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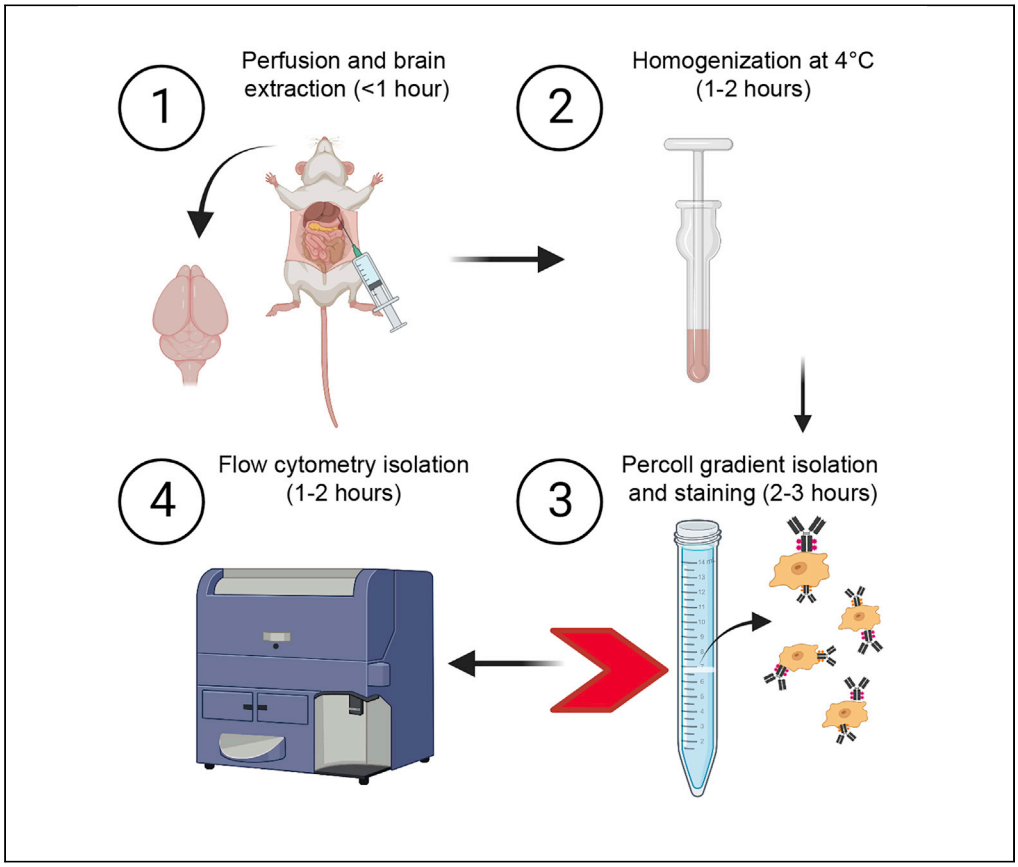
Using mechanical homogenization to isolate microglia from mouse brain tissue to preserve transcriptomic integrity



Numerous approaches have been developed to isolate microglia from the brain, but procedures using enzymatic dissociation at 37°C can introduce drastic transcriptomic changes and confound results from gene expression assays. Here, we present an optimized protocol for microglia isolation using mechanical homogenization. We use Dounce homogenization to homogenize mouse brain tissue into single-cell suspension. We then isolate microglia through Percoll gradient and flow cytometry. Isolated microglia exhibit a gene expression pattern without the changes induced by heated enzymatic digestion.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Optimized protocol for mouse microglia isolation without an enzymatic dissociation step

Using Dounce homogenization at 4°C to generate single-cell suspension

Isolation of microglia through Percoll gradient and flow cytometry

Can preserve transcriptomic integrity and is suitable for multiple sequencing endpoints

Herron et al., STAR Protocols 4, 101670
December 16, 2022
https://doi.org/10.1016/j.xpro.2022.101670

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Using mechanical homogenization to isolate microglia from mouse brain tissue to preserve transcriptomic integrity

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https://doi.org/10.1016/j.xpro.2022.101670

SUMMARY

Numerous approaches have been developed to isolate microglia from the brain, but procedures using enzymatic dissociation at 37°C can introduce drastic transcriptomic changes and confound results from gene expression assays. Here, we present an optimized protocol for microglia isolation using mechanical homogenization. We use Dounce homogenization to homogenize mouse brain tissue into single-cell suspension. We then isolate microglia through Percoll gradient and flow cytometry. Isolated microglia exhibit a gene expression pattern without the changes induced by heated enzymatic digestion.
For complete details on the use and execution of this protocol, please refer to Clayton et al. (2021).

BEFORE YOU BEGIN

Institutional permissions

All experimental procedures described in this protocol were approved by Institutional Animal Care and Use Committee of the Boston University School of Medicine. Since this protocol involves use and sacrifice of live vertebrates, any readers performing this protocol will need to acquire approvals from the relevant institutions.

Can be made prior to day of procedure

Prepare the blocking and FACS buffer before starting the procedure
(Refer to Materials and equipment for buffer recipe).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit, for UV excitation (1:300 working dilution)	Thermo Fisher Scientific	L23105
CD11b Monoclonal antibody (M1/70), PE-Cyanine7, eBioscience™ (1:300 working dilution)	Thermo Fisher Scientific	25-0112-82 RRID:AB_469588
PerCP/Cyanine5.5 anti-mouse Ly-6C antibody (1:300 working dilution)	BioLegend	128012 RRID:AB_1659242

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Alexa Fluor® 647 anti-mouse CX3CR1 antibody (1:300 working dilution)	BioLegend	149003 RRID:AB_2564272
Tmem119 Monoclonal antibody (V3RT1GOsz), PE, eBioscience™ (1:300 working dilution)	Thermo Fisher Scientific	12-6119-82 RRID:AB_2848262
PE anti-P2RY12 antibody (1:300 working dilution)	BioLegend	848003 RRID:AB_2721644
FCRLS rat monoclonal, APC (1:300 working dilution)	Butovsky Lab	N/A
Chemicals, peptides, and recombinant proteins		
Hanks' Balanced Salt Solution (1X), without calcium, with magnesium, with phenol red	Fisher Scientific	AAJ67609K2
Bovine Serum Albumin	MilliporeSigma	A9418-50G
Percoll PlusCytiva Percoll™ PLUS Centrifugation Media	Fisher Scientific	45-001-753
Gibco™ PBS (10X), pH 7.4	Fisher Scientific	70-011-069
Experimental models: Organisms/strains		
C57BL/6J mice (male/female, age and developmental stage may vary)	The Jackson Laboratory	000664
Other		
Propette pipette controller with gravity mode	Morganville Scientific	PRO1100
GenClone 12–104, 10mL Serological Pipets Sterile, Individually Wrapped, 50/Bag, 200 Pipets/Unit	Genesee Scientific	12-104
Eppendorf® Centrifuge 5804/5804R	MilliporeSigma	EP022628146
DWK Life Sciences Kimble™ Kontes™ Dounce Tissue Grinders (pestles A and B)	Fisher Scientific	K885300-0007
Falcon® 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap, 25/Pack, 500/Case	Corning Life Sciences	352235
Falcon™ 15 mL Conical Centrifuge Tubes	Fisher Scientific	05-527-90
Falcon 50mL Conical Centrifuge Tubes	Fisher Scientific	14-959-49A
Micro-dissecting scissors	MilliporeSigma	S3271
Micro-dissecting forceps	MilliporeSigma	F4142
Fisherbrand™ Sterile Syringes for Single Use	Fisher Scientific	14-955-460
Med Vet International Exel Needle, 20G x 1", Hypodermic, 100/Box	Fisher Scientific	50-209-2532
Integra™ Miltex™ Sterile Standard Scalpels	Fisher Scientific	12-460-451
Fisherbrand™ Premium Microcentrifuge Tubes: 1.5mL	Fisher Scientific	05-408-129
Low Protein Binding Microcentrifuge Tubes	Thermo Fisher Scientific	90410

MATERIALS AND EQUIPMENT

Our lab uses an Eppendorf 5804R centrifuge with S-4-72 rotor with swinging buckets, but any centrifuge that offers acceleration, brake and temperature control with swinging buckets that can accommodate 15-mL Falcon tubes will suffice.

Note: Validation must be performed if reagents are used from alternative suppliers.

Preparation of blocking buffer

- Dissolve bovine serum albumin (BSA) directly in a bottle of 500 mL of 1X HBSS. Vortex. This step is best performed at room temperature the day before experimentation to ensure proper dissolution of BSA. This buffer can be stored at 4°C for up to one month.

Solution	Amount	Final concentration
1X Hanks' balanced salt solution (HBSS)	500 mL	98%
BSA	10 g	2%

Blocking of Falcon 15mL tubes

- To prevent adhesion of microglia to the walls of the Falcon tubes in which the staining and centrifugation will take place, the tubes must be blocked for 1–2 h at 4°C with the blocking buffer. To do this, fill the Falcon 15-mL tubes into which the final Percoll interphase layer will be pipetted with 10mL of blocking buffer. Blocking buffer can be re-used for this purpose upwards of 5 times in less than a month (filtering the recycled buffer is recommended when close to 1 month after generation).

Preparation of FACS buffer

- Dissolve 1 *g* BSA directly in a bottle of 500 mL of 1X HBSS. Vortex. This step is best performed at room temperature the day before to ensure proper dissolution of BSA. Alternatively, dilute one part of blocking buffer (after its filtration) in 9 parts of 1X HBSS. This buffer can be stored at 4°C for up to one month.

Solution	Amount	Final concentration
1X HBSS	500 mL	99.8%
BSA	1 g	0.2%

To be made fresh day of procedure

Preparation of isotonic Percoll

- Mix 10% 10X PBS with 90% Percoll plus

Solution	Amount	Final concentration
PBS (10X)	Varies with size of preparation	10%
Percoll Plus	Varies with size of preparation	90%

Note: Percoll can be store at room temperature indefinitely. 1X HBSS, and 10X PBS should be taken out of the refrigerator and placed at room temperature (~20°C–25°C) prior to mixing and be kept at room temperature.

Note: Out of 50 ml of isotonic Percoll, you can generate 50 ml of 70% Percoll solution and 40ml of 37% Percoll solution, allowing for 8 gradients. 5 mL of 70% Percoll and 5 mL of 37% Percoll is required per sample for the gradient step. We recommend using one hemisphere per gradient for optimal results, but an entire adult mouse brain can be applied to one Percoll gradient.

Preparation of 70% Percoll solution

- Mix 30% HBSS with 70% isotonic Percoll

Note: 5 mL of 70% Percoll is required per sample for the gradient step.

⚠ CRITICAL: All elements for the Percoll gradients should come to at room temperature prior to mixing.

Solution	Amount	Final concentration
HBSS (1X)	Varies with size of preparation	30%
Isotonic Percoll	Varies with size of preparation	70%

Preparation of 37% Percoll solution

- Mix 63% HBSS with 37% isotonic Percoll at room temperature

Note: 5 mL of 30% Percoll is required per sample for the gradient step.

Solution	Amount	Final concentration
HBSS (1X)	Varies with size of preparation	63%
Isotonic Percoll	Varies with size of preparation	37%

STEP-BY-STEP METHOD DETAILS

Perfusion, dissection, and homogenization of mouse brain tissues

Timing: around 20 min per mouse for steps 1-9 (perfusion and homogenization)

This section describes how to properly perfuse, dissect, and homogenize brain tissue into a suitable single cell suspension without excessive loss of microglia through Dounce homogenization at 4°C.

To reduce the procedure time, consider enrolling multiple participants for the homogenization step of this protocol. These steps do not need to be carried out within the confines of a biological safety cabinet unless the downstream application is culture of isolated microglia (not recommended). If cell culture is the downstream application, perform all steps where the cell solution is exposed to the atmosphere in a Class II Biological Safety Cabinet.

1. Perfuse adult mouse transcardially with 20 mL of ice-cold HBSS and dissect entire brain or brain region of interest (see note) with razor blades on ice and keep in ice-cold HBSS until homogenization. For a visual reference on transcardial perfusion with a syringe, please see Method Video S1.

   Note: Following the perfusion of mouse with ice-cold HBSS, microdissection of brain regions of interest and whole brain razor blade chopping should be performed on a petri dish embedded in wet ice.

   Note: Meninges do not need to be removed at this step, meningeal removal will be accomplished via discontinuous Percoll gradient centrifugation.

   Note: A perfusion rate of 10 mL/min is recommended to prevent vessel rupture and ensure a thorough perfusion.

   Note: Mice below 2 months of age may not require 20mL to achieve a thorough perfusion, although there is minimal risk in over-perfusing, so we recommend using 20mL regardless of age, with the exception of neonates which cannot be perfused, and brains can be directly extracted following euthanasia.

   Note: This protocol can be applied to brain regions as small as an adult mouse hippocampus, although experience with the protocol is recommended as the Percoll layers and cell pellets will be difficult to identify with such a limited input.

Note: If performing this procedure on multiple mice, brains should be dissected and kept in ice-cold HBSS (7 mL) until razor blade chopping and homogenization. This volume of HBSS is ideal for downstream homogenization. Do not proceed directly to homogenization after dissection.

Note: Our experience finds that one adult mouse forebrain hemisphere is the ideal amount of tissue for this procedure to achieve the most efficient yield. This procedure can be completed with an entire brain, however in our experimental design, another hemisphere is used for drop fixation or freshly snap frozen. It is recommended to process by half brains for beginners and move to whole brain when protocol is mastered.

Note: Chopping once or twice sagittally and 3–4 times coronally will reduce the time homogenization takes in the next portion of this protocol and will contribute to a successful homogenization.

Note: In our experiments, the cerebellum is excluded on account of transcriptional distinction of microglia within this brain region. Depending on the downstream experiment, the experimenter may wish to remove the cerebellum for microglia isolation. This protocol can be performed with the cerebellum intact.

2. Dump entire contents of one brain in HBSS into a clean ice-cold Dounce homogenizer pre-rinsed with ice-cold HBSS.

Note: Clean glass homogenizers with soap and water, then rinse 3x with ample distilled water. Finally, ensure that the homogenizers are rinsed with ice-cold HBSS before brain homogenization to ensure proper tonicity within the homogenizer environment.

3. Very gently and slowly begin to homogenize the brain tissue with the A pestle (or loose pestle).
   a. Push down on the tissue with the pestle until brain tissue is slightly expelled up and around the sides of the pestle foot and in-between the wall of the homogenizer.
   b. Gently twist to separate the expelled tissue from the rest of the brain tissue.

Note: For a guide on how to homogenize brain tissue with pestle A, see Method Video S2.

⚠ CRITICAL: The quality of homogenization is the single most important factor in determining downstream cell quality. Because this is a mechanical dissociation, it is imperative to be gentle and slow when beginning the homogenization.

4. Continue homogenizing gently with pestle A until all remaining chunks can bypass the foot of pestle A upon depression and retraction with minimal resistance. When the mixture is similar in appearance to the image shown in Figure 1A, begin homogenization with pestle B.
5. Begin homogenization with pestle B.

Note: Similar to pestle A, moving too fast or applying too much force with the pestle will apply excessive shear force and will destroy microglia. Therefore, it is critical to apply slow, even strokes with pestle B. For a visual reference on the proper homogenization technique to follow with pestle B, please see Method Video S3.

6. Homogenize with pestle B until barely any visible chunks are visible.

Note: Some myelin debris will be impossible to homogenize into a single cell suspension but ensure that no pink brain tissue chunks are visible. Consult Figure 1B for a visual guide to what can be considered thorough homogenization.

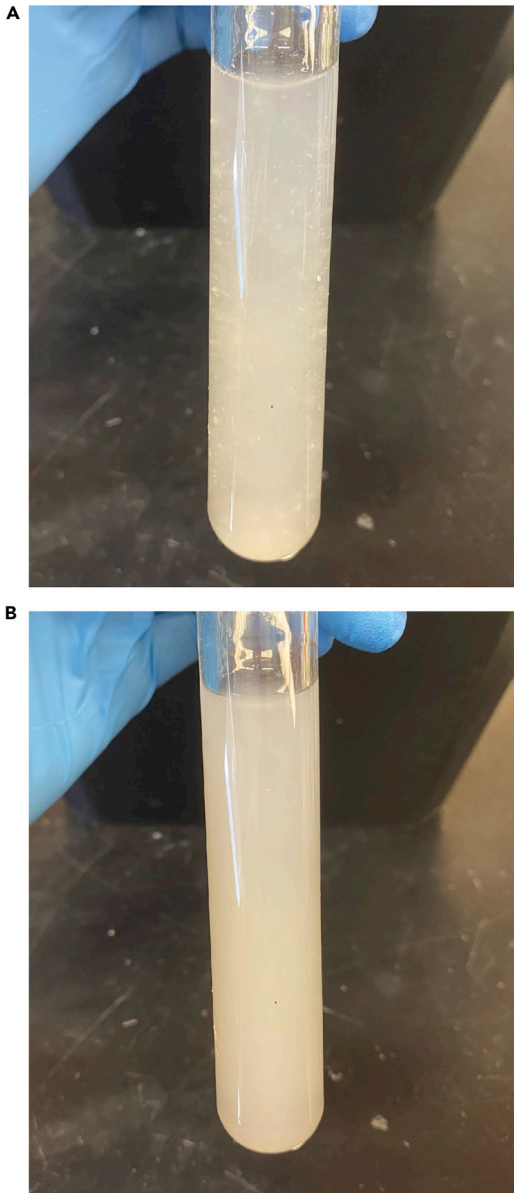

Figure 1. Representative images of the homogenization solution after completion of pestles A and B

(A) Once the homogenization mixture reaches the consistency shown in this image, the suspension is ready to begin homogenization with pestle B.

(B) Once the suspension reaches the consistency shown in panel B, the suspension is ready to be poured into a 15 mL tube for centrifugation.

⚠ CRITICAL: In addition to maintaining cell viability, it is critical that as close to a single cell solution as possible is achieved. Doublets and other large cell aggregates will be excluded upon cell sorting, which can reduce microglia yield. However, over-homogenization will also reduce the viability of the preparation and should be avoided.

7. Once thoroughly homogenized, dump contents into a fresh, pre-chilled Falcon 15mL tube.
8. Rinse homogenizer with ice-cold HBSS (7 mL) and do five slow passes with both pestles A and B. Add the contents of this wash step to the tube containing the homogenization mixture.

Note: If interested in analyzing homogenous mixture, 500 μL can be taken, centrifuged to pellet the cells and snap frozen for downstream applications.

9. Rinse the homogenizer with 7-mL ice-cold HBSS and apply vigorous strokes (10 times) with both pestles A and B to ensure no stuck cells which could be added to downstream homogenizations.
   a. Discard the HBSS. Repeat the homogenization process for the remaining brains.
10. When all samples are homogenized, pellet the mixture by centrifuging at 350 *g* at 4°C for 5 min.

Note: Given that a slow and careful homogenization is key to the success of this protocol, each brain can take upwards up 15 min to complete with an average of 5 min per homogenization step. Therefore, if many samples are being used in one experiment, multiple people homogenizing can expedite this protocol.

11. If not already prepared, prepare however many Falcon tubes equal to the number of samples in the experiment by adding 5 mL of 70% Percoll to the 15-mL Falcon tubes and leaving them at room temperature.

Note: Ensure that 70% Percoll and 37% Percoll solutions are prepared and at room temperature prior to this step.

12. Remove tubes from centrifuge and dump out the supernatant, taking care not to disturb the pellet, and place in a rack holder.

Note: For a visual reference of what the homogenate will look like after centrifugation, please refer to Figure 2.

Percoll gradient layering and centrifugation

This section describes how to properly layer Percoll solutions, isolate microglia residing at the Percoll interphase layer and washing/reconstitution of the microglia cell pellet in preparation for antibody application.

Timing: ~45 min

Note: Ensure that the centrifuge is set to fast temp ~23°C as soon as you remove the pelleted samples from step 12

13. Re-suspend the homogenized pellet in 5mL of 37% Percoll solution.

Note: Ensure a thorough resuspension without destroying cells by pipetting. A thorough resuspension will ensure that microglia clumped to other cell types will not get pulled away from the interphase layer. We recommend initially re-suspending with one mL of 37% Percoll with a P1000 and adding the additional 4 mL of Percoll with a pipette controller.

14. Slowly and carefully layer the 37% Percoll resuspension on top of the 70% Percoll layer previously prepared in step 11.

Note: For a visual reference of what the separated layers should look like prior to centrifugation, please refer to Figure 3.

⚠ CRITICAL: Mixing of the layers will destroy the interphase separation. To avoid this, use the gravity setting of the pipette and very slowly apply the first milliliter of the 37% layer. Hold the tube containing the 70% Percoll at a 65° angle initially to slow liquid travel down

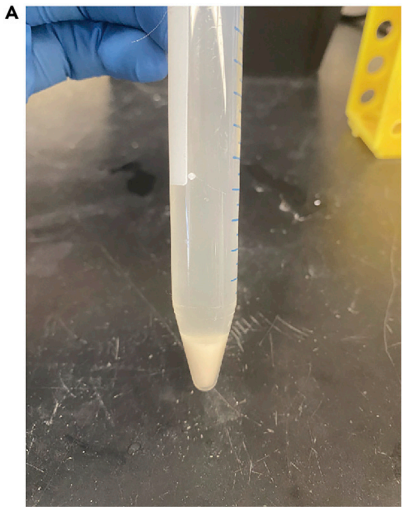

Figure 2. Representative image of the suspension solution after centrifugation

(A) Representative image of what the suspension will look like following the short 300 g spin down. A relatively clear supernatant with a robust pellet should be visible.

the tube. Adjust the angle of the tube to vertical (90°) as you add more 37% Percoll. For a visual guide of this process, please refer to Method Video S4.

15. Once all samples are layered, carefully return them to the centrifuge in the respective swinging bucket and set the centrifuge settings to 23°C, 25 min, 800 *g*, acceleration 3 and brake 1.

Note: Given the minimal brake, this step will take around 30 min total.

Note: Remove tubes from centrifuge very gently so as not to disturb the interphase layer.

Note: Set the centrifuge temperature to 4°C immediately after removing the tubes.

16. While these tubes are spinning, prepare the necessary number of Falcon 15-mL tubes by dumping out the blocking buffer and filling the tubes with 8 mL of FACS buffer.
17. Bring all tubes back to the workspace and, using a P1000, collect the interphase layer of cells. Collect between 2 to 3 mL, expelling into the appropriately labeled Falcon 15-mL tube containing FACS buffer.

Note: For a visual reference of what the layer should look like, please refer to Figure 4 and Method Video S5. For a visual guide of the vacuuming procedure, please see Method Video S6.

Note: The interphase layer may look like a hazy disc and should appear exactly at 5 mL. After vacuuming this layer with your pipette, the separation between the two layers should become glassy.

Note: While it is not necessary, it may be helpful to aspirate the layer of myelin debris which will be on top of the 37% layer, with either an aspirator connection or a P1000 to facilitate interphase layer collection.

18. Pellet the cells by centrifuging at 4°C, 350 *g* for 5 min.

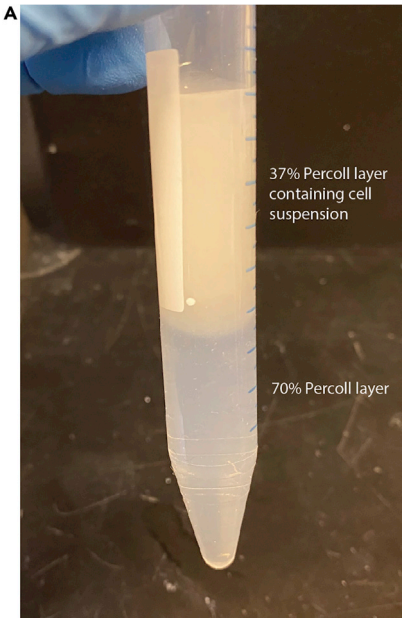

Figure 3. Representative images of the Percoll layer
(A) Image of the 37% Percoll layer with cells suspended within it layered on top of the 70% Percoll layer. It is important to prevent mixing of these two layers to ensure a proper interphase layer in the Percoll centrifugation step.

19. Following centrifugation, aspirate or remove all but 300 μL of liquid from the Falcon 15-mL tubes and re-suspend the pellet.

Note: At this point forward, it will be difficult to identify the pellet and gauge whether the pellet is re-suspended. Ensure even and thorough resuspension by agitating all sides of the bottom of the Falcon 15-mL tubes for subsequent resuspension steps with at least 10 pumps of a P1000.

Note: fill an extra 15-mL Falcon tube with 300 μL of liquid to give you a reference of the volume to keep in your experimental tubes.

Staining and flow cytometry isolation procedure

This section describes proper application of flow cytometry antibodies to cells isolated via density gradient centrifugation, cell washing, and collection strategy of cells via flow cytometry.

Timing: ~1–3 h depending on number of samples

Note: Prior to fluorescence activated cell-sorting, use compensation beads stained with CD11b-PEcy7 and Ly6C-PercpCy5.5 separately to set up compensation and gating. For a negative control for live/dead microglia, induce membrane lysis by exposing cells to a hypertonic solution or take one sample and vigorously homogenize to induce cell death. For a visual reference of poor homogenization technique leading to a greater number of dead microglia, please refer to Method Video S7 which demonstrates a very fast and aggressive homogenization where a high amount of pressure is applied with pestle A and B, brain tissue is crushed and the fast pestle movement results in shear forces and cell lysis.

20. Create a master-mix of CD11b-PEcy7, Ly6C-PercpCy5.5 and LIVE/DEAD UV Stain at a concentration of 1:150 for each antibody/dye in FACS buffer, with 300 μL of volume for each sample being accounted for. i.e., - If there are 3 samples, create a 1 mL solution (300 μL × 3 + 100 μL pipette error compensation) of 1:150 LIVE/DEAD, CD11b-PEcy7, Ly6C-PercpCy5.5 (6.6 μL each).

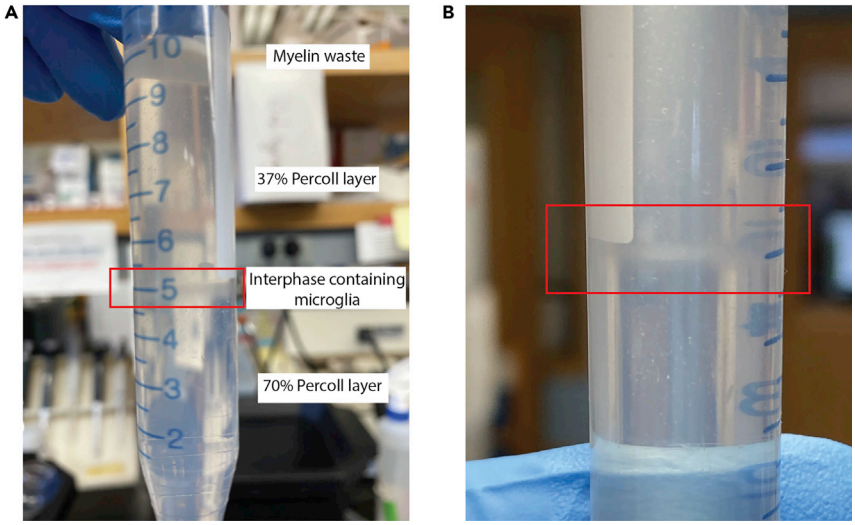

Figure 4. Representative images of the microglia layer on top of the 70% Percoll layer following the long centrifugation step

(A) Image of a microglia layer highlighted with a red box, which appears as an opaque thin layer at 5 mL, which corresponds to the amount of 70% Percoll in the tube.

(B) Image of the same layer highlighted with a red box (A).

21. Add 300 μL of staining master mix to each Falcon 15 tube containing the re-suspended pellet of myeloid cells. Leave on ice, covered from light for 15 min.
22. To wash the cells, fill the Falcon 15-mL tubes with 8 mL ice-cold FACS buffer.
23. Centrifuge at 4°C, 350 *g* for 5 min.
24. Aspirate the supernatant, leaving only 2–300 μL remaining in the tube. Re-suspend the pellet.
25. Using a P1000, transfer the re-suspended cell solution from the Falcon 15-mL tubes to the filter cap FACS sorting tubes, being sure to expel the suspension through the filter caps.
26. Fill a FACS collection tube (1.5 mL) with 300 μL of ice hold HBSS.
27. Using a cell sorter, isolate singlet LIVE/dead negative, Ly6C negative, CD11b positive cells. For a reference of gating set-up, please refer to Figure 5.

EXPECTED OUTCOMES

Using the above method, we have been able to consistently recover ~200,000 microglia per hemisphere from adult mouse brains at ~90% viability. Using CD11b and Ly6C as markers to separate between infiltrating macrophages results in a purity of microglia, which is over 99%, which we have confirmed by co-staining with the microglia specific marker Fc receptor-like molecule (FCRLS) (Figure 5) (Butovsky et al., 2014). FCRLS is a microglia specific marker, but due to the lack of commercial availability and low level of exclusion of $Ly6C^{-}$, $CD11b^{+}$ cells, the use of this antibody is not included in this protocol. RNA sequencing reveals dramatic preferential expression of microglia specific markers, but not glial or neuron enriched proteins (Figure 6). This protocol is applicable to a wide variety of mouse ages and has been successfully completed on neonates and aged mice. We have successfully completed this protocol on mice between the ages of P0 and 12 months of age.

LIMITATIONS

Warm versus cold tissue dissociation cellular yield

The major limitation of this protocol compared to conventional methods of brain tissue dissociation is the reduced viability or surviving cells due to the aggressive nature of mechanical dissociation compared to enzymatic dissociation. Indeed, another STAR protocol detailing a similar method of microglia isolation cites the difficulties in obtaining a robust live cell population of microglia using

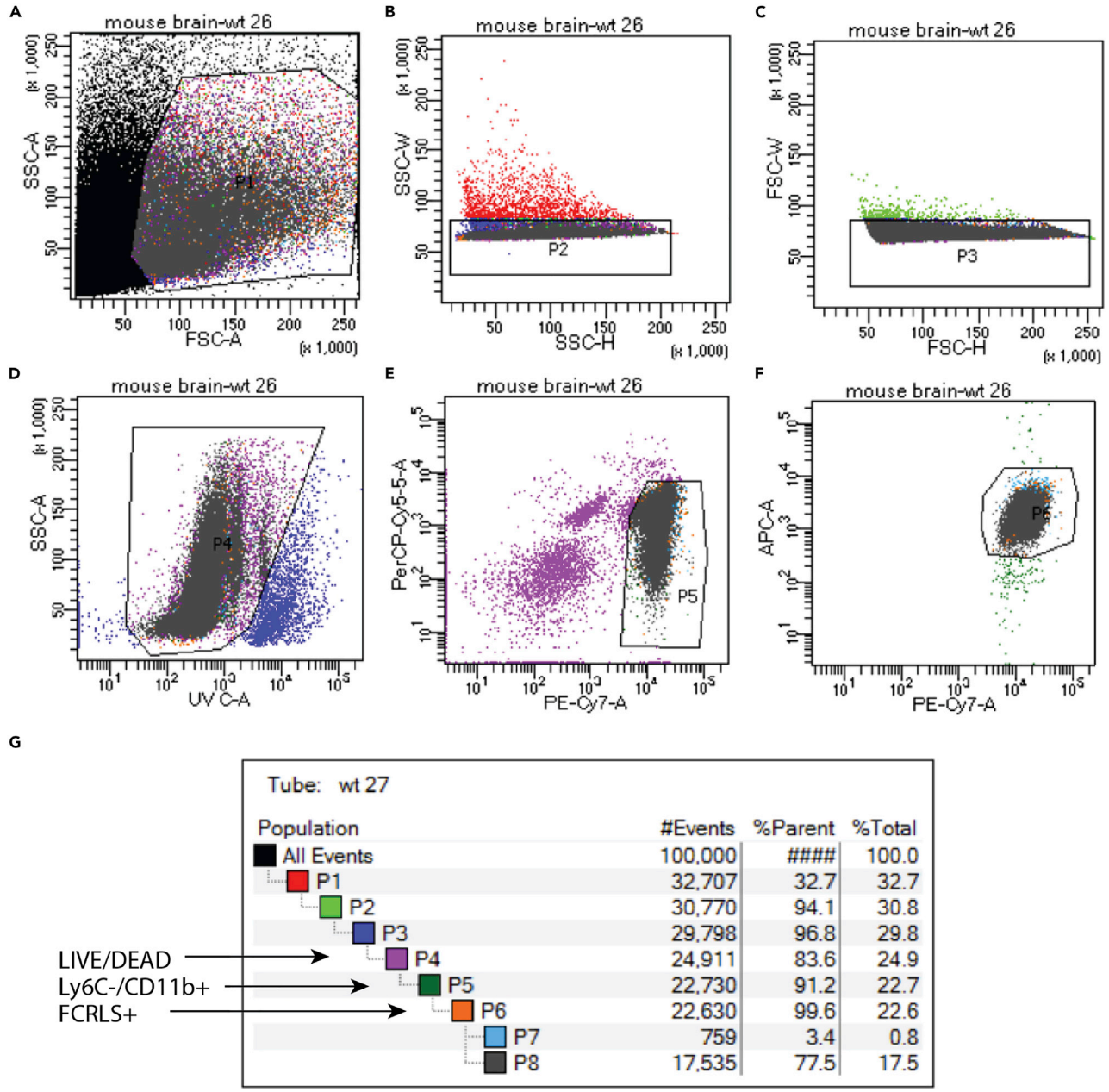

Figure 5. FACS plot depicting the gating strategy used to sort microglia

(A) Side Scatter (SSC)-Area, Forward Scatter (FSC)-Area to remove debris (B) SSC-Width, SSC-Height for exclusion of doublets (C) FSC-Width, FSC-Height to remove debris (D) SSC-A, LIVE/DEAD-UV to exclude dead cells (E) Ly6C-PercpCy5.5-A, CD11b-PE-Cy7-A to exclude peripheral macrophages and include microglia (F) FCRLS-APC-A, CD11b-PE-Cy7-A to confirm microglia specificity.

(G) Details the number and percentage of cells gathered from each gate.

mechanical/cold dissociation (Bordt et al., 2020). However, multiple studies have pointed out that enzymatic digestion can lead to substantial artifacts in transcriptome analysis of isolated central nervous system cells (Mattei et al., 2020; Hrvatin et al., 2018; Wu et al., 2017; Marsh et al., 2022; Van Hove et al., 2019; Van den Brink et al., 2017). While both mechanical and enzymatic digestion are certain to result in some level of transcriptomic deviation from microglia *in situ* transcriptomic profile, large temperature shifts like those experienced by cells in an enzymatic digestion protocol have been shown to elicit widespread transcriptional changes within short time windows (Mahat et al.,

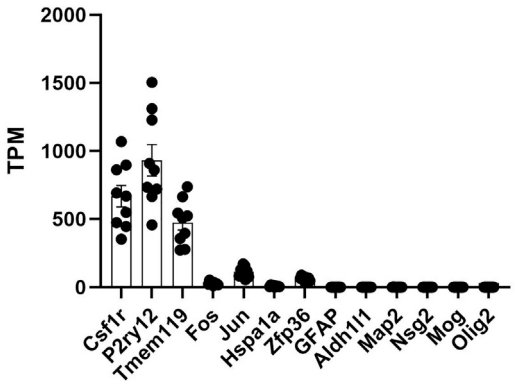

Figure 6. RNAseq data from microglia isolated using the described protocol

Transcripts per million (TPM) for microglia specific genes including *Csf1r, P2ry12* and *Tmem119* were enriched, while genes for stress induced by warm dissociation including *Fos, Jun, Hspa1a,* and *Zfp36* were minimally expressed. Markers for other CNS cell types such as astrocytes (*GFAP, Aldh1l1*), Neurons (*Map2, Nsg2*) and Oligodendrocytes (*Mog, Olig2*) were negligible (N=9 mice).

2016). Additionally, enzymatic digestion may result in altered presence of cell surface markers, both by digestion by enzymes within the dissociation buffer and altered membrane trafficking at temperatures when the cells are strongly metabolically active at physiological temperatures. Consistent expression of cell surface markers is essential for downstream applications such as flow cytometry. The goal of this protocol is to provide experimenters with a reliable method of obtaining microglia from mouse brain tissue without exposure to enzymatic dissociation steps. Given the limitation of cell number resulting from this protocol however, downstream application is limited to assays which do not require a large amount of cellular input such as RNA sequencing or single-cell RNA sequencing, precluding use in protein-based assays which may require more cellular material.

Single-cell sequencing

One difficulty that we have personally encountered when using this protocol for single cell sequencing is achieving a high enough concentration of cells required for library preparation. Library preparation for single cell sequencing requires an optimal range of cell concentration per μL. For example, the most current 10x genomics platform requires a cellular concentration of between 700 and 1200 cells per μL. While our protocol generates sufficient numbers of microglia to achieve this concentration, initial suspension preparations resulted in an insufficient number of live cells when assessed by trypan blue staining. We found that many cells were being lost following the centrifugation step after sorting on the flow cytometer. We addressed this issue by using Eppendorf protein lo-bind tubes which have a coating to prevent adhesion of proteins and cells to the insides of the tubes. This remedied our issue of cells lysing following resuspension of centrifugation after sorting, likely because previously microglia may have been sticking to the walls of the cell tube and ripping apart when re-suspended. Therefore, when using this protocol with the intention of performing single-cell sequencing, we recommend using Eppendorf lo-bind tubes as the receiver tube for cell sorting. For RNA sequencing and qPCR, cells can be pelleted at high centrifugal force and snap frozen, reducing the concern of maintaining cell viability after sorting.

Isolation of cell types besides microglia

While Dounce homogenization works well for microglia under 4°C conditions, this homogenization technique has limitations when applied to other CNS cell types. The major drawback to this mechanical dissociation protocol is that the Dounce homogenization process results in the loss of other CNS cell types. The exact reason for this is unknown, but it may be due to differences in myeloid cells to withstand aggressive sheer forces and maintain membrane viability under mechanical homogenization conditions. Therefore, we do not believe that this protocol should be used when profiling all cell types of the CNS as it may result in selection bias for myeloid cells, and in loss of other cell types of interest.

Ly6C expression in microglia

While we have found that this protocol works well with any mouse age from neonates to aged mice, we have found that the Ly6C expression profile can change in microglia with age. This can pose an

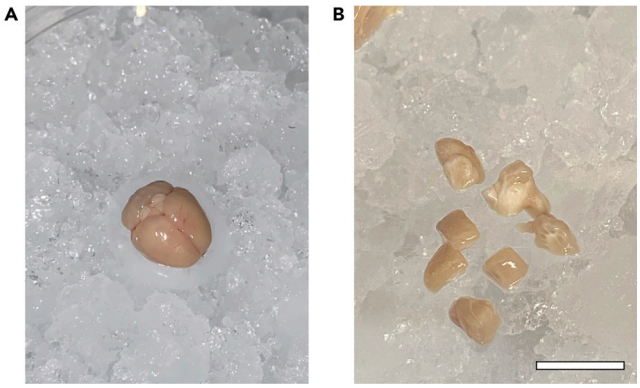

Figure 7. Representative image of properly perfused mouse brain and dissection in preparation for homogenization
(A) Image of properly perused brain removed from mouse carcass.
(B) Representative image of brain segmented into smaller pieces for more efficient homogenization. Scale bar=1 cm.

issue when using a previously saved and optimized flow cytometer gating protocol for the isolation of microglia (which is common) with mouse ages different than the age of the microglia used for the initial protocol set up. At younger ages, the Ly6C expression tends to be higher than that in older microglia. The biological reason for this is not explored in this protocol, however it is our recommendation that when profiling age points which has not been profiled before, a fluorescence-minus-one (FMO) sample, which contains cells from multiple animals and is stained for every marker in the panel except one, is brought to the flow cytometer to set up the appropriate gating strategy. FMO samples are common in setting up FACS sorting protocols, and more details of this technique can be supplied by FACS guides or consulting FACS technicians. They are used to set the upper boundary for background signal on the omitted label, and thus to identify and gate positive populations in multicolor experiments. For example, to generate an FMO sample to set up an appropriate $Ly6C^{-}$ gate, create a separate tube which contains cells from multiple animals (to avoid confounds from selection bias of a single animal) stained with LIVE/DEAD and CD11b only. Around 10 μL from the 300 μL resuspension from step 20 from each sample should be a sufficient number of cells to set up the correct gate. Run this sample on the flow cytometer and set the gate threshold at the cusp of the $Ly6C^{-}$ cells, which should be all cells since the conjugated antibody was excluded from the stain for this tube.

Additionally, there is evidence that Ly6C expression may be augmented in microglia in certain disease models (Getts, et al., 2008). When designing the antibody panel to be used to collect microglia from a novel disease model, we encourage researchers to incorporate one or more microglia specific markers which can be used to rule out unexpected expression levels of Ly6C (discussed further in "Exclusion of microglia specific flow cytometry marker" section of "Limitations").

Limited applicability for cell culture applications

In our experience, this protocol can be used to collect microglia for cell culture purposes, but the extended incubation period at 4°C and stressful flow cytometry sorting procedure results in reduced capacity of cells to re-adjust to cell culture conditions and thrive. Therefore, this protocol is not recommended for cell culture, but is better suited for applications where the cells can be kept at 4°C until the point of lysis, such as qPCR, RNA-seq and Single-Cell RNA-seq.

Exclusion of microglia specific flow cytometry marker

Given that the conjugated FCRLS antibody used in this protocol was produced in-house by the Butovsky laboratory and is not commercially available, it is not included as a component of this protocol and is only referenced to highlight the purity of the population isolated using only $CD11b^{High}/Ly6C^{Low}$ cells from mice in our experience. Based on gene signature studies examining

cells isolated through this antibody panel and validated commercial kits and protocols which rely solely upon CD11b selection (Bordt, et al., 2020), we expect that sufficient purity can be achieved with CD11b selection alone but include Ly6C as a method of further enhancing purity. However, the authors recommend the use of a microglia specific antibody to validate the purity of the $CD11b^{High}/Ly6C^{Low}$ population in novel age or disease model mice to ensure microglial purity, particularly in disease models where there is a possibility of peripheral macrophage infiltration into the brain parenchyma. There are several commercially available microglia specific antibodies suitable for flow cytometry including CX3CR1, P2RY12 and TMEM119 which are listed in the materials table of this protocol that may be useful for this purpose.

TROUBLESHOOTING

Problem 1

Low cell yield and/or less than 80% of cells are LIVE/DEAD negative.

Potential solution

The most common cause of low yield using this protocol is due to incorrect homogenization of brain tissue. The homogenization step is the most critical step for ensuring a robust number of live microglia for downstream collection. A gentle, slow homogenization and resuspension are critical to reduce the number of lysed cells resulting from mechanical dissociation. If you are experiencing low cells numbers (i.e., <20,000 cells) or you are observing less than 80% of your cells are considered alive by the LIVE/DEAD staining gate, try one or all the following troubleshooting points below:

- (Before you begin) Ensure that the tubes into which microglia are collected after the Percoll gradient isolation step are blocked properly. Cell loss can result from myeloid cells sticking to the walls of the Falcon tubes.
- (Step 1) Ensure that the brain is chopped into at least 8 smaller pieced before beginning homogenization. It may be helpful to chop the brain into even smaller pieces, potentially close to mincing the tissue, although we have had success with 6–8 equally sized chunks to begin the homogenization. For an example of the brain chopping that works well for our experiments, please consult Figure 7.
- (Steps 3–9) Apply less pressure and move the pestle even more slowly than in previous trails during the Dounce homogenization step. The video illustrating a proper homogenization technique can be found in Methods Videos S2 and S3. If you believe that you are replicating this technique accurately, homogenize even more gently than the example in the video.
- (Steps 3–9) Ensure that the homogenization is done with the glass Dounce homogenizer somewhat embedded in wet ice to keep to solution inside cool.
- (Steps 13, 19, 25) Perform the resuspension steps downstream of the homogenization more gently. If problems persist, cut 3–5 mm off the tip of the P1000 tip used for resuspension to reduce shear forces generated by a smaller opening.

Problem 2

No visible Percoll interphase layer.

Potential solution

A lack of a visible, opaque white layer of cells following the Percoll centrifugation usually indicates that the homogenization was too aggressive, and that too many cells were lysed and not separated based on density in the Percoll gradient. To troubleshoot this issue, please refer to Problem 1 above. Despite a non-robust interphase layer, often there are still cells within the layer, but simply a lower amount. Depending on the downstream application, enough cells can still be acquired from the layer, but great care will need to be taken to ensure that the correct layer is taken. Collecting cells from the right location in the gradient without a robust layer will require careful identification of the interphase between the two different Percoll solutions. This can be visualized by holding the tube directly under a light source, and you should be able to see a glassy layer which represents the

top of the 70% Percoll layer. The myeloid cells should be sitting directly on top of this layer. Please refer to Figure 4 and Method Videos S5 and S6 for identification of the layer within the tube and proper vacuuming technique. Collect 2mL form the 37% gradient from this area, taking care to not disturb/collect the 70% layer. Another potential issue is that during the layering step the two Percoll solutions mixed. To troubleshoot this, please refer to the possible solutions below:

(Before you begin, step 14, 15) Ensure that the Percoll and Percoll gradient centrifugation step occur at room temperature. This is a necessary deviation from 4°C, as the lower temperature affects the Percoll viscosity and can interfere with proper interphase formation. Ensure that the Percoll solutions are made at room temperature and that the Percoll gradient centrifugation occurs at 22°C.

(Step 14) Ensure that the first 1–2 mL of the 37% layer are applied very slowly, at a rate of 1mL per 10 s. Additionally, tilt the tube to a 45° angle and apply the first 2mL of the 37% Percoll layer this way, which will avoid direct dropping into the 70% layer and prevent mixing.

(Step 15) If the acceleration is not set to a lower setting (3/9 on our centrifuge) the rapid acceleration can disturb the Percoll layers and cause them to mix. Ensure that the acceleration is set to less than or equal to 33% of the maximum acceleration.

(Step 15) Similarly, if the deceleration is too fast, the Percoll layer will be disturbed. Ensure that the brake is set to no more than 10% of the maximum. No brake can be used, but this can result in excessively long spin down times.

Problem 3

Large pellet of red blood cells.

Potential solution

(Step 1) Following the Percoll centrifugation you may observe a red pellet at the bottom of the 70% layer. These are erythrocytes and are indicative of a poor perfusion. Many of these cells will be separated from microglia during the Percoll gradient step, but other peripheral myeloid cells will be contained in the interphase layer. These should be sorted out based on Ly6C positivity, however, many peripheral myeloid cells could result in contamination of your microglia sample. Following perfusion, the brain should appear pale pink with no visible blood vessels (Figure 7). If this is not the case, transcardial perfusion techniques should be examined for efficacy. Please refer to Method Video S1 for an example of efficient transcardial perfusion using a syringe.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tsuneya Ikezu, Ikezu.Tsuneya@mayo.edu.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101670.

ACKNOWLEDGMENTS

This work was supported in part by National Institute of Health (United States) F31 AG071106-01A1 and T32 GM008541-24 (S.H.), R01 AG066429, R01 AG072719, R01 AG067763, RF1 AG054199, and R01 AG054672 (T.I.). We would like to thank Oleg Butovsky laboratory for providing FCRLS antibody to use for the validation of this isolation protocol. In addition, we would like to thank the Flow Cytometry Core at Boston University School of Medicine for the cell sorting service.

AUTHOR CONTRIBUTIONS

S.H., J.D.C., and C.M. conceived the manuscript and conducted experiments. S.H. wrote the manuscript; J.C.D., C.M., and T.I. supervised the authors and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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