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TITLE:

Combined Mechanical and Enzymatic Dissociation of Mouse Brain Hippocampal Tissue

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SUMMARY:

This neural cell dissociation protocol is intended for samples with a low amount of starting material and yields a highly viable single-cell suspension for downstream analysis, with optional fixation and staining steps.

ABSTRACT:

This neural dissociation protocol (an adaptation of the protocol accompanying a commercial adult brain dissociation kit) optimizes tissue processing in preparation for detailed downstream analysis such as flow cytometry or single-cell sequencing. Neural dissociation can be conducted via mechanical dissociation (such as using filters, chopping techniques, or pipette trituration), enzymatic digestion, or a combination thereof. The delicate nature of neuronal cells can complicate efforts to obtain the highly viable, true single-cell suspension with minimal cellular debris that is required for single-cell analysis. The data demonstrate that this combination of automated mechanical dissociation and enzymatic digestion consistently yields a highly viable (>90%) single-cell suspension, overcoming the aforementioned difficulties. While a few of the steps require manual dexterity, these steps lessen sample handling and potential cell loss. This manuscript details each step of the process to equip other laboratories to successfully dissociate small quantities of neural tissue in preparation for downstream analysis.

INTRODUCTION:

The hippocampus was first described by a Bolognese anatomist, Giulio Cesare Aranzio, in the 1500's[1]. In naming this newfound structure, Aranzio was likely inspired by its uncanny resemblance to the seahorse of the genus *Hippocampus*[1]. The hippocampus is involved in stress responses but is widely known for its role in learning and memory. More specifically, the hippocampus is responsible for the encoding and retrieval of declarative and spatial memory[1].

The hippocampus, or hippocampus proper, is divided into the CA1 (cornu ammonis), CA2, and CA3 subfields[1]. Compared to the rest of the nervous system, the hippocampus has several unique defining characteristics, including its plasticity and potential for ongoing neurogenesis[2]. Neurogenesis is the process of the proliferation and differentiation of neural stem cells, followed by their integration into the pre-existing neuronal network. Neurogenesis is restricted to the subgranular zone of the dentate gyrus and subventricular zone of the lateral ventricles (and the olfactory bulbs)[3]. While neurogenesis is abundant in embryogenesis, it is a lifelong process[3,4]. As such, this discussion will focus on adult neurogenesis in the hippocampus.

The subventricular and subgranular zones are neurogenic niches containing ependymal and vascular cells, as well as immature and mature lineages of neural stem cells[5]. Microglia contribute to these niches as immune cells to regulate neurogenesis[6]. Neural progenitor cells are nonstem cell progenies of neural stem cells[7]. Three types of neural progenitors are present in the subventricular zone: radial glia-like type B cells, type C transit-amplifying progenitors, and type A neuroblasts[3,8]. The slowly dividing type B neural progenitor cells in the subventricular zone can differentiate into rapidly dividing type C cells[8]. Subsequently, type C cells differentiate into type A cells[8]. These neuroblasts migrate through the rostral migratory stream to the olfactory bulb before differentiating into interneurons or oligodendrocytes[9]. These olfactory bulb interneurons are key to olfactory short-term memory, and associative learning, whereas the oligodendrocytes myelinate axons of the corpus callosum[9]. The majority of adult neurogenesis occurs in the subgranular zone of the dentate gyrus, where radial type 1 and nonradial type 2 neural progenitors are found[3]. Most neural progenitor cells are destined to become dentate granule neurons and astrocytes[10]. Connected by gap junctions, astrocytes form networks to modulate plasticity, synaptic activity, and neuronal excitability[5]. As the primary excitatory neuron of the dentate gyrus, granule cells provide input from the entorhinal cortex to the CA3 region[11].

Neural stem cell populations can be isolated using immunomagnetic or immunofluorescent isolation strategies[12,13]. Neural tissue is particularly difficult to dissociate; efforts to do so often result in samples with poor cell viability and/ or fail to produce the necessary single-cell suspension for downstream analysis. Neural dissociation can be conducted via mechanical dissociation (such as using filters, chopping techniques, or pipette trituration), enzymatic digestion, or a combination of techniques[14,15]. In a study evaluating neural dissociation methods, the viability and quality of manual mechanical dissociation by pipette trituration versus combinations of pipette trituration and digestion with various enzymes were compared[15]. Quality was graded based on the amount of cell clumps and DNA or subcellular debris in the prepared suspension[15]. Suspensions of glial tumors subjected to manual mechanical dissociation alone had significantly lower cell viability than treatments with dispase or a combination of DNase, collagenase, and hyaluronidase[15]. Volovitz et al. acknowledged the variation in viability and

quality between the different methods and emphasized that inadequate dissociation may reduce the accuracy of downstream analysis[15].

In a separate study, the authors compared over 60 different methods and combinations of dissociation of cultured neuronal cells[14]. These methods included eight different variations of manual mechanical dissociation by pipette trituration, a comparison of incubation with five individual enzymes at three different intervals, and various combinations of mechanical dissociation with enzymatic digestion or the combination of two enzymes[14]. None of the mechanical methods yielded a single-cell suspension[14]. Four of the single enzyme treatments, ten of the combination enzymatic treatments, and four of the combinations of mechanical dissociation with enzymatic digestion yielded a single-cell suspension[14]. Enzymatic digestion with TrypLE followed by Trypsin-EDTA most effectively dissociated samples[14]. Incidentally, samples treated with TrypLE and/or Trypsin-EDTA tended to form gelatinous clumps[14]. While this study was performed on cultured cells, it speaks to the shortcomings of pipette trituration or enzymatic digestion alone.

Side-by-side comparisons of manual versus automated mechanical dissociation are lacking. However, one group ran flow cytometry to compare manual and semi-automated mechanical dissociation of whole mouse brains in conjunction with commercial papain or trypsin enzymatic dissociation kits[16]. Processing with the dissociator more consistently yielded viable cells[16]. Following dissociation, the authors also isolated Prominin-1 cells, neuronal precursor cells, and microglia[16]. For two of the three isolated cell populations, the purity of the isolated cells was slightly higher when samples were processed with the dissociator, as compared to manually[16]. Reiß et al. noted that person-to-person variability in pipetting technique hinders reproducibility of viable cell population yield in tissue dissociation[16]. The authors concluded that automated mechanical dissociation standardizes sample processing[16].

The method of dissociation outlined in this manuscript is a combination of fully automated mechanical dissociation and enzymatic digestion, using solutions accompanying a commercial adult brain dissociation kit[17]. Unlike standard protocols, this optimized protocol reduces sample manipulation, yields a highly viable single-cell suspension, and is intended for processing minimal amounts of starting tissue.

PROTOCOL:

Experiments were conducted in accordance with the ethical standards approved by the Institutional Animal Care and Use Committee at UAMS. 6-month-old female C57Bl6/J wild-type mice were purchased and group-housed (4 mice per cage) under a constant 12 h light/dark cycle.

1. Preparation of reagents

1.1. Prepare fixable live/dead stain stock solution. Reconstitute the fluorescent stain with 20 µL of dimethyl sulfoxide (DMSO).

1.2. Wrap the vial in foil, label it as “Reconstituted”, and store it at -20 °C for up to six months.

1.3. Prepare a 0.9% saline solution with heparin. Dilute the contents of one vial of heparin sodium (10,000 USP units per 10 mL) in 1 L of double-distilled water (ddH_2O).

1.4. Prepare enough for approximately 45 mL per animal and store at 4 °C for up to one week.

1.5. Make 1% paraformaldehyde (PFA).

1.5.1. In a fume hood, heat a hot plate to 50 °C. In a Microwave, heat 100 mL of ddH_2O in a glass beaker to approximately 60 °C. Add a magnetic stir bar and transfer to the hot plate.

1.5.2. In the fume hood, weigh out 1 g of PFA and add to the beaker of ddH_2O. Add 0.1125 g of NaOH crystals and mix until dissolved (5–10 min).

1.5.3. Add 0.4 g of $NaPO_{4-}$ monobasic and mix until dissolved (2–5 min). Vacuum filter the solution and adjust pH to 7.4 with HCl and NaOH.

1.5.4. Cool on ice or at 4°C for 30 min before storing.

NOTE: Aliquots of 1.5 mL can be stored at -20 °C for one year. Avoid freeze-thaw cycles. If, after thawing, the solution becomes cloudy or a precipitate has formed, the solution should not be used.

CAUTION: Toxic, flammable. Always work with PFA under a ventilated hood wearing proper personal protective equipment.

1.6. Resuspend lyophilized Enzyme A with 1 mL of Buffer A. Do not vortex the solution.

NOTE: Enzyme A and Buffer A are reagents in the commercial Adult Brain Dissociation Kit [17].

1.7. Divide Enzyme P into aliquots of 50 µL and resuspend Enzyme A into 10 µL aliquots. Per kit instructions, store at -20 °C for up to six months. Avoid freeze-thaw cycles.

2. Day of experiment

2.1. Cool the tabletop centrifuge to 4 °C.

2.2. Place aliquot(s) of PFA in the fridge for gradual thawing.

2.3. Place the reconstituted live/dead stain in the dark (e.g., a drawer) to thaw at room temperature.

2.4. Prepare the bovine serum albumin (BSA) Buffer. Add 0.5 g of BSA to 100 mL of 1x Dulbecco's phosphate-buffered solution without calcium and magnesium (D-PBS), pH 7.2.

2.5. Add a stir bar and mix on a stir plate for 30 min. Transfer to 50 mL conical tubes and store at 4 °C.

NOTE: Always use freshly prepared BSA buffer.

2.6. Prepare live/dead stain working dilution. Add 1 µL of the reconstituted live/dead stain stock solution to 360 µL D-PBS and store it in the dark (e.g., a drawer or box) at room temperature. Prepare 50 µL of the working dilution per sample.

3. Perfusion

3.1. Place the saline solution with heparin on ice.

3.2. Turn on oxygen, set the flowmeter indicator ball on the small animal anesthesia vaporizer system to 1 L/min. Ensure there is adequate oxygen pressure and isoflurane.

3.3. Adjust the vaporizer dial to 3.5% (for induction and maintenance).

3.4. Prime the perfusion pump lines with the saline/heparin solution. Set the speed to 6 mL/min.

3.5. Place the mouse in the induction chamber, turn on the breather, and wait several minutes until the mouse is unresponsive. Confirm sufficient depth of anesthesia through the absence of pedal withdrawal to noxious pinch.

3.6. Place the mouse on its back on the dissection tray with its nose in the nose cone. Pin all four paws to the tray.

3.7. Spray the animal's abdomen with 20% ethanol.

3.8. Using forceps, pinch the lower abdomen and lift the skin. Use scissors to cut through fur and skin to the bottom of the ribcage.

3.9. Make two diagonal incisions from below the ribcage toward each shoulder.

3.10. Carefully resect the diaphragm (avoiding the lungs and heart). Resect the ribcage to expose the heart.

3.11. Carefully sever any connective tissue around the heart.

NOTE: Steps 3.10–3.11 are critical; perform with proficiency and dexterity.

3.12. Use the scissors to clip the right atrium (dark lobe on the upper left of the heart). Turn off the flow of isoflurane to the breather.

3.13. Hold the heart steady with forceps. With the bevel of the butterfly needle facing up, pierce the left ventricle while keeping the needle level and parallel to the animal.

3.14. Hold the needle in place, turn on the pump, and perfuse at least 30 mL of the saline/heparin solution until the fluid leaving the heart is opaque and the liver and lungs pale in color.

NOTE: Steps 3.13–3.14 are critical; perform with proficiency and dexterity.

3.15. Turn off the pump, remove the needle, and transfer the mouse to the dissection area.

4. Dissection

4.1. Using large surgical scissors, decapitate the head.

4.2. Cut the fur from the back of the head up to the eyes. Peel the skin back to expose the skull.

4.3. Clip the skull between the eyes. Make two cuts at the back of the skull, at the 10 and 2 o'clock positions, then make one long cut (keep tips up to avoid damaging the brain) along the midsagittal line of the skull to the original cut between the eyes.

4.4. Use forceps to peel the two halves of the skull away to the sides. Use a spatula to remove the brain and place it into a 60 mm glass Petri dish on ice filled with cold D-PBS (**Figure 1**).

4.5. Use a scalpel or razor to separate each hemisphere. Then remove the olfactory bulbs and cerebellum.

4.6. Use forceps to remove the midbrain until the hippocampus is exposed.

4.7. Secure the brain with forceps. Using a second set of forceps, gently tease the hippocampus out of each hemisphere, and transfer both hippocampi to a labeled 1.5 mL tube containing cold D-PBS.

4.8. Place the sample tube containing the two hippocampi from the mouse on ice.

5. Prepare Enzyme Mix 1 and 2 for each sample

NOTE: For volumes greater than 2 mL, use a 10 mL serologic pipette; for volumes, 200 µL–2 mL, use a 1000 µL pipette; for volumes, 21–199 µL, use a 200 µL pipette; for volumes, 2–20 µL, use a 20 µL pipette; for volumes under 2 µL, use a 0–2 µL pipette.

5.1. For each sample, thaw one aliquot each of Enzyme P and Enzyme A at room temperature.

5.2. For Enzyme mix 1, combine 50 µL of Enzyme P and 1900 µL of Buffer Z in a labeled C Tube (**Table of Materials**).

5.3. For Enzyme mix 2, add 20 µL of Buffer Y to the thawed 10 µL aliquot of Enzyme A.

6. Adult brain dissociation protocol[17]

NOTE: When working with samples, tubes should be placed in a tube rack at room temperature while BSA and D-PBS remain on ice unless otherwise noted.

6.1. Switch on the dissociator.

6.2. Use forceps to transfer the hippocampi tissue pieces to the C Tube.

6.3. Transfer 30 µL of Enzyme mix 2 into the C Tube. Twist the cap until tension is felt, then tighten until it clicks.

6.4. Place the C Tube upside down into a position of the dissociator; the sample will be assigned the **Selected** status (**Figure 2**). Secure the heater over the C Tube.

6.5. Press the folder icon, select **Favorites** folder, scroll to and select the **37C_ABDK_02** program. Click on **OK** to apply the program to all selected C tubes, then tap on **Start** (**Figure 2**).

6.6. Label one 50 mL conical tube per sample.

6.7. Place a 70 µm cell strainer on each 50 mL conical tube and wet with 2 mL of BSA buffer.

6.8. Upon completion of the program, remove the heater and the C tube from the dissociator.

6.9. Add 4 mL of BSA buffer to the sample and apply the mixture to the cell strainer on the 50 mL conical tube.

6.10. Add 10 mL of D-PBS to the C Tube, close it, and swirl the solution gently. Apply it to the cell strainer on the 50 mL conical tube.

6.11. Discard the cell strainer and the C Tube. Centrifuge the suspension at 300 x *g* for 10 min at 4 °C. Then, aspirate and discard the supernatant.

7. Debris removal

7.1. Resuspend the pellet with 1550 µL of cold D-PBS and transfer the suspension to a labeled 15 mL conical tube.

7.2. Add 450 µL of cold Debris Removal Solution (**Table of Materials**) and pipette up and down (do not vortex).

7.3. Gently overlay 1 mL of cold D-PBS on top of the cell suspension, keeping the tip against the wall of the conical tube. Repeat until the total overlay is 2 mL.

NOTE: This step is critical; perform with proficiency and dexterity.

7.4. Centrifuge at 3000 x *g* for 10 min at 4 °C with full acceleration and full brake.

NOTE: If the phases are not clearly separated, repeat steps 7.2–7.3. Centrifuge a final time at 1000 x *g* for 10 min at 4 °C.

7.5. The suspension should now consist of three distinct layers (**Figure 3**). Aspirate the topmost layer. Sweep the pipette tip back and forth to aspirate the white middle layer. Remove as much of the middle layer as possible without disturbing the bottommost layer.

NOTE: This step is critical; perform with proficiency and dexterity.

7.6. Add 2 mL of cold D-PBS and pipette up and down to mix.

7.7. Centrifuge at 1000 x *g* for 10 min at 4 °C with full acceleration and full brake. Aspirate and discard the supernatant. Resuspend the pellet in 1 mL of BSA buffer.

NOTE: Cells can be resuspended in the appropriate buffer then magnetically labeled and isolated in preparation for single-cell sequencing at this point.

8. Cell count

8.1. Perform cell counting as per the manufacturer's protocol of available cell counter (one option is noted in the **Table of Materials**)

9. Live/dead stain

9.1. Centrifuge the remaining 900 µL (from 7.7) at 1000 x *g* for 10 min at 4 °C with full acceleration and full brake.

9.2. While the sample is spinning, label one flow tube per sample and wrap it in foil to limit light exposure.

9.3. Aspirate and discard the supernatant.

9.4. Resuspend the pellet in 50 µL of diluted live/dead stain (previously prepared).

NOTE: This step should be performed in a low-light setting. Turn off overhead room lights to achieve this.

9.5. Transfer each sample to the corresponding labeled flow tube and incubate at room temperature for 8–10 min in the dark (e.g., a drawer or box).

9.6. Add 500 µL of BSA buffer and centrifuge at 1000 x *g* for 10 min at 4 °C with full acceleration and full brake.

9.7. Aspirate and discard the supernatant.

NOTE: The pellet may not be visible; leave a small amount of buffer behind so as not to unintentionally aspirate the pellet. Cells can be resuspended in the appropriate buffer, blocked, and stained with cell-specific antibodies at this point. See **Supplemental File 1** for sample protocol[18].

10. Fixation (optional)

10.1. Resuspend the pellet in 200 µL of 1% PFA (previously prepared). Incubate for 15 min at 4 °C.

10.2. Wash by adding 500 µL of D-PBS and centrifuge at 300 x *g* for 10 min at 4 °C.

10.3. Aspirate the supernatant.

NOTE: The pellet may not be visible; leave a small amount of buffer behind so as not to unintentionally aspirate the pellet.

10.4. Resuspend the pellet in 200 µL of D-PBS and store at 4 °C for up to 3 days.

11. Flow cytometry

11.1. Label the filter caps on the new tubes.

11.2. Using a 1 mL pipette, pipette each sample onto the filter cap.

11.3. Centrifuge briefly at 200 x *g* at 4 °C, only allowing the centrifuge to reach 200 x *g* before stopping the run.

11.4. Proceed to flow cytometry core for downstream analysis.

REPRESENTATIVE RESULTS:

Samples were processed with a flow cytometer at a core facility, and the resulting data were evaluated with a software package for flow analysis. Previously, compensation controls were analyzed—the live/dead stain and negative control. If multiple fluorochromes are used, fluorescence minus one (FMO) controls and single-stain controls should be prepared for each antibody. Compensation for spectral overlap for the experimental samples was calculated based on the analyzed controls. For cell population identification, a hierarchical gating strategy was used. The primary gate excluded debris in the forward scatter (cell size) versus side scatter (granularity) plot[19,20]. Subsequently, the dead cells were excluded (**Figure 4**, **Figure 5**, **Figure 6**, **Supplemental Figure 1**, **Supplemental Figure 2**, **Supplemental Figure 3,** and **Supplemental Figure 4**). The following gate excluded cells positive for Myelin Basic Protein (**Supplemental Figure 4**). Of the remaining cells, density plots of cells positive for each fluorochrome were created (**Supplemental Figure 4**). The frequency of each neuronal cell population was calculated out of the third gate (**Supplemental Figure 4**). Samples that were processed with manual mechanical dissociation[21] and enzymatic digestion yielded a substantially lower population of cells of interest (**Supplemental File 2**[21], **Figure 4**, and **Supplemental Figure 1**). Conversely, both fixed and fresh samples prepared via automated mechanical dissociation and enzymatic digestion returned a population of cells of interest several-fold larger (**Figure 5**, **Figure 6**, **Supplemental Figure 2**, and **Supplemental Figure 3**).

FIGURE AND TABLE LEGENDS:

Figure 1: Mouse brain. (**A**) Properly perfused. (**B**) Non-perfused.

Figure 2: Selected status in step 6.4.

Figure 3: Tri-layered suspension in step 7.5: Buffer (top layer), cell debris, debris removal solution, and cells (bottom layer).

Figure 4: Representative analysis of fixed samples processed using a combination of manual dissociation by Pasteur pipette trituration and enzymatic digestion. First of two samples processed simultaneously. (**A**) Percentage of cells that are the cellular population of interest. (**B**) Percentage of the population of interest that are live cells.

Figure 5: Representative analysis of fresh samples processed using a combination of automated mechanical dissociation and enzymatic digestion. First of two samples processed simultaneously. (**A**) Percentage of cells that are the cellular population of interest. (**B**) Percentage of the population of interest that are live cells.

Figure 6: Representative analysis of fixed samples processed using a combination of automated mechanical dissociation and enzymatic digestion. First of two samples processed simultaneously. (**A**) Percentage of cells that are the cellular population of interest. (**B**) Percentage of the population of interest that are live cells.

Supplemental Figure 1: Representative analysis of fixed samples processed using a combination of manual Pasteur pipette trituration dissociation and enzymatic digestion. Second of two samples processed simultaneously. (**A**) Percentage of cells that are the cellular population of interest. (**B**) Percentage of the population of interest that are live cells.

Supplemental Figure 2: Representative analysis of fresh samples processed using a combination of automated mechanical dissociation and enzymatic digestion. Second of two samples processed simultaneously. (**A**) Percentage of cells that are the cellular population of interest. (**B**) Percentage of the population of interest that are live cells.

Supplemental Figure 3: Representative analysis of fixed samples processed using a combination of automated mechanical dissociation and enzymatic digestion. Second of two samples processed simultaneously. (**A**) Percentage of cells that are the cellular population of interest. (**B**) Percentage of the population of interest that are live cells.

Supplemental Figure 4: Representative analysis of stained and fixed samples processed using a combination of automated mechanical dissociation and enzymatic digestion. (**A**) Percentage of cells that are the cellular population of interest. (**B**) Percentage of the population of interest that are live cells. (**C**). MBP^- Cells. (**D**). PSA-$NCAM^+$ cells (Neuronal Precursor Cells). (**E**). $ACSA2^+$ cells (Astrocytes). (**F**). $CD31^+$ cells (Endothelial). (**G**). $CD11b^+$ cells (Microglia).

Supplemental File 1: Staining protocol. A sample staining protocol for immunostaining of cell surface markers.

Supplemental File 2: Adapted manual mechanical and enzymatic dissociation protocol. This method is adapted from a previously published protocol[21].

DISCUSSION:

Several steps in this neural dissociation protocol require proficient technique and dexterity—perfusion, supernatant aspiration, and myelin removal. Throughout the perfusion process, the internal organs must remain intact (aside from removing the diaphragm and clipping the heart); this includes avoiding the upper chambers of the heart with the butterfly needle. While the amount of saline with heparin needed varies, transparent fluid flowing from the heart indicates the process is complete. The brain must be completely and properly perfused, at which point it will appear off-white (**Figure 1**). With perfusion, the red blood cell removal step becomes extraneous, eliminating excess manipulation of the samples that can result in cell loss. Subsequently, the debris removal step requires a steady hand. For the centrifugation to result in clearly defined layers following the D-PBS overlay (**Figure 3**), the layers must not be disturbed in transit or while pipetting. Additionally, when aspirating the top two layers, enough suspension must be removed to eliminate excessive cell debris while still leaving a large enough sample behind. This is a key step as dead cells are more likely to bind nonspecifically and become autofluorescent[22,23], further stressing the importance of choosing a method that consistently results in high cell viability. Finally, when aspirating the supernatant, the pellet may not always

be visible. A small amount of residual supernatant must be left to ensure the sample is not accidentally discarded.

There are advantages and disadvantages to fixing the samples. Not all antibody markers are compatible with fixation, limiting downstream analysis depending on the cell populations of interest. Also, using overly concentrated PFA or leaving the cells in the fixative for an extended period of time can result in autofluorescence and false-positive readings, thereby confounding the results[24,25]. By using a 1% PFA solution and minimizing the exposure of the cells, the likelihood of false-positive readings is greatly reduced. As this procedure is detailed and has many timing variables, using fresh cells places labs under strict time constraints to ensure the cells remain viable. Fixation preserves the cell structure for next-day analysis.

Single-cell analysis can provide key insights into treatment efficacy, cell function, and disease or treatment mechanisms of action. Example methods include single-cell DNA and RNA sequencing[26,27], cytometry by time-of-flight[22,28], flow cytometry, and immunohistochemistry. With single-cell mRNA sequencing, gene expression at the time of sample collection can provide cell-type-specific insights[26]. For instance, a research group performed single-cell RNA sequencing on D1 and D2 dopamine receptors expressing medium spiny neuron subtypes from dorsomedial striatum[27]. The group redefined the transcriptome of medium spiny neurons by detecting novel subtype-specific marker genes and identifying genes that were previously and incorrectly reported to be differentially expressed due to a lack of single-cell resolution[27]. Ho et al. highlighted the potential of single-cell RNA sequencing in discovering cell type-specific drug targets[27]. With single-cell DNA sequencing, changes in gene expression can be described by measuring DNA and histone modifications, chromatin accessibility, and chromatin conformation[26]. In measuring single nucleus DNA methylation, Liu et al. constructed a single-cell DNA methylome atlas of 45 mouse brain regions and identified 161 neuronal cell types[29]. Sample preparation for single-cell sequencing is more intricate, especially the isolation of single-cells and debris removal. Mattei et al. examined the effect of enzymatic and mechanical dissociation on transcriptomic and proteotype profiling, noting that neural dissociation methods inherently introduce a level of bias[30]. Several groups have noted the importance of working efficiently, dissecting on ice, and using transcriptional inhibitors[26,30,31]. Mattei et al. also identified affected genes and proteins to inform analysis[30]. However, these techniques still provide detailed insights into cellular building blocks that are unmatched by bulk-tissue transcriptomics[26,27].

Flow cytometry is a powerful analytic tool that can simultaneously identify and measure parameters of single-cell populations using fluorescent probes. Some applications of flow cytometers include cell cycle analysis, cell sorting, viability, phenotyping, cell proliferation, and functional analyses[32,33]. Most of these applications utilize surface staining due to the accessibility of cell surface proteins. These proteins can be stained to identify specific cell populations based on lineage, developmental stage, and function[19,32,33]. For example, samples can be surface stained to identify populations of astrocytes, endothelial cells, neuronal precursor cells, and microglia [34]. A primary advantage when staining surface proteins of live cells is being able to sort the cells while retaining the option to conduct further downstream analysis[34]. While surface staining techniques are fairly standard, intracellular staining is a more delicate procedure. With

intracellular flow cytometry, the cells must be fixed and permeabilized before staining to allow the antibody to cross the cell membrane[20,32–34]. Ideally, the cell morphology will remain intact; however, permeabilization risks protein denaturation, which would negatively impact antibody detection[22,34]. Some methods of further downstream analysis are no longer an option once the cells are fixed[20]. While the downsides to intracellular staining are more pronounced than surface staining, the former allows detection and analysis of intracellular molecules that would otherwise not be plausible. Additionally, cell surface and intracellular staining procedures may be coupled to definitively identify certain cell types or assess additional parameters simultaneously[20,28,34].

There are several methods of neural dissociation that can be used to prepare the single-cell suspension required for cellular analysis, though they are not equally effective. Compared to standardized kits and the aforementioned techniques, this particular method of neural dissociation is intended for processing small quantities of tissue, yields a highly viable single-cell suspension (>90%), and streamlines the experiment. With this protocol, other labs are equipped to perform neural dissociation in a reliable and reproducible manner.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

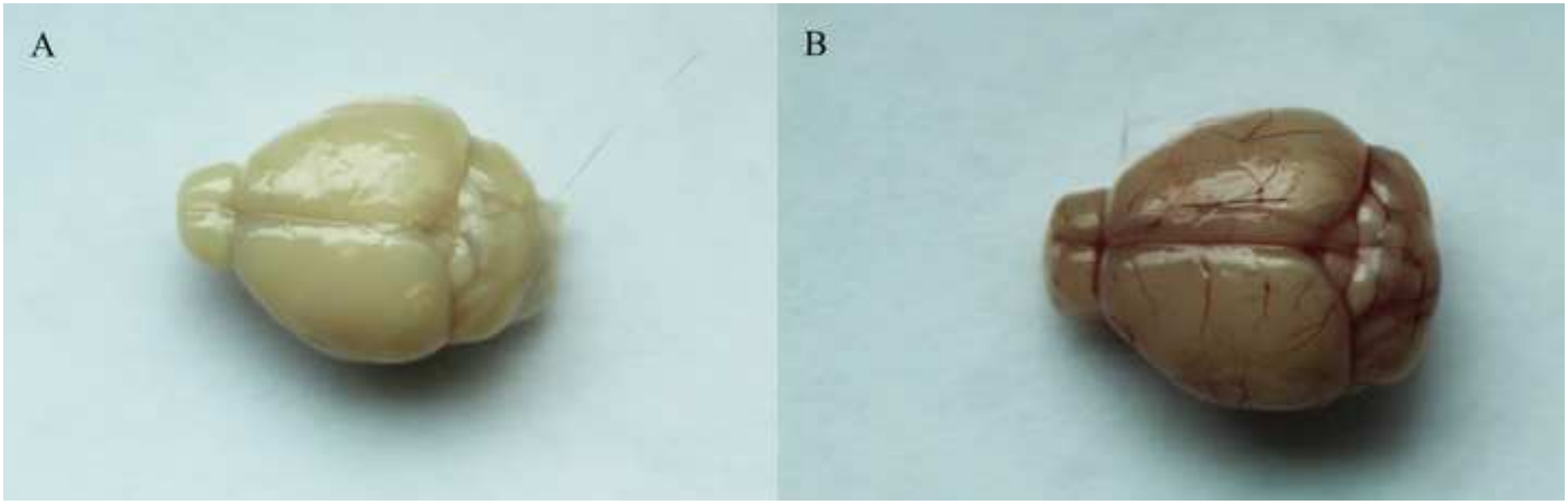

Figure 2

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Figure 3

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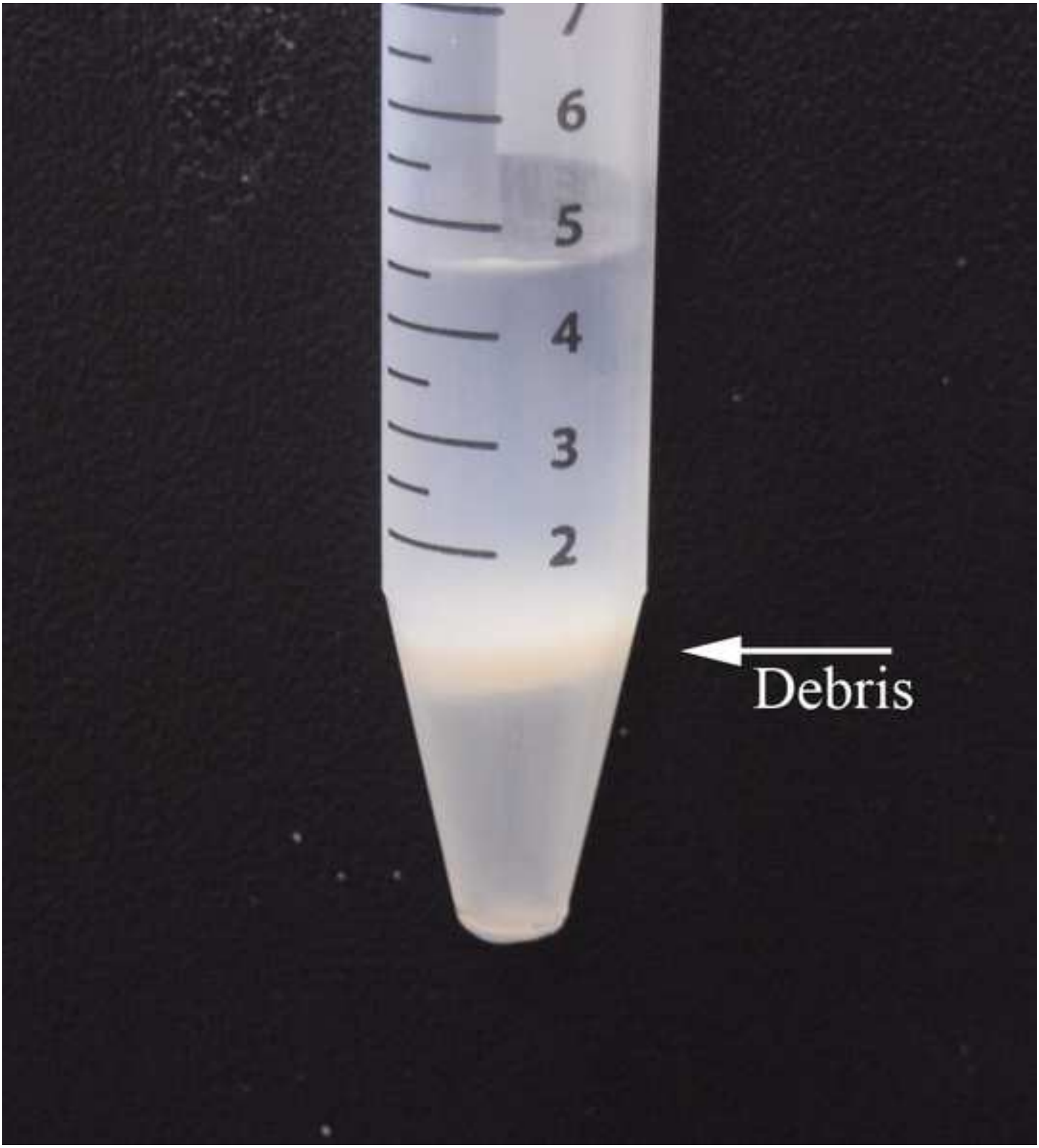

Figure 4

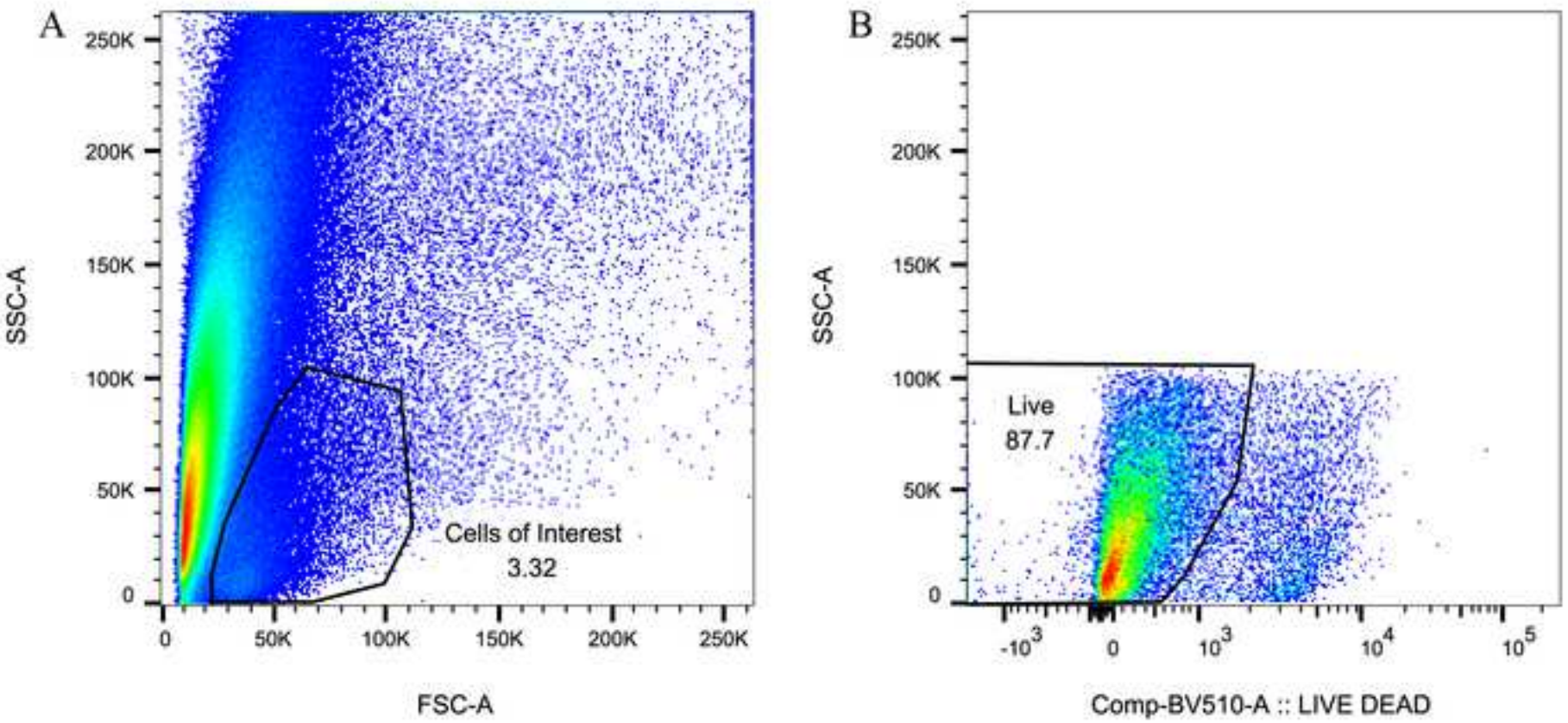

Figure 5

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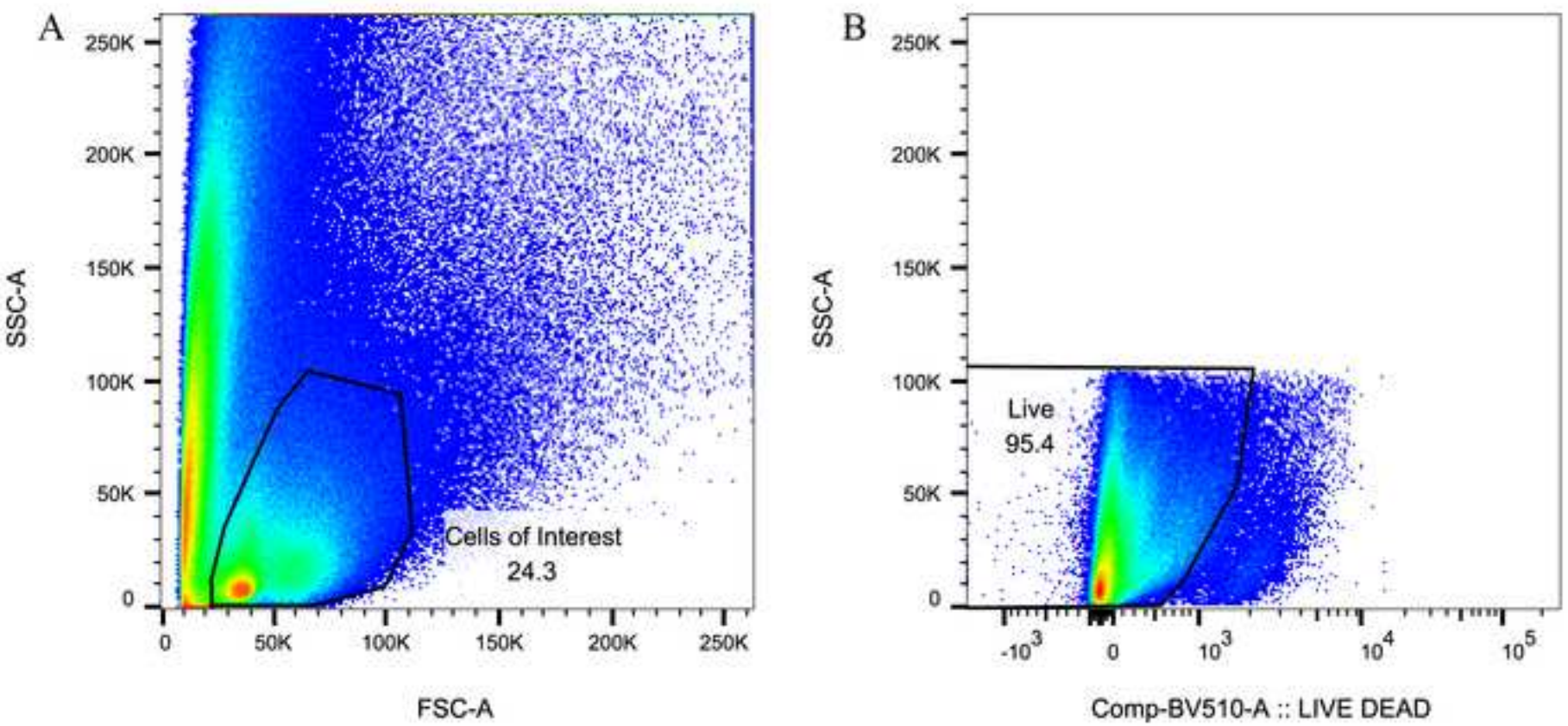

Figure 6

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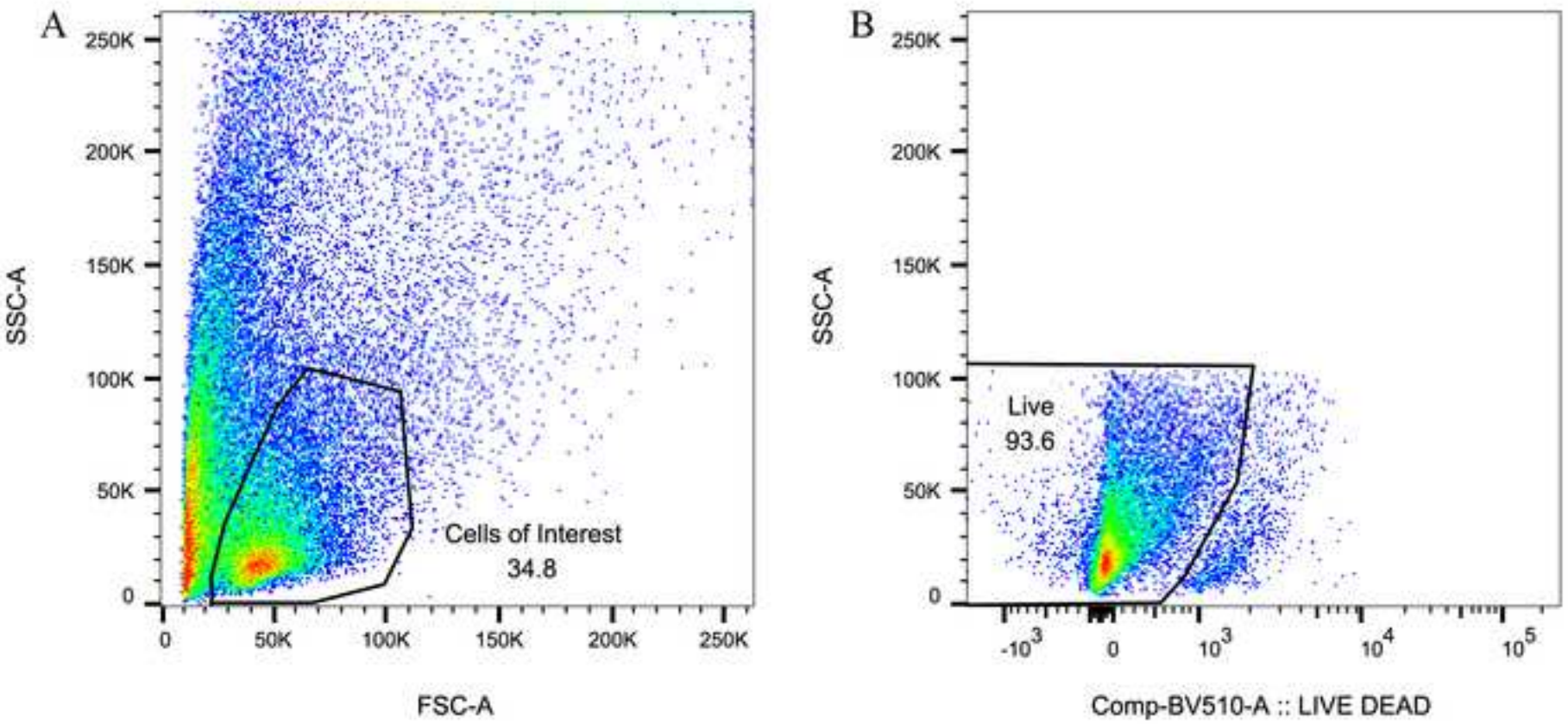

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23 August 2021

Prof. Ronald Myers
Editor-in-Chief, *Journal of Visualized Experiments*

Dear Dr. Myers:

Re: Combined Mechanical and Enzymatic Dissociation of Mouse Brain Hippocampal Tissue: ***Revised***

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please confirm whether the corresponding author is Antiño R. Allen as mentioned in the manuscript. The editorial manager shows it as Madison Trujillo.
The corresponding author is Antiño R. Allen.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Octo Dissociator, etc.
4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.
5. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks
6. Line 109-110: Please add these lines after the ethical clearance statement in line 101-102 and remove the section "Animals and study design".
This information has been moved as recommended.

7. Line 113-114: Please mention the live/dead stain used in this study (use generic terms).
The stain is now described as fixable and fluorescent.

8. Line163-165: Is the flow rate of isoflurane the same during induction and maintenance. Please specify.

A rate of 3.5% was used until step 3.13 and has now been noted as such.

9. Line 185-188: Please specify the flow rate used for perfusion.
A flow rate of 6 mL/min has been noted.

10. Line 232: Is the whole tissue transferred to the c-tube?
For one brain, there are two hippocampi—one from each hemisphere. The hippocampi in their entirety (whether they are whole or in pieces due to substandard dissection technique) should be transferred to the C tube. If two samples are pooled, then both sets of hippocampi should be transferred to the C tube. Please refer to step 4.7.

11. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
12. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
All of the figures are original and produced by our lab.

13. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
14. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.
15. Please remove ")" symbol from the figure labels of the Figures.
Figure legends have been amended.

16. Figure 4/5/6/ supplemental figures: Please specify what the numbers in the panel represent in the Figure Legends.
Figure legends have been amended.

<u>Reviewers' comments:</u>
Reviewer #1:

Manuscript Summary:
Here the authors describe a protocol for the dissection of the mouse hippocampus and subsequent preparation of single neuron suspensions. The protocol appears to mostly follow the manufacturer instructions for neuron dissociation from a commercial kit. It would be helpful to know if the authors have modified this in some way, but overall the protocol is well-described. My primary concern is that the representative results just show viable cells—these could easily be all microglia rather than neurons, as the protocol claims. It is important that the authors show some representative results that their protocol successfully captures neurons. For example, include some neuronal staining by flow cytometry. Below are some additional points to improve clarity.

Major Concerns:

1. The introduction seems a bit disjointed to me. It would be helpful if the authors better described how this method is different from previous similar methods. Authors discussed this some in the second half of the intro, but they should include more specific information about which enzymes/mechanical methods were tested, and how did this inform (or at least relate to) the current protocol?

In the introduction, further emphasis was placed on the challenges of neural dissociation and manual dissociation detailed. Additionally, a comparison of manual versus semi-automated mechanical dissociation was cited. What separates this method of dissociation from other methods is discussed at the end of the introduction as well as in the conclusion.

2. Section 6.5 - Authors should outline the criteria to replicate this program.

The 37C_ABDK_02 program is a 30-minute program in which the samples are processed at 37 °C. The company notes that for this program, there are 1140 rounds per run. Rounds per run is equivalent to the total number of rpm achieved in the programmed run. Further information regarding this program is considered proprietary.

3. Figures 4-6 - I'm not entirely sure what I am supposed to take away from these figures. It would be helpful if the authors cited them individually rather than lumping them all together like this.

The figure legends have been updated. Figure 4 shows analysis of fixed samples processed with manual dissociation by trituration and enzymatic digestion.

Figure 5 and 6 compare analysis of fixed versus fresh samples processed with fully automated mechanical dissociation and enzymatic digestion.

4. Representative results, Lines 364-366 "Samples that were processed… …of interest (Figure 4, Supplemental Figure 1)." - Without more context as to what manual mechanical dissociation and enzymatic protocol was used, this statement and associated figure aren't meaningful.

Samples that were processed manually were done so with Pasteur pipette trituration. This is now clarified in the figure legends and the manual protocol has been included as a supplemental file.

5. Discussion - Authors should discuss any limitations of their protocol. For instance, they discuss intracellular versus extracellular flow cytometry staining, but then show no actual staining. I assume this protocol strips the axons/dendrites during the dissociation process, could the authors comment on what kind of staining is possible with this protocol.

A sample staining protocol has been added in the supplemental materials. Representative data has been added as Supplemental Figure 4.

6. Authors should show some sort of staining to prove that their protocol isolates neurons.

A sample staining protocol has been added in the supplemental materials. Representative data has been added as Supplemental Figure 4.

Minor Concerns:

1. Section 2.1.2 - What does USP mean?

USP indicates that a product meets the United States Pharmacopeia standards and is commonly included on drug labels.

2. Section 2.2.1 -It would be helpful to clarify what kind of centrifuge is needed here—a microcentrifuge? One for 50 ml conical tubes? 15 ml?

A standard tabletop centrifuge with swinging buckets is used. This is now noted in section 2.1. Please refer to table of materials.

3. Section 3.1 - Some context is needed here. I think authors are referring to an isoflurane vaporizer anesthesia unit, but it is unclear.
More information was added to section 3.1 and the table of materials.

4. Section 3.3 - Is this referring to a perfusion pump?
Clarification was added to section 3.3.

5. Section 3.6 - Is 20% ethanol correct? 70% would be standard.
20% ethanol was used in this step instead of 70% ethanol. The ethanol was used to wet the fur to prevent shedding, not as a disinfectant.

6. Section 5.0 - It seems unnecessary to specify what size pipettor to use.
Previously, reviewers asked the authors to specify what size pipette was used, citing that tip diameter can affect cell integrity.

7. Section 8.5 - Is there something critical about counting cells in this manner? Could it be done using the more universal Trypan blue staining?

8. Figures 5 and 6 have the same figure legend.
Please refer to figure legends- Figure 5 is representative analysis of **fresh** samples whereas figure 6 is that of **fixed** samples.

9. Figure 3 - I'm having a hard time seeing what the authors are trying to demonstrate here. It would be helpful if authors added some sort of labels to this.
A label has been added to the figure denoting the debris layer and the figure legend has been updated.

10. Table of Materials - Authors should include information regarding surgery/dissection (ie, tools, perfusion pump).
Additional supplies were added to the table of materials.

Reviewer #2:
Comments:
The manuscript entitled "Combined Mechanical and Enzymatic Dissociation of Mouse Brain Hippocampal Tissue" by Madison Trujillo et al. described a detailed hippocampal tissue dissociation protocol by using the combination of automated mechanical dissociation and enzymatic digestion. This process will reduce technical variations between different samples compared to manual dislocation. We like to add few suggestions:

1. In step 4.0 dissection part, please mention that all the dissection procedures should be done on ice.
This is now clarified in step 4.4.

2. In step 6.5, the dissociation program which contains 37℃incubation would influence the cell's RNA transcription. Please mention transcriptional inhibitor options.
This is now addressed in the discussion.

3. In the step 2.1.1 and step 9.4, the stock and working concentration of live/ dead stain is not clear.
Changes were made to steps 1.1 and 3.5.

4. In the flow cytometry section, the example of antibody staining should be added to make the protocol more practical.
A sample staining protocol has been added in the supplemental materials.

Minor concerns：
1. In the cell count section, as most of the labs have a different counter or counting methods, the description of how to use the counter is redundant. you can refer user

manual.
2. In the description of Figures 2 and 3, which step it related to should be added in the content.
A note has been added to the figure legends as well as the steps in the protocol.

Reviewer #3:
Manuscript Summary:
The title of the manuscript is in full compliance with the stated protocol. This manuscript is devoted to the evaluation of the combined method of dissociation of the mouse hippocampus. The described protocol can be used in the presence of a very small amount of starting material, which is important under experimental conditions when studying fundamental issues. Interestingly, the authors show the effectiveness of the method under different conditions. All stages of the protocol are clearly described with the necessary details. It is indicated that this method can be effectively used both for further in vitro experiments and for the analysis of an immediately fixed cell suspension.

Major Concerns:
no

Minor Concerns:
63 - In the introduction, add information about the properties and capabilities of the neural progenitor cells and neural stem cells.
Descriptions and additional information about the various types of neural progenitor cells has been added.

110 - What is the reason that mice were planted four in a cage? Please indicate the size of the cage.
The cages are 7.62 x 5.13 x 15.01 inches. Standards of animal husbandry are outlined by the Animal Welfare Act. Up to five mice can be group housed. We housed four mice per cage, based on the size of the treatment groups in an effort to standardize conditions.

113 - What is the volume of the vial?
Vial of freeze-dried dye were purchased. We reconstitute the dye in the original vials, which can hold approximately 1 mL.

121 - Specify the volume of ddH2O.
Noted that a volume of 100 mL was used.

122 - Indicate the amount (wt) of PFA and the approximate time to complete dissolution.
Amounts of chemicals and approximate timing is now detailed in 1.3.

124 - Add: If after thawing the solution becomes cloudy or a precipitate has formed, then the solution should not be used.
This is now noted.

140 - "for gradual thawing".
This is now noted.

155 - Does this procedure take place in a special operating room or a general laboratory room?
The dissection and perfusion are conducted in surgical laboratory room or general laboratory room, both of which approved for animal use.

161 - How much is the pump filled with saline? Completely, half, a third, a quarter? What temperature should the solution be?
Step 3.1 was added to note that the solution should be on ice. In step 3.4, it is now

specified that the pump lines should be filled.

164 - Indicate the approximate time required for the mouse to not respond.
It is now indicated that the mouse will become nonresponsive within a few minutes.

170 - Why was 20% ethanol used, and not 50% or 70%, which is usually used in laboratories for external decontamination?
20% ethanol was used in this step instead of 70% ethanol. The ethanol was used to wet the fur to prevent shedding, not as a disinfectant.

188 - Not only the liver turns pale, but also the lungs.
Step 3.15 has been updated.

204 - Have you tried using HBSS (without Ca, Mg)?

205 - A scalpel can also be used to separate the cerebral hemispheres and separate the olfactory bulb and cerebellum.
Traditionally our lab uses a razor blade to separate the hemispheres but we have noted that a scalpel can be used as well.

232 - Should the pieces of the hippocampus or the whole hippocampus be transferred to tube C?
For one brain, there are two hippocampi- one from each hemisphere. The hippocampi in their entirety (whether they are whole or in pieces due to substandard dissection technique) should be transferred to the C tube. If two samples are pooled, then both sets of hippocampi should be transferred to the C tube. Refer to step 4.7.

245, 249 - What temperature should the BSA buffer be? (+4oC or RT).
Amended note under 6.0 Adult brain dissociation protocol.

252 - What temperature should the D-PBS be?
Amended note under 6.0 Adult brain dissociation protocol.

263 - "pipette up and down" = resuspend?
The sample was resuspended in the previous step. After debris removal solution is added, the sample is gently mixed by pipetting up and down.

318 - (e.g., a drawer or box).
This is now noted.

343 - Why is it possible to save up to 3 days?
As the cells are fixed, they can be processed at a later time. After three days, the samples begin to disintegrate or dry out.

370 - Figure 1: B) … or unperfused brain.
Changed to “non-perfused”.

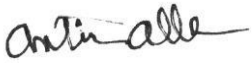
371 - Figure 2: Selected status in program.
The figure legend has been updated to “Selected status in step 6.4.”

372 - Figure 3: Layered cell suspension.
The figure legend has been updated to “Tri-layered suspension in step 7.5: Buffer (top layer), cell debris, debris removal solution and cells (bottom layer).”

374, 376, 378, 381, 383, 386 - Figure …: A) Cell Population of Interest.
The figure legends have been updated. If further changes are recommended, please specify what you are looking for.

We hope you and the reviewers will find this paper of interest and look forward to your comments. Thank you for considering this manuscript for publication in *Journal of Visualized Experiments*.

Sincerely,



Antiño R. Allen, Ph.D.
Associate Professor

Supplemental Figure

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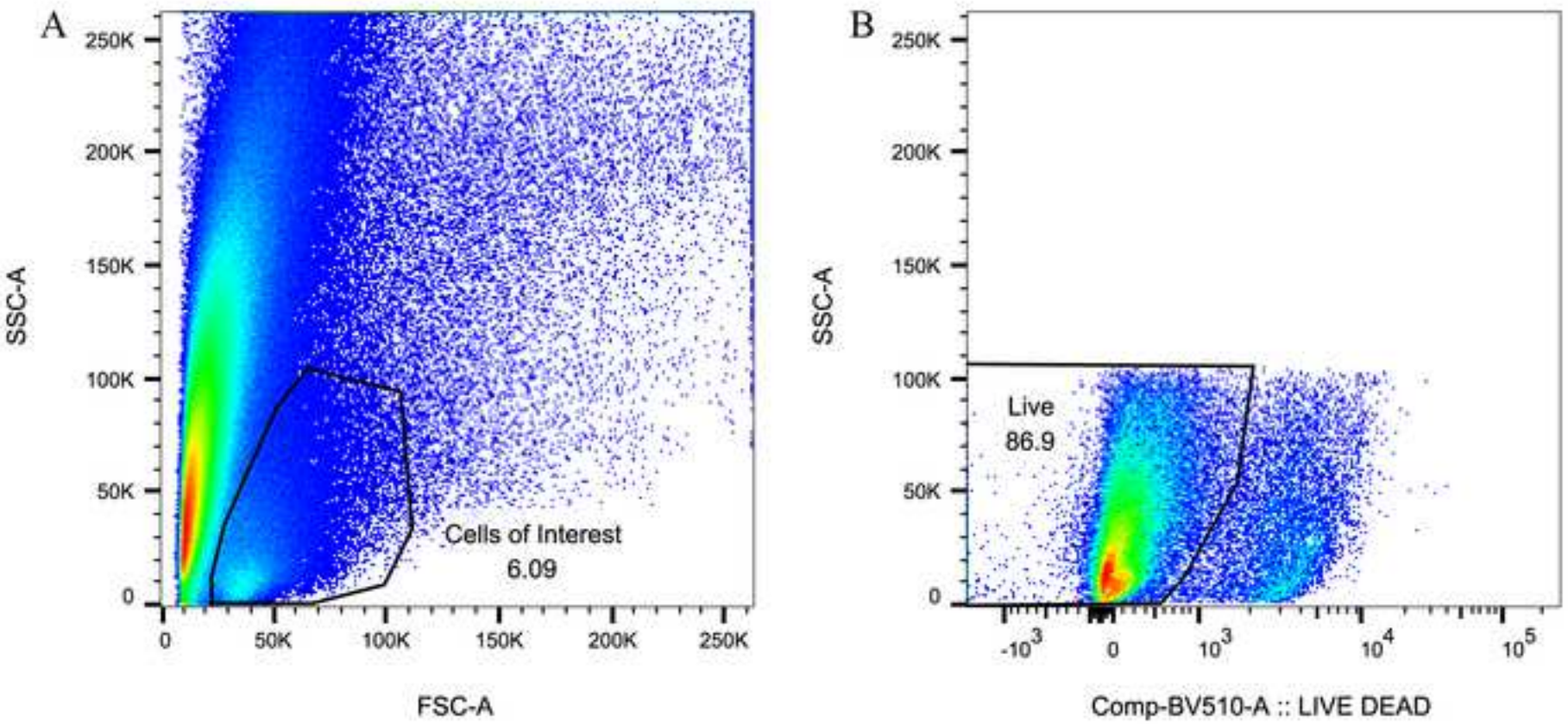

Supplemental Figure

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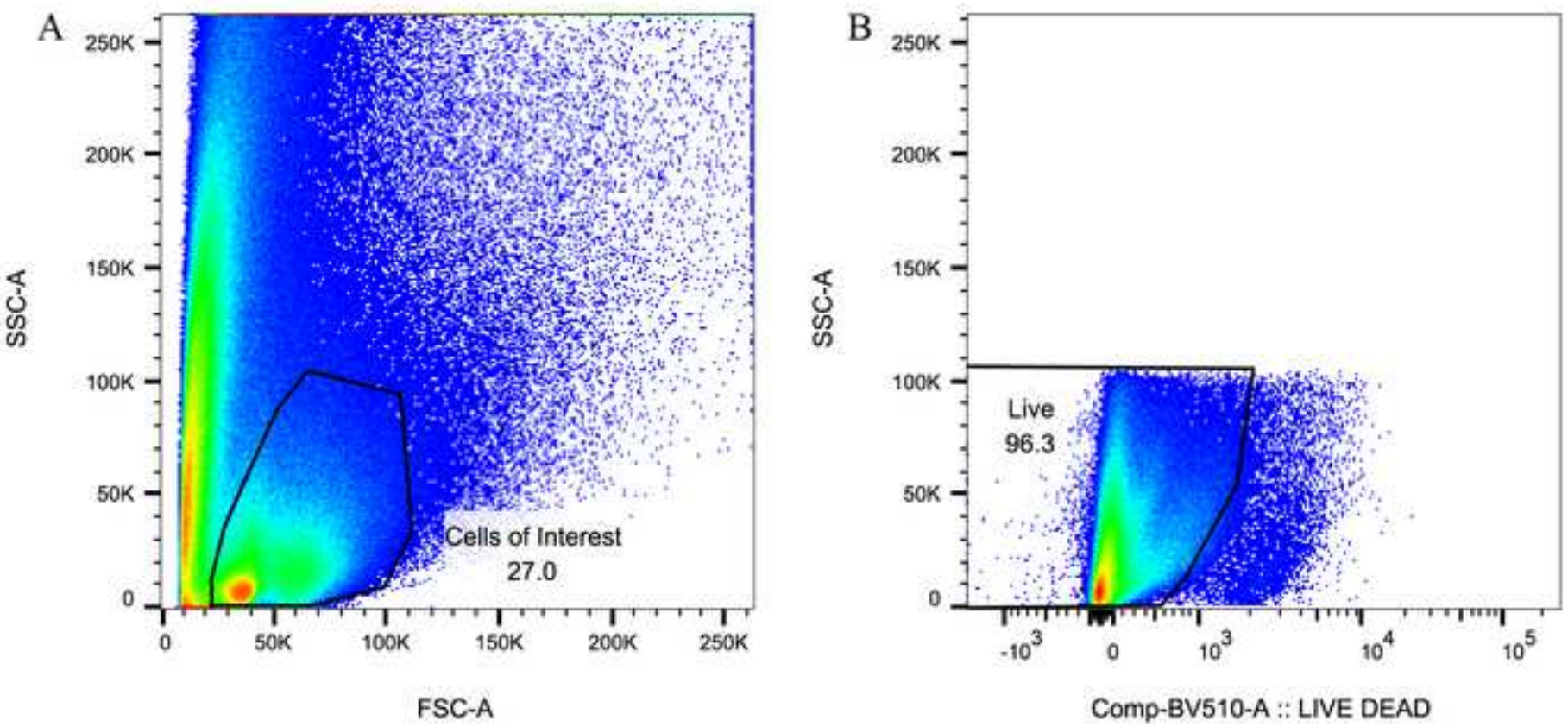

Supplemental Figure

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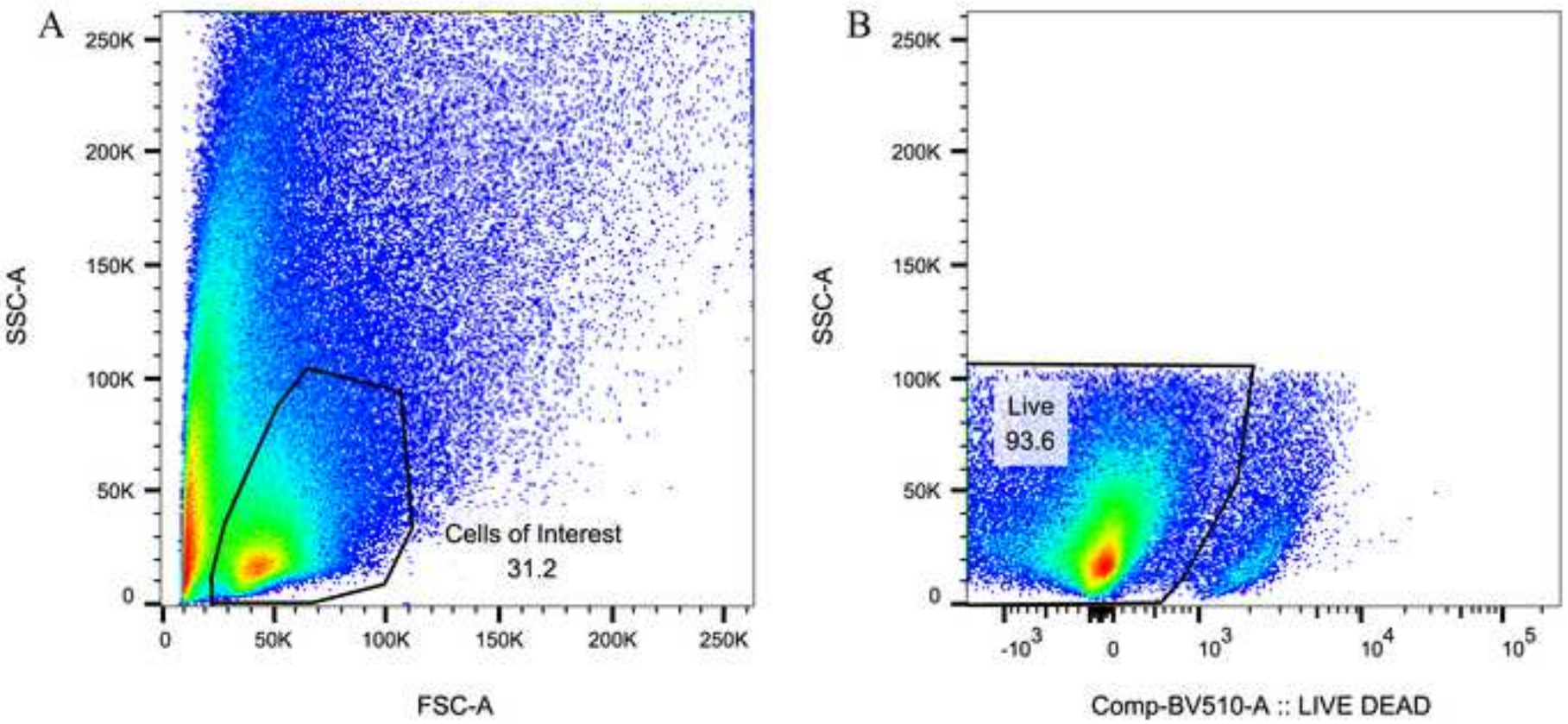

Supplemental Figure

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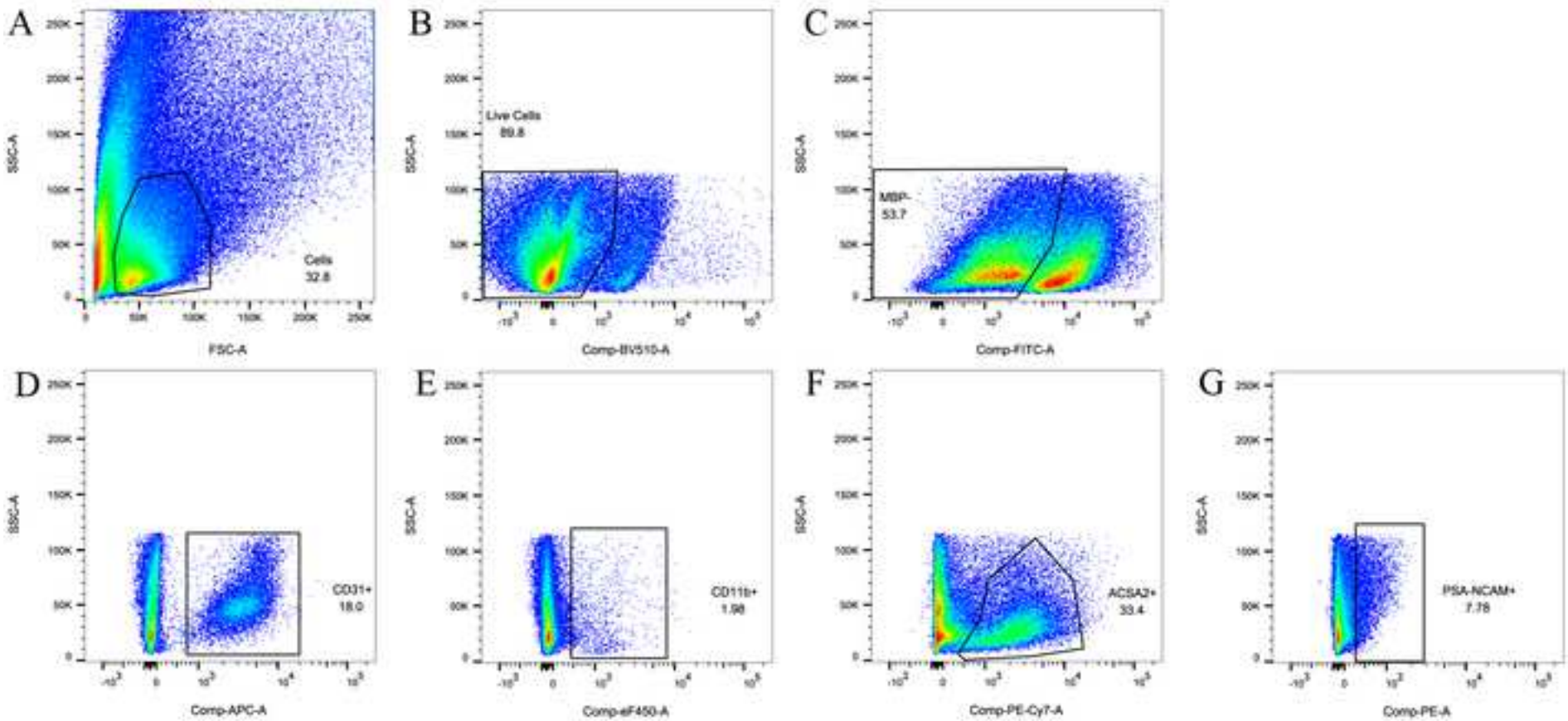

Protocol adapted from those accompanying commercial markers[18].

1.0 Blocking (continuing from 9.7)

1.1 Resuspend the pellet in 98 µL of BSA buffer.

1.2 Add 2 µL of FcR block.

1.3 Incubate for 30 min at 4 °C.

1.4 Add 1 mL of BSA buffer and centrifuge at 300 x *g* for 10 min at 4 °C.

1.5 Aspirate supernatant.

2.0 Antibody staining

2.1 Resuspend the pellet in 90 µL of BSA buffer.

2.2 Add 2 µL of FcR block.

2.3 Add 1 µL of each antibody (Anti-CD31-APC, CD11b-VioBlue, Anti-ACSA-2-PE-Vio770, Anti-PSA-NCAM-PE, Anti-MBP) and mix well by pipetting up and down after each addition.

2.4 Incubate for 10 min at 4 °C.

2.5 Add 1 mL of BSA buffer and centrifuge at 300 x *g* for 10 min at 4 °C.

2.6 Aspirate supernatant.

2.7 Resuspend the pellet in 98 µL of BSA buffer.

2.8 Add 2 µL of FcR block. Add 1 µL of FITC

2.9 Incubate for 10 min at 4 °C.

2.10 Add 1 mL of BSA buffer and centrifuge at 300 x *g* for 10 min at 4 °C.

2.11 Aspirate supernatant.

2.12 Proceed to 10.0 Fixation or 11.0 Flow Cytometry.

Biotec, M. *Cell surface flow cytometry staining protocol*, <https://www.miltenyibiotec.com/US-en/applications/all-protocols/cell-surface-flow-cytometry-staining-protocol-pbs-bsa-1-50.html> (2021).

NOTE: This method is adapted from the Schwarz video article published in Jove in May 2015[21].

1.0 Preparation for Tissue Collection (Continuing from **2.5 Day of Experiment**)

a. Fire polish 3 glass Pasteur pipettes in decreasing diameters
    i. 1 = 6.5 mm diameter
    ii. 2 = 5.5 mm diameter
b. Turn on water bath to 37 °C

2.0 Perform 3.0 Perfusion – 4.7 Tissue Dissection

3.0 **Prepare Enzyme Mix 1**

3.1 Prepare Enzyme Mix 1

3.1.1 Mix 50 µl of Enzyme P with 1,900 µl of Buffer Z for each sample

3.2 Place Enzyme Mix 1 in the water bath at 37 °C

4.0 Manual Dissociation

4.1 Use a 1 mL pipette tip to smash the tissue into as small of pieces as possible.

4.2 Using a microcentrifuge, spin the samples at 300 x *g* for 2 min at room temperature. Aspirate and discard supernatant.

4.3 Add 1,900 µl of warm Enzyme Mix 1 to each sample.

4.4 Incubate the samples for 15 min in the 37 °C water bath, inverting the tubes several times every 5 min.

4.5 Prepare Enzyme Mix 2 in a 200 µl tube.

4.5.1 Mix 20 µl of Buffer Y with 10 µl of thawed Enzyme A

4.6 Add 30 µl of Enzyme Mix 2 to each sample. Invert gently. Do not vortex.

4.7 With a fire polished 6.5 mm diameter Pasteur pipette, dissociate the sample by triturating up and down 30 times. Avoid bubbles.

4.8 Incubate for 15 min in the 37 °C water bath, invert tubes several times every 5 min.

4.9 With a fire polished 5.5 mm Pasteur pipette, dissociate the sample by triturating up and down 30 times. Avoid bubbles.

4.10 Again, using a fire polished 5.5 mm Pasteur pipette, dissociate the sample by triturating up and down 30 times. Avoid bubbles.

4.11 Incubate the samples for 10 min in the 37 °C water bath, invert tubes every 5 min

4.12 Apply single-cell suspension to a 70-µm cell strain placed in a 50 mL conical tube.

4.13 Apply 10 ml of D-PBS to the filter. Discard filter.

4.14 Centrifuge the sample at 300 x *g* for 10 min at room temperature. Aspirate and discard supernatant.

4.15 Proceed to **7.0 Debris Removal**

Schwarz, J. M. Using fluorescence activated cell sorting to examine cell-type-specific gene expression in rat brain tissue. *J Vis Exp*, e52537, doi:10.3791/52537 (2015).