

# For All the Primate FANS: Optimized Isolation of Nuclei from Frozen Postmortem Primate Brain for Fluorescence-Assisted Nuclei Sorting (FANS)

Elaine E. Guevara<sup>a,1,\*</sup>, Begün Erbabab<sup>b,1</sup>, Gregory M. Cresswell<sup>c</sup>, Melissa K. Edler<sup>d,e</sup>, and Chet C. Sherwood<sup>b</sup>

<sup>a</sup>Department of Evolutionary Anthropology, Duke University, Durham, NC, USA; <sup>b</sup>Department of Anthropology and Center for the Advanced Study of Human Paleobiology, The George Washington University, Washington DC, USA; <sup>c</sup>School of Medicine and Health Sciences, The George Washington University, Washington DC, USA; <sup>d</sup>Department of Anthropology, School of Biomedical Sciences, and Brain Health Research Institute, Kent State University, Kent, OH, USA; <sup>e</sup>Department of Comparative Medicine, University of Texas at MD Anderson Cancer Center, Houston, TX, USA

Epigenetic alterations are cell type-specific and require methods like single cell sequencing and cell type sorting by flow cytometry. These methods often rely on the availability of fresh tissue, yet postmortem frozen tissue is typically the only material available from non-experimental subjects, including humans and other nonhuman primates (NHP). Many insights can be gained from analysis of these precious samples. To this end, we developed a protocol for isolating intact nuclei from small starting amounts of postmortem frozen chimpanzee (*Pan troglodytes*) cerebral cortex tissue. Isolated nuclei can be input directly into single cell epigenomics protocols like ATAC-seq or can be immunostained for enrichment of neuronal nuclei via fluorescent-activated nuclei sorting (FANS) followed by bulk epigenetic methods like methylome sequencing. We adapted and optimized this protocol based on existing human brain tissue protocols. Our protocol specifically addresses challenges presented by postmortem frozen NHP brain tissue, including high levels of myelin debris and reduced RNA integrity. We include key steps and troubleshooting guidance to improve nuclei quality and sorting outcomes, and we also discuss limitations and considerations for researchers interested in using these methods.

\*To whom all correspondence should be addressed: Elaine E. Guevara, email: [eg197@duke.edu](mailto:eg197@duke.edu); ORCID: <https://orcid.org/0000-0003-1480-474X>.

Abbreviations: FANS, Fluorescent-Activated Nuclei Sorting; FACS, Fluorescence-Activated Cell Sorting; AO, Acridine Orange; PI, Propidium Iodide; IACUC, Institutional Animal Care and Use Committee; FSC-A, Forward Scatter Area; FSC-H, Forward Scatter Height; GEM, Gel Beads-in-Emulsion; WGBS, Whole Genome Bisulfite Sequencing; E:I, Excitatory to Inhibitory Neuron Ratio; NHP, Non-human Primates.

Keywords: methylation, single cell genomics, chimpanzee, flow sorting, cortex, nonhuman primate

Author Contributions: CCS conceived of the study and supervised the research. All authors designed the experiments. BE, EEG, and MKE conducted the nuclei isolation and immunostaining experiments. GMC performed flow cytometry analysis. All authors read and approved the final manuscript. CCS and BE were supported by National Science Foundation DRL-2219759; CCS, EEG, and MKE were supported by National Institutes of Health R01AG067419, R01AG087945, and R01HG011641; EEG was supported by National Science Foundation BSC-2127961. <sup>1</sup>Co-first author.

CC BY-NC 4.0 This is an open access article distributed under the terms of the Creative Commons CC BY-NC license, which permits use, distribution, and reproduction in any medium, provided the original work is properly cited. You may not use the material for commercial purposes.

## INTRODUCTION

Epigenetic analyses can reveal important insights into development, aging, disease, and environmental plasticity. In the brain, epigenetic mechanisms are implicated in synaptic plasticity, learning and memory, and neurodegenerative diseases. These mechanisms include DNA and histone modifications and regulation by non-coding RNAs, which collectively modulate gene expression critical for neuronal function and plasticity [1,2]. For instance, DNA methylation and histone acetylation have been shown to play pivotal roles in memory formation and synaptic function [3]. Additionally, epigenetic regulation is essential for neurogenesis, influencing the proliferation and differentiation of neural progenitor cells [2]. Disruptions in these epigenetic processes are associated with various neurodevelopmental and neurodegenerative disorders [1].

However, for cellularly heterogeneous tissues like the brain, such insights are limited with traditional genomic methods in which signal is combined across cell types with different functions, like astrocytes and other glia, as well as diverse neuronal subtypes. As such, many research questions require cell type-specific analysis, which in turn require laboratory methods to isolate single cells or populations of homogeneous cell types. In recent years, the development of methods such as single cell sequencing and cell type-specific sorting via flow cytometry have flourished [4]. These methods have been eagerly adopted and results from analyses using these methods have underscored the crucial importance of analyzing epigenetic modifications at cell type resolution [5-8].

Nevertheless, there are important limitations to current methods. Many perform best with freshly harvested tissue. Yet inaccessible tissues, like brain, are often impossible to obtain immediately upon death from subjects other than laboratory animals, including humans and nonhuman primates (NHP). In particular, our closest living relatives, the great apes, offer unique opportunities to provide insight into human-specific neurodevelopmental patterns and neurodegenerative conditions, and tissue samples from them are often rare and opportunistically collected [9-12].

Several protocols for isolating nuclei from frozen brain have been developed [13-16]. However, these protocols require relatively high starting tissue amounts (ie, several hundred milligrams) that exceed the size of some brain regions of interest. In addition, they require specialized lab equipment like an ultracentrifuge that may not be available in many university departments. Recently, 10X Genomics (Pleasanton, California, USA) released a nuclei isolation kit that requires smaller amounts of tissue (~25mg) and standard laboratory equipment [17]. However, this kit is not specific to frozen brain tissue, and we

observed that its direct application to frozen postmortem chimpanzee cortex tissue led to suboptimal outcomes, including reduced nuclei integrity, limited signal resolution during flow cytometry, and elevated debris levels, which collectively hindered efficient and accurate sorting. These limitations likely reflect species- and tissue-specific challenges. Among commonly studied specimen types, frozen postmortem primate brain tissue represents one of the most technically challenging sources to process. Primate brains have a higher proportion of white matter than those of other mammals like rodents [18], the postmortem collection of tissue from apes often takes longer due to their size, and adult brains, especially those of older adults, are likely to contain high levels of intrinsic debris that can compromise downstream experimental workflows.

Our current protocol integrates previously developed protocols and optimizes key steps, including modified lysis conditions, enhanced filtration, and additional wash steps to obtain sufficient nuclei yield and integrity from small amounts of starting tissue (~25mg) while reducing debris. We also include optional immunostaining steps for enrichment of neuronal nuclei by fluorescent-activated nuclei sorting (FANS) using flow cytometry that reduces background fluorescence and address autofluorescence. While isolated nuclei may be used directly for single-cell ATAC-seq and RNA-seq workflows, such as 10X Genomics library preparation, without sorting, isolating neurons is desirable as it is compatible with “bulk” epigenomic methods like methylome sequencing. We also address inadvertent cell subtype selection bias during isolation and sorting, which, to our knowledge, has not been previously discussed. While our protocol is tailored specifically to the unique demands of postmortem NHP samples, we also provide troubleshooting tips and discussion of technical challenges that can aid in tailoring the protocol to other species (Figure 1).

## METHODS

### *Subjects*

Subjects were captive chimpanzees that lived in Association of Zoos and Aquariums-accredited zoos or US research facilities and died of natural causes unrelated to this research. Frozen brain specimens were obtained from the National Chimpanzee Brain Resource ([www.chimpanzeebrain.org](http://www.chimpanzeebrain.org)). All procedures with postmortem tissue followed the National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at The George Washington University (IACUC protocol A2024-095).

### *Tissue Dissections*

Hemisected whole brain slabs samples were

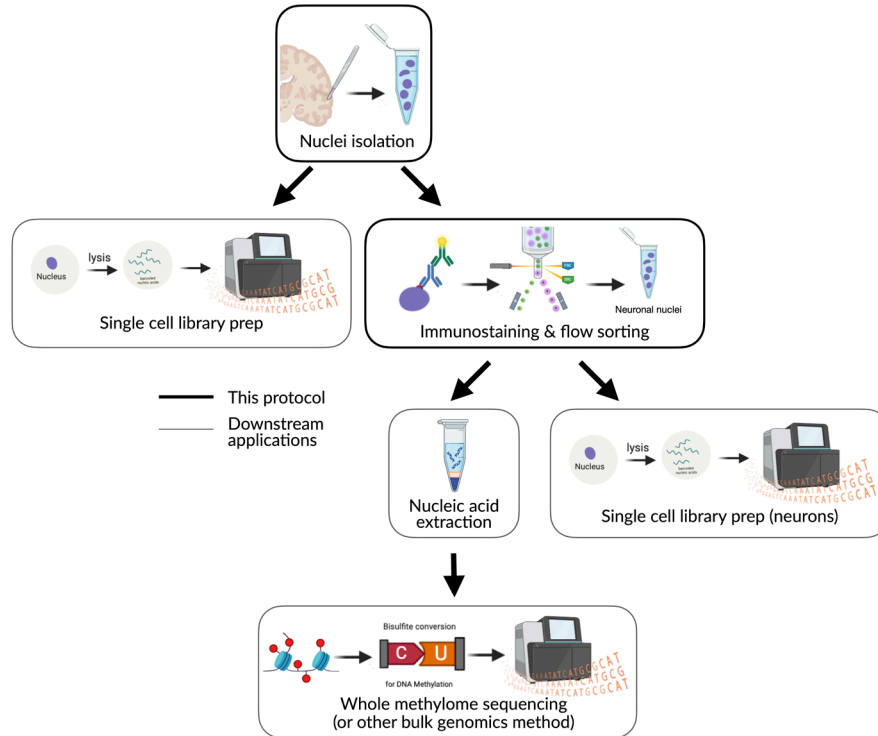


Figure 1. Overview of protocol highlighting downstream epigenetic methods. Created using BioRender.

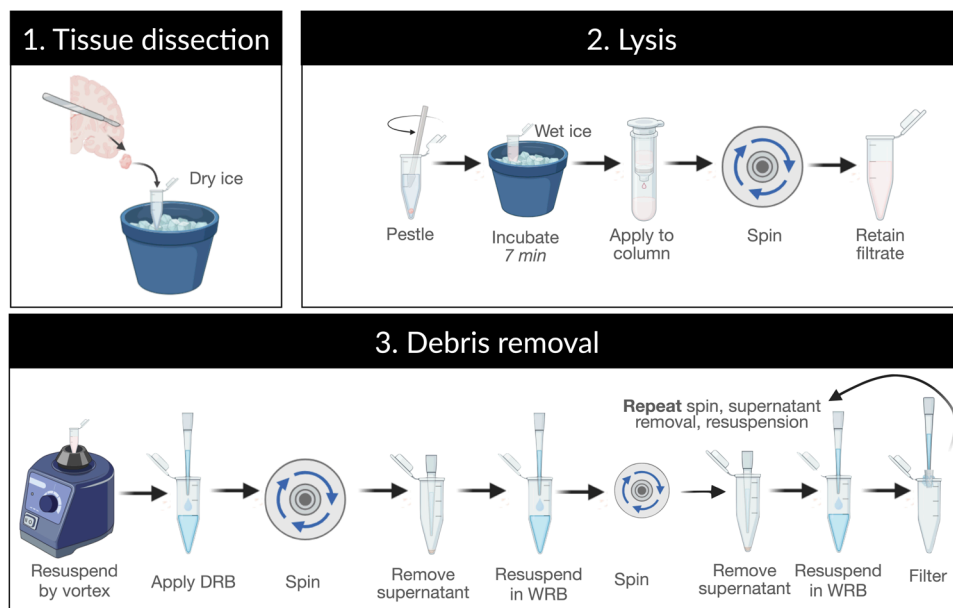
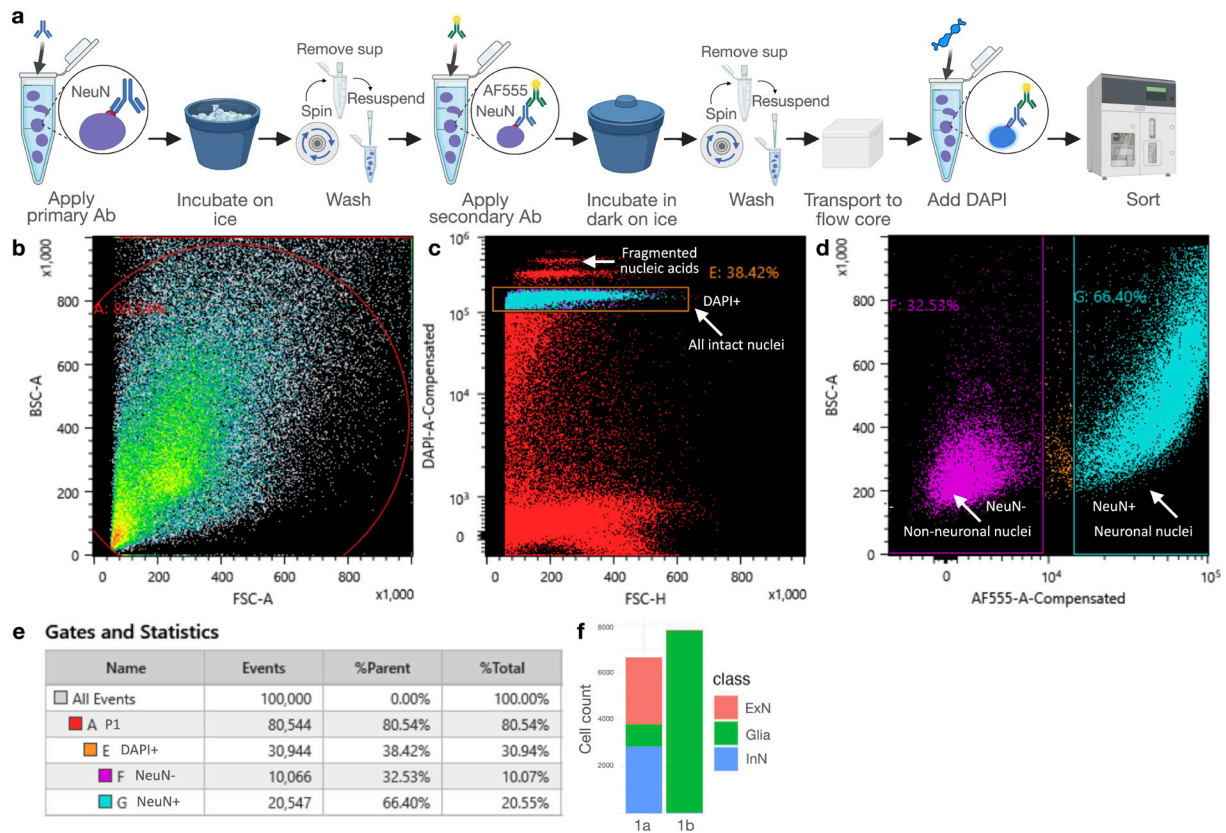


Figure 2. Protocol flowchart of isolation. Created using Biorender.

stored long term at  $-80^{\circ}\text{C}$ . Samples from the cerebral cortices were microdissected on dry ice using a 2 mm biopsy punch, yielding  $\sim 10\text{--}25$  mg tissue per punch. Then, we weighed  $\sim 25\text{--}50$  mg of frozen tissue per reaction. These specimens were transferred to 1.5 mL microcentrifuge tubes prechilled on dry ice.

### Nuclei Isolation

We used the 10X Genomics Chromium Nuclei Isolation with RNase Inhibitor Kit (PN-1000494, 10X Genomics, Pleasanton, California, USA) to isolate nuclei. We optimized the lysis time and number of wash steps (details given in detailed protocol; Figure 2) and added a filtration step using a Flowmi cell strainer (#H13680-



**Figure 3. Immunostaining workflow & FANS gating.** Panel a created using Biorender.

0040, Bel-Art, Wayne, NJ, USA).

### Quantification

Nuclei suspensions were quantified on the Countess 3 FL (A49866, Thermo Fisher, Waltham, MA, USA) automated cell counter with green fluorescent protein (GFP) and red fluorescent protein (RFP) EVOS cubes. Approximately 5  $\mu$ L of nuclei suspension was combined in a 0.2 mL tube with 5  $\mu$ L Invitrogen ReadyCount Red/Green Viability Stain (#A49905, Thermo Fisher, Waltham, MA, USA) and pipetted up and down to mix. The Red/Green Viability Stain contains the green stain Acridine Orange (AO) and red stain Propidium Iodide (PI). While AO and PI both stain nucleic acids, AO can pass through cell membranes and stain both cells and nuclei. PI is membrane-impermeable, so it only stains nuclei after cell lysis. A successful nuclei isolation will have a majority of double-stained or RFP-labeled nuclei, which is the most relevant count for downstream procedures.

### Immunostaining

Following quantification, we subdivided nuclei suspension into 2 mL microcentrifuge tubes for an unstained control, secondary antibody only control, and sample (full stain; see Detailed Protocol below; Figure 3a). We stained

sample nuclei with a primary antibody for the neuronal marker NeuN (Hexaribonucleotide Binding Protein-3). We incubated nuclei with the NeuN primary antibody for 30 min on ice after which we removed unbound antibody by centrifugation for 5 min at 400 rcf to pellet nuclei followed by supernatant removal and resuspension in blocking buffer. We then added the secondary antibody to samples and secondary only controls and incubated for 15 minutes in the dark on ice. We removed unbound secondary antibody by washing twice as described (see Detailed Protocol below), and samples were kept dark on ice until flow sorting.

### Flow Sorting

Samples were maintained on ice until sorting. We analyzed the samples on the Sony SH800Z Cell Sorter in the Flow Cytometry Core. Initially, we analyzed the unstained control to determine appropriate voltage and establish a negative gate. We added 1  $\mu$ L 0.1mg/mL DAPI (#D1306, Thermo Fisher, Waltham, MA, USA) to the remaining unstained control and our secondary-only control, and then ran these controls to establish a DAPI gate (all nuclei) and background threshold for the secondary antibody channel. Next, we added DAPI to our sample and collected nuclei inside the DAPI gate above the background threshold. Post-sort, nuclei were collected in

**Table 1. Key Resources for Nuclei Isolation**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chromium Nuclei Isolation Kit with RNase Inhibitor (16 rxns)	10x Genomics	PN-1000494
10% BSA in DPBS	Millipore-Sigma	A1595
1X PBS (no calcium, no magnesium)	Corning	21-040-CV
AO/PI ReadyCount Green/Red Viability Stain	Invitrogen	A49905
Countess Chamber Slides	Thermo Fisher Scientific	C10228
RNase Zap RNase Decontamination Solution	Invitrogen	AM9780
Flowmi Cell Strainer Tip	SP Bel-Art	H13680-0040

centrifuge tubes pre-filled with 200  $\mu$ L chilled blocking buffer. See Figure 3b-c for gating.

We validated our NeuN- and NeuN+ gates by collecting both positive and negative populations and sequencing 10X 3'-RNA-seq libraries generated from each (Figure 3f).

### Downstream Applications

Sorted neuronal nuclei, as well as unsorted suspensions, can be used in single-cell ATAC and RNA-seq library prep. Neuronal nuclei can also be put into bulk genomics protocols, such as DNA extraction for DNA methylation profiling. For nucleic acid extraction, a kit optimized for small inputs, such as from buccal swabs, should be used as the number of sorted nuclei contain fewer nucleic acids than many sample types (eg, primary tissue). For example, we used the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany). For one sample, we further assessed feasibility of downstream whole genome DNA methylation profiling using gDNAs extracted from sorted neuronal nuclei as input. Enzymatically converted DNA libraries were prepared at the Duke Sequencing and Genomics Technologies Core Facility using the NEBNext® Enzymatic Methyl-seq Kit (New England Biolabs, USA). Enzymatic conversion of unmethylated cytosines to thymines causes less damage to DNA compared with bisulfite conversion [19] and is recommended for methylome sequencing from low DNA samples. The library was sequenced on the Illumina Nova Seq 10X (25B flow cell) and ~300 000 000 reads were obtained. The PCR duplication rate was 13.8% and we obtained an average of >7X coverage of genome-wide CpGs.

## DETAILED PROTOCOL

### Before you begin

#### Institutional Permissions

All procedures involving postmortem NHP tissue must comply with institutional and ethical regulations. This protocol uses frozen chimpanzee brain tissue ob-

tained in accordance with approved National Institute of Health (NIH) and Institutional Animal Care and Use Committee (IACUC) guidelines for research on great ape materials.

### Biosafety Considerations

Treat all postmortem tissues as potentially infectious. Use standard personal protective equipment including double gloves, laboratory coat, and protective eyewear. Retrieve frozen tissue samples from  $-80^{\circ}\text{C}$  and keep on dry ice until processing.

### Consumables and Equipment

Reagents required in each step of the protocol are given in tables throughout (Tables 1,2).

- Refrigerated microcentrifuge with swinging bucket rotor (with a maximum speed: 16 000 rcf)
- Vortex mixer
- Countess 3 FL (A49866, Thermo Fisher) or equivalent fluorescence cell counter/FACS machine compatible with Alexa Fluor 555 detection
- Wet and dry ice
- Ice buckets (2 wet, 1 dry)
- RNase-free microcentrifuge tubes, 15 mL and 50 mL conical tubes, and tube racks
- 

### Step 1: Nuclei isolation

#### Preparation

**Time:** ~45 min

- Pre-chill centrifuges and rotors to  $4^{\circ}\text{C}$ .
- Prepare wet and dry ice in advance.
- Decontaminate work surfaces and equipment with RNase removal solutions (eg, RNase Zap).
- Thaw Reducing Agent B on ice.
- Label all sample tubes in advance.
- Chill pestles and tubes on dry ice.
- Prepare all buffers fresh using molecular biology-grade reagents and keep on ice.
- Chill all plasticware and reagents on ice.

- Minimize freeze-thaw cycles of sensitive reagents like enzymes and antibodies by aliquoting stock solutions.
- Vortex Lysis Reagent and Debris Removal Reagent, Reducing Agent B, and Surfactant A briefly before adding.

#### Buffer recipes

##### A. Lysis Buffer (per reaction; prepare fresh on wet ice)

- 500  $\mu$ L Lysis Buffer
- 0.5  $\mu$ L Reducing Agent B
- 5  $\mu$ L Surfactant A

##### B. Debris Removal Buffer (per reaction; prepare fresh on wet ice)

- 500  $\mu$ L Debris Removal Reagent
- 0.5  $\mu$ L Reducing Agent B

##### C. Wash and Resuspension Buffer (per reaction; prepare fresh)

- 2625  $\mu$ L 1x PBS (without calcium and magnesium)
- 300  $\mu$ L 10% BSA
- 75  $\mu$ L RNase Inhibitor

#### Procedure

**Time:** ~1.5 h

1. Transfer ~25-50 mg of frozen tissue from  $-80^{\circ}\text{C}$  to a pre-chilled 1.5 mL sample dissociation tube on dry ice.
2. Place the tube immediately on wet ice.
3. Add 300  $\mu$ L Lysis Buffer (prepared fresh on wet ice). Wait 1 min for temperature adjustment, and then homogenize tissue using a pre-chilled plastic pestle until the tissue is fully dissociated.
4. Add an additional 200  $\mu$ L Lysis Buffer and pipette up and down gently 10x.
5. Incubate on ice for 7 min.
6. Pipette the lysate into a pre-chilled Nuclei Isolation Column seated in its collection tube.
7. Centrifuge at 16 000 rcf for 20 sec at  $4^{\circ}\text{C}$ . Discard the column and retain the flowthrough.
8. Vortex the collection tube at 3200 rpm for 10 sec to resuspend nuclei.
9. Centrifuge at 500 rcf for 3 min at  $4^{\circ}\text{C}$ . Carefully discard supernatant.
10. Resuspend pellet in 500  $\mu$ L Debris Removal Buffer. Pipette mix at least 15x.
11. Centrifuge at 700 rcf for 10 min at  $4^{\circ}\text{C}$ . Discard supernatant.
12. Resuspend pellet in 1 mL Wash and Resuspension Buffer. Pipette mix gently 15x.
13. Centrifuge at 500 rcf for 5 min at  $4^{\circ}\text{C}$ . Discard supernatant.
14. Resuspend in 800  $\mu$ L Wash and Resuspension Buffer. Mix gently 15x.
15. Filter suspension through a Flowmi cell strainer into a clean tube.

16. Centrifuge again at 500 rcf for 5 min at  $4^{\circ}\text{C}$ . Discard supernatant.
17. Resuspend final nuclei pellet in 200  $\mu$ L Wash and Resuspension Buffer for counting and staining.

#### Step 2: Quantification

##### Procedure

**Time:** ~30 min

1. Gently pipette to mix the nuclei suspension. If the tube has been sitting, briefly spin down and resuspend.
2. Aliquot 5  $\mu$ L of the nuclei suspension into a 0.2 mL tube. Note that you may want to quantify each suspension in duplicate for accuracy.
3. Add 5  $\mu$ L AO/PI viability stain or PI. Mix by pipetting.
4. Load the 10  $\mu$ L of stained nuclei onto a Countess Chamber Slide. Label the slide and protect from light.
5. Insert the slide into the Countess 3 FL (or equivalent) equipped with RFP or Texas Red and GFP filters.
6. Focus and quantify total nuclei under the RFP or Texas Red channel, as this identifies DNA-containing nuclei.
7. At this step, nuclei can go straight into single-cell ATAC or RNA-seq library prep or can be stained to enrich for neurons via cell sorting by flow cytometry.

#### Step 3: Immunostaining

**Time:** ~70 min

##### Preparation

Prepare the controls to support gating and compensation:

- Unstained nuclei control
- DAPI-only control
- Secondary antibody-only control (Alexa Fluor 555)

Each control should be processed alongside stained samples, on ice, and shielded from light. Blocking buffer should be prepared fresh for each experiment. Blocking buffer components should be kept chilled on ice during preparation, and the buffer should be maintained on ice throughout the experiment.

##### Buffer Recipes

##### Blocking Buffer Recipe (per reaction)

-Prepare fresh for each reaction.

- 3930  $\mu$ L Nuclease-free water
- 500  $\mu$ L 10x PBS, pH 7.4
- 500  $\mu$ L 10% BSA in RNase free water
- 50  $\mu$ L Goat Serum

**Table 2. Key Resources for Immunostaining**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
10% BSA in DPBS	Millipore-Sigma	A1595
10X DPBS (no calcium, no magnesium)	Thermo Fisher	14200-075
1X PBS (no calcium, no magnesium)	Corning	21-040-CV
Molecular Grade Nuclease-free Water	Thermo Fisher	AM9937
RNase Zap RNase Decontamination Solution	Invitrogen	AM9780
Goat Serum	Sigma-Aldrich	G9023
Anti-NeuN Antibody (neuronal marker)	Abcam	ab177487
Goat Anti-Rabbit Antibody (Alexa Fluor 555)	Abcam	ab150078
DAPI	Thermo Fisher	D1306
RNase Inhibitor	Promega	N2615

- 25  $\mu$ L RNase inhibitor (40U/ $\mu$ l)

**Procedure**

1. Transfer the 50-200  $\mu$ L nuclei suspension to a RNase-free microcentrifuge tube.
2. Bring the sample volumes to 1000  $\mu$ L by adding Blocking Buffer.
3. Incubate 10 min.
4. Add primary antibody (eg, anti-NeuN, rabbit, unconjugated) at 1:500 dilution.
5. Incubate all of the tubes on ice for 30 min in the dark.
6. Following incubation, centrifuge all samples at 400 rcf for 5 min at 4°C.
7. Remove the supernatant leaving ~50  $\mu$ L of buffer above the pellets and pipette with 1 mL Blocking Buffer. Resuspend the pellets by gently pipetting up and down 5 times.
8. Add secondary antibody (eg, Goat anti-Rabbit Alexa Fluor 555) at 1:1000 dilution.
9. Incubate 15 min in the dark.
10. Centrifuge at 400 rcf for 5 minutes at 4°C.
11. Remove the supernatant leaving ~50  $\mu$ L of buffer above the pellets and pipette with 1 mL Blocking Buffer. Resuspend the pellets by gently pipetting up and down 5 times.
12. Centrifuge all samples at 400 rcf for 5 min at 4°C, then remove the supernatant leaving ~50  $\mu$ L of buffer above the pellets and pipette with 500  $\mu$ L Blocking Buffer. Resuspend the pellets by gently pipetting up and down 5 times.
13. Resuspend in 500  $\mu$ L Wash and Resuspension Buffer. Keep on ice and protect from light.

**Step 4: Fluorescence-Activated Nuclei Sorting (FANS)**

**Time:** ~1.5-2 h

1. Transport samples to the Fluorescence-Activated Cell Sorting (FACS) facility on ice and

protected from light.

2. Mix each sample with DAPI (1:1000) and allow 2-3 min to stain before placing into the sorter.
3. Run controls to establish voltages and compensation settings.
4. Gate on singlets, DAPI+ nuclei, and Alexa Fluor 555+ events.
5. Exclude debris and doublets.
6. Sort nuclei into chilled tubes containing 200  $\mu$ L Wash and Resuspension Buffer for cushioning.
7. Centrifuge sorted samples at 500 rcf for 5 min at 4°C.
8. Discard supernatant and snap-freeze the pellet or proceed to library preparation.

**DISCUSSION AND CRITICAL POINTS****Sample Quality**

Nuclei yield varies across individuals, which may be due to differences in tissue quality, brain region, species, or age. For example, postmortem interval and exposure to freeze-thaw cycles likely affect nuclei integrity and success in isolating high-quality nuclei as a result. Density of various cell types also vary by cortical region [20]. For example, in NHP, the visual cortex has a higher density of neurons while the prefrontal cortex exhibits relatively low density [21]. Neuron density also varies considerably across mammal species [22]. Subject age can also have a substantial influence on nuclei recovery. Animals under a few years of age have a higher density of neurons [23]; thus, the same tissue mass from an infant is expected to yield more neuronal nuclei than from an adult. Conversely, adults have more myelin than subadults and older adults tend to have more degraded myelin [24,25]. Thus, myelin debris is expected to increase with age. Both of these observations are consistent with our anecdotal observations.

### Removing Myelin Debris

Brain is considered a high debris tissue because of the large amount of lipid myelin material present, particularly in the cerebral cortex. Excessive debris remaining in a nuclei suspension can impede downstream procedures, including accurate quantification, increasing the risk of clogs in 10X library prep, and inefficient sorting. Myelin fragments are difficult to remove as many approaches to chemical removal can damage fragile nuclei, making debris removal optimization one of the most crucial factors in protocol success. We offer several recommendations for minimizing myelin debris without compromising nuclei integrity.

First, during dissections, take special care to avoid white matter and start with an initial tissue volume at the low end of the recommended input range for the isolation kit (~15-20 mg). To ensure recovery of sufficient nuclei, we recommend undertaking nuclei isolations from 2-3 small tissue specimens per sample in parallel. Replicates can be pooled after the isolation step. Note that a smaller input often leads to the absence of a visible nuclei pellet following centrifugation steps. Simply leave ~200  $\mu$ L buffer at the bottom of the tube. We found that pellet visibility was a reflection of myelin debris more than a reflection of nuclei quantity or quality. Relatedly, due to the lack of a visible pellet and the relatively low centrifugation speeds required to avoid undue stress to fragile nuclei, we strongly recommend using a swinging bucket rather than fixed-angle microcentrifuge rotor for all centrifugation steps to facilitate supernatant removal without disturbing the pellet.

Physical filtering of the isolates is crucial to removing myelin debris. Although many cell strainers are available, we found Flowmi filter tips performed significantly better than other options due to ease of use, reduced sample loss, and reduced straining time. This latter point is especially important for downstream analyses including RNA, as RNA degrades quickly, and maintaining low sample temperature is difficult during straining.

### Time Management

Nuclei are very fragile once the cell membrane is lysed in the first steps of the isolation protocol. As such, the amount of time nuclei are sitting isolated prior to nucleic acid isolation in downstream protocols (ie, sit time) must be minimized. Excessive sit time can lead to nuclear membrane degradation (ie, “blebbing”), which can result in nucleus deformation and leakage of nuclear contents. Possible downstream consequences of blebbing include reduced sort efficiency and, in 10X library prep, GEM (Gel Beads-in-Emulsion) formation, overall decreased nuclei and nucleic acid yields, and poorer data quality. We recommend researchers limit each isolation session

to 4-6 isolation reactions per person to preserve sample integrity.

### Antibody Selection

Nuclear markers are required for staining nuclei. Many commonly used cell type-specific primary antibodies are cytoplasmic or cell membrane markers. While these antibodies may be used for staining fixed tissue or cells, they will not stain nuclei or work in FANS. Take care to ensure the antibodies selected are nuclear markers, like the neuronal marker, NeuN. Titrating and validating antibodies is essential. Flow cytometry typically requires higher primary antibody concentrations than immunohistochemical staining procedures in fixed tissue samples, but we recommend trying a few concentrations when starting with a new antibody. Antibodies for other brain cell types, like astrocytes, oligodendrocytes, and microglia, will require their own validation and optimization.

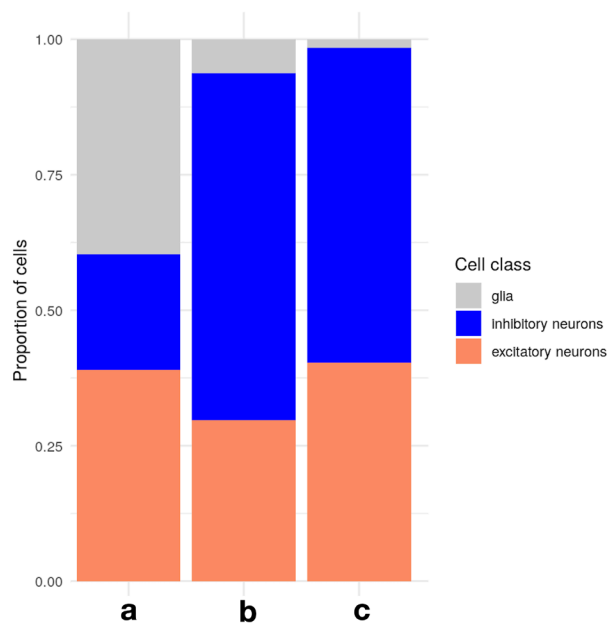
Pre-conjugated or separate secondary antibodies may be used. Pre-conjugated antibodies have the benefit of minimal background staining and decreased immunostaining time. However, using a fluorescent-labeled secondary antibody in place of a fluorescent-tagged primary antibody may offer advantages in terms of signal brightness as multiple secondary antibodies can bind to different epitopes on a single primary antibody, leading to an amplification of the fluorescent signal. We initially tried a pre-conjugated NeuN antibody, but found a two-step staining protocol with a primary antibody followed by a fluorescent secondary improved staining brightness and downstream sorting. Specifically, after initial trials we used a rabbit anti-NeuN primary antibody (ab177487, Abcam, Cambridge, UK) and Alexa fluor 555-conjugated goat anti-rabbit secondary antibody (ab150078, Abcam, Cambridge, UK).

NHP brain tissue shows variable but overall high autofluorescence in the approximate range of Alexa-488. Autofluorescence increases with age due to the accumulation of lipofuscin (Gray and Woulfe, 2005). Thus, we recommend selecting fluorophores outside this emission range to avoid impeding the ability to sort stained nuclei from background autofluorescence.

Nuclei may be stained with multiple antibodies at once, allowing for recovery of multiple cell types from the same tissue sample and maximizing the information that can be obtained for the same subject. Note, however, that additional single-color controls must be prepared in experiments using multiple fluorophores to simultaneously sort more than one cell type. Sorters also vary in the number of cell populations they can sort from the same sample, so sorter capability must be considered in the design of more complex experiments.

**Table 3. List of Solutions for Some Common Problems**

Problem	Possible Cause	Suggested Solution
Low nuclei yield	Incomplete homogenization or insufficient thawing	Thoroughly dissociate tissue on ice; allow proper thawing transition from dry to wet ice
High debris background	Tissue degradation or minimal washing	Add additional wash/debris removal steps; filter twice if needed; decrease initial tissue weight
Poor staining or low signal	Antibody degradation or incorrect dilutions	Use fresh aliquots; confirm dilutions; protect from light; try alternative antibody batch or company; switch to using non-conjugated primary antibody in combination with a conjugated secondary antibody in case a conjugated primary antibody was being used for staining
High background in controls	Non-specific secondary binding	Increase blocking time; reduce antibody concentrations; increase wash steps; exclude white matter during dissection; validate all antibodies used; filter twice if needed; decrease initial tissue weight



**Figure 4. Proportion of major cell classes by sorting condition of samples collected from Inferior Frontal Gyrus region of an adult chimpanzee brain. a.** Unsorted nuclei. **b.** NeuN positive sorted nuclei using 100 µm nozzle. **c.** NeuN positive sorted nuclei using 130 µm nozzle.

#### Inadvertent Cell Type Selection

We observed a low proportion of excitatory neurons in our samples and infer that the relatively large nuclei of these neurons are disproportionately lost, most notably during immunostaining and/or sorting due to increased sit time and/or mechanical stress from additional centrifugations and flow cytometry. The expected ratio of excitatory to inhibitory neurons (E:I) in the cortex is ~3 to 1; however, several of our NeuN-sorted samples exhibited closer to a one-to-one ratio between these two major classes of neurons. To narrow down what procedures

most affect selective disappearance of excitatory neurons, we partitioned isolated nuclei for one individual's inferior frontal gyrus sample straight into 10X RNA-seq library preparation (Chromium Next GEM Single Cell 3' Kit v3.1) followed by immunostaining. The immunostained nuclei were further divided into nuclei sorted with 100 µm and 130 µm flow cytometer nozzle diameters. Although nuclei are small, a larger nozzle size may reduce pressure on nuclei. Sorted neuronal nuclei were then put into the same 10X RNAseq library preparation. Samples were sequenced on the Illumina NovaSeq X at the Duke Sequencing and Genomics Technologies Core Facility. The unsorted sample showed an E:I ratio of ~1.8, while samples stained and sorted with both the 100 µm and 130 µm nozzle showed E:I < 1 (Figure 3f and 4). These results indicate that while our protocol successfully enriches for neuronal nuclei, the relative proportion of neuronal subtypes should be interpreted with caution.

#### Expected Outcomes

- Recovery of 2000 to 10 000 sorted nuclei from ~15-45 mg tissue with a total of ~20 000 to 50 000 events.
- Distinct NeuN+ and NeuN- populations with clean gating profiles
- High-quality nuclei suitable for single nuclei RNA-seq, WGBS, and related methods

#### Limitations

- Results depend on tissue quality, postmortem interval, regional variability, and subject age
- Antibody performance may vary by batch or supplier
- Protocol optimization may be necessary for use with other NHP species

### Troubleshooting

We have listed several troubleshooting approaches in Table 3.

**Acknowledgments:** We thank Jack Villani and Kam-wing Jair of the George Washington University Genomics Core, Carles Moreno Soriano of GW Microbiology, Immunology, and Tropical Medicine, Leon Grayfer and Michael Massiah of The George Washington University Biology, Ellora Haukenfrers of Duke Molecular Genomics Core, Devi Swain Lenz and Sarah Cleaton of the Duke Sequencing and Genomics Technologies Core Facility, Genevieve Konopka for laboratory support and technical guidance.

**Funding:** This project was supported by the National Science Foundation (BSC-2127961, DRL-2219759), funding provided to the National Chimpanzee Brain Resource (NIH NS092988), and the National Institutes of Health (R01AG067419, R01AG087945, R01HG011641).

### REFERENCES

- Men J, Wang X, Zhou Y, Huang Y, Zheng Y, Wang Y, et al. Neurodegenerative diseases: epigenetic regulatory mechanisms and therapeutic potential. *Cell Signal*. 2025 Jul;131(111715):111715.
- Yao B, Christian KM, He C, Jin P, Ming GL, Song H. Epigenetic mechanisms in neurogenesis. *Nat Rev Neurosci*. 2016 Sep;17(9):537–49.
- Singh P, Paramanik V. DNA methylation, histone acetylation in the regulation of memory and its modulation during aging. *Front Aging*. 2025 Jan;5:1480932.
- Qu HQ, Kao C, Hakonarson H. Single-cell RNA sequencing technology landscape in 2023. *Stem Cells*. 2024 Jan;42(1):1–12.
- Lake BB, Chen S, Sos BC, Fan J, Kaeser GE, Yung YC, et al. Integrative single-cell analysis of transcriptional and epigenetic states in the human adult brain. *Nat Biotechnol*. 2018 Jan;36(1):70–80.
- Zhang Y, Amaral ML, Zhu C, Grieco SF, Hou X, Lin L, et al. Single-cell epigenome analysis reveals age-associated decay of heterochromatin domains in excitatory neurons in the mouse brain. *Cell Res*. 2022 Nov;32(11):1008–21.
- Rahman MF, McGowan PO. Cell-type-specific epigenetic effects of early life stress on the brain. *Transl Psychiatry*. 2022 Aug;12(1):326.
- Armand EJ, Li J, Xie F, Luo C, Mukamel EA. Single-cell sequencing of brain cell transcriptomes and epigenomes. *Neuron*. 2021 Jan;109(1):11–26.
- Bianchi S, Stimpson CD, Duka T, Larsen MD, Janssen WGM, Collins Z, Bauernfeind AL, Schapiro SJ, Baze WB, McArthur MJ, Hopkins WD, Wildman DE, Lipovich L, Kuzawa CW, Jacobs B, Hof PR, Sherwood CC. Synaptogenesis and development of pyramidal neuron dendritic morphology in the chimpanzee neocortex resembles humans. *Proc Natl Acad Sci U S A*. 2013 Jun 18;110 Suppl 2(supplement\_2):10395–401. <https://doi.org/10.1073/pnas.1301224110>.
- Sherwood CC, Gordon AD, Allen JS, Phillips KA, Erwin JM, Hof PR, et al. Aging of the cerebral cortex differs between humans and chimpanzees. *Proc Natl Acad Sci USA*. 2011 Aug;108(32):13029–34.
- Edler MK, Sherwood CC, Meindl RS, Hopkins WD, Ely JJ, Erwin JM, et al. Aged chimpanzees exhibit pathologic hallmarks of Alzheimer's disease. *Neurobiol Aging*. 2017 Nov;59:107–20.
- Hopkins WD, Li X, Roberts N, Mulholland MM, Sherwood CC, Edler MK, et al. Age differences in cortical thickness and their association with cognition in chimpanzee (*Pan troglodytes*). *Neurobiol Aging*. 2023 Jun;126:91–102.
- Mendizabal I, Berto S, Usui N, Toriumi K, Chatterjee P, Douglas C, et al. Cell type-specific epigenetic links to schizophrenia risk in the brain. *Genome Biol*. 2019 Jul;20(1):135.
- Policicchio S, Davies JP, Chioza B, Burrage J, Mill J, Dempster E. Fluorescence-activated nuclei sorting (FANS) on human post-mortem cortex tissue enabling the isolation of distinct neural cell populations for multiple omic profiling. 2020 Oct 6 [cited 2025 Mar 29]; Available from: <https://www.protocols.io/view/fluorescence-activated-nuclei-sorting-fans-on-huma-bmh2k38e.pdf>
- Matevossian A, Akbarian S. Neuronal nuclei isolation from human postmortem brain tissue. *J Vis Exp* [Internet]. 2008 Oct 1;(20). Available from: <https://pmc.ncbi.nlm.nih.gov/articles/PMC3233860/>
- Grant LB. Isolation of Nuclei from Adult Human Brain Tissue for 10x Genomics Platform. 2019 Mar 13 [cited 2025 Mar 29]; Available from: <https://www.protocols.io/view/isolation-of-nuclei-from-adult-human-brain-tissue-y6rfzd6.pdf>
- Genomics 10x. Chromium Nuclei Isolation Kit: Sample Prep User Guide. Document Number: CG000505, Rev A [Internet]. 2022. Available from: [https://cdn.10xgenomics.com/image/upload/v1660261285/support-documents/CG000505\\_Chromium\\_Nuclei\\_Isolation\\_Kit\\_UG\\_RevA.pdf](https://cdn.10xgenomics.com/image/upload/v1660261285/support-documents/CG000505_Chromium_Nuclei_Isolation_Kit_UG_RevA.pdf)
- Ventura-Antunes L, Mota B, Herculano-Houzel S. Different scaling of white matter volume, cortical connectivity, and gyrification across rodent and primate brains. *Front Neuroanat*. 2013 Apr;7:3.
- Hoppers A, Williams L, Ponnaluri VK, Sexton B, Saleh L, Campbell M, et al. Enzymatic methyl-seq: next generation methylomes. *J Biomol Tech*. 2020 Aug;31 Suppl:S15.
- Collins CE, Turner EC, Sawyer EK, Reed JL, Young NA, Flaherty DK, et al. Cortical cell and neuron density estimates in one chimpanzee hemisphere. *Proc Natl Acad Sci USA*. 2016 Jan;113(3):740–5.
- Collins CE, Airey DC, Young NA, Leitch DB, Kaas JH. Neuron densities vary across and within cortical areas in primates. *Proc Natl Acad Sci USA*. 2010 Sep;107(36):15927–32.
- Herculano-Houzel S. The human brain in numbers: a linearly scaled-up primate brain. *Front Hum Neurosci*. 2009 Nov;3:31.
- Rabinowicz T, de Courten-Myers GM, Petetot JM, Xi G, de los Reyes E. Human cortex development: estimates of neuronal numbers indicate major loss late during gestation. *J Neuropathol Exp Neurol*. 1996 Mar;55(3):320–8.

24. Miller DJ, Duka T, Stimpson CD, Schapiro SJ, Baze WB, McArthur MJ, et al. Prolonged myelination in human neocortical evolution. *Proc Natl Acad Sci USA*. 2012 Oct;109(41):16480–5.
25. Safaiyan S, Kannaiyan N, Snaidero N, Brioschi S, Biber K, Yona S, et al. Age-related myelin degradation burdens the clearance function of microglia during aging. *Nat Neurosci*. 2016 Aug;19(8):995–8.