ABSTRACT
There are various approaches in which one can isolate microglia from murine brains, such as immunomagnetic, density gradient, FACS and differential adhesive methods. In this procedure a modified flask-tapping approach was used due to its simplicity and reproducibility. Our protocol requires only a single step to isolate the microglia from the mixed cell population. Once the microglia were isolated, we characterized cell purity, microglial morphology and phagocytic activity. The single-step protocol, without the need for additional astrocyte or oligodendrocyte separation, allows microglial cells to be used immediately for experimental purposes. The protocol is low-cost and can be performed in any lab with standard cell-culture equipment.

METHOD SUMMARY
In this protocol we describe a simplified and low-cost approach to the isolation of mouse microglia with high purity. The procedure can be easily performed in any lab equipped with standard tissue-culture equipment and scaled up to meet the need for large cell numbers. The protocol is versatile; the microglia can be used for experimentation both in isolation and among the mixed glial populations.

TWEETABLE ABSTRACT
A single-step, low-cost protocol for isolation of primary microglia for immediate experimental purposes #microglia #siue

KEYWORDS:
flow cytometry ● fluorescence ● microglia ● primary cell ● tissue culture

Immortalized cell lines are standard research tools in many different fields of biomedical research. Cell lines are easy to maintain, low-cost and yield an unlimited supply of cells for experimental use; thus they are often used in place of primary cells [1]. However, cell lines are not without disadvantages. The immortalization process [2] alters phenotype and normal functioning of the cells, and long-term serial passaging can cause genetic drift and phenotypic variation leading to problems with data reproducibility [3,4]. The mouse BV2 cell line [5] is a commonly used model for brain microglia [6] but may inaccurately mimic characteristics of primary cells [6,7]. Primary mouse microglia are usually considered a much better model because they reflect the natural physiological system. A researcher wishing to pursue the isolation of primary microglia may choose among several methods that yield high purity and total cell numbers but are technically demanding and may require costly equipment such as immunomagnetic separation [8,9], density gradients [10,11] or FACS [12,13]. Other approaches take advantage of the natural adhesive properties of microglia as a means to separate them in culture [14,15].

Materials & methods
Materials
Heat-inactivated fetal bovine serum; Dulbecco’s modified Eagle medium with 4.5 g/l glucose, L-glutamine and sodium pyruvate; phosphate-buffered saline (PBS) without calcium and magnesium; trypsin–ethylenediaminetetraacetic acid solution; and 75-cm² cell culture flasks were purchased from Corning (NY, USA). Penicillin–streptomycin–amphotericin B solution and bovine fibronectin solution were purchased from MP Biomedicals (OH, USA). Culture media were prepared by adding 10% fetal bovine serum and penicillin–streptomycin–amphotericin B to the Dulbecco’s modified Eagle medium and then filtering the mixture through 0.22-μm filter cups. Rat anti-CD11b (clone M1/70) labeled with fluorescein isothiocyanate (FITC) and mouse anti-GFAP (clone 1B4) labeled with Alexa Fluor® 647 were purchased from BD Biosciences (NJ, USA). Hoechst 33258 DNA stain and Alexa Fluor 488 phalloidin were purchased from Thermo Fisher Scientific (MA, USA). The FITC-tagged Aβ1–42 peptide was obtained from Bachem (CA, USA). Trypsin from porcine pancreas, trypsin inhibitor, DNase I and lipopolysaccharides from Escherichia coli O111:B4 were purchased from Millipore Sigma (MO, USA).
Figure 1. Representative images of the steps in the mouse microglia isolation procedure. (A) A dissected brain on weigh paper from a P0–P3 stage mouse. The hemispheres were separated, and each hemisphere was then rolled across the surface of the weigh paper to remove the meningeal layers. Scale bar = 1 cm. (B) Mixed cell population 5 h after addition to the culture flask. Scale bar = 50 μm. (C) Mixed cell population 6 days after addition to the culture flask. Arrows indicate the appearance of microglia. Scale bar = 50 μm. (D) Isolated microglia after 24-h attachment in a 35-mm culture dish. Scale bar = 50 μm. (E) DNA and (F) actin staining of isolated microglia on glass coverslips coated with fibronectin. Scale bar = 50 μm.

Animals
In the procedure, we used the senescence accelerated mouse prone-8 (SAMP8) strain of mice; however, any mouse strain can be used as a microglia source. The SAMP8 mouse strain exhibits behavioral, morphological and neurochemical changes identified with cognitive decline as well as microglia-associated neuroinflammation [16,17]. Mice were housed in rooms with a 12-h light/dark cycle (20–22°C) with water and food available ad libitum. All experiments were conducted in accordance with the institutional approval of the animal use subcommittee, which subscribes to the NIH Guide for Care and Use of Laboratory Animals. The colony is derived from siblings provided by Dr. Takeda (Kyoto University, Japan).

Isolation of mouse microglia
Isolation aligns with previous methods with modifications [15]. Procedures were performed in a sterile environment. The 75-cm² flasks were prepared by the addition of 5 ml poly-L-lysine solution (0.1 mg/ml in sterile water) and then incubation at room temperature for 30 min. The poly-L-lysine solution was removed, the flask washed once with 5 ml sterile water, and then dried before use. In this protocol, primary microglia were isolated from mouse pups ranging from P0 to P3. Two or three pups were removed from cages, and body temperature was maintained with a heating blanket. The pups were decapitated with a razor blade consistent with the American Veterinary Medical Association Guidelines for the Euthanasia of Animals (2020 Edition). The brain was extracted using forceps and submerged in 30 ml cold dissection medium (Hanks’ balanced salt solution with calcium, magnesium, glucose, 10 mM HEPES and penicillin–streptomycin–amphotericin B) and washed for 2 min to remove blood cells and monocyte contamination. The brain was then placed on weigh paper (Figure 1A) and the hemispheres were separated using a razor blade. Each hemisphere was then carefully rolled across the weigh paper to remove the meningeal layers. The hemispheres were rolled until meningeal layers were completely removed. The brain tissue was separated into small pieces on the weigh paper using forceps and then any remaining blood particulates were removed. The tissue pieces were placed in a 50-ml conical tube with 5 ml cold dissection medium to ensure tissue hydration. Once all the tissue was in the conical tube, the volume of dissection medium was increased to 30 ml, 1.5 ml trypsin solution (2.5%) was added and then the 50-ml conical tube was gently mixed. The tube was then placed into a 37°C water bath for 15 min.

During the 15-min period of incubation, the tubes containing the trypsin and brain tissue were inverted once per minute to facilitate digestion. Next, 1.5 ml of trypsin inhibitor (1 mg/ml) was added and mixed gently for 1 min; then 750 μl of DNase I (10 mg/ml) was added, gently mixed and then centrifuged for 5 min at 400 × g at 20°C. After centrifuging, the supernatant was removed and replaced with 5 ml of culture medium at 37°C. The cell pellet was resuspended using a 1-ml transfer pipette and then transferred to a 15-ml conical tube. The 15-ml tube was centrifuged for 5 min at 400 × g at 20°C. After centrifugation, the supernatant was removed and replaced with 5 ml culture medium at 37°C. The cell pellet was resuspended using a 1-ml transfer pipette and then transferred into a T-75 cell culture flask.
(pretreated with poly-L-lysine) containing 10 ml of culture medium, making a total volume of 15 ml in the flask. The flask was placed into a 5% CO₂ cell incubator at 37°C. After 5 h incubation, the mixed cell population had settled on the bottom of the flask and attached (Figure 1B). Tissue debris and apoptotic cells settled on the top surface of the attached mixed cell population.

The next day (day 1), the culture medium was removed and replaced with 15 ml fresh culture medium, and the flask containing the mixed cell population was incubated for an additional 4 days. On day 5 the culture medium was replaced. On day 6, microglia were visible on top of the mixed cell layer and ready for harvest (Figure 1C). At this point of the procedure, the microglia were weakly attached to the underlying mixed cell monolayer. To remove the microglia from the rest of the cell mixture, the flask was tapped on the edge of a bench top three times. After tapping, the flask was visually inspected for the detached microglia. Additional tapping may increase microglia detachment but may lead to contamination of the microglial cells with other cell types. Alternatively, the microglia may be detached in a more reproducible manner by placing the flask on a rocker or shaker. The culture medium containing the detached microglia was harvested, pelleted by centrifugation at 800 × g for 5 min and resuspended in 2 ml culture medium. Alternatively, the medium can be carefully removed from the flask and replaced with a small volume of fresh culture medium before tapping; the detached microglia are harvested in the small volume and can be used without the need for centrifugation. The isolated microglia can be seeded into a new culture dish (Figure 1D), added to coverslips for fluorescent staining and microscopy procedures (Figure 1F) or analyzed by flow cytometry (Figure 2) or other methods. From our experience, after 24 h attachment, the isolated microglia could not be removed from tissue culture surfaces using a standard trypsin–ethylenediaminetetraacetic acid solution; cell removal required scraping.

**Flow cytometry**

Flow cytometry was used to assess the purity and phagocytic activity of the isolated microglia. For purity measurement, 100 μl isolated microglia were placed in 1.5-ml microcentrifuge tubes, then 1:100 dilution rat anti-CD11b labeled with FITC and 1:100 dilution mouse anti-GFAP labeled with Alexa Fluor 647 were added and the tubes were incubated for 30 min at 37°C with 0.01% Triton™ X-100 from Thermo Fisher Scientific (MA, USA). After incubation, the tubes were removed and 50 μl was sampled from each tube by flow cytometry to measure the mean fluorescence intensity. For the 30-min phagocytic activity assay, 100 μl of isolated microglia were placed in 1.5-ml microcentrifuge tubes, 100 nM FITC-labeled Aβ1–42 peptide was added, and the samples were incubated for 30 min at 37°C. The samples were analyzed by flow cytometry to measure the mean fluorescence intensity. For the 24-h phagocytic activity assay, 100 nM FITC-labeled Aβ1–42 peptide was added to mixed cell populations in six-well plates on day 6 of the isolation procedure. The peptide was incubated with the mixed cell populations for 24 h at 37°C. After incubation, the plates were tapped to isolate the microglia, and the cells were analyzed by flow cytometry.

**Microscopy**

Epifluorescence microscopy was used to perform isolated microglial nucleus and actin staining and FITC-labeled Aβ1–42 peptide uptake experiments. For this, 18 × 18 mm glass coverslips were coated with 50 μg/ml bovine fibronectin for 1 h at 37°C and then washed once with sterile PBS. The fibronectin-coated coverslips were immediately placed in a 35-mm dish and the microglia, suspended in culture medium, were added. For microglial nucleus and actin staining, the cells were incubated for 24 h to allow attachment and spreading to the fibronectin, then fixed in PBS containing 4% paraformaldehyde and 0.1% Triton X-100 for 60 min, and finally washed in water. The coverslips were blocked in 1% bovine serum albumin solution for 20 min and then stained with Hoechst 33258 DNA stain and Alexa Fluor 488 phalloidin for 20 min at room temperature. The coverslips were washed with water for 20 min and mounted onto glass slides. The nucleus and actin images were acquired with a Leica DMi8 inverted microscope (Leica, Wetzlar, Germany) fitted with a 63× oil immersion lens and 12-bit charge-coupled device monochrome camera. For the FITC-labeled Aβ1–42 peptide uptake experiments, isolated microglia were added to 35-mm culture dishes, peptide was added and incubated for 24 h, and the microglia were imaged with a 10× dry lens. All images were acquired and processed using Metamorph imaging software from Molecular Devices (CA, USA).

**Data analysis**

For the flow cytometric analysis, an event threshold was set at 750,000 forward scatter height and a gate was drawn around the intact cell population in the forward scatter height versus side scatter height plots. All experiments were repeated a minimum of three times. In the 24-h FITC-Aβ1–42 peptide uptake experiments measured by flow cytometry, the data were normalized, averaged and analyzed by one-way analysis of variance and Tukey’s multiple comparison post hoc test.

**Results & discussion**

In this simplified procedure, removal of the meningeal layers was easily achieved by rolling the brain hemispheres on paper (Figure 1A) rather than removal under a stereo microscope using forceps [18]. After trypsin digestion on day 0, the mixed cell population was added to a standard tissue culture flask or plate (Figure 1B). On day 6 (Figure 1C), the microglia were ready for harvest by tapping the flask on a bench top. The isolated cells can be used directly in microscopy staining procedures (Figure 1E & F) or other assays. We assessed the purity of the isolated microglia by measuring the expression of CD11b and GFAP [19]. Expression of CD11b is a definitive marker of brain microglia [20,21]. Consistently among repeated procedures, the isolated cells displayed homogenous appearance in culture (Figure 1D) and stained >90% CD11b-positive and <1% GFAP-positive (Figure 2). The cell morphology was nonramified and ameboid, which is likely
the result of the presence of 10% serum in the cell medium [22] and the near postnatal period of the pups [23]. Although purity was high, the total yield per mouse ranged from 50,000 to 100,000 cells, which is lower than other reported procedures [10,24]. Tapping the flask to detach the microglia is a crucial step in the procedure and may need optimization to achieve the desired balance between purity and yield. In our lab, three gentle taps on a bench top were sufficient to detach the microglia from the underlying mixed cell population. Additional tapping may increase the total microglia yield; however, the increased yield may be at the expense of purity. Other factors, such as the presence of colony-stimulating factor 1 in the culture medium after isolation, may promote microglia proliferation [25], thereby increasing the cell numbers available for experimental purposes.

We determined whether the microglia, both isolated and among the mixed cell population, retained intact Aβ1–42 peptide uptake and response to lipopolysaccharide. As shown in Figure 3, the isolated microglia dose-dependently associated with FITC-Aβ1–42 peptide after 30 min incubation, as measured by flow cytometry. We also performed peptide uptake experiments at 24 h on both isolated microglia and microglia among the mixed cell population. Isolated microglia were placed in a culture dish and incubated with 100 or 300 nM FITC-Aβ1–42 peptide. After 24 h, we observed the association of peptide fluorescence at both concentrations (Figure 4A). In the mixed cell population experiments, peptide and lipopolysaccharide were added on day 6 of the procedure. After a 24-h incubation
Figure 3. Uptake of Aβ1–42 peptide by isolated microglia after 30 min. The microglia were harvested on day 6 and immediately incubated in suspension with the indicated concentrations of fluorescein isothiocyanate-labeled Aβ1–42 peptide for 30 min and analyzed by flow cytometry. Control = 0 nM peptide. The mean fluorescence intensity of representative samples is reported within each histogram plot.

period, the microglia were isolated by gentle tapping and then analyzed for FITC fluorescence by flow cytometry (Figure 4B). Both the low (0.83 ng/ml) and the high (8.3 ng/ml) lipopolysaccharide concentrations increased peptide uptake above the control.

Conclusion

In conclusion, we detail here a simplified and low-cost procedure for isolating mouse microglia which can be performed in any lab with standard cell-culture equipment. The isolated cells were of high purity and were functional in peptide uptake assays and response to lipopolysaccharide. The beginning of the isolation procedure (day 0) requires a few hours of work, and then the remaining days of the procedure require little effort – only medium changes and centrifugation of isolated cells before analysis. In addition to its simplicity, the procedure has other advantages. We recommend removing the meningeal layers by rolling the brain hemispheres on weigh paper rather than using forceps under a dissection microscope [18], which is more tedious. Microglia can be used in experimentation while in isolation or when they are among the mixed cell population. After treatments in the mixed population, the microglia can be isolated by tapping. The single-step purification is particularly useful because purified microglia attached to glass or plastic surfaces are difficult to remove without mechanical cellular damage. We chose newborn pups due to the greater number of brain microglia; however, although not tested directly, we believe our procedure is extendable to adult brains. The only serious limitation of this procedure is that purity is gained at the expense of overall yield. Nevertheless, we believe this simplified procedure can be useful to many labs with a research interest in neuroinflammation, neurodegenerative disease or basic microglia biology.

Future perspective

As the mechanisms of microglia in neuroinflammation, neurodegeneration and neuropsychiatric disease become increasingly understood in detail, microglia will be the target of future therapies. A demand for preclinical cellular models is thus expected to increase
Figure 4. Microglia uptake of Aβ1–42 peptide after 24 h. Isolated microglia in six-well plates were incubated with 0 (control), 100 or 300 nM fluorescein isothiocyanate-labeled Aβ1–42 peptide for 24 h and then imaged for green fluorescence. Bar graph: mixed cell population samples were incubated with 100 nM fluorescein isothiocyanate-labeled Aβ1–42 peptide for 24 h in the presence of the indicated ng/ml concentrations of LPS. After the 24-h incubation period, the microglia were harvested by tapping and the samples were analyzed by flow cytometry. Three experiments were averaged and normalized to control (0 ng/ml LPS fluorescence channel 1 height average value).

*p < 0.05.

LPS: Lipopolysaccharide; ns: Not significant.

as well. The use of primary microglia is not intended to replace cell lines, but will provide a path toward a more physiological system. Simplified procedures for primary cell isolation will facilitate an increase in laboratory participation in these much-needed research areas.

Author contributions
N Scott and J Schober: designing experiments, performing experiments, data interpretations, writing and reviewing the manuscript. K Witt: procedure development, providing resources, data interpretation, writing and reviewing the manuscript.

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Ethical conduct of research
All experiments were conducted in accordance with the institutional approval of the animal use subcommittee, which subscribes to the NIH Guide for Care and Use of Laboratory Animals.

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