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Nuclei isolation of multiple brain cell types for omics interrogation.

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Abstract

We present a nuclei isolation protocol for genomic and epigenomic interrogation of multiple cell type populations in the human and rodent brain. The nuclei isolation protocol allows cell type-specific profiling of neurons, microglia, oligodendrocytes and astrocytes, which is compatible with fresh and frozen samples obtained from either resected or postmortem brain tissue. This two-day procedure consists of tissue homogenization with fixation, nuclei extraction and antibody staining followed by fluorescence-activated nuclei sorting (FANS) and does not require specialized skillsets. Cell type-specific nuclei populations can be used for downstream omic-scale sequencing applications with an emphasis on epigenomic interrogation such as histone modifications, transcription factor binding, chromatin accessibility and chromosome architecture. The nuclei isolation protocol enables translational examination of archived healthy and diseased brain specimens through utilization of existing medical biorepositories.

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Author contributions

A.N., J.C.M.S, and C.K.G. conceptualized the study; A.N. and J.C.M.S. optimized the methodology with input from B.R.F.; A.N. acquired and analyzed nuclei ChIP-seq and ATAC-seq datasets; A.N., J.C.M.S., B.R.F. and C.K.G. wrote the manuscript.

Competing interests

The authors declare no competing interests

Key references using this protocol

Nott, A. et al. Brain cell type–specific enhancer–promoter interactome maps and disease-risk association. Science 366, 1134–1139 (2019). doi: 10.1126/science.aay0793

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Key data used in this protocol

Nott, A. et al. Brain cell type–specific enhancer–promoter interactome maps and disease-risk association. Science 366, 1134–1139 (2019). doi: 10.1126/science.aay0793

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Introduction

There has been intense interest in elucidating mechanisms that link human non-coding genetic variation to risk of neurodegenerative and psychiatric diseases. Towards this effort, we describe a nuclei isolation protocol that has allowed characterization of the epigenome of specific cell types in the human brain[1,2]. Many brain-related diseases have a degree of heritability and are characterized as complex polygenic disorders. These encompass neurodevelopmental disorders such as Autism Spectrum Disorders (ASD), psychiatric conditions including schizophrenia, bipolar disorder and major depressive disorder, and neurological disorders such as Alzheimer's disease (AD), Parkinson's disease, Multiple Sclerosis, and epilepsy. Genome-wide association studies have identified genetic variants that are associated with increased risk of developing these disorders; however, the function of these disease-risk variants is largely unknown[3]. Disease-risk variants often reside outside of genes in DNA regions called enhancers that are thought to regulate gene expression[4]. Upon binding combinations of regulatory proteins called transcription factors, enhancers integrate environmental signals and induce gene expression programs that guide cell type-specific identity and responses[5]. Chromatin marks of enhancer activity have been key for inferring dysregulated gene expression programs in AD[6,7] and ASD[8]. Prior work has focused on analysis of bulk tissue, potentially masking epigenomic changes that occur in rare cell types. For example, microglia, the immune cells of the brain, have been implicated in aging-associated disorders; however, they constitute only 5% of cells in the brain and have been difficult to detect by bulk tissue analysis. In addition, altered epigenomics of bulk tissue may reflect changes in the proportions of particular cell types. For example, an increased percentage of microglia due to a loss of neurons may incorrectly register as microgliosis by bulk RNA-seq. In addition, brain cells are not easily accessible from living individuals. To overcome these obstacles, we have established a cell type-specific nuclei isolation protocol for frozen tissue samples, which are widely available from surgical resections and/or biorepositories[1]. This protocol has been utilized to define gene regulatory elements of neurons, microglia, oligodendrocytes and astrocytes from pediatric human cortical tissue[1]. We found that genetic variants associated with increased risk of AD were largely confined to microglia enhancers, while genetic variants associated with psychiatric disorders were enriched in neuronal enhancers[1]. The nuclei isolation approach was further utilized to generate interactome maps that connect disease-risk variants located in enhancers to gene promoters[1]. Integrative analysis of promoter-enhancer interactome maps has revised and expanded the gene repertoire influenced by AD-risk variants and also revealed the probable cell types in which they function[1]. The protocol is compatible with both fresh and frozen tissue from brain resections[1] or postmortem autopsies[2]. The nuclei isolation protocol has been used with success on postmortem tissue to identify cell type-specific brain somatic mosaic variants using massive parallel amplicon sequencing[2]. Ultimately, this approach permits cell type-specific utilization of archived specimens, including banked disease biopsies, that had previously been difficult to analyze. Furthermore, our nuclei isolation protocol allows cell type-specific interrogation of model systems such as murine and organoid models of brain diseases. The ability to isolate nuclei of microglial origin is a major advance, as it permits interrogation of the neuroinflammatory components of brain-related disorders and traumas.

Applications of the method

The nuclei isolation approach can be used to isolate cell type-specific nuclei populations from frozen or fresh brain tissue[1]. This includes nuclei from neurons, microglia, oligodendrocytes and astrocytes. Our protocol allows interrogation of human brain tissue from resected and postmortem sources, as well as from murine brain tissue. Nuclei can be used to identify chromatin features, such as active promoter and enhancer regions, and to identify the binding sites of specific transcription factors through chromatin immunoprecipitation-sequencing (ChIP-seq)[9]. Our protocol for nuclei isolation from cell type of origin can be utilized for assessment of chromatin architecture through approaches such as chromosome conformation capture techniques[10], proximity ligation assisted ChIP (PLAC)-seq[11] and HiChIP[12]. In addition, nuclei can be used to study open regions of chromatin using a modified assay for transposase-accessible chromatin by sequencing (ATAC-seq) protocol for fixed tissue[13]. In theory, following reverse cross-linking, our nuclei isolation protocol can also be utilized for transcriptomic profiling. Our protocol was optimized for ChIP-seq applications; however, tissue fixation can be omitted when the quality of the tissue is high, and the nuclear proteins used to distinguish target cell types are stable.

The nuclei isolation approach will be of interest to researchers investigating specific cell types in the brain using model organisms and archived human tissue. The major advantage of this protocol is that our approach does not rely on genetically tagging cell types of interest through endogenous expression of fluorescent nuclear markers. Since our method bypasses the requirement for additional genetic manipulation, it is highly amenable for use with human tissue and other model systems. Furthermore, our protocol will be informative to investigators interested in rare cell types that may be missed through analysis of bulk tissue. These cells include microglia, the resident immune cells of the brain, and specialized glial cells called astrocytes. These under-represented cell types are altered during disease and it is thought that they may play a major role in the development and progression of brain disorders[14,15]. Microglia represent approximately 5% of the total cell population in the brain and are challenging to detect using bulk approaches. Disease conditions can alter the proportional representation of particular cell types, which further complicates the interpretation of bulk tissue. Thus, enrichment of nuclei from specific cell types will provide an invaluable tool for researchers in the field of brain disorders.

Experimental design

Biological replicates

The nuclei isolation protocol has been optimized for isolation of nuclei from brain cell types from human and mouse brain tissue for downstream applications requiring DNA. As a guide, for experiments using mice that are considered to be almost genetically identical, it would be expected that replicates of 3 per group would be sufficient. For experiments using human tissue, the sample number that is required will need to consider human genetic variation between the biological replicates, as well as the experimental question that is being explored. For example, a comparison between different brain cell types may require a smaller sample group of 4 to capture the majority of cell type specificity. However, an

experimental question that compares two different conditions within a single cell type, such as microglia in health versus disease, will ideally require sample sizes of 20 or more per group; although previously publications have made similar comparisons using group sizes of 10[7,16].

Tissue preparation, dissociation and fixation

Brain tissue is homogenized and fixed with 1% (wt/vol) formaldehyde in phosphate buffered saline (PBS) followed by a glycine quench to halt the formaldehyde fixation (Fig. 1). Repeated removal of brain samples from −80 °C storage can have a detrimental effect on sorting nuclei from sensitive cell types such as microglia. To maintain nuclei integrity for large samples that need to be used repeatedly, it is suggested to divide large tissue samples into ~150 mg aliquots, which should be performed on dry ice to prevent thawing of the sample. For applications relating to ChIP-seq, while 1% (wt/vol) formaldehyde is adequate for ChIP-seq of most histone modifications, alternative fixation methods may be required for ChIP-seq of certain transcription factors and will need to be optimized for each ChIP antibody. A common fixation approach for transcription factors is a double fixation using disuccinimidyl glutarate (DSG) followed by formaldehyde (see Troubleshooting Step 10 for details). The DSG/formaldehyde double fixation has been tested using our nuclei isolation protocol in mice and does not affect the yield of PU.1-positive (a marker for microglia) nuclei, however, other fixation approaches may reduce nuclei yields and will need to be individually tested.

Isolation of nuclei

Homogenates are washed, dounced and enriched for single nuclei using a sucrose cushion. The inclusion of the sucrose gradient is to reduce the level of myelin and to remove small debris particles and will improve the efficiency and speed of nuclei isolation by FANS (Fig. 1). The stringency of the sucrose cushion will be a compromise over reducing the amount of debris in the sample versus the yield of nuclei that will be collected. For example, a higher molarity of sucrose will provide a more stringent sucrose cushion that removes more debris from the sample but may trap more nuclei within the debris fraction and lower the final yield. The appropriate stringency of the sucrose cushion will need to be optimized for different sample sources by testing a range of sucrose molarities followed by FANS to determine nuclei yields (see Troubleshooting step 29 for details).

Antibody staining of nuclei

For human and mouse brain tissues, nuclei are immunostained overnight (or up to two nights) at 4 °C with antibodies against the following cell type-specific nuclear markers: NeuN for neurons, PU.1 for microglia and OLIG2 for oligodendrocytes (Fig. 1). Currently, there is a lack of usable antibodies for nuclear astrocytic markers. However, LIM/Homeobox 2 (LHX2) has been shown to be highly expressed in astrocytes and moderately expressed in neurons. Thus, astrocyte nuclei have been isolated using a combinatorial flow cytometry gating strategy of NeuN-negative and LHX2-positive nuclei isolated from human brain tissue. Using DAPI as a nuclear stain, immuno-labeled single nuclei are isolated by fluorescence activated nuclei sorting (FANS; Fig. 2).

The protocol as outlined here for antibody staining of nuclei has been optimized for three to four colors (DAPI, Alexa Fluor 488, PE, and Alexa Fluor 647) using the MoFlo Astrios cell sorter. However, the protocol can be adjusted as needed to answer different experimental questions by adding more or different antibodies. We advise before a new antibody is added, to 1) adjust the staining panel for possible spectral overlap; 2) include proper isotype controls; 3) check the flow cytometer's technical parameters such as whether a UV-laser is available; and 4) perform compensation. For example, if the user would like to expand the number of antibodies and perform labeling of PU.1, OLIG2, NeuN, and LHX2 in one experiment, a different secondary antibody for LHX2 should be used that does not overlap with Alexa Fluor 488, PE, and Alexa Fluor 647 spectra should be used. Depending on whether a UV-laser is available or not, a secondary antibody Goat anti-rabbit IgG (H+L) BV421 (BD Biosciences; 565014) or Goat anti-rabbit IgG (H+L) APC/Cy7 (Invitrogen; A-21039), respectively, can be used (at step 59). Another possible approach would be to directly conjugate the Rabbit anti-LHX2 to a secondary antibody coupled to a fluorophore (BV421, APC/Cy7 or others) if desired.

A common issue is the lack of PU.1 nuclei staining; this is often due to compromised tissue quality such as from postmortem sources. Ideally the tissue quality should be improved by using samples with low postmortem delays that have been snap frozen and properly stored at −80 °C. In addition, the PU.1 antibody concentration can be increased to 1:100 and the staining incubation time can be extended up to 48 hours for postmortem samples, which may improve the nuclei yield for problematic samples (see Troubleshooting step 78 for details).

High background fluorescence may be observed in the unstained control (no antibody staining) for a fraction of the nuclei. This may occur following oxidation of the tissue due to poor tissue quality and is a common issue for postmortem samples. To prevent collection of autofluorescent nuclei, it is suggested to generate dot plots using two channels and to avoid using histograms alone. The presence of autofluorescent nuclei should be tested using the unstained control and will likely appear as a positive signal in both channels whereas target nuclei will be positive in a single channel.

Downstream applications for cell type nuclei populations

Cell type-specific nuclei populations are then used for downstream applications[1,2], which include identification of chromatin features and transcription factor binding sites by ChIP-seq, identification of open chromatin regions by ATAC-seq, and assessment of chromatin conformation by PLAC-seq and HiChIP. The approach for fixation of tissue that is described in this nuclei isolation protocol is compatible for most ChIP-seq protocols[9], as well as for PLAC-seq[11] and HiChIP[12]. ATAC-seq works best with non-fixed samples, however, reverse-crossing and Proteinase K digestion of the sample immediately after Tn5 tagmentation can be applied for fixed samples[13]. This approach is suitable for fixed nuclei isolated from high quality tissue, as we have shown for surgically resected brain tissue[1]. However, ATAC-seq data quality from human postmortem tissue may be compromised due to prolonged postmortem intervals, changes in tissue pH, and repeated freeze-thaw cycles.

For RNA-seq transcriptomic profiling, cross-linking also has to be reversed. We have isolated RNA using a combined reverse-crosslinking and Proteinase digestion at 55 °C for 3

hours, as published[17,18]. The resulting RNA will be highly fragmented due to the formaldehyde fixation, which can be quantitatively determined using a bioanalyzer. Because the RNA is already fragmented, we recommend proceeding with an rRNA depletion procedure (such as riboZero), followed by whole-transcriptome analysis using total RNA-Seq. We find that rRNA depletion is less efficient when using RNA extracted from fixed samples and that RNA-seq libraries from these samples will contain higher than expected rRNA contamination (40% or higher) and will therefore require deeper sequencing. Alternatively, it is possible to proceed with messenger RNA (mRNA)-seq, however, due to the fragmentation of the samples there will be a large 3' bias that may compromise the complexity of the sequencing libraries. For single nuclei RNA-seq, protocols have been developed that are compatible for samples fixed with 1% formaldehyde[19,20].

Expertise needed to implement the protocol

Human brain tissue prior to fixation is treated as a laboratory hazard that includes potential exposure to bloodborne pathogens and agents. This work will require specialized biosafety training, which will include the use of appropriate Personal Protective Equipment (PPE) in a Class II A2 Biosafety Cabinet according to institutional guidelines. In addition, brain tissue obtained from patients requires informed consent under a protocol approved by an Institutional Review Board. Lastly, the sorting of nuclei cell type populations will require specialized expertise in operating a FACS machine. For most institutions, cell sorting can be performed with technical assistance provided by a core facility who should be consulted prior to initiating the protocol.

Comparison with other methods

The nuclei isolation protocol was an adaptation of previous protocols for isolation of neuronal nuclei for ChIP-seq[21–24]. The nuclei protocol presented here has been modified to be completed with greater speed and to capture additional cell types including microglia and astrocytes, which have previously proven difficult to isolate from human tissue. We have further demonstrated that the nuclei isolation protocol is compatible with ATAC-seq[1], PLAC-seq[1] and whole genome sequencing[2], as well as ChIP-seq as previously reported. Nuclei isolation strategies have recently been published using alternative markers; IRF8 for microglia[25,26] and SOX10 for oligodendrocytes[26]. Yields reported for these alternative markers are 400,000 IRF8 +ve nuclei and 1M SOX10 +ve nuclei per 500 mg of human postmortem cortex[26], which are comparable to yields we have achieved using the markers PU.1 and OLIG2, respectively (see Anticipated Results). Nuclei isolation protocols that do not include fixation[22–24,26] often require an ultracentrifugation step and may require further optimization for staining of sensitive nuclear markers in compromised samples; for example, staining for particular transcription factors in postmortem tissue can be problematic using unfixed tissue.

Nuclei isolation strategies have been utilized to overcome the technical difficulties associated with obtaining intact cells from postmortem and/or frozen archived brain tissue. However, sorting of microglia, astrocytes and endothelial cells using non-nuclear markers from postmortem tissue has recently been reported using ethanol fixation[27].

Limitations

A relative weakness of this approach is that the cell type-specific markers used for FANS may capture heterogeneous cell populations. For example, PU.1 is a master transcription factor for myeloid cells and will capture myeloid populations including peripherally derived macrophages. In the brain, microglia are the major PU.1-expressing cell population. Our epigenomic data obtained from PU.1 nuclei[1] highly correlates with that derived from live 'ex vivo' microglia of high purity[28] and is distinct from PU.1 nuclei of peripheral myeloid cells obtained from a schwannoma tumor (Fig. 3). In addition, our nuclei isolation approach can be adapted for downstream single cell analysis of cell type-enriched populations. For example, protocols have been published for single nuclei RNA-seq using samples fixed with 1% formaldehyde[19,20] (as described in the Experimental Design section). Lastly, neurons, microglia, oligodendrocytes and astrocytes do not completely encapsulate all of the major cell types in the brain. Additional cell types and cell states can be explored, potentially by incorporating combinations of multiple nuclear markers. These could include endothelial cells and pericytes of the blood brain barrier, which is compromised during neurodegeneration[29,30]; and the distinction between excitatory and inhibitory neuronal subtypes. Cell states of interest could include homeostatic microglia and disease-associated macrophages, which have been identified as associated with amyloid plaques in AD[31–33]. Future testing of additional nuclear markers will allow isolation of these nuclei cell type populations and cell states associated with disease.

Materials

Biological Materials:

- Fresh or fresh-frozen tissue from human or mouse brain. Human brain tissue was obtained with informed consent under a protocol approved by the UC San Diego and Rady Children's Hospital Institutional Review Board (IRB # 171361). For all study participants, informed consent was obtained from the parents and when appropriate assent from the patient. The Vestibular Schwannoma tumor was obtained from a 15-year-old female Hispanic patient. All animal procedures were approved by the University of California San Diego Institutional Animal Care and Use Committee in accordance with University of California San Diego research guidelines for the care and use of laboratory animals. For mouse brain tissue, we used a whole C57BL/6 mouse brain from an 8–10-week-old animal that has been rapidly dissected and snap frozen. For mouse experiments that require only PU.1+ nuclei, the cerebellum is excluded prior to tissue dissociation to reduce sorting time, as the cerebellum is a neuronal-rich brain region.

CAUTION Brain tissue obtained from patients requires informed consent under a protocol approved by an Institutional Review Board.

CAUTION Animal experiments should be performed according to the relevant institutional and national guidelines and regulations.

Reagents:

- Dulbecco's PBS (DPBS) solution (Thermo Fisher Scientific; MT21031CV)

- Formaldehyde, 37% by Weight (Thermo Fisher Scientific; F79–1) **! CAUTION** Combustible liquid; harmful if swallowed, in contact with skin or if inhaled; causes serious eye irritation; causes skin irritation; may cause an allergic skin reaction; may cause cancer; may cause genetic defects; may cause respiratory irritation.
- Disuccinimidyl glutarate (DSG; ProteoChem; c1104)
- Dimethyl Sulfoxide (DMSO; MilliporeSigma; D2650) **! CAUTION** Combustible liquid; may cause eye and skin irritation in susceptible persons. May be harmful by inhalation or if swallowed.
- UltraPure DNase/RNase-Free Distilled Water (Thermo Fisher Scientific; 10977023)
- Glycine (MilliporeSigma; 4810)
- 1M Tris-HCl, pH 8.0 (Thermo Fisher Scientific; 15568025)
- 0.5M EDTA, pH 8.0 (Thermo Fisher Scientific; 15575020)
- 1M $MgCl_2$ (Thermo Fisher Scientific; AM9530G)
- Sucrose (Thermo Fisher Scientific; S6500)
- Triton X-100 (MilliporeSigma; T8787) **! CAUTION** Causes serious eye damage; harmful to aquatic life with long lasting effects.
- 1,4-Dithiothreitol (DTT; Thermo Fisher Scientific; BP172–5) **! CAUTION** Harmful if swallowed; causes skin irritation; causes serious eye irritation; may cause respiratory irritation.
- Bovine serum albumin (BSA; MilliporeSigma; A3059)
- Mouse anti-mouse/human NeuN Alexa Fluor 488 antibody [clone A60] (MilliporeSigma; MAB377X) https://scicrunch.org/resolver/AB_2149209
- Mouse anti-human PU.1 PE antibody [clone 7C6B05] (BioLegend; 658010) https://scicrunch.org/resolver/AB_2616863
- Rat anti-mouse PU.1 PE antibody [clone 7C2C34] (BioLegend; 681308) https://scicrunch.org/resolver/AB_2629618
- Rabbit anti-mouse/human PU.1 PE antibody [clone 9G7] (Cell signaling Technology; 81886S) https://scicrunch.org/resolver/AB_2799984
- Rabbit anti-mouse/human OLIG2 Alexa Fluor 647 antibody [clone EPR2673] (abcam; ab225100) https://scicrunch.org/resolver/AB_10861310
- Rabbit anti-human LHX2 (unconjugated) antibody (abcam; ab219983) https://scicrunch.org/resolver/AB_2868535
- Goat anti-rabbit IgG (H+L) Alexa Fluor 647 (Thermo Fisher Scientific; A21244) https://scicrunch.org/resolver/AB_2535812

- 4’,6-Diamidino-2-Phenylindole, Dilactate (DAPI; BioLegend; 422801) **! CAUTION** DAPI is a potential mutagen and should be handled with care.

Equipment:

- Class II A2 Biosafety Cabinet (Purifier® Logic®+ Class II A2 Biosafety Cabinet, Labconco®)
- Forceps
- Single-edged razor blades (Thermo Fisher Scientific; 12640)
- Corning Falcon Petri Dishes (Thermo Fisher Scientific; 08–757-100D)
- Kontes Cordless Motor (VWR; KT749540–0000)
- Kontes RNase-Free Pellet Pestle Grinders for 1.5 ml microtubes (VWR; KT-749521–1590)
- 1 ml wide bore pipette tips (Thermo Fisher Scientific; 21–236-2)
- 1.5 ml LoBind microtubes (Thermo Fisher Scientific; 13698791 / Eppendorf; 022431021)
- Falcon 15 mL conical centrifuge tubes (Thermo Fisher Scientific; 05–538-59A)
- Falcon 50 mL conical centrifuge tubes (Thermo Fisher Scientific; 14–432-22)
- Sterile vacuum bottle-top filters, 0.22 μm pore size (Thermo Fisher Scientific; SCGPS01RE)
- Syringe filter unit, 0.22 μm pore size (MilliporeSigma; SLGP033R)
- 50 ml Syringe without needle (Thermo Fisher Scientific;13–689-8)
- Rocking shaker platform (Reliable Scientific; model 55D)
- Refrigerated centrifuge for 15 ml and 50 ml tubes (Eppendorf Centrifuge; 5810R)
- Wheaton™ Dounce Tissue Grinders (Thermo Fisher Scientific; 06435A)
- Falcon cell strainers, 70 μm pore size (Thermo Fisher Scientific; 08–771-2)
- Refrigerated centrifuge for microfuge tubes (Eppendorf Centrifuge; 5424R)
- P2, P20, P200, and P1000 Pipettes (Gilson Pipetman; VWR; 76207–552)
- Drummond Original Portable Pipet-Aid Pipet Controller (Thermo Fisher Scientific; 1368119)
- Falcon Round-Bottom Polypropylene 5 ml Tubes (FACS tube; Thermo Fisher Scientific; 14–959-11A)
- Falcon Test Tube with Cell Strainer Snap Cap, 35 μm pore size (Thermo Fisher Scientific; 08–771-23)

- Beckman Coulter MoFlo Astrio EQ cell sorter (Alternatives: BD Influx or FACSAria II cell sorters)

Reagent setup:

Fixative Solution (1% (wt/vol) formaldehyde in DPBS): Add 270.27 μl 37% (wt/vol) formaldehyde to 9.73 ml DPBS (final concentration 1% (wt/vol) formaldehyde) and invert mix. Make fresh and use immediately. **! CAUTION** Prepare fixative solution in a chemical fume hood and wear appropriate PPE including lab coat, gloves and safety glasses. Dispose of materials contaminated with formaldehyde according to institutional guidelines.

2 mM DSG Solution: Dissolve 25 mg of disuccinimidyl glutarate in 3.83 mL DMSO. Add 34.45 mL of DPBS to this solution and mix to make the 2 mM DSG solution. Make fresh 2 mM DSG solution on day of use; safely discard excess 2 mM DSG solution. **! CAUTION** DMSO: combustible liquid; may cause eye and skin irritation in susceptible persons. May be harmful by inhalation or if swallowed.

1 M DTT stock solution: Dissolve 3.085 g DTT in 20 ml UltraPure water. Aliquot in 1ml and store at −20 °C for up to 3 months. **! CAUTION** Harmful if swallowed; causes skin irritation; causes serious eye irritation; may cause respiratory irritation.

0.1 M DTT working stock solution: Dilute 0.1 ml 1 M DTT stock solution in 0.9 ml UltraPure water. Store at −20 °C for up to 1 month.

2.5 M glycine stock solution: Dissolve 9.38 gram of glycine in 30 ml UltraPure water. Adjust final volume to 50 ml with UltraPure water. Syringe filter into a 50 ml tube and store at room temperature for up to 1 month.

10% (vol/vol) Triton X-100 stock solution: Add 5 ml Triton X-100 to 45 ml UltraPure water in a 50 ml tube and rock at around 30 cycles per minute at room temperature until in solution. Store protected from light at 4 °C for 6 months.

NF1 buffer: Add 5 ml 1 M Tris-HCl pH 8.0, 1 ml 0.5 M EDTA, 2.5 ml 1 M $MgCl_2$, 50 ml 1 M sucrose and 25 ml 10% (vol/vol) Triton X-100 to 416.5 ml UltraPure water. This corresponds to 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$, 0.1 M sucrose and 0.5% (vol/vol) Triton X-100, respectively. Store at 4 °C for up to 3 months.

1.2 M sucrose stock solution: Dissolve 102.69 gram of sucrose in 150 ml UltraPure water. Adjust final volume to 250 ml with UltraPure water. Filter sterilize into an autoclaved glass bottle. Check solution for contamination before each use. Uncontaminated sucrose solution can be stored at 4 °C for up to 3 months.

Sucrose cushion: Supplement 1.2 M sucrose solution with 10 mM Tris-HCl pH 8.0, 3 mM $MgCl_2$ and 1 mM DTT. The table below can be used as a guide for making 5 ml, 10 ml and 20 ml sucrose cushions. Prepare fresh on ice and use immediately. **! CAUTION** DTT is harmful if swallowed; causes skin irritation; causes serious eye irritation; may cause respiratory irritation.

Stock solutions	Final conc.	5 ml cushion	10 ml cushion	20 ml cushion
1.2 M sucrose	1.17 M	4.88 ml	9.76 ml	19.52 ml
1 M Tris-HCl, pH 8.0	10 mM	50 µl	100 µl	200 µl
1 M $MgCl_2$	3 mM	15 µl	30 µl	60 µl
100 mM DTT	1 mM	50 µl	100 µl	200 µl

10% (wt/vol) BSA stock solution in DPBS: Dissolve 5 gram of BSA in 40 ml DPBS. Adjust final volume to 50 ml with DPBS. Syringe filter sterilize 10% (wt/vol) BSA solution into a clean 50 ml tube. Store at 4 °C for up to 1 month.

FANS buffer: Add 5 ml 10% (wt/vol) BSA stock solution and 100 µl 0.5 M EDTA to 45 ml DPBS and store at 4 °C for up to 1 week (DPBS, 1% (wt/vol) BSA, 1 mM EDTA).

5 mg ml^{-1} DAPI stock solution: Dissolve 10 mg in 2 ml UltraPure water to make a 5 mg ml^{-1} DAPI stock solution. Store protected from light at −20 °C for up to 6 months. **! CAUTION** DAPI is a potential mutagen and should be handled with care.

0.5 mg ml^{-1} DAPI working stock solution: Add 10 µl 5 mg ml^{-1} DAPI stock solution to 90 µl UltraPure water to make a 0.5 mg ml^{-1} DAPI working stock solution. Store protected from light short term at 4 °C. **! CAUTION** DAPI is a potential mutagen and should be handled with care.

Procedure

Preparation of reagents and equipment · Timing ~15 min

1. Prepare 10 ml Fixative Solution in a 15 ml tube per sample and prepare 5 ml sucrose cushion per sample, fresh on the day of use as described in the Reagents Setup section.
2. Ensure that sufficient working solutions are available for the 2.5 M glycine stock solution, NF1 buffer, FANS buffer and 0.5 mg ml^{-1} DAPI working stock solution as described in the Reagents Setup section. These reagents should be maintained on ice or at 4°C.
3. Initiate airflow in biosafety cabinet for homogenization of human tissue.
4. Set centrifuges to 4 °C.

Tissue preparation • Timing ~30 min—Δ CRITICAL Brain tissue should remain frozen until homogenization of sample (step 10).

5. Pre-cool forceps, razor blade and petri dish on dry ice.
6. Transfer the frozen brain tissue from its storage container to the petri dish on dry ice using the cooled forceps and avoid contact of the tissue with the warmth of your hands.

! CAUTION Human tissue should be handled as pathogenic material prior to fixation (step 12). Handling of human tissue should be performed with appropriate PPE in a Class II A2 Biosafety Cabinet according to institutional guidelines.

7. Using the cooled razor, cut approximately 100 mg of brain tissue.

8. Using the cooled forceps, transfer the 100 mg brain tissue to a 1.5 ml tube and store on dry ice.

Δ CRITICAL STEP When using the tissue dissociation approach described in steps 10–14, the brain tissue size should not exceed ~150 mg in a 1.5 ml tube to avoid loss of material during homogenization.

9. Store any remaining brain tissue that is not needed at −80 °C. **? TROUBLESHOOTING**

Tissue dissociation and fixation • Timing ~15 min

10. Add 0.3 ml Fixative Solution to 100 mg brain tissue in a 1.5 ml tube. **? TROUBLESHOOTING**

! CAUTION Use Fixative Solution in a chemical fume hood (or biosafety cabinet if working with human material) and wear appropriate PPE including lab coat, gloves and safety glasses.

11. Immediately homogenize brain tissue in the fixative solution using the cordless motor with an RNase-Free Pellet Pestle Grinder. The pestle grinder should only be engaged after immersion into the fixative solution to avoid spraying.

Δ CRITICAL STEP Brain tissue should be homogenized immediately after adding fixative solution. Tissue that remains in the fixative solution will become increasingly difficult to homogenize over time. Tissue should appear uniformly homogenized without large chunks. This step should take less than 5 min to avoid over-fixation of the tissue.

12. Transfer the 0.3 ml homogenate to 9.7 ml Fixative Solution in a 15 ml tube using a 1 ml wide-bore pipette tip (total 10 ml volume). Optional: wash out the 1.5 ml tube with Fixative Solution to maximize the amount of homogenate transferred to the 15 ml tube.

13. Rock the homogenate at around 30 cycles per minute at room temperature for 10 min.

14. Add 0.5 ml 2.5 M glycine (final concentration 0.125 M glycine) to the homogenate and rock at around 30 cycles per minute at room temperature for 5 min.

Δ CRITICAL STEP Addition of glycine quenches the formaldehyde and prevents over fixation of the sample, which could be critical for downstream applications.

Isolation of nuclei • Timing ~2 hr

15. Spin the homogenate at 1,100xg for 5 min at 4 °C.

16. Remove the supernatant and resuspend the pellet with 10 ml ice-cold NF1 buffer (first wash).
17. Spin the homogenate at 1,100xg for 5 min at 4 °C.
18. Remove the supernatant and resuspend the pellet with 10 ml ice-cold NF1 buffer (second wash).
19. Spin the homogenate at 1,100xg for 5 min at 4 °C.
20. Remove the supernatant and resuspend the pellet with 5 ml ice-cold NF1 buffer.
21. Incubate the homogenate on ice for 30 min.

PAUSE POINT Homogenates can be incubated on ice for up to 2 hours without compromising the sample quality.

22. Transfer 5 ml homogenate to a 7 ml glass Dounce.
23. Pass the loose pestle through the homogenate 20 times or until large chunks have disappeared.
24. Pass the tight pestle through the homogenate 5 times.
25. Transfer the homogenate through a 70 μm strainer into a 50 ml tube.
26. Pass an additional 15 ml NF1 buffer through the 70 μm strainer into the same 50 ml tube (total volume 20 ml).
27. Underlay the 5 ml sucrose cushion below the homogenate by slowly pipetting the sucrose cushion underneath the homogenate onto the bottom of the 50 ml tube with a 5 ml serological pipette.

Δ CRITICAL STEP Underlay the sucrose cushion with care to ensure an interphase is maintained between the sucrose cushion at the bottom of the 50 ml tube and the homogenate on top. We have tested the nuclei isolation using different tissue sources and would suggest adjusting the sucrose cushion as follows: 1.2 M sucrose cushion for human resected cortical tissue, 1 M sucrose cushion for human postmortem cortical tissue and 1.2 M for whole mouse brain.

28. Spin at 3,900xg (or a maximum speed of 4,960xg) for 30 min at 4 °C with brakes on ‘low’.
29. The upper homogenate phase should appear almost clear. The interphase should contain a white layer of myelin. The nuclei should be visible as a white/beige pellet below the sucrose cushion. **? TROUBLESHOOTING**
30. Remove the upper aqueous phase and the interface using an aspirator or a 25 ml pipette.
31. Gently remove the sucrose cushion with a P1000 pipette.
32. Resuspend the nuclei pellet with 1 ml NF1 and transfer to a 15 ml tube.
33. Add 9 ml NF1 buffer and partially resuspend the pellet by pipette mixing to wash the nuclei.

34. Spin at 1,600xg for 5 min at 4 °C to pellet the nuclei. **CRITICAL STEP** For isolation of astrocyte nuclei proceed directly to step 47; for other cell types, proceed immediately to Step 35.

Antibody staining of nuclei for neurons, myeloid cells/microglia and oligodendrocytes • Timing ~16 hr

35. Resuspend the nuclei pellet with 10 ml FANS buffer.

36. Spin the nuclei at 1,600xg for 5 min at 4 °C.

37. Gently resuspend the nuclei in 0.3 ml FANS buffer using a P1000 pipette.

38. Prepare a FACS tube with 0.3 ml FANS buffer for the ‘unstained’ control and add 3 μl of nuclei (from step 37).

39. For each antibody being used, prepare a FACS tube with 0.3 ml FANS buffer for ‘Single Stain’ controls and add 3 μl of nuclei (from step 37).

40. Transfer the remaining ~0.3 ml nuclei from step 37 to a FACS tube for staining with antibodies for neurons, myeloid cells/microglia and oligodendrocytes (referred to as ‘Total Stain’).

41. Myeloid/microglia, neuronal and oligodendrocyte nuclei can be isolated from the same sample by staining for PU.1, NeuN and OLIG2 respectively. Add antibodies to single stain controls and total stain samples using the antibodies and dilutions in Table 1 for human nuclei and using the antibodies and dilutions in Table 2 for mouse nuclei.

Critical Step A lack of PU.1 nuclei staining may be an issue for compromised tissue sources such as human postmortem tissue. If this occurs, PU.1 antibody concentration can be increased to 1:100 and the staining incubation time extended up to 48h to potentially improve PU.1 nuclei yields.

42. Incubate nuclei with antibodies overnight at 4 °C.

PAUSE POINT Antibody incubation can be performed between 1 hour or 24 hours. Little difference is observed between short and longer incubations with antibodies tested other than for PU.1. A minimum overnight incubation is suggested for PU.1 staining when using nuclei from compromised samples such as postmortem tissue and can be extended up to 48 hours.

43. Next day, add 4 ml FANS buffer to nuclei.

44. Spin at 1,600xg for 5 min at 4 °C.

45. Resuspend unstained and Single Stain controls in 0.3 ml FANS buffer. Resuspend the Total Stain sample in 1 ml FANS buffer. Resuspend nuclei by pipetting at least 10 times.

46. Proceed with flow cytometry starting at Step 66.

Antibody staining of nuclei for astrocytes • Timing ~16 hr

47. Resuspend the nuclei from step 34 with 10 ml FANS buffer.

48. Spin the nuclei at 1,600xg for 5 min at 4 °C.

49. Gently resuspend the nuclei in 0.3 ml FANS buffer using a P1000 pipette.

50. Prepare a FACS tube with 0.3 ml FANS buffer for the 'unstained' control and add 3 μl of nuclei (from step 49).

51. For each antibody being used, prepare a FACS tube with 0.3 ml FANS buffer for 'Single Stain' controls and add 3 μl of nuclei (from step 49).

52. Transfer the remaining ~0.3 ml nuclei from step 49 to a FACS tube for staining with antibodies for neurons and astrocytes (referred to as 'Total Stain').

53. Astrocyte nuclei are sorted as NeuN-negative and LHX2-positive nuclei. To stain human samples, add antibodies to Single Stain controls and Total Stain samples using the antibodies and dilutions in Table 3.

Δ CRITICAL STEP Astrocytes and oligodendrocytes cannot be stained together as described in this protocol, unless an alternative fluorophore is used for either LHX2 or OLIG2 staining (see Experimental Design section for further details).

54. Incubate nuclei with antibodies overnight at 4 °C.

55. Next day, add 4 ml FANS buffer to Single Stain controls and Total Stain nuclei samples.

56. Spin at 1,600xg for 5 min at 4 °C.

57. Resuspend unstained and NeuN Single Stain controls in 0.3 ml FANS buffer. Resuspend nuclei by pipetting at least 10 times. Set aside until step 66.

58. Resuspend the LHX2 Single Stain control and Total Stain sample in 0.5 ml FANS buffer and mix by pipetting at least 10 times.

59. Dilute 1 μl Goat anti-rabbit IgG (H+L) Alexa Fluor 647 (Thermo Fisher Scientific; A21244) in 9 μl DPBS and mix to generate anti-rabbit IgG – 647 1:10 working solution.

60. Add 1.25 μl anti-rabbit IgG – 647 1:10 working solution (from step 59) to 0.5 ml LHX2 Single Stain control and Total Stain sample (from step 58).

61. Leave on ice in dark for 30 min.

PAUSE POINT Nuclei can be incubated in secondary antibody for up to 2 hours without compromising the sample quality.

62. Add 4 ml FANS buffer to the LHX2 Single Stain control and Total Stain sample.

63. Spin at 1,600xg for 5 min at 4 °C.

64. Resuspend the LHX2 Single Stain control in 0.3 ml FANS buffer and resuspend the Total Stain sample in 1 ml FANS buffer. Resuspend nuclei by pipetting at least 10 times.

65. Proceed with flow cytometry as described from Step 66.

Flow cytometry of nuclei cell type populations • Timing ~4 hr per 100 mg tissue

66. Pass nuclei (from Step 46 or 65) through a 35 μm cell strainer snap cap into a new FACS tube.

67. To counterstain the nuclei with 0.5 μg ml^{-1} DAPI, add 1 μl 0.5 mg ml^{-1} DAPI working stock solution to 1 ml Total Stain sample. **! CAUTION** DAPI is a potential mutagen.

68. Count nuclei using a hemocytometer and dilute to ~1 million nuclei per ml.

Δ CRITICAL STEP Nuclei that are too concentrated may block the sorter and delay collection. However, counting nuclei can be problematic due to the large amount of debris that remains during the isolation procedure. As an estimate, nuclei generated from ~100 mg brain tissue is diluted in ~4 ml FANS buffer for sorting. Bring extra FANS buffer to the cell sorter to allow further dilution of the sample as appropriate.

69. Prepare materials to take to the sorter. These should include the control and stained samples, clean FACS tubes for collection, extra FANS buffer and 0.5 μg ml^{-1} DAPI.

70. Sort fixed nuclei using a 70 μm or 100 μm nozzle. The 70 μm nozzle is recommended for increasing the speed of sorting fixed nuclei. However, the 70 μm nozzle will be more prone to clogging the FACS machine compared to the 100 μm nozzle. If the sample clogs the FACS machine, the nuclei will need to be diluted with additional FANS buffer.

71. Use the unstained control and the Single Stain controls to set up the compensation for the FACS sorter.

Δ CRITICAL STEP Assistance is required to operate the FACS machine except for experienced users.

72. (Optional) Add 0.5 mg ml^{-1} DAPI at 1:1000 to the unstained and Single Stain controls to use for setting up the gates described in steps 73 – 79 before running the Total Stain sample. **? TROUBLESHOOTING**

73. Using the Total Stain sample, generate a dot plot of forward scatter (FSC)-Height versus DAPI-height-log to select for DAPI-positive nuclei (Fig. 2a).

74. Generate a dot plot of side scatter (SSC)-Height versus SSC-Area to select for single nuclei and exclude doublets (Fig. 2b).

75. Generate a dot plot of SSC-Height versus SSC-Width for further selection of single nuclei and exclusion of doublets (Fig. 2c).

76. Generate a dot plot of NeuN-488 and PU.1-PE for identifying neuronal and myeloid/microglia nuclei (Fig. 2d).

77. Generate a dot plot of NeuN-488 and OLIG2–647 for identifying neuronal and oligodendrocyte nuclei (Fig. 2e).

78. Sort NeuN-positive (neuron), OLIG2-positive (oligodendrocyte) and PU.1-positive (myeloid/microglia) events into separate FACS tubes containing 500 μl FANS buffer kept at 4 °C. We recommend using a high purity sorting mode. **? TROUBLESHOOTING**

79. For samples that are stained for astrocyte nuclei, generate a dot plot of NeuN-488 and LHX2–647. Astrocyte nuclei are sorted as NeuN-negative and LHX2-positive events (Fig. 2f).

80. The purity of nuclei populations can be assessed by reanalyzing the sorted nuclei. For reanalysis, take 5% of the sorted nuclei and resuspend in 300 μl FANS buffer in a new FACS tube; then analyze the diluted nuclei using the same parameters that were set for the original sort. Following reanalysis of high purity nuclei populations, greater than 95% of the nuclei will fall within the original gates used for sorting.

Nuclei storage for downstream applications • Timing ~20 min

81. Adjust sorted nuclei samples to 1% (wt/vol) BSA using the 10% (wt/vol) BSA in DPBS stock solution and pipette mix. For example, for a final sort volume of 2 ml, add 0.2 ml 10% (wt/vol) BSA in DPBS.

Δ CRITICAL STEP Addition of BSA improves spinning down of nuclei after FACS sorting and will improve the final yield of nuclei recovered.

82. Spin at 1,600xg for 15 min at 4 °C to collect nuclei for further processing.

83. Remove FANS buffer.

PAUSE POINT Downstream processing can be performed immediately, or the nuclei pellet can be snap-frozen for storage at −80 °C. We have been able to generate high quality H3K27ac ChIP-seq data using NeuN nuclei sorted from postmortem tissue and stored at −80 °C for 2.5 years. Storage of sorted nuclei for longer has not been tested and may be feasible.

Δ CRITICAL STEP Nuclei can be stored at −80 °C prior to applications that require fixed nuclei as the starting material, such as protocols for ChIP-seq[9], PLAC-seq[11], HiChIP[12] and HiC[34]. Nuclei should ideally be used immediately for ATAC-seq applications using a protocol specific for fixed material[13].

Troubleshooting

Step: 9

Problem: Tissue quality deteriorates over time due to repeated handling of tissue.

Possible Reason(s): Repeated handling and removal of tissue from the freezer can compromise the quality of the tissue. This may still occur even if the tissue is not allowed to completely thaw.

Possible solution(s): When preparing fresh tissue for freezing or when handling frozen samples for the first time, aliquot tissue as adequately sized pieces for a single experiment (~100–150 mg) in separate tubes to avoid repeat removal of the entire specimen.

Step: 10

Problem: ChIP-seq is required for transcription factors that are difficult to detect using mild fixation such as 1% (wt/vol) formaldehyde.

Possible Reason(s): Whereas histones are tightly bound to DNA, particular transcription factors may be part of large chromatin complexes and require additional fixation.

Possible Solution(s): Homogenize the sample in 0.3 ml 2 mM DSG solution in a 1.5 ml tube. Transfer homogenate to 9.7 ml 2 mM DSG solution in a 15 ml tube (total 10 ml volume). Rock at around 30 cycles per minute at room temperature for 30 min. Add 270.27 µl 37% (wt/vol) formaldehyde to the 10 ml homogenate to make a 1% (wt/vol) formaldehyde solution and rock at around 30 cycles per minute for a further 10 min. Quench the sample with 0.125 M glycine as described in step 14 and proceed with the remainder of the protocol. Depending on the cell type and transcription factor of interest, the DSG concentration and incubation time may need to be adjusted.

Step: 10

Problem: Light fixation as described in our protocol leads to preservation of unstable proteins like PU.1 but interferes with other applications such as RNA-seq.

Possible Reason(s): Our protocol has been optimized for ChIP-seq and not for RNA-seq. LHX2 and PU.1 are unstable proteins and omitting the fixation step may lead to loss of PU.1 and LHX2 signal.

Possible Solution(s): If transcriptomic profiling is required, cross-linking has to be reversed according to published protocols or with modifications[17]. For certain proteins of interest such as NeuN and OLIG2, no fixation is required. We suggest adding RNase inhibitors (e.g. 0.2 U μl^{-1} RNasin, Promega; N2111) and Protease inhibitors (e.g. 1X final conc. Roche cOmplete Protease Inhibitor Cocktail; MilliporeSigma; 11697498001) to each buffer. Alternatively, for high quality tissue samples it may be possible to omit the fixation step to sort unfixed nuclei; for unfixed samples it is recommended to adjust the density of the sucrose cushion to 1M or lower.

Step: 29

Problem: No nuclei pellet is visible below the sucrose cushion after spinning.

Possible Reason(s): The nuclei are trapped at the interphase and did not pellet below the sucrose cushion. This may occur due to differences in species used, tissue source and quality.

Possible Solution(s): Reduce the density of the sucrose cushion by decreasing the molarity of the sucrose from 1.2 M to 1 M or lower. We have tested the nuclei isolation using different tissue sources and have adjusted the sucrose cushion as follows: 1.2 M sucrose cushion for human resected cortical tissue, 1 M sucrose cushion for human postmortem cortical tissue and 1.2 M for whole mouse brain.

Step: 29

Problem: No material is trapped at the interface after spinning.

Possible Reason(s): The sample may have low levels of myelin and debris, in which case you can proceed as normal to wash the nuclei pellet. It is also possible that the debris and myelin are not trapped at the interphase and have pelleted with the nuclei. This will generate a larger than expected pellet and will take longer to sort on the FACS machine. This may occur due to differences in species used, tissue source and quality.

Possible Solution(s): Increase the density of the sucrose cushion by increasing the molarity of the sucrose from 1.2 M to 1.4 M or higher. Increasing the molarity of the sucrose cushion leads to increased removal of debris/myelin and thus increases the purity of the nuclei pellet. A possible caveat, is a reduced yield of nuclei per cell population.

Step: 71

Problem: High background fluorescence observed in the unstained control (with no antibody staining).

Possible Reason(s): Poor tissue quality can lead to high background fluorescence signal, or autofluorescence, which may be a consequence of increased oxidation of the tissue. This is more problematic for postmortem samples compared to freshly resected tissue.

Possible Solution(s): Autofluorescence in postmortem samples is detected in multiple channels, including 488, 647 and PE. To avoid collecting autofluorescent nuclei, generate dot plots using two channels to set your gates (even when performing a single stain), rather than using histograms. The autofluorescent nuclei will appear as positive in both channels whereas target nuclei will be positive in a single channel.

Step: 76 – 79

Problem: High background fluorescence observed with antibody staining.

Possible Reason(s): High background signal may be caused by non-specific antibody binding, which may occur if the antibody concentration used is too high.

Possible Solution(s): For new antibodies, performing a titration is required to determine the optimal concentration of the antibody. Isotype controls are needed to differentiate non-specific background signal from specific antibody signal.

Isotype control antibodies suggested for use with primary antibodies listed in this protocol:

Primary Antibody	Isotype Antibody
Mouse anti-mouse/human NeuN Alexa Fluor 488 antibody [A60] (MilliporeSigma; MAB377X) https://scicrunch.org/resolver/AB_2149209	Mouse IgG1, κ Isotype Ctrl Alexa Fluor 488 antibody [MOPC-21] (MilliporeSigma; FCMAB310A4) https://scicrunch.org/resolver/AB_10806480
Mouse anti-human PU.1 PE antibody [7C6B05] (BioLegend; 658010) https://scicrunch.org/resolver/AB_2616863	Mouse IgG1, κ Isotype Ctrl (ICFC) PE antibody [MOPC-21] (BioLegend; 400139) https://scicrunch.org/resolver/AB_4934438
Rat anti-mouse PU.1 PE antibody [7C2C34] (BioLegend; 681308) https://scicrunch.org/resolver/AB_2629618	Rat IgG2a, κ Isotype Ctrl PE antibody [RTK2758] (BioLegend; 400507) https://scicrunch.org/resolver/AB_326530
Rabbit anti-mouse/human OLIG2 Alexa Fluor 647 antibody [EPR2673] (abcam; ab225100) https://scicrunch.org/resolver/AB_10861310	Rabbit IgG, Isotype Ctrl Alexa Fluor 647 antibody [EPR25A] (abcam; ab199093) https://scicrunch.org/resolver/AB_2818935
Rabbit anti-human LHX2 (unconjugated) antibody (abcam; ab219983) https://scicrunch.org/resolver/AB_2868535	Rabbit IgG Isotype Ctrl (unconjugated) antibody (abcam: ab171870) https://scicrunch.org/resolver/AB_2687657

Step: 78

Problem: Zero or few PU.1-positive microglia/myeloid nuclei were detected by FACS after antibody staining.

Possible Reason(s): The most common cause of no PU.1 staining is due to poor tissue quality. This is particularly problematic with long postmortem intervals for autopsy samples or poor tissue handling during acquisition and freezing.

Possible Solution(s): For postmortem tissue, sample selection criteria should include low postmortem intervals if possible. Ideally samples should be kept on ice immediately upon availability and should be snap frozen as soon as possible. Samples should be immunostained overnight at 4°C; if staining is poor but detectable it is possible to increase the concentration of PU.1 antibody to 1:100 and to extend the antibody incubation time up to 48 hours.

Step: 76–79

Problem: Gradual loss of antibody staining of specimens after repeated freeze-thaw cycles.

Possible Reason(s): Repeated freeze-thaw events lead to protein degeneration.

Possible Solution(s): Aliquot specimens appropriately (e.g. ~150 mg aliquots in 1.5 ml tubes) to avoid repeated freeze-thaw cycles and thereby reduce protein degradation.

Timing

Steps 1 – 4, Preparation of reagents and equipment: ~15 min

Steps 5 – 9, Tissue preparation: ~30 min

Steps 10 – 14, Tissue dissociation and fixation: ~15 min

Steps 15 – 34, Isolation of nuclei: ~2 hr

Steps 35 – 46, Antibody staining of nuclei for neurons, microglia and oligodendrocytes: ~16 hr

Steps 47 – 65, Antibody staining of nuclei for astrocytes: ~16 hr

Steps 66 – 80, Flow cytometry of nuclei cell type populations: ~4 hr per 100 mg tissue

Steps 81 – 83, Nuclei storage for downstream applications: ~20 min

Anticipated Results

We have presented a protocol to isolate nuclei of brain cell type populations for neurons, microglia, oligodendrocytes, and astrocytes using FANS from frozen resected cortical tissue of epilepsy patients[1]. From ~150 mg resected cortical brain tissue from pediatric epilepsy patients, we routinely isolate ~1 M neuronal nuclei, ~400,000 myeloid/microglia nuclei, ~1.5 M oligodendrocyte nuclei and ~100,000 astrocyte nuclei. From ~120 mg rapid autopsy human postmortem cortical brain tissue, we routinely isolate ~600,000 neuronal nuclei, ~100,000 myeloid/microglia nuclei, ~900,000 oligodendrocyte nuclei. The number of myeloid/microglia nuclei can be highly variable depending on tissue quality. Obtaining myeloid/microglia nuclei from postmortem tissue requires a short postmortem interval (PMI) and rapid freezing to ensure robust detection of PU.1-positive nuclei. If possible, samples with a PMI under 6 hours should be selected; if available, samples from rapid autopsies would be prioritized that have a PMI around 3 hours. A longer PMI of over 24 hours and/or inappropriate tissue handling will lead to a low-to-zero yield of PU.1-positive nuclei, while other cell type populations will still be detectable. A higher yield of PU.1 nuclei from postmortem tissue may be achievable by increasing the antibody concentration and extending the staining incubation time up to 48 hours. High complexity ATAC-seq and H3K27ac ChIP-seq libraries can be generated with 200,000 and 500,000 nuclei respectively, according to published protocols[1,9,28,35,36].

The nuclei isolation protocol can used to isolate nuclei from rodent brains with ease when using tissue that has been rapidly dissected and snap frozen. A whole C57BL/6 mouse brain from an 8–10-week-old animal yields approximately 3 M neuronal nuclei, 1.8 M oligodendrocyte nuclei and 300,000 – 400,000 myeloid/microglia nuclei. The latter compares to 250,000 – 300,000 live microglia cells isolated from one C57BL/6 mouse brain using a slow mechanical dissociation on ice[37]. Myeloid/microglia nuclei were isolated using two different fixation procedures, single fixation with 1% formaldehyde and a double fixation with 2 mM DSG followed by 1% formaldehyde (see Steps 10–14 and Troubleshooting Step 10 respectively) without a noticeable difference in yield. However,

other fixation approaches may result in differences in myeloid/microglia nuclei yield and will need to be individually optimized.

As discussed in the limitations, the PU.1-positive nuclei population represent a heterogeneous group of myeloid cells. The PU.1-positive nuclei datasets were directly compared to equivalent datasets for live *ex vivo* microglia, isolated at high purity from freshly resected tissue by FACS[28]. We further compared these to nuclei generated from a Vestibular Schwannoma, a tumor of the peripheral nervous system, that is enriched for peripheral-derived myeloid cells distinct from microglia. Transcription factors required for general myeloid cell identity, including PU.1 (*SPI1*) and IRF8, are expressed in all three datasets (Fig. 3a, b). In contrast, the SALL1 transcription factor, thought to be critical for microglia identity compared to other myeloid populations, is detected in both PU.1-nuclei and *ex vivo* microglia but is absent in the peripheral-derived myeloid population (Fig. 3c). Pearson's correlation analysis of the ATAC-seq and H3K27ac ChIP-seq signal distribution demonstrates the similarity of the microglia nuclei data with that of the *ex vivo* live microglia and its distinction from the peripheral-derived myeloid population (Fig. 3d, e). These datasets suggest that the PU.1-positive nuclei populations isolated from human cortical tissue predominantly represent microglia over other myeloid populations.

ATAC-seq and ChIP-seq analysis

ATAC-seq and ChIP-seq datasets for *ex vivo* microglia and brain-derived PU.1 nuclei were previously published[1,28]. Each sample within ATAC-seq and ChIP-seq datasets were generated using a distinct patient. ChIP-seq peaks were called with HOMER for each H3K27ac sample using the parameters -style histone -minDist 1000 -size 500 against the corresponding inputs. ATACseq peaks were called with HOMER for each sample using the parameters -style factor -minDist 200 -size 200; no inputs were used. Pooled tags from all replicates for each group were used for UCSC track visualization. To quantify the Pearson's correlation, peaks for each group were merged using HOMER's mergePeaks, and tag counts normalized to 10 million were extracted at merged peaks using HOMER's annotatePeaks. Pearson's correlation was calculated using log2(normalized Tags + 1) using the cor function from stats (version 3.4.0) in RStudio (version 1.0.143). Heatmaps were visualized using pheatmap (version 1.0.12).

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data Availability

The processed ATAC-seq and H3K27ac ChIP-seq tracks for the Schwannoma myeloid cells has been made available as part of a previously published UCSC genome browser session (hg19) containing ATAC-seq, ChIP-seq, and PLAC-seq datasets for brain cell types[1]: https://genome.ucsc.edu/s/nottalexi/glassLab_BrainCellTypes_hg19. ATAC-seq and ChIP-seq datasets for *ex vivo* microglia and brain-derived PU.1 nuclei were previously published[1,28] and are available on dbGap (https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs001373.v2.p2)

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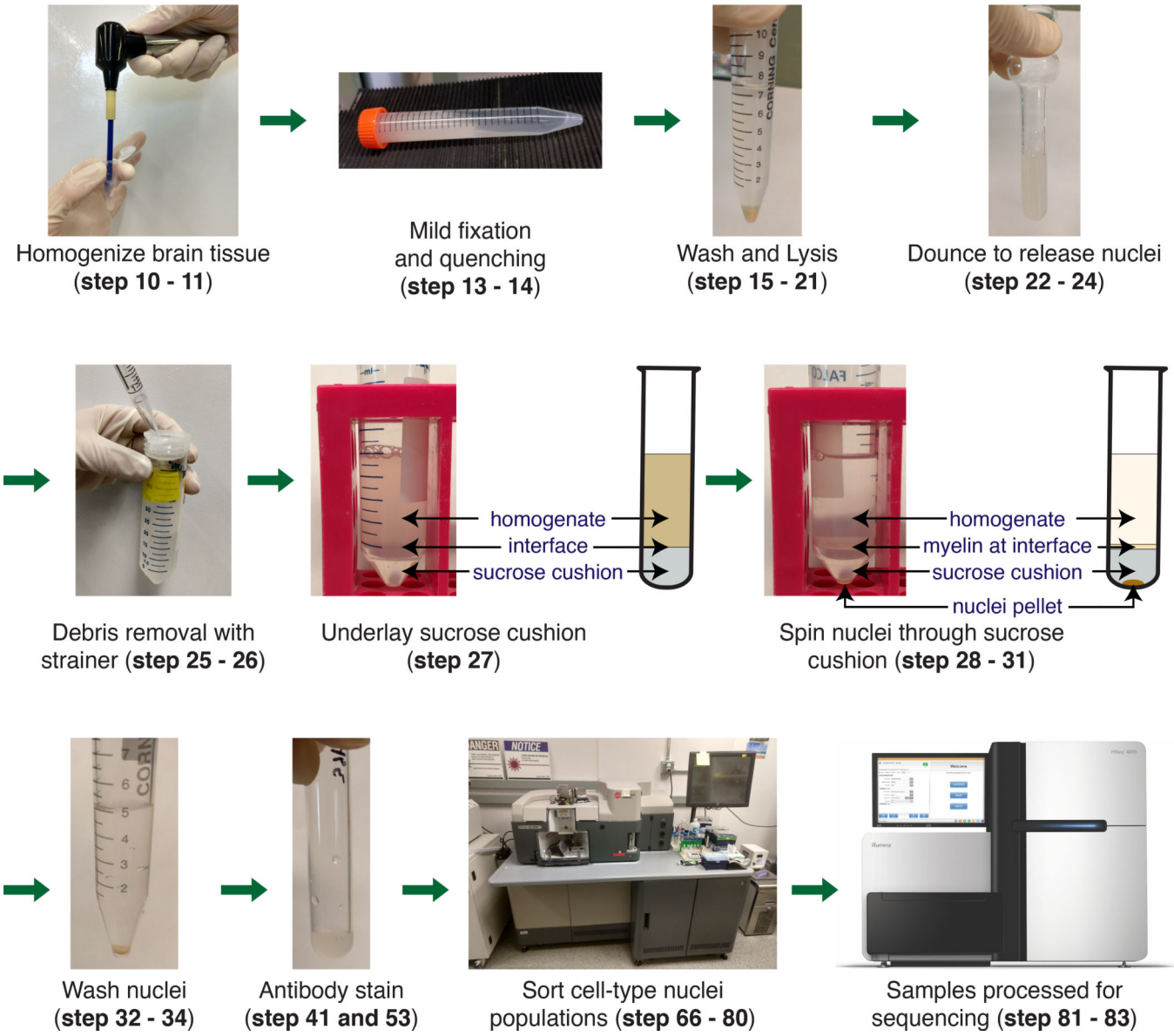

Fig. 1 |. Schematic of cell type-specific nuclei isolation from frozen brain tissue.

The brain is homogenized in 1% (wt/vol) formaldehyde in DPBS (steps 10 – 11). The homogenate is fixed for 10 mins followed by a 5 min quenching (steps 13 – 14). Homogenate is washed and then lysed in NF1 buffer on ice for 30 min (steps 15 – 21). Nuclei are released by douncing (steps 22 – 24). To remove large debris the homogenate is passed through a 70 μm strainer (steps 25 – 26). To remove the myelin a sucrose cushion is first underlayed (steps 27). The nuclei are then pelleted by centrifugation and the myelin remains at the interphase (steps 28 – 31). The nuclei pellet is washed with NF1 buffer and FACS buffer (steps 32 – 34). The nuclei are stained for cell type-specific markers (steps 41 and 53). Nuclei populations are isolated by sorting on a FACS machine (steps 66 – 80). Nuclei are processed for downstream sequencing applications (steps 81 – 83).

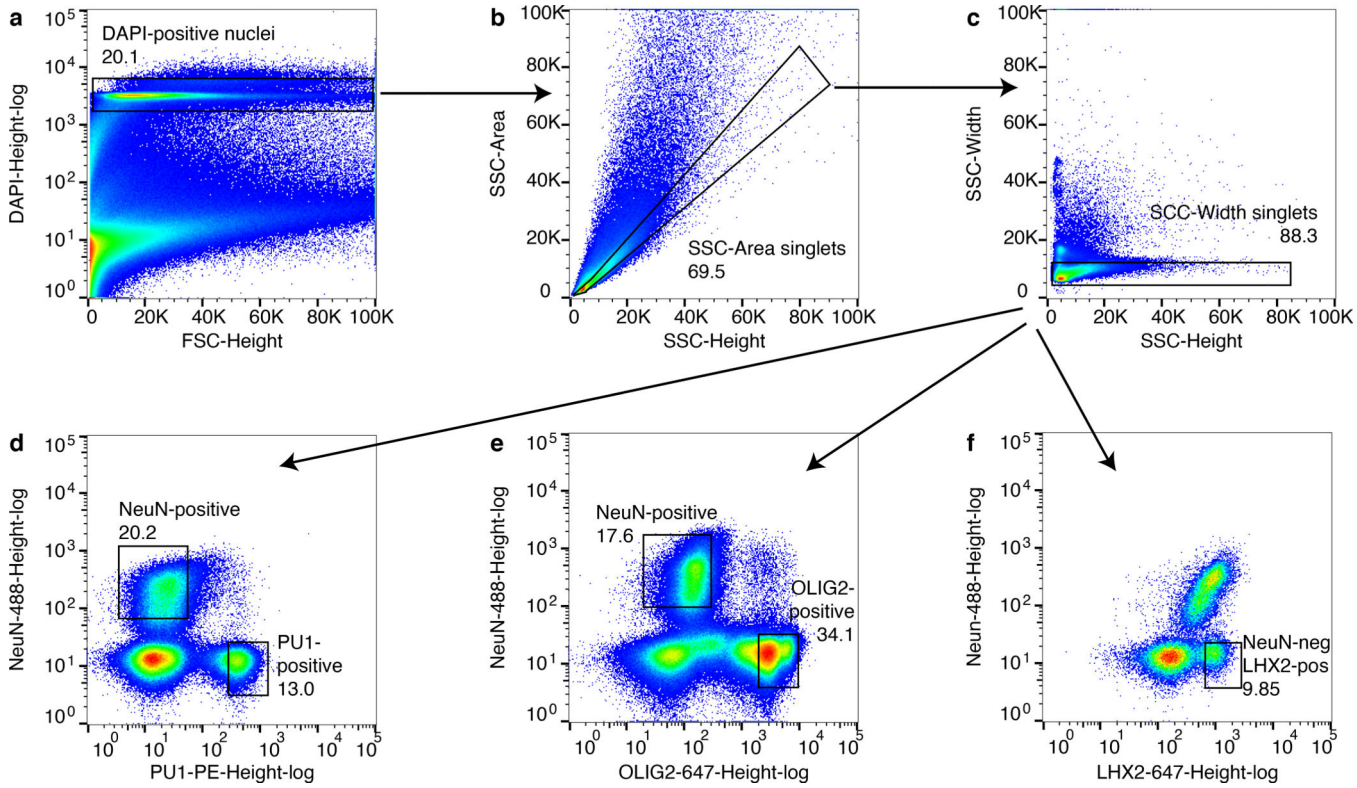

Fig. 2 |. FANS gating strategy for sorting of cell type nuclei populations.

Data representation of NeuN, PU.1 and OLIG2 nuclei isolated from cortical tissue of a pediatric epilepsy patient[1]. (a-f) Representative FACS plots generated on the MoFlo Astrios and visualized with FlowJo V10 with percentage of parent gates for each population (steps 73 – 79). (a) DAPI positive nuclei are selected (step 73) and (b - c) doublets are excluded (74–75). (d) Gating for NeuN-positive neuronal nuclei and PU.1-positive myeloid/microglia nuclei (step 76) and (e) NeuN-positive neuronal nuclei and OLIG2-positive oligodendrocyte nuclei (step 77). (f) Gating for NeuN-negative; LHX2-positive astrocyte nuclei (step 79).

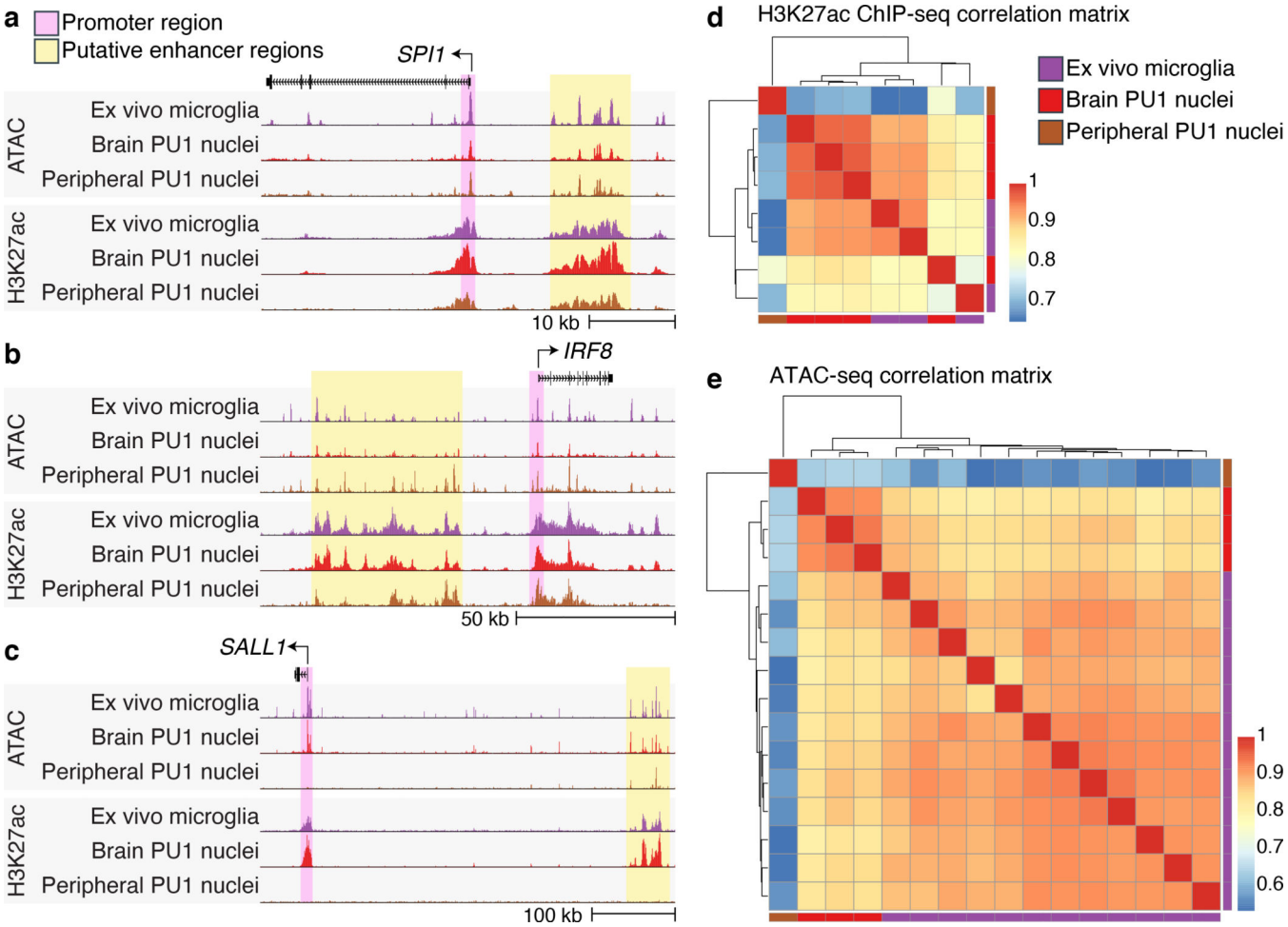

Fig. 3 |. Enhancer atlases from *ex vivo* microglia and PU.1 nuclei.

(**a - c**) Representative UCSC browser view of ATAC-seq and H3K27ac ChIP-seq from *ex vivo* microglia[28], brain-derived PU.1 nuclei[1] and peripheral-derived PU.1 nuclei at the (**a**) *SPI1* locus, (**b**) *IRF8* locus and the (**c**) *SALL1* locus. (**d**) Pearson's correlation of H3K27ac ChIP-seq signal from *ex vivo* microglia[28] (N = 3), brain-derived PU.1 nuclei[1] (N = 4) and peripheral-derived PU.1 nuclei (N = 1). (**e**) Pearson's correlation of ATAC-seq signal from *ex vivo* microglia[28] (N = 12), brain-derived PU.1 nuclei[1] (N = 3) and peripheral-derived PU.1 nuclei (N = 1).

Table 1 |

Antibodies used for staining human nuclei of neuronal, myeloid/microglia and oligodendrocyte origin

	Mouse anti-SPI1 (PU.1)-PE (0.1 mg ml^{-1}) BioLegend; 658010 [clone 7C6B05], https://scicrunch.org/resolver/AB_2616863	**Mouse anti-NeuN-Alexa Fluor 488 (1 mg ml^{-1}) MilliporeSigma; MAB377X [clone A60], https://scicrunch.org/resolver/AB_2149209**	**Rabbit anti-OLIG2-Alexa Fluor 647 (0.5 mg ml^{-1}) Abcam; ab225100 [clone EPR2673], https://scicrunch.org/resolver/AB_10861310**
unstained	none	None	none
PU.1 Single Stain	1:500	none	none
NeuN Single Stain	none	1:2,500	none
Olig2 Single Stain	none	none	1:2,500
Total Stain	1:500	1:2,500	1:2,500

Table 2 |

Antibodies used for staining mouse nuclei of neuronal, myeloid/microglia and oligodendrocyte origin

	Rat anti-SPI1 (PU.1)- PE (0.2 mg ml^{-1}) BioLegend; 681308 [clone 7C2C34 https://scicrunch.org/resolver/AB_2629618	**Mouse anti-NeuN-Alexa Fluor 488 (1 mg ml^{-1}) MilliporeSigma; MAB377X [clone A60], https://scicrunch.org/resolver/AB_2149209**	**Rabbit anti-OLIG2-Alexa Fluor 647 (0.5 mg ml^{-1}) Abcam; ab225100 [clone EPR2673], https://scicrunch.org/resolver/AB_10861310**
unstained	none	none	none
PU.1 Single Stain	1:100	none	none
NeuN Single Stain	none	1:2,500	none
Olig2 Single Stain	none	none	1:2,500
Total Stain	1:100	1:2,500	1:2,500

Table 3 |

Antibodies used for staining human nuclei of astrocyte origin

	Mouse anti-NeuN-Alexa Fluor 488 (1 mg ml^{-1}) MilliporeSigma; MAB377X [clone A60], https://scicrunch.org/resolver/AB_2149209	**Rabbit anti-LHX2; unconjugated (0.1 mg ml^{-1}) abcam; ab219983, https://scicrunch.org/resolver/AB_2868535**
unstained	none	none
NeuN Single Stain	1:2,500	none
LHX2 Single Stain	none	1:500
Total Stain	1:2,500	1:500