

Quantification of Microglial Engulfment of Synaptic Material Using Flow Cytometry

Bilge Ugursu¹, Susanne A. Wolf^{1,2}

¹ Psychoneuroimmunology, Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association ² Experimental Ophthalmology, Charité Universitätsmedizin Berlin

Corresponding Author

Susanne A. Wolf

susanne.wolf@charite.de

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Abstract

Microglia play a pivotal role in synaptic refinement in the brain. Analysis of microglial engulfment of synapses is essential for comprehending this process; however, currently available methods for identifying microglial engulfment of synapses, such as immunohistochemistry (IHC) and imaging, are laborious and time-intensive. To address this challenge, herein we present *in vitro* and *in vivo** assays that allow fast and high-throughput quantification of microglial engulfment of synapses using flow cytometry.

In the *in vivo** approach, we performed intracellular vGLUT1 staining following fresh cell isolation from adult mouse brains to quantify engulfment of vGLUT1⁺ synapses by microglia. In the *in vitro* synaptosome engulfment assay, we used freshly isolated cells from the adult mouse brain to quantify the engulfment of pHrodo Red-labeled synaptosomes by microglia. These protocols together provide a time-efficient approach to quantifying microglial engulfment of synapses and represent promising alternatives to labor-intensive image analysis-based methods. By streamlining the analysis, these assays can contribute to a better understanding of the role of microglia in synaptic refinement in different disease models.

Introduction

Microglia are the resident immune cells of the central nervous system (CNS)¹. They constantly scan their microenvironment and provide surveillance^{1,2}. Moreover, they frequently interact with synapses and mediate a fine-tuning of the

synaptic activity³. Thus, they have emerged as a key player in the process of synaptic refinement.

The role of microglia in synaptic refinement through the engulfment of synapses has been shown by various research groups^{3,4,5,6,7}. Disruptions in this process can contribute to the pathology of neurodevelopmental and

neurodegenerative disorders such as schizophrenia and Alzheimer's disease⁸. Aberrant synaptic refinement by microglia has already been detected in various murine models of neurological disorders^{5,9,10}. Therefore, identification of distinct mechanisms underlying microglial engulfment of synapses is paramount to understanding the pathophysiology of neurodevelopmental and neurodegenerative disorders⁸.

Targeting microglial engulfment of synapses holds great potential for both intervening in disease progression and gaining insights into the underlying mechanisms of neurodevelopmental and neurodegenerative disorders. To facilitate such investigations, there is a need for fast and high-throughput approaches. Current methodological approaches encompass *in vivo*, *ex vivo*, and *in vitro* assays that enable the detection of synaptic material within microglia. Generally, the detection of microglial engulfment of synapses relies heavily on immunohistochemistry (IHC) and microscopy-based approaches^{5,6,11}, which are labor-intensive and show limitations in analyzing a large number of microglia.

Given these technical limitations, the exploration of alternative methodologies is imperative. To overcome this, we have optimized a flow cytometry-based approach, which enables an efficient, unbiased, and high-throughput analysis of microglial engulfment of synapses. We chose the hippocampus as the main region of interest due to its high degree of synaptic remodeling and plasticity¹², but the protocol can be adapted to various brain regions. While flow cytometry has already been used in previous studies to detect microglial engulfment of synapses^{13,14,15}, we herein provide a step-by-step methodology employing a currently commercially available, fluorophore-conjugated vGLUT1 antibody. We, moreover, provide a complementary *in vitro* approach for high-

throughput screening of microglial engulfment of synaptic material by using crude synaptosomes.

Protocol

A general view of the experimental procedure is graphically illustrated in **Figure 1A**. All experiments involving the handling of living animals used were performed in strict accordance with the German Animal Protection Law and were approved by the Regional Office for Health and Social Services in Berlin (Landesamt für Gesundheit und Soziales, Berlin, Germany). The mice were group-housed in ventilated cages under standard laboratory conditions with a 12:12 h light/dark cycle at the animal core facility of the Max Delbrück Center for Molecular Medicine (MDC). Food and water were provided *ad libitum*. See **Table 1** for the composition of buffers and reagents and the **Table of Materials** for details related to all reagents, instruments, and materials used in this protocol. For the vGLUT1-specific assay, we used the term *in vivo** throughout the manuscript to acknowledge that flow cytometry requires tissue homogenization and cell isolation, and microglia exhibit approximately 95% viability after the isolation procedure (**Figure 1B** and **Supplementary Figure S1**). Therefore, they retain their ability to engulf synaptic material *ex vivo*, for a short period, until the fixation. Thus, the quantification of vGLUT1⁺ microglia comprises both *in vivo* and short-term *ex vivo* engulfment until the fixation step.

1. Intracellular vGLUT1 staining for the detection of *in vivo** engulfment of glutamatergic synapses by microglia

NOTE: The following cell isolation procedure is adapted from¹⁶. All steps of cell isolation should be carried out on ice.

1. Anesthetize the mice using intraperitoneal injection of pentobarbital. Perfuse the mice intracardially with 10

mL of ice-cold Dulbecco's Phosphate Buffered Saline (DPBS) for ~2 min.

NOTE: One mouse is used per sample (n).

2. Take the brain out of the skull and preserve it in 1 mL of neural cell medium.

NOTE: Neural cell medium, such as Hibernate-A medium, is used to ensure high viability of cells following the tissue dissociation process.

3. Transfer the brain to a Petri dish filled with 1 mL of ice-cold neural cell medium and dissect the hippocampi as described previously¹⁷.
4. Transfer the hippocampi to a Dounce homogenizer filled with 1 mL of neural cell medium and dissociate the tissue using the loose pestle with approximately ~25 gentle strokes.
5. Place a 70 µm strainer on a 5 mL polypropylene tube and add 500 µL of neural cell medium. Transfer the tissue homogenate to the 5 mL polypropylene tube through the strainer.
6. Rinse the Dounce homogenizer 2x with 1 mL of cold neural cell medium and centrifuge the samples at 400 × g for 8 min.
7. Aspirate the supernatant and resuspend the pellet in 500 µL of ice-cold DPBS via gentle pipetting. Ensure a homogeneous suspension and complete the final volume to 1.5 mL by using DPBS.
8. Add 500 µL of isotonic Percoll solution to the sample, resuspend it gently, and overlay it with another 2 ml of cold DPBS.
9. Centrifuge the samples at 3,000 × g for 10 min with full acceleration and no brake. Aspirate the top layer as well as the myelin disk in the middle phase.

NOTE: All following centrifugation steps are carried out at 4 °C if not specified otherwise.

10. Add 4 mL of cold DPBS and centrifuge the samples at 400 × g for 10 min. Aspirate the supernatant and resuspend the cells in 100 µL of fixable viability staining solution and incubate the samples for 30 min at 4 °C.
11. Add 1 mL of cold DPBS to the sample and centrifuge the samples at 300 × g for 5 min. Discard the supernatant and add 100 µL of CD16/CD32 staining solution (1/200 in FACS buffer). Vortex for ~5 s and incubate for 10 min at 4 °C.

NOTE: CD16/CD32 staining is a pretreatment to minimize non-specific binding of antibodies to FcR-bearing cells, such as microglia, prior to applications such as flow cytometry.
12. Add 1 mL of FACS buffer to the sample and centrifuge at 300 × g for 5 min. Aspirate the supernatant and add 100 µL of staining master mix-I (1/100 anti-CD11b/ anti-CD45 + 1/200 anti-Ly6C/ anti-Ly6G in 1x FACS Buffer). Incubate the samples for 20 min at 4°C in the dark.
13. Add 1 mL of FACS buffer to the sample and centrifuge at 300 × g for 5 min.
14. Resuspend the pellet in 250 µL of fixation buffer. Incubate at 4 °C for 25 min.
15. Add 2 mL of 1x permeabilization (PERM) Buffer and centrifuge 300 × g for 5 min.
16. Discard the supernatant and add 100 µL of vGLUT1 or isotype control staining solution. Vortex for ~5 s and incubate the samples at 4 °C for 50 min.
17. Add 2 mL of 1x PERM Buffer and centrifuge 300 × g for 5 min. Discard the supernatant and add 2 mL of FACS buffer to the samples.

18. Centrifuge at $300 \times g$ for 5 min and discard the supernatant. Resuspend the cells in 250 μ L of FACS buffer and pass the samples through a 40 μ m strainer filter.

19. Analyze vGLUT1 fluorescence intensity from single/viable/CD11b⁺⁺/CD45⁺ microglia using flow cytometry. Use spleen macrophages as a negative control for each experiment.

1. Isolate splenocytes by gently compressing minced spleen tissue through a 70 μ m strainer filter twice. Rinse the filters with 40 mL of DPBS and collect the suspension in a 50 mL conical tube.
2. Centrifuge $350 \times g$ for 10 min and resuspend the resultant pellet in a 1 mL solution of red blood cells lysis buffer. Incubate for 10 min on ice.
3. Add 10 mL of DPBS to the sample following the incubation and centrifuge at $350 \times g$ for 10 min.
4. Proceed with the staining steps explained between steps 1.11 and 1.17.

NOTE: The gating strategy is provided in **Supplementary Figure S2** to define spleen macrophages as CD11b⁺⁺/CD45⁺⁺/Viable cell population.

5. Gating strategy (**Figure 1**)
 1. Primary gate: Adjust the forward Scatter Area (FSC-A) [x-axis] and Side Scatter Area (SSC-A) [y-axis] to include the microglia population in the gated area and exclude the cellular debris.
 2. Adjust the forward Scatter Area (FSC-A) [x-axis] and Forward Scatter Height (FSC-H) [y-axis] to exclude doublets. Singlets appear as a diagonal on this dot plot.

3. Adjust CD11b-PECy7 [y-axis] and CD45-APC [x-axis] and gate the population with a high surface level of CD11b and medium-level of CD45 as microglia.

4. Exclude dead cells in the FITC[y-axis] negative gate. OPTIONAL: Also exclude cells that are positive for Ly6C- and Ly6G-FITC in the FITC-negative gate to exclude CNS-associated macrophages from the analysis.

NOTE: In contrast to live cells, dead cells with compromised membranes allow the fixable viability dye to enter the cytoplasm, which increases the amount of protein labeling¹⁸. Thus, dead cells will be brighter than live cells, which are included in the defined gate.

5. Adjust CD45-APC [x-axis] and vGLUT1-PE [y-axis]; the population that is above the threshold gate, where there are no positive events detected in the spleen sample (internal biological negative control, **Figure 1E**) is regarded as the vGLUT1-positive fraction in the sample.

2. Detection of *in vitro* engulfment of crude synaptosomes by microglia

1. Crude synaptosome preparation and pHrodo Red labeling

NOTE: All the following steps should take place on ice.

1. Follow steps 1.1 to 1.2.
2. Transfer the brain to a Petri dish filled with 1 mL of ice-cold neural cell medium and carefully dissect the hippocampi. Always keep the Petri dish on ice. Use hippocampi for microglia isolation in the next step.

3. Transfer the rest of the brain (excluding the cerebellum and olfactory bulb) to a Dounce homogenizer filled with 1 mL of synaptic protein extraction reagent and gently dissociate the tissue using the loose pestle with approximately ~30 strikes. Supplement the one tablet protease inhibitor per 10 mL of the extraction reagent and isolate synaptosomes according to the manufacturer's instructions.

NOTE: Synaptic protein extraction reagents, such as SynPER¹⁹, are used to prepare synaptosomes that contain biologically active pre- and postsynaptic proteins.
 4. Dissolve the crude synaptosome pellet in 500 μ L of 0.1 M Na₂CO₃ solution. Stain the synaptosome sample with 10 μ L of 0.2 mM pHrodo Red. Incubate the crude synaptosome samples at room temperature (24-25 °C) for 1.5 h with gentle agitation.
 5. Add 1 mL of cold DPBS onto the sample, centrifuge for 1 min at full speed (20,815 \times g), and aspirate the supernatant.
 6. Repeat step 2.1.5 for 7x in total to remove unbound excessive pHrodo Red from the samples.
 7. After the last centrifuge, perform a standard BCA assay to quantify the protein concentrations of the sample.
 8. Optional: snap-freeze synaptosome samples in DPBS with 5% DMSO using liquid nitrogen and preserve them for 3 weeks at -80 °C. Cover the tubes with aluminum foil to keep the light exposure minimum.
2. *In vitro* crude synaptosome engulfment assay using freshly isolated adult microglia
 1. Prepare aCSF and equilibrate it with 95% O₂:5%CO₂ for 30 min.

NOTE: For the steps 2.2.2-2.2.4, follow the manufacturer's instructions for preparation of papain-based digestion solution.
 2. Add 4 mL of aCSF to **vial 2** in the papain kit. Place the vial in a 37 °C water bath for ~10 min until the papain solution appears clear.
 3. Add 400 μ L of aCSF to **vial 3** in the papain kit. Mix gently for ~10 times by slow pipetting.
 4. Add 200 μ L from **vial 3** to **vial 2** (reconstructed at step 2.2.3). Save the rest of the **vial 3**.
 5. Take the hippocampi dissected in step 2.1.2 and mince the dissected hippocampi by using a scalpel.
 6. Transfer the minced hippocampi to a tissue dissociator tube filled with 2 mL of enzyme solution prepared in step 2.2.5. Place the tube into the tissue dissociator and run the program: **37C_ABDK_01** (takes ~30 min).
 7. Place the samples in a water bath at 37 °C for ~20 min and triturate the mixture every 5 min using a 1 mL pipet without making any bubbles.

NOTE: This process should be continued until the tissue is fully dissociated and appears completely homogeneous to ensure an efficient dissociation. All following centrifugation steps are carried out at 4 °C if not specified otherwise.
 8. Carefully remove the cloudy cell suspension to a new 15 mL tube and centrifuge at 300 \times g for 5 min.

9. During this 5 min period, prepare the following wash mix (5 mL) per sample; add 500 μ L of reconstituted albumin-ovomuroid inhibitor solution provided in the papain kit to 4.5 mL of aCSF. Add the remaining solution in vial 3 from step 2.2.5 to the wash mix.
10. Discard the supernatant from step 2.2.8 and immediately resuspend the cell pellet in the wash mix solution.
11. Pass the sample through a 70 μ m filter to a new 5 mL microcentrifuge tube. Centrifuge the samples at 300 \times g for 5 min.
12. Proceed with the Percoll gradient centrifugation step explained previously in steps 1.7-1.9.
13. Resuspend the cells carefully in MACS staining buffer by pipetting slowly up and down. Incubate the samples for 15 min at 4 $^{\circ}$ C.
14. Add 1 mL of MACS buffer to each sample and centrifuge at 300 \times g for 8 min.
15. Resuspend the cells in 500 μ L of MACS buffer.
16. Place the positive selection columns in the magnetic separator. Equilibrate the columns by rinsing them with 3 mL of MACS Buffer.
17. Gently mix and apply 500 μ L of the cell suspension onto the column. Wash the columns 3x with 3 mL of MACS Buffer.
18. Remove the columns from the magnetic separator and place them on 15 mL conical tubes. Add 5 mL of MACS buffer onto the column and immediately flush out the cells using a plumper. Centrifuge the samples at 300 \times g for 10 min.
19. During this period, prepare 20 mL of 40% FBS in DPBS. Prewarm 1 mL of DMEM per sample up to 37 $^{\circ}$ C in a water bath.
20. Dissolve the final cell pellet in 1 mL of prewarmed DMEM. Seed around \sim 150,000-200,000 cells in 500 μ L of prewarmed DMEM per well in a 24-well plate. As a control, seed a similar number of cells in 1-2 extra wells. Check the confluency of cells in all the wells using a light microscope.
NOTE: If the target brain region is hippocampus or comparably small brain regions, 5 mice can be pooled per sample (n) to isolate \sim 150,000 microglia. For the whole brain, 1 mouse per n will suffice to obtain similar numbers of cells using both isolation protocols. Alternatively, \sim 40,000 cells can be plated in 96-well plates in 100 μ L final volume to start the engulfment assay. This reduces the number of cells analyzed but also reduces the number of mice used per n. Protein deprivation due to lack of FCS in DMEM will trigger phagocytosis.
21. Incubate the plate for 1-2 h in the incubator (37 $^{\circ}$ C and 5% CO₂).
NOTE: This step aims for the cells to recover from the stress-prone effects of the isolation procedure prior to the start of the functional engulfment assay.
22. Take 250 μ L of medium out of each well very slowly, add 250 μ L of fresh prewarmed DMEM to each well, and add 3 μ g of pHRedo Red-labeled synaptosomes on the top. Check the cell confluency in all the wells using a light microscope.
 1. For the negative control wells, add the same amount of unlabeled synaptosomes to the extra well seeded with cells.

2. For test wells, ensure that well 1 contains only cells; well 2 contains cells+ unlabeled synaptosomes; well-3 contains cells+ 3 μ g of pHrodo Red; well 4 contains DMEM + 3 μ g of pHrodo Red.
23. Incubate the cells with synaptosomes for 2 h in the incubator (37 °C and 5% CO₂).
24. Take out the medium and wash the wells with cold DPBS. Add 200 μ L of Trypsin/EDTA solution per well to detach the cells for 35 s.
25. Add 1 mL of 40% FBS in DPBS per well and transfer the cells to a 5 mL polypropylene tube through the strainer. Keep both the plate and the tube on ice during this process to facilitate the detachment of cells.
26. Wash each well 2x using 500 μ L of ice-cold DPBS. Centrifuge the collected samples at 500 \times g for 5 min.
27. Resuspend the cells in the staining solution containing 1/200 CD16/CD32 in 100 μ L of FACS buffer and incubate for 10 min on ice.
28. After the incubation, add CD11b and CD45 to the staining solution with a final concentration of 1/100 from each. Incubate the samples for 20 min at 4 °C in the dark.
29. Wash the samples with 1 mL of FACS buffer and centrifuge them at 300 \times g for 10 min.
30. Resuspend the pellet in 250 μ L of FACS buffer and record at least 100,000 total events using flow cytometry. Analyze pHrodo Red fluorescence intensity from CD11b⁺⁺/CD45⁺ microglia.
31. Gating strategy (**Figure 2C**)

1. Adjust the primary gate: Forward Scatter Area (FSC-A) [x-axis] and Side Scatter Area (SSC-A) [x-axis] to include the microglia population in the gated area and exclude the cellular debris.
2. Adjust the forward Scatter Area (FSC-A) [x-axis] and Forward Scatter Height (FSC-H) [y-axis] to exclude doublets. Singlets appear as a diagonal on this dot plot.
3. Adjust CD11b-PECy7 [y-axis] and CD45-APC [x-axis] and gate the population with high surface level CD11b and medium-level of CD45 as microglia.
4. Calculate the median fluorescence intensity of pHrodo-PE from this population. Use the same cells incubated with unlabeled synaptosomes as the negative control.

Representative Results

In this project, we optimized and presented two protocols to measure *in vivo** and *in vitro* engulfment of synapses by microglia. In the first protocol, we focused on *in vivo** engulfment of vGLUT1-positive synapses. As a starting point, we used a previously published protocol¹⁴. However, the FACS antibodies used in this protocol are discontinued and we added many optimization steps as well as a novel method for microglia isolation¹⁶. That is why the protocol presented here is worth sharing with the scientific community as a comprehensive update to the protocols that are already published.

To quantify microglial engulfment of synapses, we used C57BL/6N male mice aged 11-14 weeks. The hippocampus was selected as the main region of interest due to its high degree of synaptic remodeling and plasticity¹². We analyzed

%vGLUT1-positive microglia as well as microglia-specific vGLUT1-PE fluorescence intensity (MFI) in the hippocampus of C57BL/6N mice. Spleen macrophages derived from the same animals were used as a biological negative control per experiment. We tested the vGLUT1 antibody by demonstrating a higher vGLUT1-PE fluorescence signal from the hippocampal microglia compared to the isotype control and spleen macrophages (**Figure 1B-E**)

Furthermore, we compared the microglial engulfment of synapses in the cerebellum as well as in the olfactory bulb (as another reference for high synaptic plasticity)²⁰. We found a higher vGLUT1 fluorescence signal in the microglia from the olfactory bulb and a lower signal in the cerebellum compared to the hippocampus (**Figure 1F**). The lowest signal intensity was detected in the spleen macrophages, serving as the internal negative control (**Figure 1E**). Additionally, we used *Vglut-IRES-Cre/ChR2-YFP* mice to test the immunoreactivity of our vGLUT1 antibody. YFP is expressed by the glutamatergic neurons of these mice, indicating that the YFP-positive population should also include a vGLUT1-positive fraction. Using this staining protocol, we detected 98.7% of the YFP-positive population as vGLUT1-positive, validating the efficiency of our antibody (**Supplementary Figure S3**).

Overall, these results validate the efficiency of the vGLUT1 antibody and the presented staining protocol. We demonstrate that this protocol and the antibody can be confidently used to quantify *in vivo** engulfment of synapses

in a high-throughput and fast manner compared to other experimental approaches.

Moving on to the *in vitro* method, we isolated adult microglia and incubated them with freshly isolated pHrodo Red-labeled synaptosomes isolated from the same animals to quantify their *in vitro* engulfment (**Figure 2A**). We labeled synaptosomes with pHrodo Red, which naturally increases the fluorescence signal in acidic surrounding pH²¹. We freshly isolated synaptosomes and exposed them to different pH values (pH = 4 and pH = 11). After confirming the increase in fluorescence signal in low pH as a proof-of-principle experiment (**Figure 2B**), we incubated these synaptosomes with freshly isolated microglia for 1.5-2 h. As a control, we incubated microglia with unlabeled synaptosomes. Next, we analyzed the pHrodo Red-PE fluorescence signal from CD11b⁺⁺/CD45⁺ microglia and observed a positive PE fluorescence, which was comparable to that obtained from synaptosomes at pH = 4 (**Figure 2C**). Thus, this method provides a fast and high-throughput analysis of the *in vitro* engulfment of synaptosomes and can be extended to amyloid plaques or the engulfment of other potential targets following necessary optimization steps. Indeed, Rangaraju et al. quantified engulfment of amyloid beta by microglia using a similar flow cytometry-based approach²². In conclusion, these two methods provide robust, efficient, and high-throughput quantification of microglial engulfment of synapses both *in vivo** and *in vitro*.

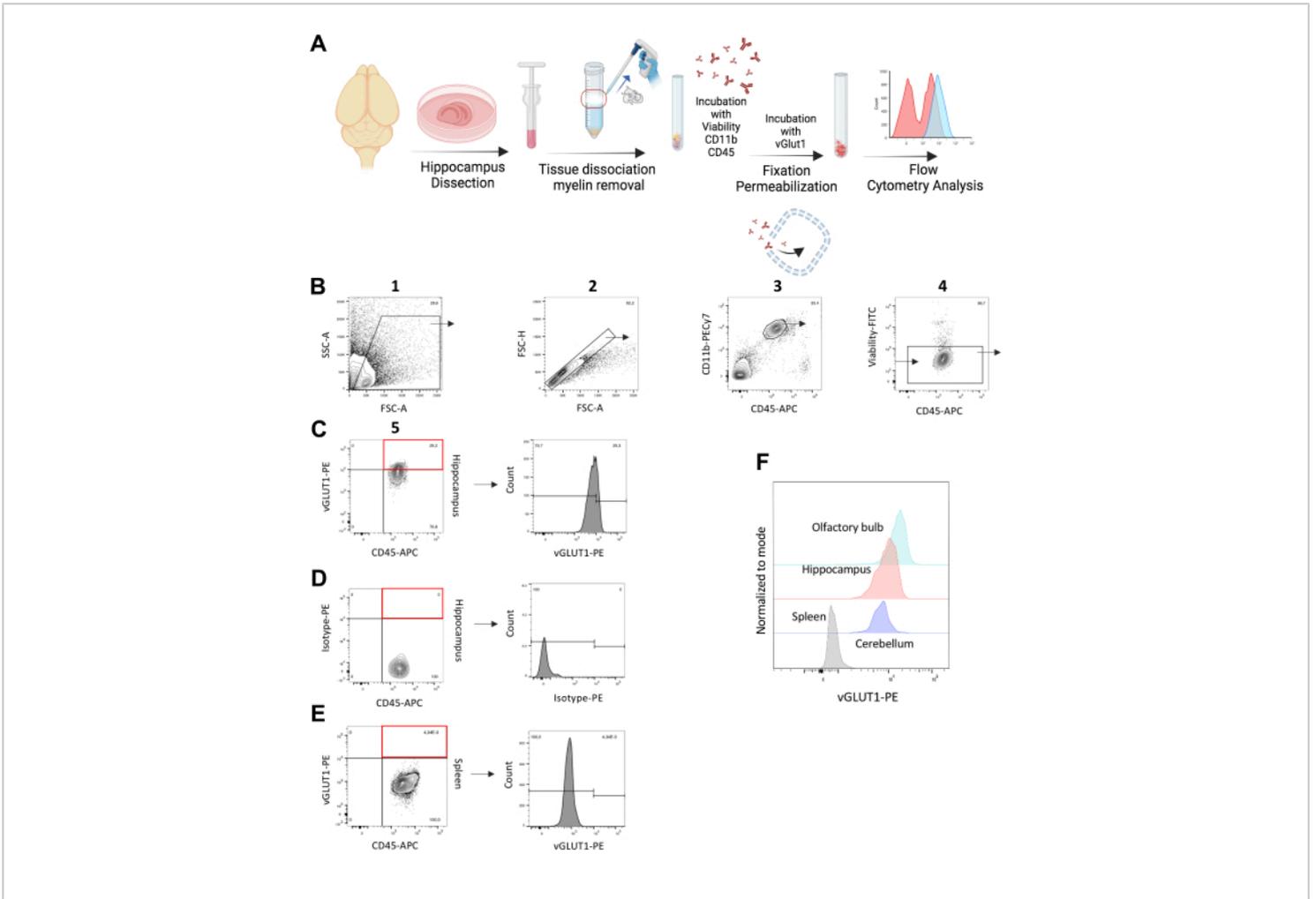


Figure 1: Analysis of microglial engulfment of vGLUT1⁺ synapses *in vivo*.* (A) Graphical illustration of the experimental workflow depicting steps of intracellular vGLUT1 staining. (B) Gating strategy to define single/CD11b⁺⁺/CD45⁺/ viable cell population from the hippocampus. This population was used to analyze vGLUT1-MFI as well as to quantify the percentage of vGLUT1⁺ microglia in the hippocampus. The gate shown with the red rectangle indicates the vGLUT1⁺ cell fraction in the total sample. (C) The histogram indicates vGLUT1-PE fluorescence intensity. (D) The gate shown with the red rectangle indicates no positive cell fraction showing Isotype-PE immunoreactivity. The histogram indicates Isotype-PE fluorescence intensity. (E) The gate shown with the red rectangle indicates no positive cell fraction showing vGLUT1-PE immunoreactivity in the spleen macrophages. The histogram indicates vGLUT1-PE fluorescence intensity. The gate indicated on the histogram starts at the level, where the vGLUT1-MFI from the spleen terminates (~10⁴) and is used to analyze the vGLUT1 positive fraction in the brain samples. (F) The overlaid histogram shows the comparison of PE fluorescence intensity of spleen macrophages (grey) and microglia from the hippocampus (red), cerebellum (purple), and olfactory bulb (light blue). [Please click here to view a larger version of this figure.](#)

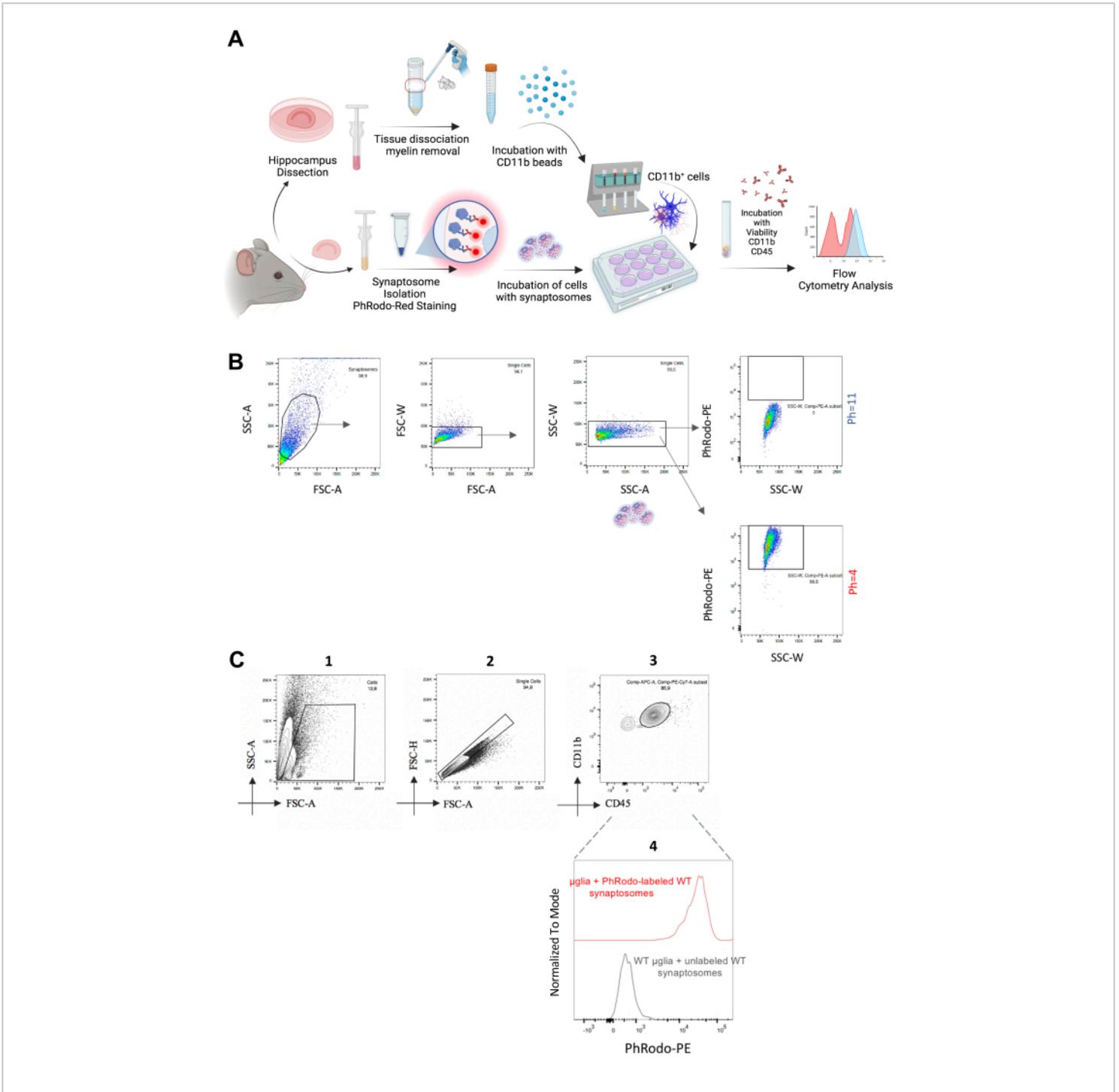


Figure 2: Analysis of microglial engulfment of synaptosomes synapses *in vitro*. (A) Graphical illustration of the experimental workflow depicting steps of the *in vitro* synaptosome engulfment assay. (B) Synaptosomes incubated at two different pH values show a low pHrodo Red-PE fluorescence signal at pH = 11 and a high pHrodoRed-PE fluorescence at pH = 4. (C) Single/CD11b⁺⁺/CD45⁺ cell population was used to analyze pHrodo Red-PE fluorescence intensity. Microglia

incubated with unstained synaptosomes were used as a negative control. [Please click here to view a larger version of this figure.](#)

Table 1: List of buffers and reagents used in this protocol.

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Supplementary Figure S1: Representative image of freshly isolated adult microglia.

Image acquired using a light microscope with 20x objective following the papain-based tissue dissociation protocol and MACS-based isolation of CD11b⁺ microglia. Scale bar = 50 μm. [Please click here to download this File.](#)

Supplementary Figure S2: Representative FACS plots demonstrating the gating strategy to define spleen macrophages.

Spleen was used as a negative control in the experiments per experimental run while testing microglial engulfment of synapses in the hippocampus. FACS plots given above define the spleen macrophages as CD11b⁺⁺/CD45⁺⁺/viable population. This population was used to set a threshold to quantify vGLUT1⁺ microglia in the brain samples that reside above this threshold gate. [Please click here to download this File.](#)

Supplementary Figure S3: Representative FACS plots demonstrating the gating strategy to test the efficiency of the vGLUT1 antibody.

(A) Graphical illustration of the experimental workflow depicting steps of the vGLUT1 staining. YFP⁺ glutamatergic neurons were used to test the immunoreactivity of the vGLUT1 antibody. (B) Gating strategy to define the YFP⁺ population from the hippocampus of Vglut-IRES-Cre//ChR2-YFP mice that were used as a positive control for testing the efficiency of the vGLUT1 FACS antibody. YFP⁺ fraction was gated to specify glutamatergic synapses. In this population, the immunoreactivity of the vGLUT1 antibody was analyzed to test the immunoreactivity

of the antibody. Compared to the (C) Isotype control; 97.9% of YFP-positive cell fraction is detected as (D) vGLUT1-positive. (E) The overlaid histogram indicates the comparison of the PE fluorescence between the isotype and vGLUT1 antibody.

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Discussion

Synaptic refinement through microglia-synapse interaction is an intriguing area of study within the field of neuroimmunology, offering promising insights into the role of microglia in neurodegenerative and neurodevelopmental disorders. In 2011; Paolicelli et al. provided evidence of the presence of synaptic material within microglia, shedding light on their involvement in the process of synaptic engulfment⁴. Another intriguing study employed time-lapse imaging and an *ex vivo* organotypic brain slice culture model and reported that microglia engage in a phagocytic process known as trogocytosis, where they engulf presynaptic structures rather than the entire synaptic structure²³. A very recent publication using a new transgenic mouse model that enables measurement of phagocytosis in intact tissue showed pruning by Bergmann-glia *in vivo* upon motor learning²⁴. Thus, there is sufficient evidence indicating the involvement of glial cells in synaptic engulfment, including microglia. However, the extent to which this microglial function impacts the dynamic, and selective process of synaptic pruning requires further evidence.

Nevertheless, the quantification of microglial engulfment of synapses serves as a valuable indicator and provides partial insight into the complex dynamics of microglia-synapse interactions, especially synaptic refinement. A comprehensive review has summarized current protocols

used to investigate microglia engulfment of synapses²⁵. We would like to emphasize that our protocols are optimized based on existing protocols that are already in use. The methods presented in this study provide fast and high-throughput quantification microglial engulfment of synapses in various dissected brain regions. Depending on the brain region, an analysis of at least 10,000 microglial cells in a maximum of two days is possible for both methodologies, making them valuable for testing multiple mouse models in parallel.

We acknowledge that the quantification of vGLUT1⁺ microglia comprises both *in vivo* and short-term *ex vivo* engulfment until the fixation step. Therefore, we suggest that our assay presents a fast and reliable way to quantify synaptic material inside microglia as an initial step prior to *in vivo* validation using approaches such as IHC.

Another disadvantage of the flow cytometry analysis is the limited availability of antibodies for synaptic markers, particularly for inhibitory synapses. It is challenging to find commercially available, directly conjugated antibodies that show a bright signal for these markers. Given the extensive optimization time required to test different antibodies targeting synaptic markers, it is important to share the well-optimized procedures with the scientific community for intracellular staining with different antibodies as we do here with this study.

Regarding data analysis in this study, we used Isotype controls as technical negative controls to account for non-specific bindings of the vGLUT1 antibody, since they provide an estimate for nonspecific binding of an antibody in a sample while optimizing flow cytometry-based assays²⁶. However, isotype controls have been mostly optimized to detect the nonspecific background signal from the surface staining procedures and are not optimal for intracellular

staining controls^{27,28}. Therefore, they should not be relied upon to distinguish between the negative and positive populations when performing intracellular staining, which involves fixation and permeabilization steps that can impact antigen detection, autofluorescence, and fluorophore brightness²⁹. Such intracellular staining procedures require the use of appropriate biological internal controls to define the positive cell population stained for an intracellular marker²⁹. Thus, considering that we use an intracellular staining protocol, we employed an internal biological negative control (spleen macrophages) and defined the boundary between the positive and negative populations according to the spleen macrophages isolated from the same mice. We distinguished the positive population above the gate, at which there are no vGLUT1 positive events from the spleen macrophages that serve as the biological negative control (**Figure 1**).

Both methods presented in this study offer great potential for initial analysis of microglial engulfment of synapses in a fast and high-throughput manner, analyzing over 10,000 cells from small brain regions and this is not achievable with standard microscopy techniques. Therefore, these methods offer a significant advantage over labor and time-intensive methods and further, provide a more comprehensive analysis of synaptic engulfment by allowing an analysis of a greater number of microglia. Additionally, the *in vitro* method presented in this study is particularly useful for testing the impact of different treatments on the microglial engulfment of synapses. It enables direct quantification of the effect of treatment on microglia without the confounding factors associated with other cell types. In addition, it serves as an indirect approach to proving a potential effect of microenvironment or other cell types on the process of synaptic engulfment. Therefore, we conclude that these methods, especially when used in parallel, offer intuitive

and advantageous alternatives for the analysis of microglial engulfment of synaptic materials.

However, the analysis of freshly isolated microglia by FACS-based phagocytic assays *ex vivo* may pose a few disadvantages. First, it is critical to employ well-optimized protocols that generate freshly isolated microglia from the adult brain while avoiding *ex vivo* activation and stress response of microglia. Dissing-Olesen et al. incorporated the use of transcriptional and translational inhibitors to overcome this issue by employing a tissue dissociation procedure at 37 °C³⁰. Mattei et al., on the other hand, presented a cold, mechanical tissue dissociation protocol to avoid inducing *ex vivo* expression of stress associated genes¹⁶ and we adapted this protocol in the first section to avoid *ex vivo* activation of stress-associated microglia response prior to intracellular vGLUT1 staining. We employed an enzymatic tissue dissociation protocol in the second section prior to the *in vitro* synaptosome engulfment assay considering the higher yield of microglia following papain-based tissue dissociation (data not shown). Microglia inevitably remain at 37 °C under culture conditions when incubated with synaptosomes, and incubation at 37 °C can indeed induce changes in microglia as common drawbacks of all *in vitro* assays and cell culture procedures. Therefore, we suggest the use of both presented protocols in parallel to reach a broader conclusion in terms of microglial engulfment of synapses.

Furthermore, it is important to carefully define the gating strategy to select CD11b⁺⁺/CD45⁺ microglia by taking into account the presence of other immune cells in the brain parenchyma that also express these markers³¹. More importantly, when choosing markers to specifically target microglia (e.g., TMEM119, P2RY12), it is important to

consider that they can undergo changes in their expression levels during pathological and inflammatory conditions³², and such changes should be considered prior to establishing the FACS panel to quantify microglial engulfment of synapses. Finally, it is essential to emphasize that neither of the methods discussed earlier, including the IHC- and microscopy-based *in vivo* approaches, can alone capture the active and selective pruning of synapses by microglia. These methods are not able to discriminate the active pruning by microglia from the passive scavenging of synaptic debris within the brain parenchyma. Therefore, when evaluating and discussing the data, it is imperative to clearly distinguish between these distinct concepts.

Disclosures

The authors declare no competing interests.

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References

1. Wolf, S. A., Boddeke, H. W., Kettenmann, H. Microglia in physiology and Disease. *Annu Rev Physiol.* **79**, 619-643 (2017).
2. Hanisch, U. K., Kettenmann, H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci.* **10** (11), 1387-1394 (2007).

3. Tremblay, M. È., Lowery, R. L., Majewska, A. K. Microglial interactions with synapses are modulated by visual experience. *PLoS Biol.* **8** (11), e1000527 (2010).
4. Paolicelli, R. C. et al. Synaptic pruning by microglia is necessary for normal brain development. *Science (New York, N.Y.)*. **333** (6048), 1456-1458 (2011).
5. Schafer, D. P. et al. Microglia contribute to circuit defects in *Mecp2* null mice independent of microglia-specific loss of *Mecp2* expression. *eLife*. **5**, e15224 (2016).
6. Schafer, D. P. et al. Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron*. **74** (4), 691-705 (2012).
7. Filipello, F. et al. The microglial innate immune receptor TREM2 is required for synapse elimination and normal brain connectivity. *Immunity*. **48** (5), 979-991.e8 (2018).
8. Salter, M. W., Stevens, B. Microglia emerge as central players in brain disease. *Nat Med*. **23** (9), 1018-1027 (2017).
9. Hong, S. et al. Complement and microglia mediate early synapse loss in Alzheimer mouse models. *Science (New York, N.Y.)*. **352** (6286), 712-716 (2016).
10. Di Liberto, G. et al. Neurons under T cell attack coordinate phagocyte-mediated synaptic stripping. *Cell*. **175** (2), 458-471.e19 (2018).
11. Bisht, K. et al. Dark microglia: A new phenotype predominantly associated with pathological states. *Glia*. **64** (5), 826-839 (2016).
12. Weerasinghe-Mudiyanselage, P. D. E. et al. Structural plasticity of the hippocampus in neurodegenerative diseases. *Int J Mol Sci*. **23** (6), 3349 (2022).
13. Aw, E., Zhang, Y., Carroll, M. Microglial responses to peripheral type 1 interferon. *J Neuroinflammation*. **17** (1), 340 (2020).
14. Brioschi, S. et al. Detection of synaptic proteins in microglia by flow cytometry. *Front Mol Neurosci*. **13**, 149 (2020).
15. Norris, G. T. et al. Neuronal integrity and complement control synaptic material clearance by microglia after CNS injury. *JEM*. **215** (7), 1789-1801 (2018).
16. Mattei, D. et al. Enzymatic dissociation induces transcriptional and proteotype bias in brain cell populations. *Int J Mol Sci*. **21** (21), 7944 (2020).
17. Jaszczyk, A., Stankiewicz, A. M., Juszczak, G. R. Dissection of mouse hippocampus with its dorsal, intermediate and ventral subdivisions combined with molecular validation. *Brain Sci*. **12** (6), 799 (2022).
18. Thermo Fisher Scientific. *Fixable viability dyes for flow cytometry*. <https://www.thermofisher.com/de/de/home/life-science/cell-analysis/flow-cytometry/flow-cytometry-assays-reagents/cell-viability-assays-flow-cytometry/fixable-viability-dyes-flow-cytometry.html> (2024).
19. Thermo Fisher Scientific. *SynPER synaptic protein extraction reagent*. https://www.thermofisher.com/order/catalog/product/87793?gclid=CjwKCAiAi6uvBhADEiwAWiyRdigrNHuDkIAVVsaW8OaC3VJNgrPEm1I64E2PBZA8X_A_0ipFr_sulxoCDm8QAvD_BwE&ef_id=CjwKCAiAi6uvBhADEiwAWiyRdigrNHuDkIAVVsaW8OaC3VJNgrPEm1I64E2PBZA8X_A_0ipFr_sulxoCDm8QAvD_BwE:G:s&s_kwcid=AL!3652!3!606658601747!e!!g!!syn_per!13368767883!123500396056&cid=bid_pca_ppf_r01_co_cp1359_pjt0000_bid00000_0se_gaw_bt_pur_con&gad_source=1#/87793 (2024).

20. Wu, A., Yu, B., Komiyama, T. Plasticity in olfactory bulb circuits. *Curr Opin Neurol.* **64**, 17-23 (2020).
21. Thermo Fisher Scientific. *pHrodo indicators for pH determination.* <https://www.thermofisher.com/de/de/home/brands/molecular-probes/key-molecular-probes-products/phrodo-indicators.html> (2024).
22. Rangaraju, S. et al. Differential phagocytic properties of CD45^{low} microglia and CD45^{high} brain mononuclear phagocytes-activation and age-related effects. *Front Immunol.* **9**, 405 (2018).
23. Weinhard, L. et al. Microglia remodel synapses by presynaptic trogocytosis and spine head filopodia induction. *Nat Commun.* **9** (1), 1228 (2018).
24. Morizawa, Y. M. et al. Synaptic pruning through glial synapse engulfment upon motor learning. *Nat Neurosci.* **25** (11), 1458-1469 (2022).
25. Morini, R. et al. Strategies and tools for studying microglial-mediated synapse elimination and refinement. *Front. Immunol.* **12**, 640937 (2021).
26. Maecker, H. T., Trotter, J. Flow cytometry controls, instrument setup, and the determination of positivity. *Cytometry. Part A: J. Int Soc Anal Cytol.* **69** (9), 1037-1042 (2006).
27. Easthope, E. *Strategies for intracellular flow cytometry success.* <https://www.biocompare.com/Editorial-Articles/582159-Strategies-for-Intracellular-Flow-Cytometry-Success/> (2022).
28. Paduano, F. *Isotype control antibodies, Key points.* <https://www.antibodies.com/primary-antibodies/isotype-control-antibodies#:~:text=Isotype%20controls%20should%20be%20used,the%20primary%20antibody%20and%20fluorophore> (2024).
29. Bio-Rad. *Flow cytometry intracellular staining controls.* <https://www.bio-rad-antibodies.com/flow-cytometry-intracellular-controls.html> (2024).
30. Dissing-Olesen, L. et al. FEAST: A flow cytometry-based toolkit for interrogating microglial engulfment of synaptic and myelin proteins. *Nat Commun.* **14**, 6015 (2023).
31. Jurga, A. M., Paleczna, M., Kuter, K. Z. Overview of general and discriminating markers of differential microglia phenotypes. *Front Cell Neurosci.* **14**, 198 (2020).
32. van Wageningen, T. A. et al. Regulation of microglial TMEM119 and P2RY12 immunoreactivity in multiple sclerosis white and grey matter lesions is dependent on their inflammatory environment. *Acta Neuropathol Commun.* **7** (1), 206 (2019).