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TITLE:

Isolation of Adult Human Astrocyte Populations from Fresh-frozen Cortex Using Fluorescence-activated Nuclei Sorting

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Astrocytes; FANS; Nuclei Isolation; Glia; Cortex; Human

SUMMARY:

We have developed a method that enriches for and isolates human astrocyte populations from fresh-frozen tissue for use in downstream molecular analyses.

ABSTRACT:

The complexity of human astrocytes remains poorly defined in primary human tissue, requiring better tools for their isolation and molecular characterization. Fluorescence-activated nuclei sorting (FANS) can be used to successfully isolate and study human neuronal nuclei (NeuN+) populations from fresh-frozen archival tissue, thereby avoiding problems associated with fresh tissue. However, efforts to similarly isolate astroglia from the non-neuronal (NeuN–) element are lacking. A recently developed and validated immunotagging strategy uses three transcription factor antibodies to simultaneously isolate enriched neuronal (NeuN+), astrocyte (paired box protein 6 (PAX6)+NeuN–), and oligodendrocyte progenitor (OLIG2+NeuN–) nuclei populations from non-diseased, fresh-frozen, postmortem human temporal neocortex.

This technique was shown to be useful for the characterization of cell type-specific transcriptome alterations in primary pathological epilepsy neocortex. Transcriptomic analyses confirmed that PAX6+NeuN– sorted populations are robustly enriched for pan-astrocyte

markers and capture astrocytes in both resting and reactive conditions. This paper describes the FANS methodology for the isolation of astrocyte-enriched nuclei populations from fresh-frozen human cortex, including tissue dissociation into single-nucleus (sn) suspension; immunotagging of nuclei with anti-NeuN and anti-PAX6 fluorescently conjugated antibodies; FANS gating strategies and quality control metrics for optimizing sensitivity and specificity during sorting and for confirming astrocyte enrichment; and recommended procurement for downstream transcriptome and chromatin accessibility sequencing at bulk or sn resolution. This protocol is applicable for non-necrotic, fresh-frozen, human cortical specimens with various pathologies and recommended postmortem tissue collection within 24 h.

INTRODUCTION:

The molecular complexity of human astrocytes remains poorly defined in primary tissue, requiring better tools for their isolation and characterization at high resolution, both in health and disease. Separation of intact human neurons and glia from their niche has proven difficult due to limited access of fresh brain tissue samples, the heavily interconnected nature of glial and neuronal processes, and inevitable cellular activation during processing, all of which limit the molecular characterization of these cell types *ex vivo*[1]. Fluorescence-activated nuclei sorting (FANS) has emerged as an alternative to live-cell sorting, enabling the dissociation and immunotagging of nuclei populations from fresh-frozen tissue. In the past decade, FANS has become widely used for isolating and molecularly characterizing human neuronal (NeuN+) nuclei populations in a variety of brain specimens and anatomical regions[1-4].

However, similar methods for isolating specific glial nuclei subpopulations from human cortex have been limited, leading to a relative lack of sophistication in the understanding of astrocyte complexity in both normal and diseased tissues. To this end, a previously published protocol was adapted for isolating human neuronal populations using FANS[4], and a method was validated to enrich for astrocytes (and for oligodendroglial progenitors) using a triple-antibody combination, capturing astrocytes in both resting and reactive conditions[5]. To specifically enrich for astrocytes in the NeuN– fraction, antibodies were used against one of two transcription factors known to be differentially expressed across astrocyte populations, PAX6 or SRY-box transcription factor 9 (SOX9)[6,7]. PAX6 is highly expressed during early fetal development within radial glia-like progenitors in germinal zones and contributes toward both neurogenesis and gliogenesis[8-11] as well as to retinal neuronal specification[12].

In the adult, PAX6 is differentially overexpressed in resting human astrocytes[6] and shows protein co-expression with glial fibrillary acidic protein (GFAP) in human epilepsy tissue astrocytes[13]. This protocol describes the simultaneous isolation of neocortical neuronal and astrocyte-enriched nuclei populations by FANS. Fresh-frozen, postmortem tissue collected from adult cortex is first mechanically and chemically dissociated. After lysis and ultracentrifugation in a sucrose gradient, the cytoplasmic and extracellular components are discarded while the nuclei are retained. Nuclei are then labeled with fluorescently conjugated nuclear antibodies corresponding to the desired target lineages and sorted using FANS. Following this approach, enrichment of astrocytes is demonstrated in the collected PAX6+NeuN– populations, validated both by a targeted qPCR panel as well as by downstream nuclear RNA sequencing.

PROTOCOL:

NOTE: The Program for the Protection of Human Subjects at the Icahn School of Medicine at Mount Sinai (ISMMS) and its Institutional Review Board (IRB) assures the ethical conduct of research and compliance with federal, state, and institutional regulations. In this study, all postmortem specimens used were de-identified, obtained under appropriate consent through the biorepository, and were exempt from "human research" designation by ISMMS's IRB (HS#14-01007). If performing downstream RNA sequencing, thoroughly treat all workspaces and tools with RNase Decontamination Solution to prevent mRNA degradation.

1. Buffer preparation

1.1. Prepare lysis buffer

1.1.1. Dissolve the following in distilled H_2O up to 50 mL: 5.47 g of sucrose, 250 µL of 1 M $CaCl_2$, 150 µL of 1 M $(CH_3COO)_2$ Mg, 10 µL of 500 mM ethylenediaminetetraacetic acid (EDTA), 500 µL of 1 M Tris-HCl (pH 8), 50 µL of non-ionic surfactant (see the **Table of Materials**), and 17 µL of 3 M dithiothreitol (DTT) (add fresh).

1.2. Prepare sucrose buffer

1.2.1. Dissolve the following in distilled H_2O up to 50 mL: 30.78 g of sucrose, 150 µL of 1 M $(CH_3COO)_2$ Mg, 500 µL of 1 M Tris-HCl (pH 8), 17 µL of 3 M DTT (add fresh).

2. Frozen tissue dissociation into single-nucleus suspension

2.1. Add 4 mL of ice-cold lysis buffer to a 7 mL glass tissue douncer (grinder), and keep on ice. Dissect approximately 200–400 mg of fresh-frozen human adult cortex, dounce approximately 50x, and transfer the tissue homogenate to a 12 mL ultracentrifuge polypropylene tube.

2.2. Using a 5 mL pipette, add 6.5 mL of ice-cold sucrose buffer to the bottom of the ultracentrifuge tube, taking care not to disturb the layer between sucrose and the tissue homogenate.

NOTE: If processing additional samples, carefully balance the tubes by weight by adding additional lysis buffer to the lighter sample. Precise balancing of the ultracentrifuge tubes is essential to prevent damage to the rotor and ensure rotation at the proper speed.

3. Ultracentrifugation

3.1. Ultracentrifuge the lysate at 101,814 × *g* for 1 h at 4 °C. Carefully aspirate the supernatant and debris without disturbing the pellet.

NOTE: A pellet may not be visible if starting with less than 200 mg of tissue.

3.2. Add 0.1% w/v bovine serum albumin (BSA) in phosphate-buffered saline (PBS) to the ultracentrifuge tubes, and incubate on ice for 10 min before resuspending the nuclei pellet.

3.3. Use a hemocytometer to visualize the intact nuclei under a microscope and to ensure the concentration of the resuspension is above 10^5 nuclei/mL before proceeding to the next step (combine 10 µL of the resuspended nuclei with 10 µL of trypan blue).

4. Antibody incubation

4.1. To the resuspended sample, add mouse anti-NeuN conjugated to AF555 (see the **Table of Materials**) and mouse anti-PAX6 conjugated to allophycocyanin (APC).

NOTE: Alternative antibodies conjugated to APC or additional antibodies conjugated to other fluorophores may be added (see discussion). Here, mouse anti-OLIG2 conjugated to AF488 was used with favorable results.

4.2. Perform 4',6-diamidino-2-phenylindole (DAPI)-only and single-color controls to set up gating parameters, using a small amount of the sample as necessary.

4.2.1. Add approximately 20 µL of the resuspended sample to an antibody solution containing only mouse anti-NeuN conjugated to AF555 (**Table of Materials**).

4.2.2. Add 20 µL of the resuspended sample to an antibody solution containing only mouse anti-PAX6 conjugated to APC.

4.2.3. Add 20 µL of the resuspended sample to a solution with no antibody (for DAPI-only control).

NOTE: If more than two antibodies are used, performing fluorescence-minus-one (FMO) control is recommended in addition to the single-color controls.

4.3. Incubate the samples and the controls with rotation in the dark for 1 h at 4 °C.

4.4. Add DAPI at 1:1000 to all the samples and controls, and proceed with sorting.

5. Fluorescence-activated nuclei sorting (FANS)

NOTE: It is recommended to use an institutional flow cytometry facility with assistance from trained personnel unless already proficient in flow cytometry/sorting techniques.

5.1. Gate as shown below.

5.1.1 First, and for each sample, gate by forward scatter (FSC-A) *vs*. side scatter (SSC-A) to include particles in the appropriate size range for nuclei, thus excluding red blood cells and debris (**Figure 1A**).

NOTE: A very clean nuclei preparation can distinguish between a smaller debris cluster and a nuclei cluster.

5.1.2. Next, and for each sample, gate by FSC-A *vs.* FSC-W (or FSC-A *vs.* FSC-H) and SSC-A *vs.* SSC-W (or SSC-A *vs.* SSC-H) to include only singlet nuclei (**Figure 1B**).

5.1.3. Next, and for each sample, gate by DAPI to include intact nuclei singlets (DAPI-high gated population), excluding debris (DAPI-low, on left of gated population) and doublets (on right of gated population) (**Figure 1C**).

5.1.4. Set further gating based on fluorescence controls, determined visually as distinct clusters on FACS plots.

5.1.4.1. Run DAPI-only control to determine background staining of populations, in the absence of antibody (**Figure 1D** and **Figure 2A**).

5.1.4.2. Run NeuN-AF555-only control to determine the cutoff for NeuN+ staining in the channel for AF555 (**Figure 2B**).

5.1.4.3. Run PAX6-APC-only control to determine the cutoff for PAX6+ staining in the APC channel (**Figure 2C**).

5.1.4.4. Run additional single-color controls if using more antibodies (**Figure 2D,E**).

NOTE: If using more than two antibodies, an FMO control is recommended to visualize any shifts in populations.

5.1.5. Once all controls have been run, draw the gates for NeuN+ and PAX6+ collections above the established thresholds.

5.1.5.1. Gate the NeuN+ population to collect neurons (**Figure 1E** and **Figure 2F**).

5.1.5.2. Gate the PAX6+ population from the NeuN– population to collect astrocytes (**Figure 1E,F** and **Figure 2F**).

5.1.5.3. Gate and collect additional glial populations (such as oligodendrocyte progenitor cells, as shown here by OLIG2+) from the NeuN–PAX6– population (**Figure 1F**).

NOTE: In case determining appropriate gating cutoffs is difficult with the flow cytometry

software due to indistinct populations, it may be helpful to modify the number of events being visualized (either increasing or reducing the number of events on the FANS plot).

5.2. Collect samples appropriately based on the intended downstream analysis.

6. Collection of FANS populations for downstream molecular analyses

6.1. For bulk RNA sequencing, collect 50,000–500,000 nuclei in PBS (see also section 7.1).

6.1.1. Add 2 mL of sucrose solution, 50 µL of 1 M $CaCl_2$, and 30 µL of 1 M $(CH_3COO)_2$ Mg, and fill with PBS up to 10 mL.

6.1.2. Invert and incubate on ice for 15 min, then centrifuge at 900 × *g* for 10–15 min at 4 °C.

6.1.3. Aspirate the supernatant, resuspend in 1 mL of RNA-extracting reagent, vortex, freeze on dry ice, and store at -80 °C.

6.1.4. Alternatively, collect the samples directly into 200 µL of the RNA-extracting reagent. Add the RNA-extracting reagent up to 1 mL after sorting, maintaining a 1:1 ratio of the reagent to sorted sample. Vortex, freeze on dry ice, and store at -80 °C.

6.2. For bulk assay for transposase-accessible chromatin using sequencing (ATAC-seq), collect 50,000–75,000 nuclei in PBS in a microcentrifuge tube coated with 5% bovine serum albumin (BSA). Freeze nuclei on dry ice/-80 °C or immediately use them for ATAC preparation.

6.3. For sn RNA-seq or ATAC-seq, collect nuclei in 0.04% BSA in PBS (see also section 7.2).

7. Pre-library preparation tips

7.1. Bulk nuclear RNA sequencing library preparation

7.1.1. After collecting in RNA extraction reagent, perform standard phenol/chloroform RNA extraction by adding phenol/chloroform and precipitating RNA from the upper aqueous layer with ethanol, followed by DNase digestion on tube (15 min).

7.1.2. Perform RNA cleanup and concentrate in a final volume of 15 µL of water.

NOTE: Using this method, representative recovery from 300 mg of adult cortex sample is ~300,000 NeuN+ nuclei (15–20 ng/µL total RNA after cleanup and concentration) and ~250,000 PAX6+ nuclei (10–12 ng/µL total RNA after cleanup and concentration). This representative yield can vary greatly based on sample quality, gating stringency, and RNA recovery.

7.1.3. Perform quantitative polymerase chain reaction (qPCR) prior to sequencing to confirm enrichment of astrocytes based on high differential expression of canonical astrocyte markers

(*GFAP, SOX9), 10-formyltetrahydrofolate dehydrogenase (ALDH1L1)*) and depletion of neuronal and other cell lineage markers (**Figure 1G**).

NOTE: Collection of the double-negative population (NeuN–PAX6–) allows for a more accurate, multitiered qPCR quality control analysis for the relative enrichment of astrocytes (**Figure 2H**).

7.1.4. Generate RNA-seq libraries using a kit recommended for low RNA integrity number values, as expected from postmortem samples.

7.2. Single-nucleus sequencing library preparation

7.2.1. Perform sn sequencing through an institutional sequencing core.

7.2.2. For nanofluidics-based processing and sequencing, use the recommended concentration of 1×10^6 cells/mL in at least 60 µL of 1x PBS + 0.04% BSA for single-nucleus RNA-sequencing (snRNA-seq) and 3×10^6 cells/mL in at least 20 µL of 1x PBS + 0.04% BSA for single-nucleus Assay for Transposase-Accessible Chromatin using sequencing (snATAC-seq).

REPRESENTATIVE RESULTS:

Nuclei were collected, frozen, and thaw-dissociated from temporal neocortex tissue with a postmortem collection time of 12 h. After the dissociation of nuclei, samples were incubated with antibodies against NeuN, PAX6, and OLIG2, and sorted according to the gating shown in **Figure 1** and **Figure 2**. Nuclei were collected from NeuN+, PAX6+NeuN–, and OLIG2+NeuN– sorted populations (**Figure 1E,F** and **Figure 2F**). A targeted qPCR panel revealed enrichment for the pan-astrocyte markers, *GFAP* and *ALDH1L1,* in the PAX6+(NeuN–) population (**Figure 1G** and **Figure 2H**). Additionally, the OLIG2+ population was enriched for the oligodendrocyte progenitor cell (OPC) marker *PDGFRA* (**Figure 1G**). Bulk RNA sequencing of the collected populations showed comparative enrichment of astrocyte markers and depletion of neuronal markers in the PAX6+(NeuN–) population (**Figure 1H**). Immunofluorescence confirmed the colocalization of PAX6 with GFAP in adult human cortical astrocytes (**Figure 1I**).

Examining single-color controls reveals distinct positive and negative populations for each marker, which enabled the setting of accurate cutoffs for collection (**Figure 2A–G**). Furthermore, astrocyte-enriched FANS isolation was compared with two different antibodies against astrocyte nuclear markers, SOX9 and PAX6. A higher percentage of nuclei events was captured by FANS using PAX6 (~13.8%) than by FANS using SOX9 (~6%) within the astrocyte-enriched gate (**Figure 2F,G**). A targeted qPCR panel revealed enrichment for *GFAP*, *PAX6,* and *SOX9* in both SOX9+(NeuN–) and PAX6+(NeuN–) populations (**Figure 2H**). Retroactively comparing the dissociation and staining of several samples with varying postmortem interval (PMI) of tissue collection revealed that a shorter PMI was associated with greater intact nuclei recovery (**Figure 3A**). Frozen tissue with a PMI of up to 24 h yielded a high rate of intact nuclei, up to 90%; at 30 h PMI, however, very few intact nuclei could be recovered (**Figure 3A**). Including an antibody-wash step in the standard protocol (which typically omits this step to

optimize recovery) did not reveal significant shifts in the separation of distinct NeuN+/– or PAX6+/– populations (**Figure 3B**).

Occasionally, poor separation of PAX6+NeuN– populations could be seen, even in samples with a high percentage of viable nuclei (**Figure 3C**), necessitating the repetition of the dissociation of the nuclei and the FANS protocol. Single-nucleus RNA-seq studies further prioritized *PAX6* as a top differentially expressed nuclear transcription factor across both protoplasmic and fibrous adult astrocyte subpopulations, not only in the neocortex, but also in the subventricular zone (SVZ) and adjacent striatum (**Figure 3D**). Comparison of NEUN/PAX6/OLIG2 triple FANS between the neocortex and striatum derived from the same brain sample showed region-specific differences in the separation of PAX6+ nuclei (**Figure 3E**). This protocol is currently validated for the neocortex only.

FIGURE AND TABLE LEGENDS:

Figure 1: Validation of neuron- and astrocyte-enriched isolation by FANS. (**A–F**) Representative examples of sequential FANS gating to exclude debris and doublets (**A–C**) and define background staining using (**D**) a DAPI-only control for the collection of (**E–F**) enriched neuronal (NeuN+), astrocyte (PAX6+NeuN–), and OPC (OLIG2++NeuN–) nuclei populations. (**G**) Minimal qPCR panel using pan-astrocyte (*GFAP*, *ALDH1L1*), neuronal (*RBFOX3*), and OPC (*PDGFRA*) markers for quality control of the collected populations (**A–G**: temporal neocortex autopsy specimen without pathology, 12 h PMI, 100,000 events shown; dotted lines represent positively gated sequential populations; solid lines represent final collection of cell-type enriched populations). (**H**) Heatmap generated from row-normalized bulk RNA sequencing data showing expression of canonical astrocyte and neuronal markers in PAX6+ and NeuN+ sorted populations (n=3 autopsy cases, temporal neocortex without pathology, PMI 12–21 h). (**I**) Representative immunofluorescence image of PAX6, GFAP, and NeuN in human neocortex. Arrows indicate astrocytes co-expressing PAX6 and GFAP. Abbreviations: FANS = fluorescence-activate nuclei sorting; DAPI = 4',6-diamidino-2-phenylindole; NeuN = neuronal nuclei; PAX6 = paired box protein 6; OLIG2 = oligodendrocyte transcription factor 2; OPC = oligodendrocyte progenitor cell; qPCR = quantitative polymerase chain reaction; GFAP = glial fibrillary acidic protein ; ALDH1L1 = 10-formyltetrahydrofolate dehydrogenase; RBFOX3 = RNA-binding protein FOX-1 homolog 3; PDGFRA = platelet-derived growth factor alpha; PMI = postmortem interval; SSC-A = side scatter-area; FSC-A = forward scatter area; SSC-W = side scatter width; FSC-W = forward scatter width; NeuN-555 = mouse anti-NeuN conjugated to AF555; PAX6-APC = mouse anti-PAX6 conjugated to allophycocyanin; OLIG2-488 = mouse anti-OLIG2 conjugated to green fluorescent dye for the 488 nm laser line.

Figure 2: Comparative isolation of astrocyte-enriched populations using PAX6 or SOX9 antibodies. (**A–E**) Single-color controls for DAPI, NeuN, PAX6, SOX9, and OLIG2 define positive/negative cutoffs in the respective fluorescence channels. (**F–G**) Comparative FANS method for astrocyte-enriched isolation using (**F**) NeuN/PAX6 or (**G**) NeuN/SOX9 antibodies. A greater percentage of events are captured by PAX6 than by SOX9 within the astrocyte-enriched gate. (**A–G**: identical postmortem non-pathological temporal neocortex specimen used for all

experiments; 12 h PMI; 10,000 events shown). (**H**) Quality control qPCR analysis confirms enrichment of astrocyte markers and depletion of non-astrocyte markers in PAX6+(NeuN–) and SOX9+(NeuN–) sorted populations from **F–G** (data normalized to *ACTB* and quantified as fold change of the NeuN+ population). Abbreviations: DAPI = 4',6-diamidino-2-phenylindole; NeuN = neuronal nuclei; PAX6 = paired box protein 6; OLIG2 = oligodendrocyte transcription factor 2; SOX9 = SRY-box transcription factor 9; qPCR = quantitative polymerase chain reaction; GFAP = glial fibrillary acidic protein ; RBFOX3 = RNA-binding protein FOX-1 homolog 3; PDGFRA = platelet-derived growth factor alpha; PMI = postmortem interval; NeuN-555 = mouse anti-NeuN conjugated to AF555; PAX6-APC = mouse anti-PAX6 conjugated to allophycocyanin; OLIG2-488 = mouse anti-OLIG2 conjugated to green fluorescent dye for the 488 nm laser line.

Figure 3: Dependent and independent metrics for successful FANS experiments. (**A**) Effect of sample collection PMI on the quality of nuclei: lower PMI enables the recovery of a greater number of intact nuclei with adequate results up to 24 h. (**B**) The inclusion of a wash step after antibody incubation does not visually appear to shift FANS populations. (**C**) Example of a failed FANS experiment with lack of distinct PAX6+(NeuN–) population (on right) despite the presence of intact nuclei and low background (on left) (postmortem temporal neocortex, non-pathological, 7 h PMI). (**D**) Heatmap representation of adult human sn RNA-seq data showing differential expression of *PAX6* and two other astrocyte nuclear factors, *SOX9* and *LHX2*, across protoplasmic and fibrous astrocyte subpopulations in both cortex and SVZ, compared to other cell types (red color gradient represents spectrum of log-normalized average gene expression values; n=3 distinct autopsy cases, 43,619 total nuclei. (**E**) Distinct brain regions (temporal neocortex *vs*. SVZ and subjacent striatum) show different patterns of NeuN/PAX6/OLIG2 separation. Abbreviations: FANS = fluorescence-activate nuclei sorting; DAPI = 4',6-diamidino-2-phenylindole; NeuN = neuronal nuclei; PAX6 = paired box protein 6; OLIG2 = oligodendrocyte transcription factor 2; GFAP = glial fibrillary acidic protein ; ALDH1L1 = 10-formyltetrahydrofolate dehydrogenase; LXH2 = LIM homeobox 2; PMI = postmortem interval; SSC-A = side scatter-area; FSC-A = forward scatter area; NeuN-555 = mouse anti-NeuN conjugated to AF555; PAX6-APC = mouse anti-PAX6 conjugated to allophycocyanin; OLIG2-488 = mouse anti-OLIG2 conjugated to green fluorescent dye for the 488 nm laser line; SVZ = subventricular zone; A(p) = protoplasmic (gray matter) astrocytes; A(f) = fibrous (white matter) astrocytes; OL = oligodendrocytes; OPC = oligodendrocyte progenitor cells; EN = excitatory neurons; MSN = medium spiny neurons; MG = microglia; BVC = blood vessel cells.

DISCUSSION:

Experimental design following the outlined protocol should be finalized after considering several biological and technical factors. Starting tissue samples are fresh-frozen, without having been fixed, and preferably have a short postmortem collection interval to maximize nuclei recovery. Based on experience, a PMI of up to 24 h allows for adequate nuclei recovery; however, a PMI of 12 h or less is preferable to optimize intact nuclei recovery. Additional factors apart from PMI, including temperature of body storage and pH levels of the tissue, may also affect nuclei yield, but were not accounted for in these studies[14]. From anecdotal experience, FANS recovery has been found to be comparable with the same tissue being stored up to five years at -80 °C, although controlled experiments were not performed to assess the

variability of storage on cell-type specific expression. Washing the sample after antibody incubation is another variable that may need consideration. In general, a wash step decreases the overall yield, but may improve the separation of distinct immunolabeled populations.

In these studies, no difference was observed in the separation of NEUN/PAX6 populations when including or excluding a post-antibody wash step, but this step may be beneficial when using other primary and secondary antibodies, especially if they are not preconjugated. Due to the variability in the sensitivity of the sorting equipment, it is necessary to perform appropriate controls at each sorting event. If excessive extracellular debris is seen in the resuspended sample when visualizing on a hemocytometer, the sample can be passed through a 40 µm filter to retain only the nuclei. If sample is limited, beads tagged with the appropriate fluorophore may also be used for single-color and FMO controls. If the samples appear to not be enriched for the desired astrocyte populations, as determined either by qPCR or sequencing results, it may be helpful to gate all NeuN– as well as PAX6+(NeuN–) populations more strictly, leaving more room between the gate cutoffs for positive and negative populations.

Most of the validation studies described here were performed using OLIG2 in addition to NeuN and PAX6, to simultaneously enrich for neuronal, astrocyte, and oligodendrocyte progenitor (OPCs) nuclei populations. It is recommended to include OLIG2 as a third lineage marker to enrich for cortical astrocytes more effectively within the NeuN– fraction. The presence or absence of FITC-conjugated OLIG2 does not appear to shift the PAX6+ population; therefore, the assumption is that experiments excluding OLIG2 will result in similar astrocyte enrichment. Of note, it is essential that the PAX6+ population be gated from the NeuN– population, as some neuronal populations express both NeuN and PAX6[15]. Astrocytes can also be sorted according to SOX9 expression; however, staining and sorting with SOX9 leads to less robust enrichment of astrocytes compared to PAX6. Although clear separation was observed between neuronal, astrocytic, and OPC lineages in the adult cortex, a similar separation was not observed between PAX6+ and OLIG2+ populations in the adult SVZ and adjacent striatum.

While it is possible that some astrocytes from the SVZ express low levels of OLIG2, the collected populations were only validated by qPCR (data not shown); hence, downstream RNA sequencing may be helpful in further defining the purity of these populations. Additionally, this protocol can be adapted to isolate nuclei from frozen fetal brain tissue and brain tumor tissue using population-specific nuclear markers. Because these tissues are considerably more cellular, it may be helpful to start with less tissue to minimize the aggregation of nuclei. When sorting aneuploid nuclei from neoplastic tissue, DAPI gating is adjusted to account for potentially higher fluorescence. It is strongly recommended that users perform a careful validation prior to performing FANS on neoplastic tissue, as the current protocol is validated for non-neoplastic samples only. Overall, the above-described protocol puts forward a validated strategy for isolating enriched astrocyte populations, adapted from previously established protocols to enrich for neurons. This methodology can be used to effectively isolate astrocyte nuclei populations from both normal and diseased cortical brain tissues for use in further molecular analyses.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

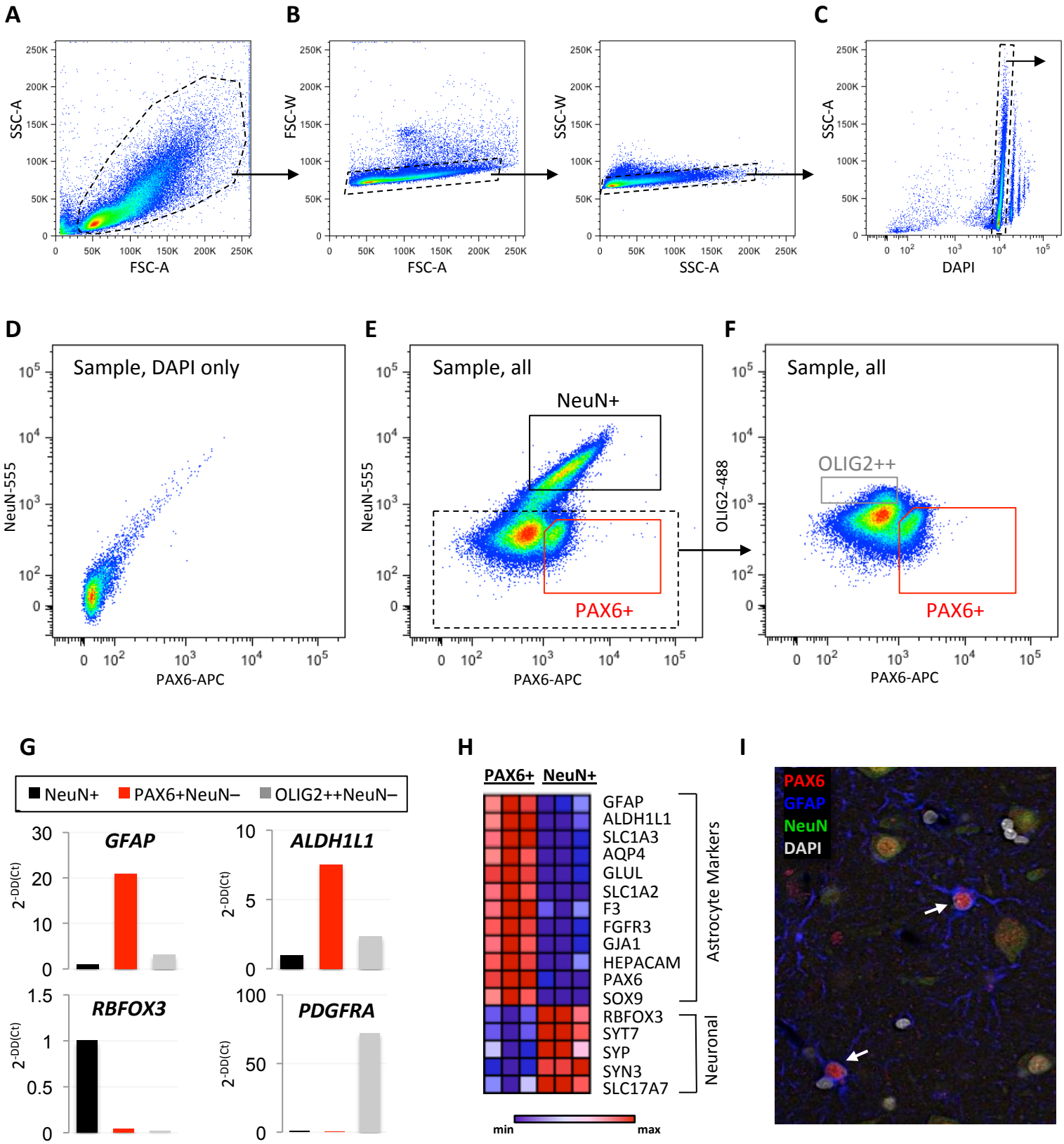
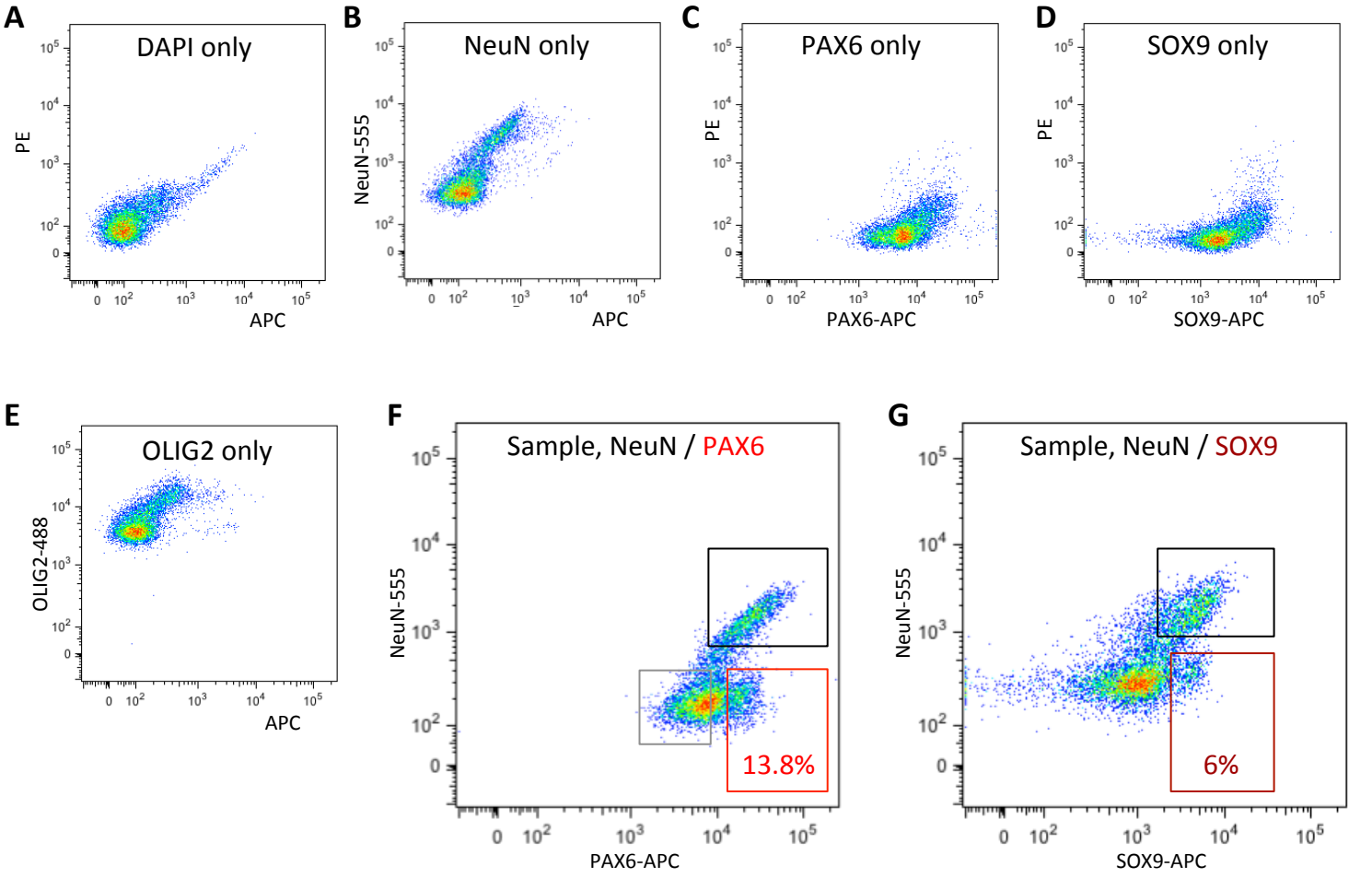


Figure 2



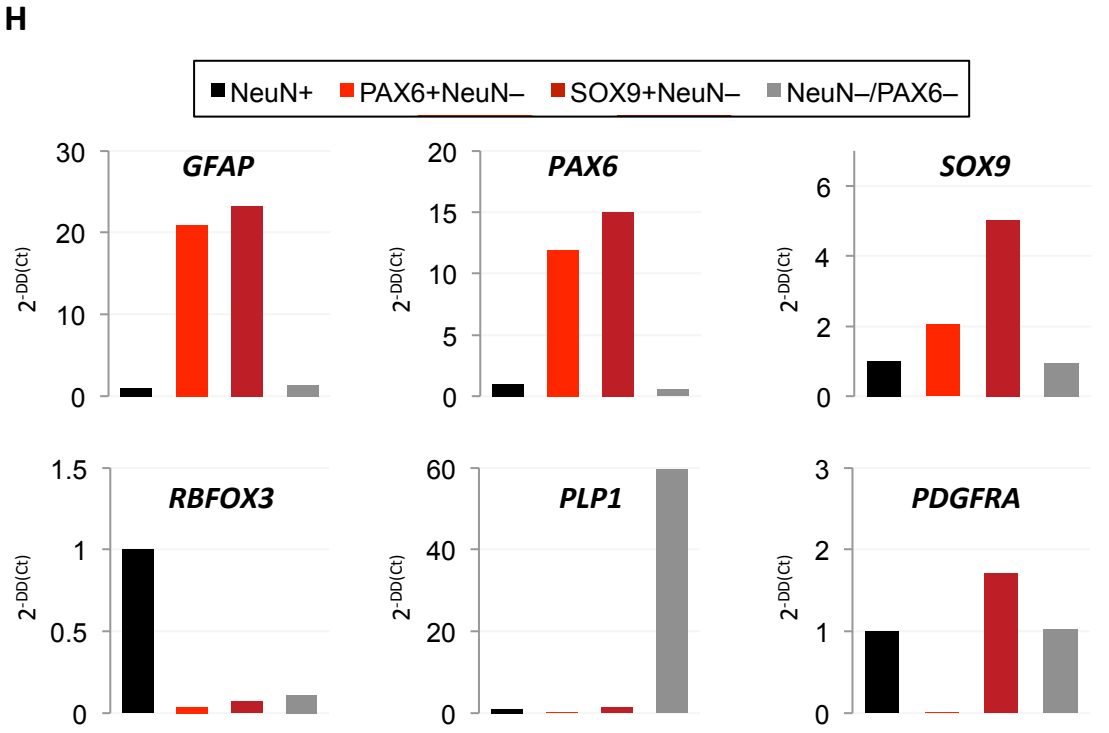
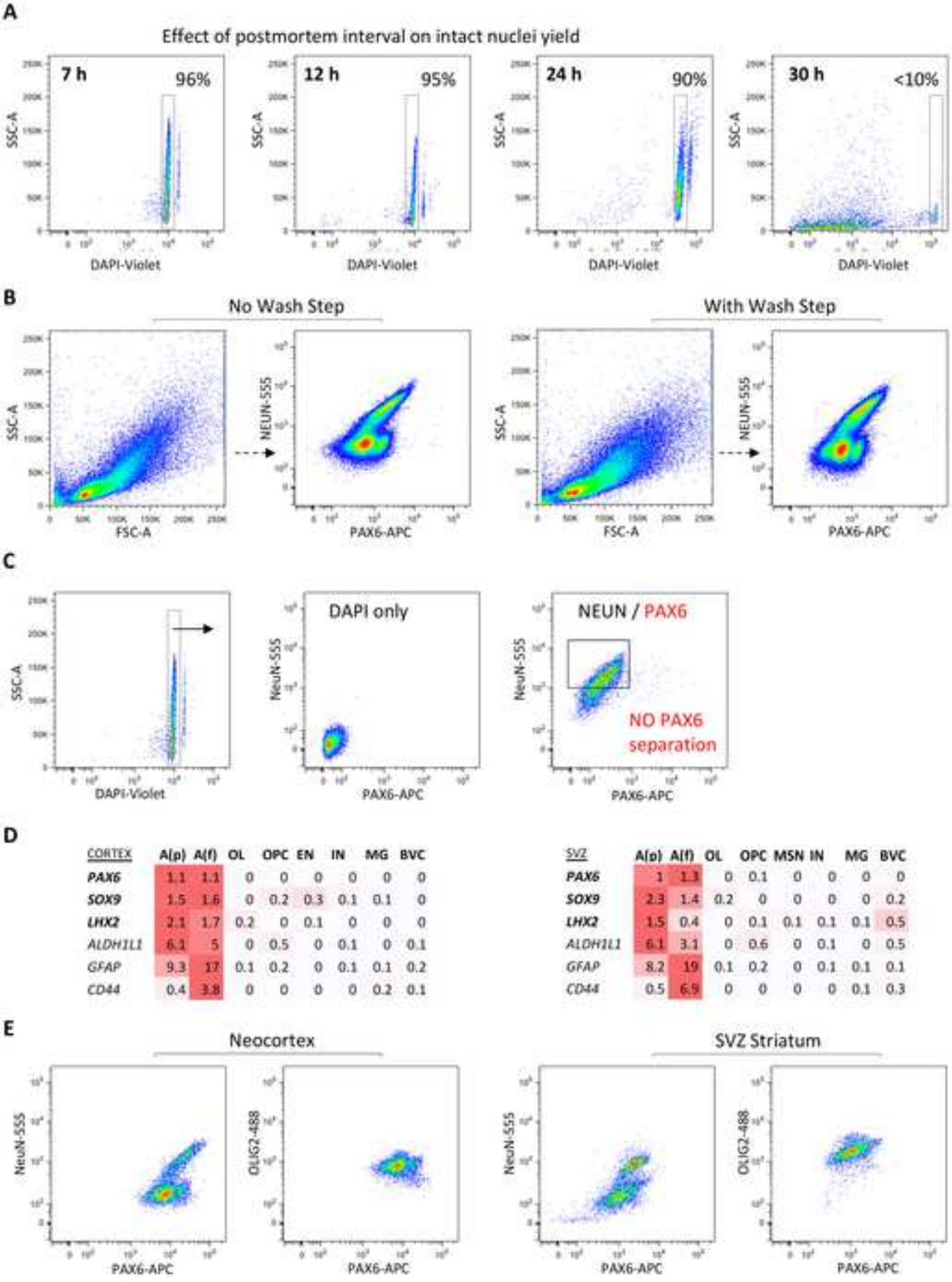


Figure 3

Figure 3



D

CORTEX	A(p)	A(f)	OL	OPC	EN	IN	MG	BVC
PAX6	1.1	1.1	0	0	0	0	0	0
SOX9	1.5	1.6	0	0.2	0.3	0.1	0.1	0
LHX2	2.1	1.7	0.2	0	0.1	0	0	0
ALDH1L1	6.1	5	0	0.5	0	0.1	0	0.1
GFAP	9.3	17	0.1	0.2	0	0.1	0.1	0.2
CD44	0.4	3.8	0	0	0	0	0.2	0.1

SVZ	A(p)	A(f)	OL	OPC	MSN	IN	MG	BVC
PAX6	1	1.3	0	0.1	0	0	0	0
SOX9	2.3	1.4	0.2	0	0	0	0	0.2
LHX2	1.5	0.4	0	0.1	0.1	0.1	0.1	0.5
ALDH1L1	6.1	3.1	0	0.6	0	0.1	0	0.5
GFAP	8.2	19	0.1	0.2	0	0.1	0.1	0.1
CD44	0.5	6.9	0	0	0	0	0.1	0.3

Table of Materials

Click here to access/download;Table of Materials;Mussa et al Materials nt changes.xls

Name of Material/ Equipment	Company
10x PBS pH 7.2	Invitrogen
ANTI-NEUN ANTIBODY CLONE A60	Millipore
ANTI-OLIG2 ANTIBODY CLONE 211	Millipore
Bovine Serum Albumin	Fisher
Bright-Line Counting Chamber	Hausser Scientific
Calcium Chloride Anhydrous	Fisher
Cell Strainers, 40 µM	SP Scienceware
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Invitrogen
DL-Dithiothreitol	Sigma
DNA Library Kit	Illumina, Nextera
DNAse I	Worthington
Dounce Tissue Grinder	WHEATON
FACS Sorter	BD Biosciences
Magnesium Acetate Tetrahydrate	Fisher
PAX6 (PAX6/496) - 100 TESTS	Novus
RNA Clean & Concentrator	Zymo Research
RNaseZap RNase Decontamination Solution	Invitrogen
SMARTer Stranded Total RNA-Seq Kit Pico Input Mammalian	Clontech Laboratories
Sucrose, crystal certified, ACS, 500 mg	Fisher
SW 41 Ti Swinging-Bucket Rotor	Beckman Coulter
Tris-HCl, 1M Solution, pH 8.0, Molecular Biology Grade, Ultrapure	Thermo Scientific
TritonX-100	Fisher
TRIzol LS Reagent	Invitrogen
TRIzol Reagent	Invitrogen
Trypan Blue Solution, 0.4%	Gibco
Ultracentrifuge	Beckman Coulter Optima XE-100
Ultracentrifuge tubes PP 9/16 X 3-1/2	Beckman Coulter
UltraPure Distilled Water (RNAse-, DNAse-free)	Invitrogen
Ultrapure EDTA	Life Technologies

Catalog Number	Comments/Description
70013073	
MAB377A5MI	mouse anti-NeuN conjugated to a fluorescent compound AF555 (excitation, 553 nm; emission, 568 nm)
MABN50A4MI	mouse anti-OLIG2 conjugated to a fluorescent compound AF488 (excitation, 499 nm; emission, 520 nm)
BP9704-100	
3110V	
C614-3	
136800040	
D1306	
43815-1G	
FC-121–1030	
LS002139	
357542	
	BD FACSAria III
M13-500	
NBP234705J	
R1013	
AM9780	
635005	Fragmentation time of 2.5 minutes, as recommended for low RIN RNA values.
S5500	
331362	
J22638AE	
BP151-500	non-ionic surfactant in lysis buffer
10296028	
15596026	reagent for isolation of RNA
15250061	
A94516	
331372	
10977023	referred to as distilled water
15576-028	

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February 26th, 2021

To:
Vidhya Iyer, Ph.D.
Review Editor, JoVE
vidhya.iyer@jove.com

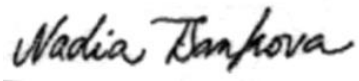
RE: Response letter for JoVE62405

Dear Dr. Vidhya Iyer and team,

We are thankful for the overall positive response to our manuscript entitled "**Isolation of adult human astrocyte populations from fresh-frozen cortex using fluorescence-activated nuclei sorting**." We appreciate all suggestions raised, which we found constructive and very helpful in further improving our study. We have addressed all suggestions, to the best of our ability, and provide itemized responses below (in blue).

We are grateful for your consideration of our manuscript for publication at *JoVE* and look forward to further communication.

Sincerely:



Nadejda Tsankova, MD/PhD

Itemized Responses:

A. Editorial comments:

Editorial Changes
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have carefully proofread the entire manuscript.

2. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

We have included an ethics statement after the discussion.

3. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We have changed the numbering of steps to include more primary steps and only up to four substeps, which now creates a workflow that is much easier to follow. We have limited each substep to 4 sentences maximum, except in 5.1.5.

4. Use appropriate SI units and abbreviations, and add a single space between the quantity and its unit. E.g. “4 oC” instead of “4C” (line 144, 188, 190), “1 M CaCl2” instead of “1M CaCl2” (line186), “60 µL” instead of “60 µL” (line228, 232), “ 12-21 h” instead of “12-21 hrs” (line 285, 295, 307), etc. Express centrifugation speeds as “..x g” instead of “.. rpm”. (e.g. line 119, 188).

We have made the requested changes.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. E.g. RNase Zap, Nexetra, SMARTer etc.

We have made the requested changes.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. E.g. lines 161-162, 172-179,224-232 etc.

We have made the requested changes.

7. Consider adding reagent compositions as a separate table which can be referenced in the protocol. This table should be uploaded separately through Editorial Manager. E.g. Lines 90-104.

8. Figure 3D: What does the color coding indicate? Please specify in the legend.

The red color gradient represents log-normalized average gene expression data from single nuclei RNA-seq experiment, which is now included in the legend.

9. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).]. Do not use “&” or “and” in the references, and do not abbreviate the journal title to conform with the JoVE style.

We have made the requested changes.

10. Please sort the Materials Table alphabetically by the name of the material. Also remove any formatting from the table.

We have made the requested changes.

B. Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Manuscript describes interesting method for isolation of astrocyte-enriched nuclei populations from fresh-frozen human cortex. Overall, the paper was well written. Authors leads the researcher step by step through isolation protocol. There are just one minor point that may need improvement as listed below.

Minor Concerns:
Add symbol "º" in the temperature values, ex 4ºC.

We appreciate the reviewer's positive appraisal of our manuscript. We have made the requested change for temperature values.

Reviewer #2:
Manuscript Summary:
Mussa et al describe a method for isolating astrocyte and neuron populations from fresh frozen adult cortex using fluorescence-activated nuclei sorting. Overall, this is a very well-written protocol that will benefit the scientific community. The authors also discuss various techniques (bulk/snRNA-seq, ATAC-seq) that can be used following the isolation method described.

We appreciate the reviewer's positive appraisal of our manuscript.

Minor Concerns:
- The authors mention that the method can potentially be used in brain tumors. Given the aneuploidy of tumors, how would this affect the FANS? A discussion about identification of single nuclei based on DNA content could be provided.

We included further text in regards to this point in the discussion section, mentioning that samples with aneuploidy may show different DAPI signal and also recommending that users perform a careful validation prior to performing FANS on neoplastic tissue.

- Can the authors discuss whether the storage time of frozen tissues may affect the results

In our anecdotal experience, we have seen comparable FANS recovery with the same tissue stored up to five years at -80C but we have never performed controlled experiment to assess the variability of storage on cell-type specific expression. We have added brief discussion to this point.

Reviewer #3:
Manuscript Summary:
This protocol allows for nuclear isolation of cortex astrocytes and their separation from neuron

and oligodendrocyte nuclei in postmortem human brain tissue. Unfortunately, validation for neocortex only is provided. Nevertheless, the protocol is a substantial advance over current cell separation approaches.

We appreciate the reviewer's critical appraisal of our manuscript.

Major Concerns:
All concerns are minor.

Minor Concerns:
1) Pax6 is a less familiar marker for astrocytes. Some discussion of its expression during development and adulthood, its regional specificity, and function are warranted.

We thank the reviewer for bringing up this important point. We now provide additional text in regards to this point in the introduction.

2) Please specify how your tissue was frozen and for how long prior to processing. The manuscript appears to refer only to the postmortem time interval before freezing.

In our anecdotal experience, we have seen comparable FANS recovery with the same tissue stored up to five years at -80C but we have never performed controlled experiment to assess the variability of storage on cell-type specific expression. We have added brief discussion to this point.

3) To be clear, please confirm that the liquid components added to the buffers in 1.1 and 1.2 will increase the total volume beyond 50 ml.

We have clarified in 1.1 and 1.2 that water is added to a total volume of 50 mL.

4) It is unclear whether 2.10.3 refers to 2.10.4-6

We have changed the numbering of steps to include more primary steps and only up to four substeps, which now creates a workflow that is much easier to follow.

5) If possible please quantify failure rate for the protocol in your hands.

Failure rate is heavily interdependent on the sorting skills of the user, the tissue quality, as well as, to some extent, on the functional fluidics of the instrument being used. It is, therefore, impossible to quantify. We estimate the failure rate below 10-20% in experienced users processing high quality tissue.