concentration of Ab fibrils is 200µM which is then diluted prior to use. Store at -20 °C. Prepare fresh working solution for each experiment.

Dear Dr. Alisha DSouza,

Thank you for your editorial comments on our protocol manuscript. We have addressed all your comments and have highlighted the appropriate changes via highlight in the revised manuscript.

Point-wise responses are listed below:
Note: In this current manuscript all recent changes are highlighted in blue and the protocol content that needs to be filmed are in yellow.

1. Minimum of 6 keywords required.
   Author response: We have addressed this issue and have now included eight keywords.

2. Please add a 50-word Summary which briefly mentions the goal of your work.
   Author response: We have now included this section at the beginning of the manuscript prior to abstract.

3. Please add a clear statement that says that your protocol was approved by your local IACUC.
   Author response: We thank the editor for pointing at this important requirement. We have now addressed this point by including a note at the beginning of the protocol.

4. Replace the product name with a generic alternative. I've flagged all further instances.
5. Remove product names from text and figure
   Author response: We have now replaced all mention of "Percoll" to "density gradient medium" throughout the manuscript including the abstract and figures.

6. Define DPBS and FBS.
   Author response: We have now replaced DPBS with 1xPBS with specific pH information to exclude produce (Dulbecco) names. We have also included expansions to these acronyms in steps 1.2 and 3.9.

7. Remove product names (LSR II).
   Author response: We have made necessary changes to steps 4.1(.2 and note) and replaced all product names to generic terminology and description.

8. Figure 2 is referenced before fig 1. Please reorder figures.
   Author response: We thank the editor for bringing this point to attention. We have now referenced Fig 1 in step 2.10 before referencing Fig 2 in step 4.2.3.

9. This cannot be filmed so I have made this a note.
   Author response: We thank the editor for making this change. Our current manuscript reflects the changes made in this section.

Thank you for your consideration,

Supriya Ramesha
Srikant Rangaraju