Isolation and Molecular Profiling of Nuclei of Specific Neuronal Types from Human Cerebral Cortex and Striatum

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Most pathological conditions of the central nervous system do not affect all cell types to the same extent. Delineation of molecular events underlying disease symptoms, including genetic, epigenetic, and transcriptional changes, thus relies on the ability to characterize a specific cell type separately from others. We have developed a methodology for the collection of nuclear RNA and genomic DNA of specific cell types from frozen post-mortem striatum and cerebral cortex. This allows deep transcriptomic profiling of specific cell populations and characterization of their genomes and epigenetic properties. The method is based on the purification of cell nuclei, followed by fluorescence-activated sorting of nuclei (FANS) labeled with nucleic acid probes or antibodies binding to targets present in specific cell types. The protocol describes in detail the procedure for isolating and labeling neuronal and glial nuclei from human brain tissue, the steps that can be taken to extract RNA and genomic DNA, a way to combine the procedure with ATAC-seq to yield information about chromatin accessibility, as well as computational measures for assessing the quality of cell type-specific RNA-seq and ATAC-seq datasets. © 2024 The Author(s). Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Tissue homogenization, isolation of cell nuclei by ultracentrifugation and formaldehyde-fixation
Basic Protocol 2: Antibody-based labeling and isolation of nuclei of specific neocortical neuron types
Support Protocol 1: Generation of ATAC-seq libraries from the nuclei of specific neuron types of the cerebral cortex
Basic Protocol 3: Nucleic acid hybridization-based labeling and isolation of nuclei of specific striatal projection neuron types
Alternate Protocol 1: Labeling and isolation of nuclei of specific striatal interneuron types
Support Protocol 2: Generation of ATAC-seq libraries from the nuclei of specific striatal neuron types
Basic Protocol 4: Extraction of genomic DNA and nuclear RNA and preparation of sequencing libraries
Basic Protocol 5: Processing and quality control of FANS-seq and ATAC-seq data

Keywords: ATAC-seq • cell type-specific RNA-seq • FANS-seq • fluorescence activated nuclear sorting • human post-mortem brain tissue

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INTRODUCTION

The human brain contains various types of cells, each with distinct roles and functions, as is evident from their diverse projection targets and signal relay mechanisms. Gene expression profiling through the analysis of mRNA molecules present in a nerve cell provides insight into how the cell functions, such as the neurotransmitters it synthesizes to communicate with other neurons. It can also be used to predict cell dysfunction in pathological conditions. Isolation of brain cells from adult brain tissue for mRNA analysis requires tissue dissociation and is compromised by loss of cell integrity. However, intact cell nuclei can be isolated with much higher yield, enabling the use of the nuclear transcriptome for cell type-specific gene expression profiling. Cell type-specificity can be achieved either by separating single nuclei into wells or droplets (single-nucleus RNA-seq, snRNA-seq) or by separating populations of nuclei based on distinguishable characteristics that can be detected by immunolabeling, for example. Gene expression profiling can then be carried out on these isolated nuclei or populations of nuclei by turning intranuclear RNA into sequencing libraries and inferring the presence of individual transcripts from high-throughput sequencing data (Lake et al., 2017; Xu et al., 2018). The most popular approach to acquiring cell type-specific data on gene expression, chromatin organization, and epigenetic markers is the analysis of single nuclei (Piwecka et al., 2023; Preissl et al., 2018; Tian et al., 2023), but current methods suffer from data sparsity, as the fidelity of detecting most transcripts, accessible sites, or methylated cytosines is low. Thus, most common snRNA-seq methods are unable to detect transcripts with medium and low expression in most nuclei analyzed, and can only detect polyadenylated transcripts. Droplet-based snRNA-seq methods must compensate for contamination with ambient transcripts present in all preparations from human tissue, as these could otherwise lead to the misinterpretation of cell types and false-positive expression changes (Caglayan et al., 2022; Slyper et al., 2020).

Methods based on the analysis of populations of nuclei separated based on a distinguishing characteristic offer an alternative approach that typically relies on immunolabeling of a nuclear epitope that is cell type-specific, followed by fluorescence-activated nuclei sorting (FANS) (Xu et al., 2018). Such approaches have been used to generate gene expression profiles and epigenetic data from neurons and glial cells of the cerebral cortex (Nott et al., 2019; Nott et al., 2021) and have been used to interrogate other cell type-specific properties of the human brain, such as the age of cells (Spalding et al., 2005). However, these broad classifications into a few different cell types leave intra-population heterogeneity unresolved.

We have developed methods for labeling and fluorescence-activated nuclear sorting (FANS) of populations of nuclei derived from specific types of neurons of the human cerebral cortex and striatum (Matlik et al., 2024; Pressl et al., 2024). The analysis of transcriptomes from populations of nuclei collected with this methodology (FANS-seq) from frozen post-mortem brain tissue results in very deep gene expression profiles of specific neuronal subtypes. This approach also enables similarly deep profiling of chromatin accessibility using the assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) (Buenrostro et al., 2013) and, for more abundant cell types,

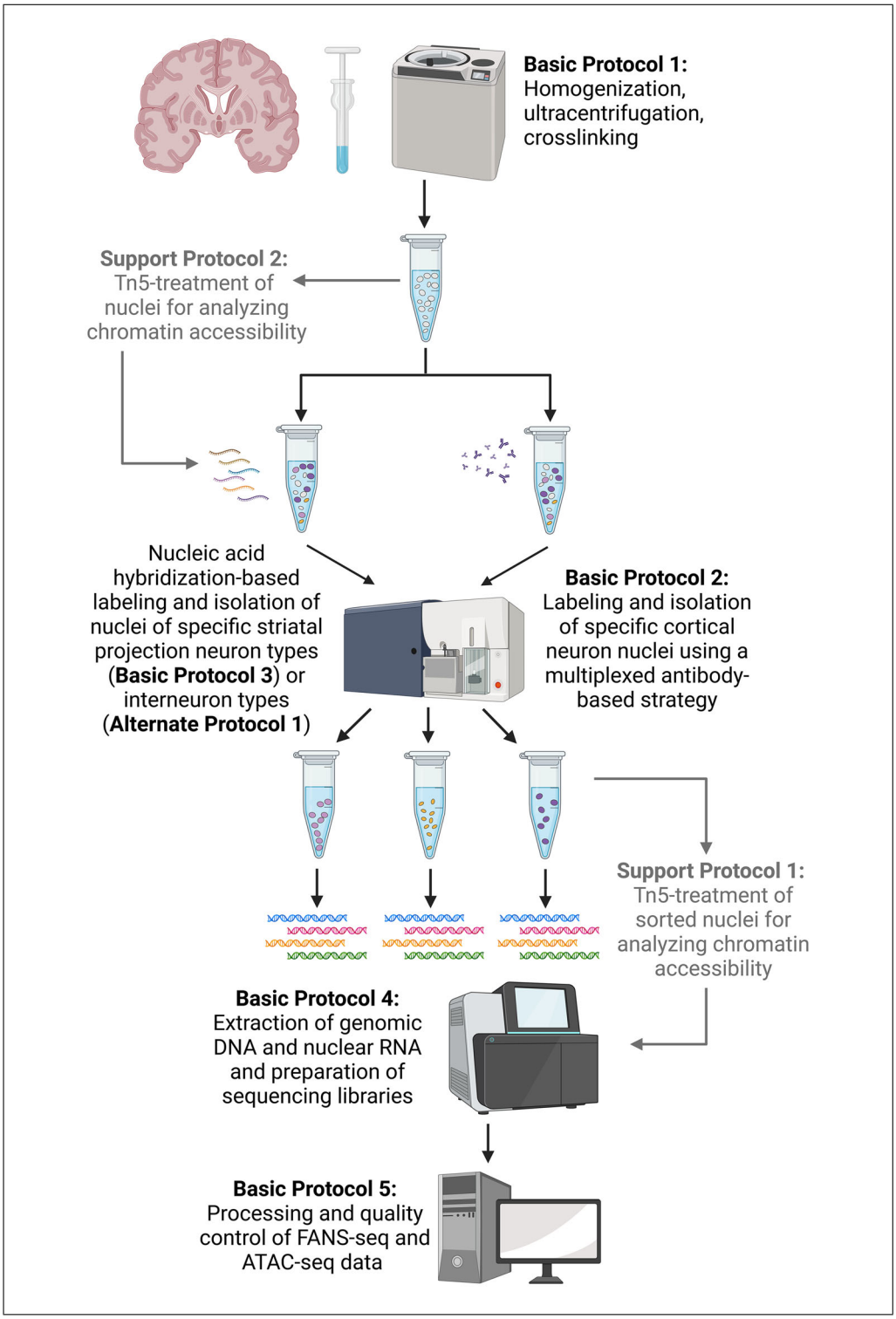

Figure 1 Schematic overview of the experimental steps for producing data on nuclear transcript abundance and chromatin accessibility in specific types of neurons in the human cerebral cortex and striatum. Created with BioRender.com.

genomic DNA methylation and hydroxymethylation (Booth et al., 2013). Overall, complementing deep FANS-seq datasets with ATAC-seq-based confirmation of gene expression enables quantitative analysis of transcripts of approx. 12,000-13,000 genes per cell type (Matlik et al., 2024; Pressl et al., 2024).

Basic Protocol 1 describes the isolation of cell nuclei from a sample of frozen brain tissue (Fig. 1). Starting from there, the procedures differ depending on whether the aim is to isolate nuclei of specific cell types from the cerebral cortex (Basic Protocol 2) or striatum (Basic Protocol 3 and Alternate Protocol 1). These protocols describe how to label the nuclei of cell types of interest, the FANS strategy and procedure, and, as Support Protocols 1 and 2 describe, how to generate cell type-specific chromatin accessibility data from these populations by preparing ATAC-seq libraries. To mitigate autofluorescence, a common issue encountered when working with human brain samples, we describe the use of True Black® for autofluorescence quenching in Basic Protocol 2. Basic Protocol 4 outlines the procedure for extracting genomic DNA and nuclear RNA and preparing sequencing libraries. Basic Protocol 5 describes how ATAC-seq and FANS-seq sequencing reads can be processed for analysis and how to assess the quality of these data.

Experiments with de-identified human tissue must be approved by the appropriate institutional review boards.

CAUTIONS

Homogenization of brain tissue can create aerosols, and since human brain tissue can contain infectious pathogens, it is best to perform the homogenization step while wearing personal protective equipment. Handling of unfixed samples should be performed in a biosafety cabinet.

STRATEGIC PLANNING

We recommend that once an aliquot of frozen brain tissue is thawed, the steps described in Basic Protocols 1 and 2 (or 1 and 3) are carried out for 2 to 3 consecutive days until the sorting process is completed and nuclei are stored at −80°C. Note that for the preparation of ATAC-seq libraries, we typically do not freeze nuclei treated with Tn5 (Support Protocol 1 or 2) but extract genomic DNA after overnight treatment with proteinase K, thus adding an extra day to the procedure. We recommend that no more than 3 tissue samples are processed simultaneously during these initial steps (Basic Protocols 1–3). More samples of stored nuclei can be processed in parallel for nucleic acid extraction and sequencing library preparation (Basic Protocol 4).

Based on our initial experiments, we noted that brain tissue samples with post-mortem interval (PMI) exceeding 40 hr rarely yield high-quality FANS-seq libraries. Therefore, the great majority of the hundreds of samples we have chosen to analyze have had a PMI <30 hr. However, even within this PMI range, brain tissue quality can be highly variable, not only between tissue donors but even between different brain regions of the same donor. We have not observed clear correlations between the quality of FANS-seq libraries and variables such as RNA integrity number (RIN) or sample storage duration, but the lack of expected FANS staining patterns is the best predictor of unacceptably low tissue quality.

BASIC PROTOCOL 1

TISSUE HOMOGENIZATION, ISOLATION OF CELL NUCLEI BY ULTRACENTRIFUGATION AND FORMALDEHYDE-FIXATION

Basic Protocol 1 describes the procedure for dissociating frozen brain tissue, separation of cell nuclei from the homogenate by ultracentrifugation, followed by formaldehyde-fixation and permeabilization steps. Formaldehyde fixation will minimize the loss of soluble proteins from the nuclei during the staining procedure, while permeabilization of the nuclear membrane allows antibodies or PrimeFlow probes to reach target molecules inside the nuclei. By the end of this procedure, the cell nuclei are ready for the staining procedures described in Basic Protocol 2, Basic Protocol 3, or Alternate Protocol 1.

Materials

Buffer D (chilled on ice, see recipe)
Buffer C (chilled on ice, see recipe)
Buffer E (chilled on ice, see recipe)
Paraformaldehyde 16% Aqueous Solution (Electron Microscopy Sciences, cat. no. 15710)
2 M Glycine (see recipe)
Permeabilization buffer (see recipe)

Bucket with ice
Kontes® 7-ml All Glass Dounce Tissue Grinder with Large & Small Pestles (DWK Life Sciences, cat. no. 885300-0007)
13.2 ml Ultra-clear ultracentrifuge tubes (Beckman Coulter, cat. no. C14293)
Ultracentrifuge with a swing-out rotor (e.g., Optima XPN with SW 41 Ti Swinging-Bucket Rotor, Beckman Coulter, cat. no. 331362) cooled to 4°C
Nuclease-free 1.5-ml microcentrifuge tubes (e.g., USA Scientific, cat. no. 1615-5500)
Cell strainer snap cap (Corning, cat. no. 352235)
Samco™ Transfer Pipettes (Thermo Fisher Scientific, cat. no. 202-1SPK)
Low Retention Microcentrifuge Tubes (e.g., Thermo Fisher Scientific, cat. no. 3448)
Refrigerating centrifuge with a swing-out rotor for 1.5-ml tubes (e.g., Eppendorf, cat. no. 5810-R) cooled to 4°C
Shaker

Tissue Homogenization

1. Thaw an aliquot of brain tissue on ice for 45–60 min.

   It can be difficult to collect the tissue aliquot from the bottom of the tube once it has thawed, so we recommend placing the tube on ice inverted.

2. Transfer tissue aliquot(s) into a Dounce tissue grinder (kept on ice) and add Buffer D (4 ml per 100–150 mg of tissue, 8 ml for larger samples).

3. Homogenize tissue on ice using **30** strokes with pestle A followed by **15** strokes with pestle B. Avoid bubble formation.

Isolation of Cell Nuclei by Ultracentrifugation

4. Pour homogenate into a 15-ml Falcon tube. Rinse the Dounce grinder with 1 ml buffer D and add to homogenate. Adjust homogenate volume to 5 ml with Buffer D.

   If 8 ml were used for douncing, divide the homogenate into two tubes.

5. Add 4.5 ml Buffer C to 5 ml homogenate. Mix by inverting 10 times.

6. Add 1 ml Buffer E to the bottom of a 13.2 ultra-clear ultracentrifuge tube and place the tube on ice.

7. Slowly layer 1 ml of the homogenate on top of Buffer E and repeat until all homogenate has been layered.

   It is important to do this slowly so that the layers do not mix.

8. Place ultracentrifuge tubes into Beckman Coulter SW41 Ti rotor buckets. Balance the buckets and cap.

9. Centrifuge with SW 41 Ti rotor at 10,000 rpm (i.e., 17,200 × *g* at rmax) for 30 min at 4°C using fastest acceleration and slowest deceleration settings.

10. Retrieve samples from buckets and place on ice. Remove the supernatant by pipetting until 0.5 ml is left. Resuspend the pellet in 1 ml Buffer D and transfer to a low-retention 1.5-ml microcentrifuge tube by filtering the suspension through a cell strainer snap cap using a transfer pipette.

11. Pellet by centrifugation (in a swing-out rotor) 1000 × *g*, 5 min at 4°C. Remove supernatant and resuspend pellet in 1 ml Buffer D.

    All centrifugation steps are carried out in a swing-out rotor. If ATAC-seq samples are going to be generated with the procedure described in Support Protocol 2, count the number of nuclei after resuspension and follow the steps listed in that protocol.

Formaldehyde-Fixation of Nuclei

12. Fix nuclei by adding 67 µl of 16% formaldehyde, mix by pipetting with a P1000, and incubate at room temperature on a shaker for 8 min (covered with foil).

13. Quench formaldehyde by adding 100 µl of 2 M glycine. Incubate at room temperature on a shaker for 5 min (covered with foil).

14. Centrifuge for 5 min at 1000 × *g*, 4°C to pellet nuclei. Remove supernatant and resuspend nuclei in 1 ml Buffer D.

15. Centrifuge for 5 min at 1000 × *g*, 4°C to pellet nuclei. Remove supernatant and resuspend nuclei in 1 ml permeabilization buffer.

16. Centrifuge for 5 min at 800 × *g*, 4°C to pellet nuclei. Remove supernatant and resuspend nuclei in 1 ml permeabilization buffer.

17. For permeabilization, incubate at room temperature on a shaker for 30 min (covered with foil).

    From this point on, the procedure differs for nuclei of the cerebral cortex (stained with antibodies according to Basic Protocol 2) and for striatal cell nuclei (stained with PrimeFlow probes according to Basic Protocol 3 or Alternate Protocol 1).

BASIC PROTOCOL 2 ANTIBODY-BASED LABELING AND ISOLATION OF NUCLEI OF SPECIFIC NEOCORTICAL NEURON TYPES

This protocol describes the materials, labeling procedure, and serial FANS strategy for the isolation of specific neocortical nuclei populations listed in **Table 1**.

The concept of our two-stage serial sorting and staining strategy includes the collection of three NeuN positive populations, NeuN+/SATB2-, NeuN+/SATB2medium, NeuN+/SATB2+, and one NeuN negative population into separate tubes, followed by another round of sorting for each of the three NeuN positive populations

Table 1 Neocortical Cell Types that can be Analyzed Using Basic Protocol 2

NeuN+/SATB2-	NeuN+/SATB2medium	NeuN+/SATB2+	NeuN-
VIP expressing Interneurons	PVALB expressing Interneurons	Layer 5a pyramidal neurons	Astrocytes
RELN expressing Interneurons	CRHBP expressing Interneurons	Layer 6a pyramidal neurons	Microglia
LAMP5 expressing Interneurons	Layer 4 granular neurons	Layer 6b pyramidal neurons	Oligodendrocytes
Layer 2/3 pyramidal neurons	Layer 5rs – region-specific Von Economo neurons or Betz Cells		Oligodendrocyte progenitor cells

(NeuN+/SATB2-, NeuN+/SATB2medium, NeuN+/SATB2+). The NeuN-negative nuclei are stained with antibodies again before another round of sorting that separates the nuclei of different glial cell types. To prevent autofluorescent nuclei from appearing falsely positive after staining, we quench lipofuscin autofluorescence prior to adding any fluorescent labels. The collected nuclei can be used for RNA and genomic DNA extraction (Basic Protocol 4) or handled according to Support Protocol 1 to generate cell type-specific ATAC-seq data. The labeling and FANS strategy can also be used to prepare for targeted single-nucleus RNA-seq analysis, for instance.

Materials

TrueBlack® Plus Lipofuscin Autofluorescence Quencher (Biotium, cat. no. 23014)
PBS (see recipe)
Wash buffer (chilled on ice, see recipe)
DAPI buffer (chilled on ice, see recipe)
AllPrep DNA/RNA FFPE Kit (Qiagen, cat. no. 80234)
NeuN polyclonal chicken primary antibody (Millipore cat. no. ABN91)
SATB2 monoclonal mouse primary antibody (Santa Cruz, cat. no. sc-81376)
BCL11B monoclonal rat antibody conjugated to FITC (Abcam, cat. no. ab123449)
TLE4 monoclonal mouse antibody conjugated to PE (Santa Cruz, cat. no. sc-365406)
SLC1A3 monoclonal mouse primary antibody (Santa Cruz, cat. no. sc-515839)
IRF8 monoclonal mouse antibody conjugated to PE (Thermo Fisher Scientific, cat. no. 12-9852-82)
OLIG2 polyclonal goat primary antibody (R&D Systems, cat. no. AF2418)
Donkey anti-Chicken A647 secondary antibody (Jackson ImmunoResearch, cat. no. 703-605-155)
Donkey anti-Mouse A594 secondary antibody (Invitrogen, cat. no. A-21203)
Donkey anti-Mouse A488 secondary antibody (Invitrogen, cat. no. A-21202)
Donkey anti-Goat A594 secondary antibody (Invitrogen, cat. no. A-11058)

Bucket with ice
Refrigerating centrifuge with a swing-out rotor for 1.5 ml tubes (e.g., Eppendorf, cat. no. 5810-R) cooled to 4°C
Shaker
Nuclease-free 1.5-ml microcentrifuge tubes (e.g., USA Scientific, cat. no. 1615-5500)
Cell strainer snap cap (Corning, cat. no. 352235)
FACS with at least a 4-way sorting option and the ability to detect Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor 647 and DAPI.
Low Retention Microcentrifuge Tubes (e.g., Thermo Fisher Scientific, cat. no. 3448)

Quenching Autofluorescence of Nuclei with Trueblack Plus Treatment

TrueBlack Plus can be omitted when autofluorescence is not an issue. If this is the case, proceed directly to primary antibody incubation.

1. Prepare TrueBlack Plus Mix: 7.5 µl TrueBlack Plus + 300 µl PBS.
2. Centrifuge nuclei (from step 17 of Basic Protocol 1) for 4 min, 1000 × *g*, 4°C.
3. Pour out supernatant and add up to 1 ml Wash buffer.
4. Centrifuge for 5 min, 1000 × *g*, 4°C.
5. Pipette off supernatant, leaving 100 µl at the bottom.
6. Add TrueBlack Plus Mix and resuspend nuclei. Incubate 5 min at room temperature.

Table 2 Antibodies and Dilutions Used in Basic Protocol 2

	Primary antibody 1	Primary antibody 2	Primary antibody 3	Primary antibody 4	Secondary antibody 1	Secondary antibody 2
Separation Sort Staining strategy	NeuN chicken 1:500	SATB2 mouse 1:100	BCL11B rat 1:100	TLE4 mouse conjugated 1:100	A647 Donkey anti-chicken 1:1000	A594 Donkey anti-mouse 1:1000
Post Sort Staining strategy	Olig2 goat 1:500	EAAT1 mouse 1:1000	IRF8 mouse conjugated 1:100		A594 Donkey anti-goat 1:1000	A594 Donkey anti-mouse 1:1000

7. Add up to 1 ml Wash buffer and mix by pipetting.
8. Centrifuge for 5 min, 1000 × *g*, 4°C.
9. Pour out supernatant.
10. Repeat the wash steps (7, 8, and 9) two more times.

Staining of Nuclei with Antibodies

11. Directly add primary antibodies to nuclei in 100 µl Wash Buffer as shown in **Table 2**.

    We use the minimum possible volume to conserve antibody while still ensuring accurate pipetting of antibody volume. For antibody dilutions between 1:100 and 1:500, we add primary antibodies to 100 µl sample for incubation.

12. Incubate at room temperature for 1 hr on the shaker on gentle rotation or at 4°C overnight.
13. Add up to 1 ml Wash buffer and resuspend.
14. Centrifuge for 3 min, 1000 × *g*, 4°C.
15. Pour out supernatant.
16. Repeat the wash steps (13, 14, and 15) two more times.
17. Adjust sample volume to 500 µl with Wash buffer.
18. Add secondary antibodies at 1:1000 dilution, as shown in **Table 2**.
19. Incubate at RT for 30 min on the shaker on gentle rotation, covered with foil.
20. Add 1 ml Wash buffer and resuspend.
21. Centrifuge for 3 min, 1000 × *g*, 4°C.
22. Pour out supernatant.
23. Repeat the wash steps (20, 21, and 22) two more times.
24. Resuspend nuclei in 500 µl DAPI buffer.

    The suspension can always be diluted with DAPI buffer if necessary.

25. Place sample on ice and filter it through a cell strainer snap cap into an empty tube compatible with the FACS sorter immediately before proceeding to FANS.

Serial FANS of Neuronal Nuclei

For a visualization of the following gating steps, refer to **Figure 2**. Gating strategy for the collection of cortical neuronal nuclei.

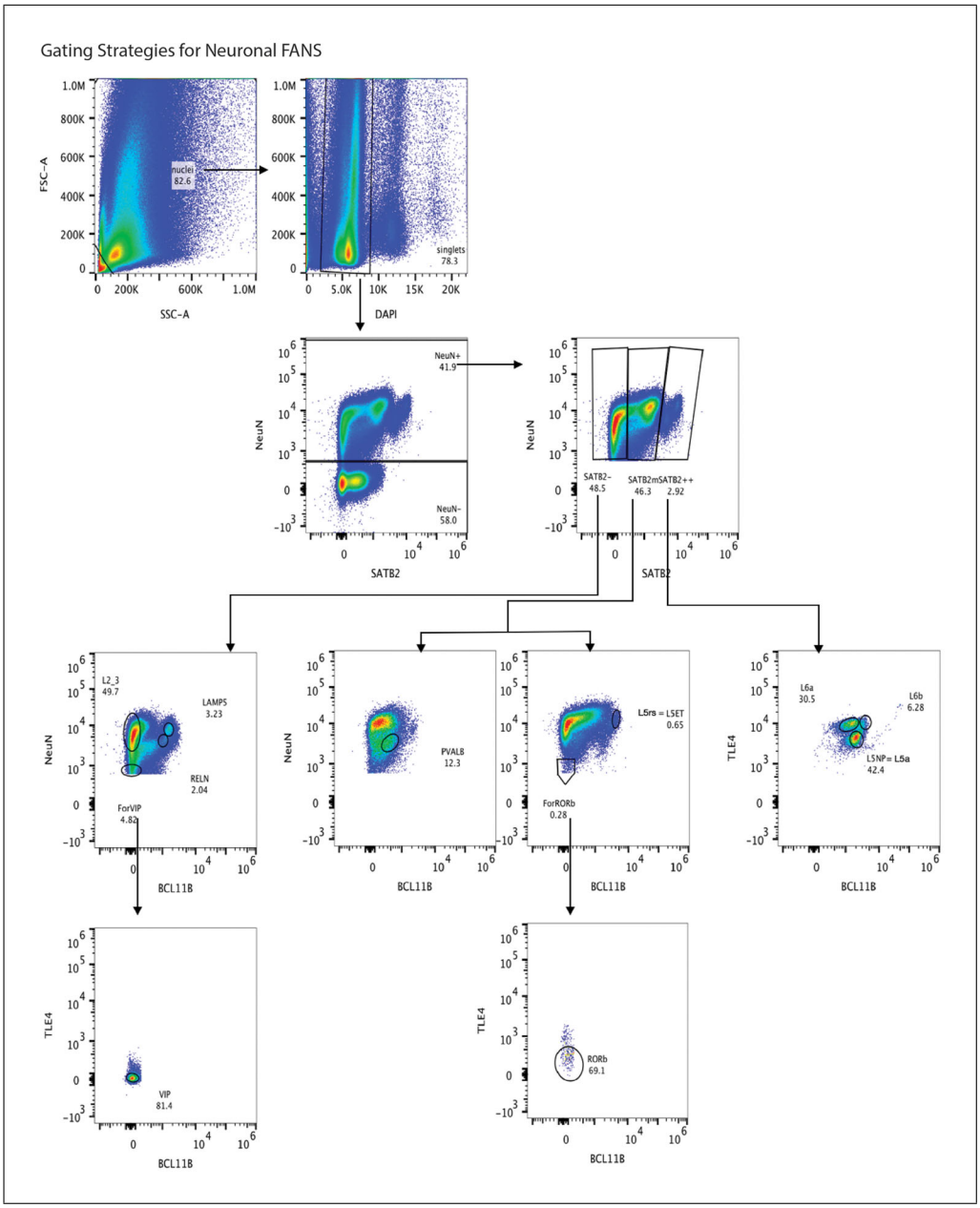

Figure 2 Gating strategy for the collection of cortical neuronal nuclei.

26. Visualize forward scatter area (FSC-A), side scatter area (SSC-A), and DAPI staining intensity on a linear scale.

27. Visualize fluorophore measurements in biexponential mode.

28. Perform particle size selection for nuclei by excluding particles below approximately 100K along the FSC-A and SSC-A axes.

29. Perform doublet discrimination by gating on the lowest positive population on the linear DAPI axis.

30. Separate NeuN-positive neuronal nuclei from NeuN-negative non-neuronal nuclei along the NeuN axis.

31. Plot NeuN-positive nuclei along the NeuN and SATB2 axes.

32. Divide populations into three NeuN-positive populations to perform your separation sort for:

    a. NeuN+/SATB2− nuclei.
    b. NeuN+/SATB2med nuclei.
    c. NeuN+/SATB2+ nuclei.
    d. NeuN− nuclei (proceed to post-staining procedure with this population, step 39)

33. Plot NeuN+/SATB2− nuclei against NeuN and BCL11B to reveal four subpopulations:

    a. NeuN+low/SATB2−/BCL11B− nuclei for VIP-expressing interneurons. This VIP-expressing population can be further purified by plotting and gating this population against nuclei that spread upwards along the TLE4 axis.
    b. NeuN+high/SATB2−/BCL11B− nuclei for CUX2 expressing cortical layer II/III excitatory neurons.
    c. NeuN+high/SATB2−/BCL11Bmedium nuclei for a RELN-expressing inhibitory neuron population.
    d. NeuN+high/SATB2−/BCL11B+ nuclei for a LAMP5-expressing inhibitory neuron population.

34. Plot NeuN+/SATB2medium nuclei along the SATB2 axis and divided into NeuN+/SATB2mediumlow and NeuN+/SATB2mediumhigh populations.

35. Plot both populations, NeuN+/SATB2mediumlow and NeuN+/SATB2 mediumhigh, against NeuN and BCL11B to reveal four subpopulations:

    a. NeuN+low/SATB2mediumlow/BCL11B+ nuclei for PVALB-expressing inhibitory neurons.
    b. NeuN+low/SATB2mediumhigh/BCL11B− nuclei for layer IV excitatory neurons. This RORb-expressing population can be further purified by plotting and gating against nuclei that spread upwards along the TLE4 axis.
    c. NeuN+high/SATB2mediumhigh/BCL11B+high is a small region-specific population, enriched for POU3F1, a transcription factor expressed by Betz cells and Von Economo neurons.
    d. NeuN+low/SATB2mediumhigh/BCL11B+high is a small sample and region-specific population expressing CRHBP.

36. Plot NeuN+/SATB2+ nuclei against TLE4 and BCL11B to reveal three subpopulations:

    a. NeuN+/SATB2+/BCL11B+/TLE4+ for HTR2C-expressing layer Va excitatory neurons.
    b. NeuN+/SATB2+/BCL11B+/TLE4+high for CTGF- and NPFFR2-expressing layer VIa excitatory neurons.
    c. NeuN+/SATB2+/BCL11B+high/TLE4+high for CTGF-expressing layer VIb excitatory neurons. This cell population is sparse, does not express NPFFR2, and specific marker genes appear variable across the different regions of the cortex.

37. FANS-seq Sample Handling: After sorting the respective populations, nuclei should be split into separate tubes if the number of sorted nuclei is greater than 100,000.

    a. If the sorted sample volume is >300 µl, you can pellet the nuclei by centrifugation (2600 × g, 10 min) and remove the supernatant, leaving at least a 50–100 µl residual volume.

    b. Add Buffer PKD (from Qiagen Allprep DNA/RNA FFPE kit) to 150 µl total volume if nuclei are in 100 µl or less. If collected nuclei are in more than 100 µl, add 50% volume of PKD. Following the addition of Buffer PKD from the Qiagen FFPE RNA isolation kit,

nuclei in Buffer PKD should be immediately placed on dry ice before sorting additional samples.

38. ATAC-seq Sample Handling: *No PKD is added to the samples, and further handling steps are described in Support Protocol 1*.

Staining NeuN-Negative Nuclei with Antibodies After the 1st Round of Sorting

39. Centrifuge NeuN− nuclei for 10 min, 1600 × *g*, 4°C.
40. Pour out supernatant and resuspend nuclei in 1 ml Wash buffer. If you have multiple tubes containing NeuN− nuclei, combine these in a new Eppendorf tube.
41. Centrifuge for 4 min, 1000 × *g*, 4°C.
42. Remove supernatant, leaving 200 µl at the bottom.
43. Directly add primary antibodies to nuclei in Wash buffer as indicated in **Table 2**.
44. Incubate at room temperature for 1 hr with gentle rotation.
45. Add up to 1 ml Wash buffer and mix.
46. Centrifuge for 3 min, 1000 × *g*, 4°C.
47. Pour out supernatant.
48. Repeat the wash steps (43, 44, and 45) two more times.
49. Add up to 500 µl Wash buffer with secondary antibodies (1:1000).
50. Incubate at room temperature for 30 min with gentle rotation, covered with foil.
51. Add up to 1 ml Wash buffer and resuspend.
52. Centrifuge for 3 min, 1000 × *g*, 4°C.
53. Pour out supernatant.
54. Repeat the wash steps (49, 50, and 51) two more times.
55. Resuspend nuclei in 500 µl DAPI buffer. *The sample can always be diluted with DAPI buffer if necessary*.
56. Place sample on ice and filter the suspension through a cell strainer snap cap into an empty tube compatible with the FACS immediately before proceeding to FANS.

FANS of Non-Neuronal Nuclei

For a visualization of the following gating steps, refer to **Figure 3**. Gating strategy for the collection of cortical non-neuronal nuclei.

57. Plot previously separated NeuN-negative post-stained nuclei along the EAAT1 and OLIG2 as well as along the IRF8 and OLIG2 axes to isolate four subpopulations:
    i. NeuN− EAAT1 (aka SLC1A3) positive nuclei for astrocytes.
    ii. NeuN− IRF8 positive nuclei for microglia.
    iii. NeuN− OLIG2 positive nuclei for oligodendrocytes.
    iv. NeuN− OLIG2 high positive nuclei for OPCs.
58. FANS-seq Sample Handling: After sorting the respective populations, nuclei should be split into separate tubes if the number of sorted nuclei is greater than 100,000.

    a. If the sorted sample volume is >300 µl, you can pellet the nuclei by centrifugation (2600 × g, 10 min) and remove the supernatant, leaving at least a 50–100 µl residual volume.

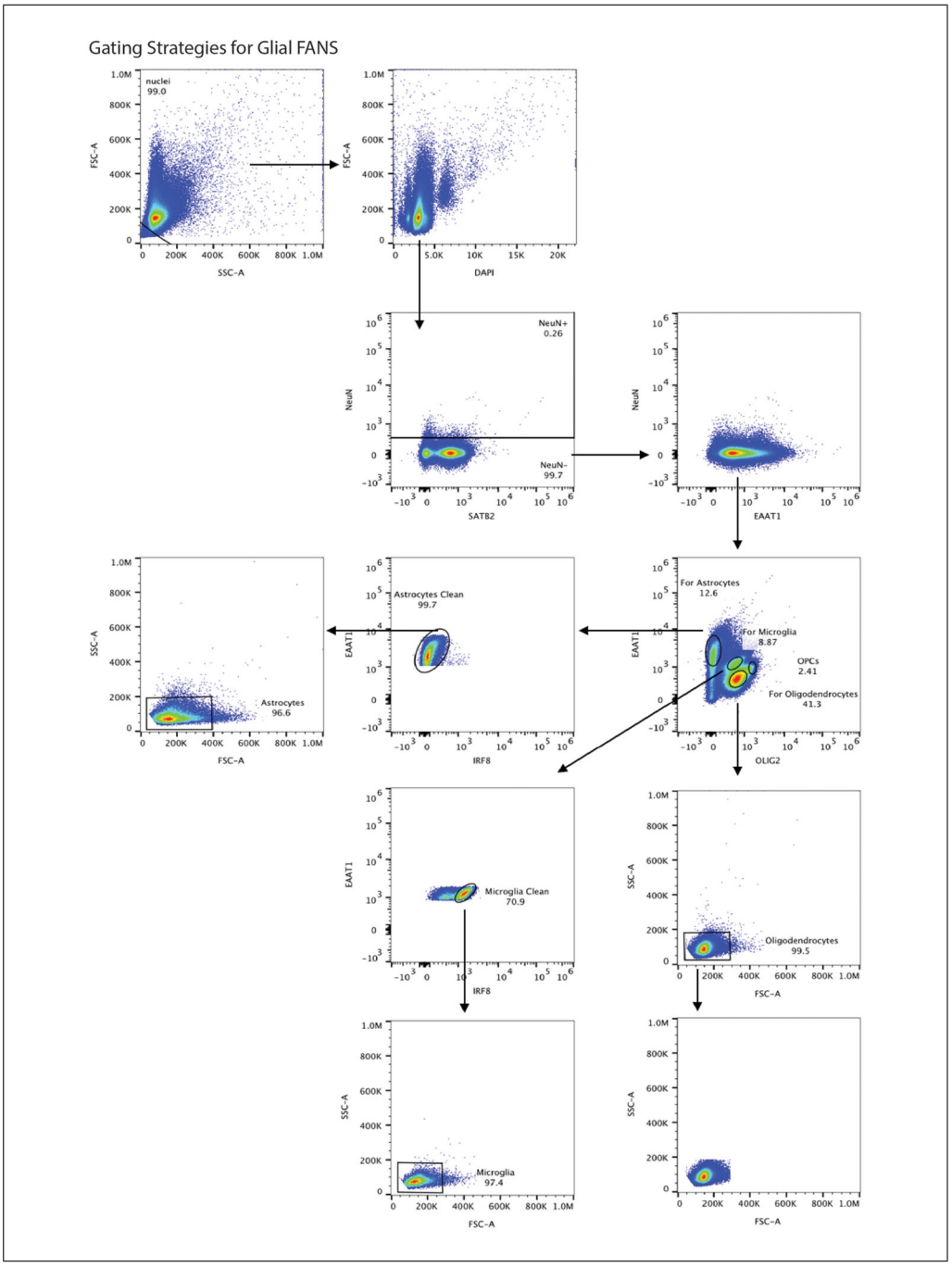

Figure 3 Gating strategy for the collection of cortical non-neuronal nuclei.

b. Add Buffer PKD (from Qiagen Allprep DNA/RNA FFPE kit) to 150 µl total volume if nuclei are in 100 µl or less. If collected nuclei are in more than 100 µl, add 50% volume of PKD. Following the addition of Buffer PKD from the Qiagen FFPE RNA isolation kit, nuclei in Buffer PKD should be immediately placed on dry ice before sorting additional samples.

59. ATAC-seq Sample Handling: No PKD is added to the samples, and further handling steps are described in Support Protocol 1.

GENERATION OF ATAC-seq LIBRARIES FROM THE NUCLEI OF SPECIFIC NEURON TYPES OF THE CEREBRAL CORTEX

Promoters of actively transcribed genes and regulatory DNA elements are more readily accessible to nuclease cleavage and adapter ligation by Tn5 transposase (Buenrostro et al., 2013). Thus, the assay for transposase-accessible chromatin with sequencing (ATAC-seq) can provide cell type-specific data that is independent of but complementary to gene expression profiling by FANS-seq.

To characterize chromatin accessibility from specific neuron types of the cerebral cortex, we developed a protocol in which the nuclei are treated with Tn5 transposase after they have been isolated by FANS (as described in Basic Protocol 2). There are two reasons for this. First, the specific pattern of staining that enables the isolation of nuclei from these cortical neuron subtypes (Fig. 2) is not completely reproducible when performed on Tn5-treated nuclei. Second, the abundance of each of the cortical neuron subtypes is relatively low. Thus, the collection of sufficient numbers of nuclei for ATAC-seq library generation would require Tn5-treatment of very large numbers of unsorted nuclei and a large amount of Tn5 (if performed before FANS). Therefore, we describe the procedure for treating cortical neuronal nuclei with Tn5 after fixation and FANS. This protocol also describes how, for ATAC-seq library preparation, the genomic DNA extracted from these populations of cell nuclei is used as template DNA in PCR amplification with primers that contain sequencing adapters and sample-specific barcodes for demultiplexing of sequencing data.

Materials

Lysis Buffer (chilled on ice, see recipe)
Illumina Tagment DNA TDE1 Enzyme and Buffer Small Kit (Illumina, cat. no. 20034197)
2×TD Buffer (chilled on ice, see recipe)
DNase/RNase-Free water
Reverse Crosslink Solution (see recipe)
MinElute Reaction Cleanup Kit (Qiagen, cat. no. 28204)
Barcoded PCR amplification primers (Supplementary Table 1 in (Buenrostro et al., 2013))
Q5 2× High Fidelity DNA Polymerase (New England Biolabs, cat. no. M0492L)
AMPure XP Reagent (Beckman Coulter, cat. no. A63881)
80% Ethanol (see recipe)

Bucket with ice
Heat block
PCR strip tubes (Millipore, cat. no. 11667009001)
PCR thermocycler
High Sensitivity D1000 ScreenTape (Agilent, cat. no. 5067-5584)
High Sensitivity D1000 Reagents (Agilent, cat. no. 5067-5585)
Agilent 4200 TapeStation System
Qubit™ 1× dsDNA HS Assay Kit (Thermo Fisher Scientific, cat. no. Q33231)
Qubit Fluorometer

Treatment of FANS-isolated Nuclei with Tn5 Transposase and Extraction of Genomic DNA

1. Centrifuge populations of sorted nuclei (step 38 of Basic Protocol 2 for neuronal populations, step 59 of Basic Protocol 2 for glial populations, no PKD added) for 5 min at 950 × *g*, 4°C.

2. Carefully remove supernatant by pipetting and resuspend pellet in 50 µl Lysis Buffer when using 50,000 nuclei or less. Increase the volume of Lysis Buffer by 10 µl for every additional 10,000 nuclei. Centrifuge for 10 min at 500 × *g*, 4°C.

3. Carefully remove supernatant by pipetting, and resuspend nuclei in Transposition Mix (50 µl when using 50,000 nuclei or less, 10 µl for every additional 10,000 nuclei):

   Transposition Mix (per 50 µl rxn)

2×TD Buffer	25 µl
Tagment DNA TDE1 Enzyme (Tn5)	2.5 µl
DNase/RNase-Free water	22.5 µl

   Prepare Transposition Mix immediately before using it to resuspend the nuclei.

4. Incubate on a heat block at 37°C for 30 min.

5. Remove sample from the heat block, add 200 µl Reverse Crosslink Solution, and mix by gentle pipetting.

6. Incubate overnight at 55°C, shaking at 500 rpm.

7. Isolate DNA using Qiagen MinElute Reaction Cleanup Kit according to MinElute® Handbook (Protocol: MinElute Reaction Cleanup Kit using a Microcentrifuge), except add 500 µl Buffer ERC for loading into MinElute column and elute into 20 µl Buffer EB.

   The samples can be kept warm (55°C) before loading onto the column to avoid precipitate formation.

Generation of ATAC-seq Sequencing Libraries

8. Proceed with PCR amplification by mixing the following reagents in a clean PCR strip tube (on ice):

Purified gDNA	10 µl (i.e., half of the purified DNA)
Nuclease-free water	10 µl
Ad1_noMX universal primer	2.5 µl (of 25 µM stock)
Ad2.* indexing primer	2.5 µl (of 25 µM stock)
Q5 2× High Fidelity DNA Polymerase	25 µl

   Use unique Ad2. indexing primer for each sample in a sequencing library pool.*

   Run the following program on a PCR thermocycler (72°C for 5 min, 98°C for 30 s, 13×[98°C for 10 s, 63°C for 30 s, 72°C for 1 min]). Use 13 amplification cycles but increase to 15 if the number of isolated Tn5-treated nuclei is less than 15,000.

9. Double-sided bead purification of PCR product:

   a. Add 0.5× (25 µl) AMPure XP Reagent beads (warmed to room temperature) to the PCR sample, pipette up and down 10× to mix thoroughly.
   b. Incubate at room temperature for 10 min.
   c. Place on magnetic rack for 5 min.
   d. Transfer supernatant to a new PCR strip tube.
   e. Add 1.3× original volume (65 µl) AMPure XP Reagent Beads, pipette up and down 10× to mix thoroughly.
   f. Incubate at room temperature 10 min.
   g. Place on magnetic rack for 5 min.
   h. Discard supernatant.
   i. Wash beads with 200 µl freshly prepared 80% EtOH, pipetting over the beads 10×, then discard as completely as possible.

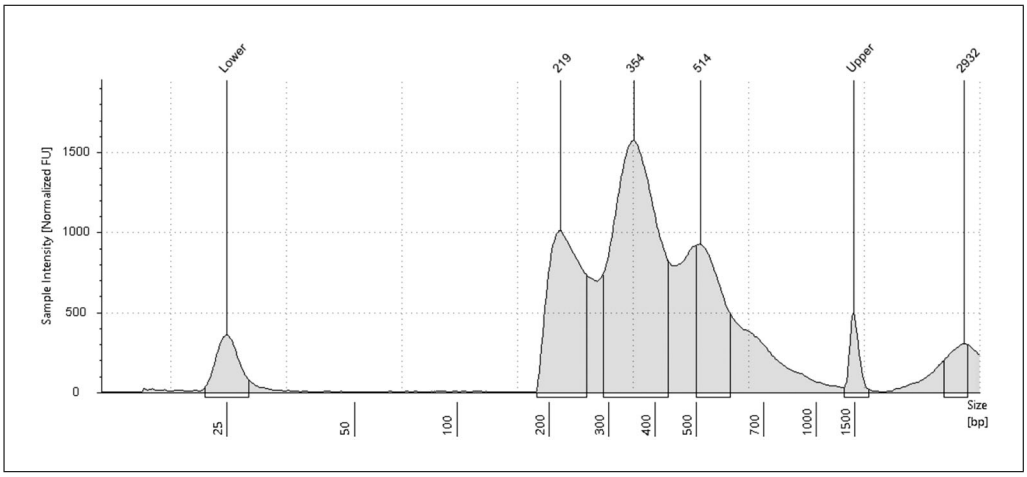

Figure 4 Representative TapeStation trace showing the size distribution of a purified ATAC-seq library generated from ~3200 nuclei of neocortical PVALB+ interneurons. High-quality libraries exhibit a nucleosomal banding pattern.

j. Allow beads to air dry with cap open for 5 min on magnet.
k. Resuspend beads in 20 µl nuclease-free H_2O, and pipette up and down 10× to mix thoroughly.
l. Incubate on magnet for 5 min.
m. Transfer 19 µl of the eluted library to a clean tube.

10. Analyze the size distribution of purified DNA fragments, e.g., on an Agilent TapeStation using High Sensitivity D1000 ScreenTape (Fig. 4).
11. Measure double-stranded DNA concentration with Qubit™ 1× dsDNA HS Assay Kit. Use Qubit-measurement results to pool libraries for sequencing.
12. Assessment of Illumina-sequencing data quality from these ATAC-seq libraries is described in Basic Protocol 5.

NUCLEIC ACID HYBRIDIZATION-BASED LABELING AND ISOLATION OF NUCLEI OF SPECIFIC STRIATAL PROJECTION NEURON TYPES

BASIC PROTOCOL 3

This protocol, along with Alternate Protocol 1, describes the materials, labeling procedure, and three mutually exclusive FANS strategies for the isolation of specific striatal neuron nuclei populations in the following combinations:

1. *DRD1*− and *DRD2*-expressing striatal medium spiny neurons (MSNs) (Basic Protocol 3)
2. Striatal *TAC3*+ interneurons, *SST*+ interneurons, *PVALB*+ interneurons, and MSNs (both MSN subtypes combined) (Alternate Protocol 1)
3. Striatal *CHAT*+ cholinergic interneurons and MSNs (both MSN subtypes combined) (Alternate Protocol 1)

The input material for these protocols is the suspension of cell nuclei collected according to Basic Protocol 1 (Fig. 1). The labeling procedure is based on the PrimeFlow kit and specific PrimeFlow probes that hybridize to nuclear RNA molecules present in specific cell types only. Labeling RNA molecules instead of nuclear protein epitopes enables the selection of labeling probes for a significantly wider list of cell-specific target genes. It has enabled separate analysis of the two subtypes of striatal projection neurons—dopamine receptor D1 (DRD1)- and dopamine receptor D2 (DRD2)-expressing MSNs. Notably, this possibility is important for users who are interested in further development of this method for other brain regions and cell types.

Basic Protocol 3 describes the staining of MSNs with PrimeFlow probes binding to *DRD1* and *DRD2* transcripts. The striatal projection neuron nuclei obtained with this procedure can be frozen until extraction of RNA and genomic DNA (Basic Protocol 4). The procedure for generating ATAC-seq datasets for chromatin accessibility analysis of these cell types is described in Support Protocol 2.

Materials

PrimeFlow™ RNA Assay Kit (Thermo Fisher Scientific, cat. no. 88-18005-210)
SUPERase-In RNase Inhibitor (Thermo Fisher Scientific, cat. no. AM2696)
Recombinant RNasin Ribonuclease Inhibitor (Promega, cat. no. N2515)
DRD1 PrimeFlow Probe Set (Alexa Fluor 647, Thermo Fisher Scientific, cat. no. VA1-3002351-PF)
DRD2 PrimeFlow Probe Set (Alexa Fluor 488, Thermo Fisher Scientific, cat. no. VA4-3083767-PF)
PPP1R1B PrimeFlow Probe Set (Alexa Fluor 568, Thermo Fisher Scientific, cat. no. VA10-3266354-PF)
DAPI buffer (chilled on ice, see recipe)
AllPrep DNA/RNA FFPE Kit (Qiagen, cat. no. 80234)

Bucket with ice
Refrigerating centrifuge with a swing-out rotor for 1.5-ml tubes (e.g., Eppendorf, cat. no. 5810-R)
Wide-orifice pipette tips e.g., ART™ Wide-Bore Filtered Pipette Tips (Thermo Fisher Scientific, cat. no. 2069GPK)
Nuclease-free 1.5-ml microcentrifuge tubes (e.g., USA Scientific, cat. no. 1615-5500)
Hybridization oven or a heat block with a lid
Cell strainer snap cap (Corning, cat. no. 352235)
FACS with at least a 4-way sorting option and the ability to detect Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor 647 and DAPI.
Low Retention Microcentrifuge Tubes (e.g., Thermo Fisher Scientific, cat. no. 3448)

Labeling of Nuclei with Primeflow Probes

1. Take the suspension of nuclei off the shaker (step 17 of Basic Protocol 1 or step 12 of Support Protocol 2) and centrifuge for 5 min at 800 × *g*, room temperature.

2. Remove supernatant by pipetting, resuspend the pellet in 1 ml PrimeFlow RNA Wash Buffer (PFRWB) by pipetting, and centrifuge for 5 min at 800 × *g*, room temperature.

3. Repeat step 2, but after the centrifugation, leave nuclei in approx. 20–50 µl and resuspend the pellet in PFRWB containing RNase inhibitors (Superase-In Ribonuclease Inhibitor and RNasin Plus RNase inhibitor diluted 1:100) so that the nuclei can be distributed into different tubes (1 to 4) as specified on Figure 5.

   (a) When the nuclei are divided between tubes as specified in Figure 5, most of the nuclei end up in the tube used for sorting (tube 4), while control tubes (1 to 3), used to position sorting gates, contain fewer nuclei. Do not exceed 5 × 10^6 nuclei per tube but perform the staining in several tubes if you have more nuclei than that.

   (b) It is important that the volume of resuspended nuclei in tubes 1–4 is as close to 100 µl as possible.

4. Warm PrimeFlow RNA Target Probe Diluent (TPD) to 40°C. Use 100 µl per tube of nuclei to be stained.

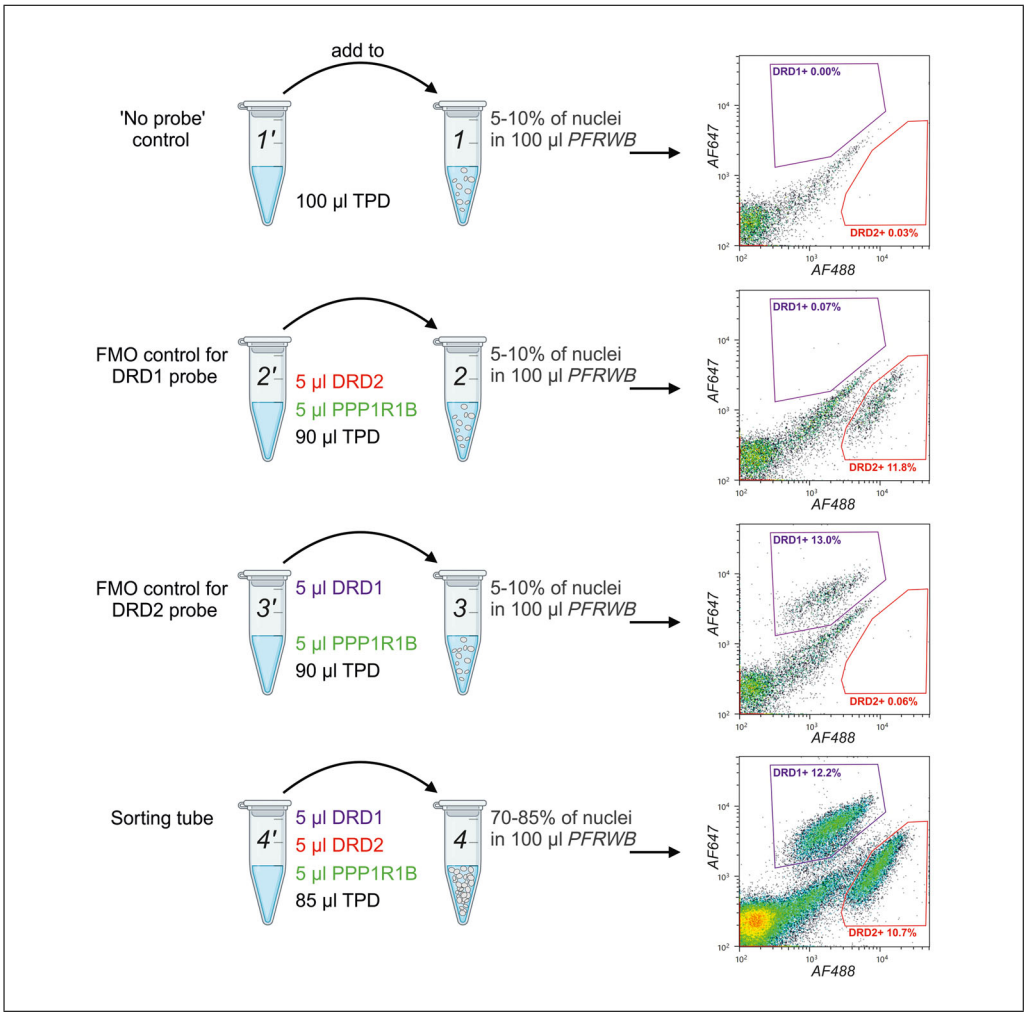

Figure 5 Probe combinations used in a FANS experiment to isolate nuclei of *DRD1*- and *DRD2*-expressing striatal MSNs. PrimeFlow probes are mixed with PrimeFlow RNA Target Probe Diluent (TPD) in tubes 1'–4'. After these mixes are warmed to 40°C, they are added to nuclei divided into tubes 1–4. FANS is performed on nuclei in tube 4. Tubes 1–3 are used as controls that help to position the sorting gates. FMO - Fluorescence Minus One, PFRWB - PrimeFlow RNA Wash Buffer.

5. Pipette 5 µl of each probe to tubes that will be used to mix probes with TPD (tubes 1'–4', Fig. 5). *The probes are stable, and the tubes do not have to be kept on ice.*

6. Bring up the volume to 100 µl by adding warm TPD (according to Fig. 5) and warm the mixes to 40°C.

7. Add 100 µl probes+TPD mix directly to the suspension of nuclei with wide-orifice tips, mix gently by pipetting, and avoid bubbles. Put the mix of nuclei and probes immediately back to the 40°C heat block before taking all your samples to the hybridization oven.

   Instead of incubation in a hybridization oven, the samples can be kept in a heat block that offers accurate and uniform heating and prevents condensation inside the tubes.

   Here and from this point on, use only wide orifice 200 µl tips for mixing resuspended nuclei with PrimeFlow reagents (i.e., Target Probe Diluent, PreAmp mix, Amp mix, and Label Probe Diluent). For resuspending pellets of nuclei after centrifugation steps, you can use regular 1-ml tips with regular orifice size but keep handling to a minimum.

8. Mix by pipetting the samples gently after 1 hr incubation.

9. After another hour of incubation (2 hr in total), add 1 ml PFRWB to each sample, mix by pipetting 1–2×, and centrifuge for 5 min at 800 × *g*, room temperature.
10. Remove supernatant and resuspend nuclei in 1 ml PFRWB. Centrifuge for 5 min at 800 × *g*, room temperature.
11. Aspirate supernatant to leave nuclei in approx. 20–50 µl and bring the volume to 100 µl by resuspending the pellet in PFRWB containing Superase-In Ribonuclease Inhibitor (diluted 1:100).
12. At this point, samples can be stored overnight in the dark at 2°C to 8°C.
13. Warm PrimeFlow RNA PreAmplification mix (PreAmp Mix) to 40°C. Use 100 µl per tube of nuclei to be stained. Add RNasin Plus RNase inhibitor to PreAmp Mix (diluted 1:100), mix by thorough pipetting and return to heat block. Pre-warm samples and PrimeFlow RNA Wash Buffer to room temperature.
14. Make sure the nuclei are in suspension and add 100 µl warm PreAmp Mix directly to the nuclei suspension for each sample and mix by pipetting. Put the mix of nuclei and probes immediately back on the 40°C heat block before taking all of your samples to the hybridization oven.
15. Incubate for 1.5 hr at 40°C.
16. Add 1 ml PFRWB to each sample, mix by pipetting 1–2×, centrifuge for 5 min at 800 × *g*, room temperature, and remove supernatant.
17. Repeat step 16 two times for a total of three washes. After the last centrifugation, leave 100 µl PFRWB on the nuclei and resuspend the pellet with wide-orifice tips.
18. Warm PrimeFlow RNA Amplification mix (Amp Mix) to 40°C. Use 100 µl per tube of nuclei to be stained. Add RNase inhibitor Amp Mix (Superase-In Ribonuclease Inhibitor and RNasin Plus RNase inhibitor diluted 1:100), mix by thorough pipetting, and return to heat block.
19. Add 100 µl warm Amp Mix directly to the nuclei suspension for each sample and mix by pipetting. Put the mix of nuclei and probes immediately back on the 40°C heat block before taking all of your samples to the hybridization oven.
20. Incubate for 1.5 hr at 40°C.

    Before the end of the incubation, warm PrimeFlow Label Probes to room temperature before mixing these (1:100) with PrimeFlow RNA Label Probe Diluent (LPD) warmed to 40°C. Add RNase inhibitors (Superase-In Ribonuclease Inhibitor and RNasin Plus RNase inhibitor diluted 1:100), mix by thorough pipetting, and return to heat block. You will need 100 µl Label Probes+LPD mix per each sample.

21. Add 1 ml PFRWB to each sample, mix by pipetting 1–2×, centrifuge for 5 min at 800 × *g*, room temperature, and remove supernatant.
22. Repeat step 21 once more for a total of two washes. After the last centrifugation, leave 100 µl PFRWB on the nuclei and resuspend the pellet with wide-orifice tips.
23. Add 100 µl warm PrimeFlow Label Probes+LPD Mix directly to the nuclei suspension for each sample and mix by pipetting. Put the mix of nuclei and probes immediately back on the 40°C heat block before taking all your samples to the hybridization oven.
24. Incubate for 1 hr at 40°C. Keep samples protected from light from this step onwards.
25. Add 1 ml PFRWB to each sample, mix by pipetting 1–2×, centrifuge for 5 min at 800 × *g*, room temperature, and remove supernatant.

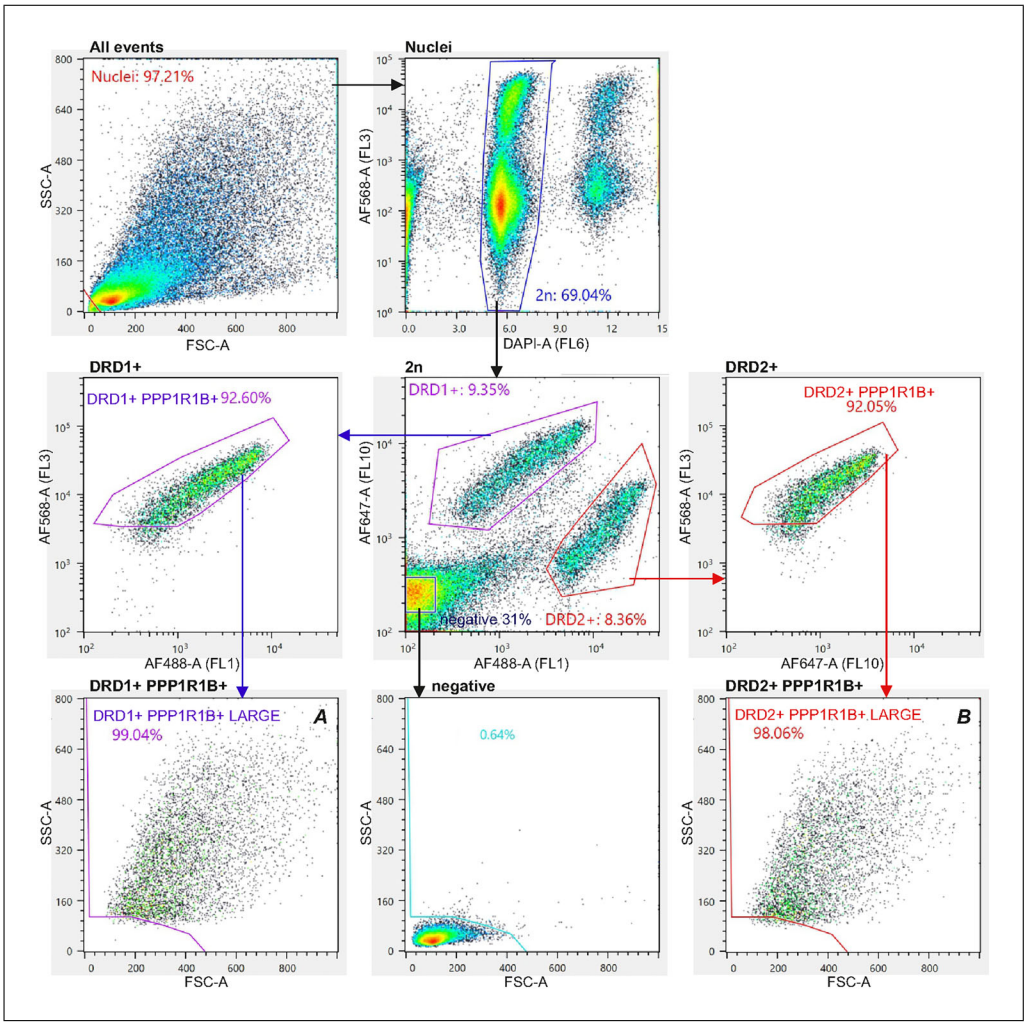

Figure 6 FANS strategy for isolating nuclei of *DRD1*- and *DRD2*-expressing striatal MSNs. After selecting nuclei from the 'SSC vs. FSC' plot (i.e., 'side scatter vs. forward scatter'), the gate on euploid DNA-containing objects (2n nuclei) so that possible aggregates of nuclei are left out. For collecting DRD2-expressing neuron nuclei, select DRD2+ (i.e., AF488+) nuclei and, to assess PPP1R1B-positivity (i.e., AF568+), display these on a plot showing signal recorded by detectors for AF647 and AF568 (red arrows). Select PPP1R1B+ (i.e., AF568+) nuclei and display these on a plot showing SSC and FSC. Select and collect nuclei that have higher SSC and FSC signals (sorting **gate B**) as MSN nuclei appear larger and more granular than glial cell nuclei or most striatal interneuron nuclei. For collecting DRD1-expressing neuron nuclei (sorting **gate A**), follow a similar strategy by selecting DRD1+, PPP1R1B+ nuclei that appear large and granular (blue arrows). All gates should be drawn so that they contain as few events as possible in samples lacking the respective DRD2-AF488, DRD1-AF647, and PPP1R1B-AF568 probes. **Gates A** and **B** should be positioned so that nuclei with low autofluorescence (from gate 'negative', mostly non-neuronal nuclei) would be excluded from these gates (middle plot in bottom row).

26. Repeat step 25, keep residual volume to about 50 µl, and place samples on ice.
27. Resuspend nuclei in 800 µl DAPI Buffer (control samples, tubes 1 to 3, in 200 µl).
28. Right before sorting, filter the nuclei suspension through a cell-strainer cap into an empty tube compatible with FACS.

FANS of Striatal Projection Neuron Nuclei

FANS can also be performed on Day 3

29. Collect DRD1+ projection neuron nuclei (sorting **gate A**) and DRD2+ projection neuron nuclei (sorting **gate B**) into low-retention microcentrifuge tubes according to the strategy depicted in Figure 6.

30. FANS-seq Sample Handling: After sorting the respective populations, nuclei should be split into separate tubes if the number of sorted nuclei is greater than 100,000.

    If the sorted sample volume is >300 µl, you can pellet the nuclei by centrifugation (2600 × g, 10 min) and remove the supernatant, leaving at least a 50–100 µl residual volume.

31. Add Buffer PKD (from Qiagen Allprep DNA/RNA FFPE kit) to 150 µl total volume if nuclei are in 100 µl or less. If collected nuclei are in more than 100 µl, add 50% volume of PKD. Following the addition of Buffer PKD from the Qiagen FFPE RNA isolation kit, nuclei in Buffer PKD should be immediately placed on dry ice before continuing with sorting additional samples.

    Handling of ATAC-seq samples is described in Support Protocol 2 (starting at step 13).

ALTERNATE PROTOCOL 1

LABELING AND ISOLATION OF NUCLEI OF SPECIFIC STRIATAL INTERNEURON TYPES

This Alternate Protocol describes labeling probes and sorting strategies for isolating nuclei of striatal interneuron subtypes. Since striatal interneurons are much rarer than MSNs, we recommend that a bigger piece of striatal tissue (≥200 mg) is used for the isolation of nuclei (Basic Protocol 1). The labeling procedure is identical to the one described in detail for Basic Protocol 3, except for the PrimeFlow probes used and the control samples needed. Figures 7 and 8 summarize the recommended control samples and the FANS strategy for isolating the nuclei of *TAC3*+ interneurons, *SST*+ interneurons, *PVALB*+ interneurons, and MSNs (both subtypes combined). Figures 9 and 10 summarize the strategy for isolating the nuclei of *CHAT*+ cholinergic interneurons and MSNs (both subtypes combined). When using good-quality tissue samples, as few as 1000 collected nuclei are sufficient to prepare high-quality FANS-seq libraries.

Additional Materials

For isolating the nuclei of *TAC3*+ interneurons, *SST*+ interneurons, *PVALB*+ interneurons and MSNs
- TAC3 PrimeFlow Probe Set (Alexa Fluor 647, Thermo Fisher Scientific, cat. no. VA1-16603-PF)
- ETV1 PrimeFlow Probe Set (Alexa Fluor 488, Thermo Fisher Scientific, cat. no. VA4-3083818-PF)
- SST PrimeFlow Probe Set (Alexa Fluor 568, Thermo Fisher Scientific, cat. no. VA10-3252595-PF)
- PPP1R1B PrimeFlow Probe Set (Alexa Fluor 568, Thermo Fisher Scientific, cat. no. VA10-3266354-PF)

For isolating the nuclei of *CHAT*+ cholinergic interneurons and MSNs
- TRPC3 PrimeFlow Probe Set (Alexa Fluor 647, Thermo Fisher Scientific, cat. no. VA1-3004835-PF)
- COL6A6 PrimeFlow Probe Set (Alexa Fluor 647, Thermo Fisher Scientific, cat. no. VA1-3014134-PF)
- PPP1R1B PrimeFlow Probe Set (Alexa Fluor 568, Thermo Fisher Scientific, cat. no. VA10-3266354-PF)

Labeling and Isolating the Nuclei of TAC3+ Interneurons, SST+ Interneurons, PVALB+ Interneurons and MSNs

Follow the procedure described in Basic Protocol 3, except use PrimeFlow probes as specified in Figure 7 (at step 5 of Basic Protocol 3) and the FANS strategy explained in Figure 8.

Labeling and Isolating the Nuclei of Striatal Cholinergic Interneurons and MSNs

Follow the procedure described in Basic Protocol 3, except use PrimeFlow probes as specified in Figure 9 (at step 5 of Basic Protocol 3) and the FANS strategy explained in Figure 10.

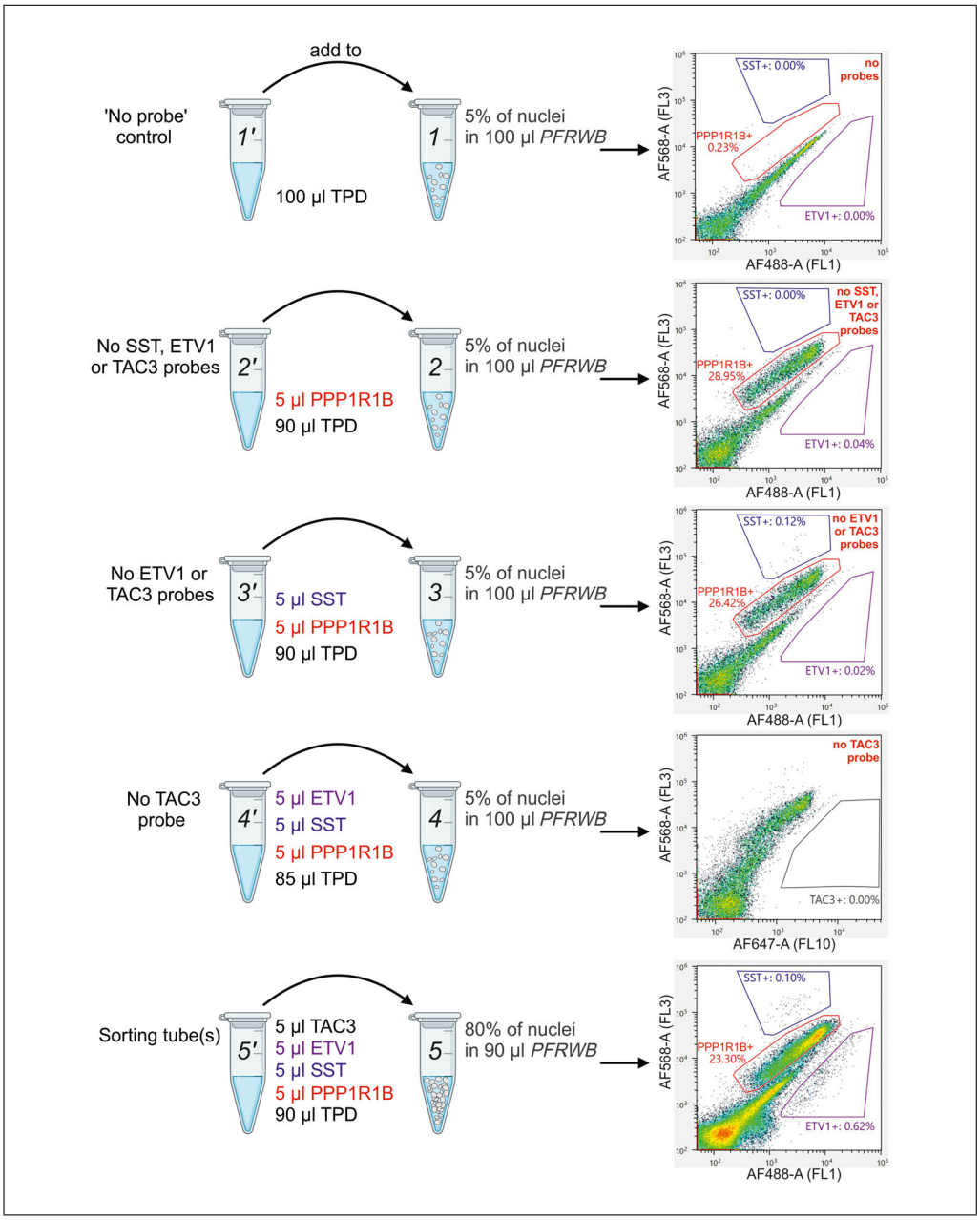

Figure 7 Probe combinations used in a FANS experiment to isolate nuclei of striatal *TAC3*+ interneurons, *SST*+ interneurons, *PVALB*+ interneurons and MSNs. PrimeFlow probes are mixed with PrimeFlow RNA Target Probe Diluent (TPD) in tubes 1'–5'. After these mixes are warmed to 40°C, they are added to nuclei divided into tubes 1–5. FANS is performed on nuclei in tube 5. Tubes 1–4 are used as controls that help to position the sorting gates. PFRWB - PrimeFlow RNA Wash Buffer.

GENERATION OF ATAC-seq LIBRARIES FROM THE NUCLEI OF SPECIFIC STRIATAL NEURON TYPES

SUPPORT PROTOCOL 2

The labeling of striatal neuron nuclei (Basic Protocol 3) is dependent on relatively long incubations at a temperature of +40°C, and these conditions might alter the native chromatin structure and result in experimental artifacts in chromatin accessibility measured by ATAC-seq. We have, therefore, developed a procedure, described here in Support Protocol 2, in which a sufficient number of cell nuclei, collected according to Basic Protocol 1, is treated with Tn5 transposase before formaldehyde fixation. These cell nuclei, carrying ATAC-seq adapters in the accessible sites of their genomes, undergo PrimeFlow-based labeling and FANS as described in Basic Protocol 3. For ATAC-seq

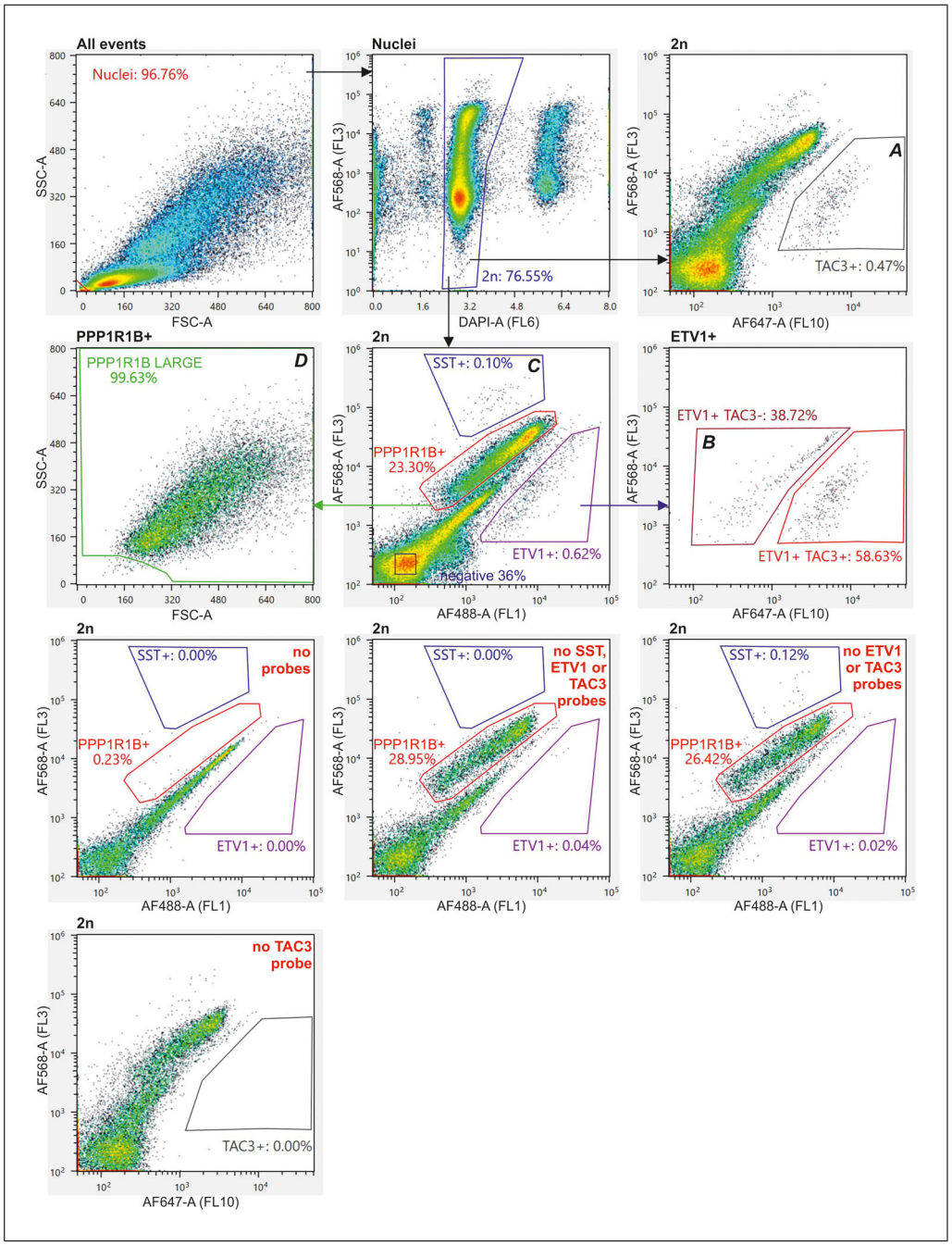

Figure 8 FANS strategy for isolating nuclei of striatal *TAC3*+ interneurons, *SST*+ interneurons, *PVALB*+ interneurons and MSNs. After selecting nuclei from the 'SSC vs. FSC' plot, the gate on euploid DNA-containing objects (2n nuclei, usually the major population) so that possible aggregates of nuclei with higher DAPI signal intensity are left out. To collect MSN nuclei, select PPP1R1B+ (i.e., AF568+) nuclei and display these on a plot showing SSC and FSC (green arrows). Select nuclei with higher SSC and FSC signals as MSN nuclei appear larger and more granular than glial cell nuclei or most striatal interneuron nuclei (sorting **gate D**). Somatostatin (SST)-positive interneuron nuclei are highly positive for AF568 (sorting **gate C**), and TAC3-expressing interneuron nuclei are labeled strongly with the TAC3 probe (i.e., AF647+, sorting **gate A**). For collecting parvalbumin-positive (PVALB+) interneuron nuclei, select ETV1+ (i.e., AF488+) nuclei, and out of those select nuclei that are not labeled with the TAC3 probe (i.e., AF647-negative, sorting **gate B**, blue arrow). All gates on labeled populations should be drawn to contain as few events as possible in samples lacking the respective SST, TAC3, ETV1, and PPP1R1B probes (two bottom rows). **Gate D** should be positioned so that nuclei with low autofluorescence (from gate 'negative', mostly non-neuronal nuclei) would be excluded (not shown).

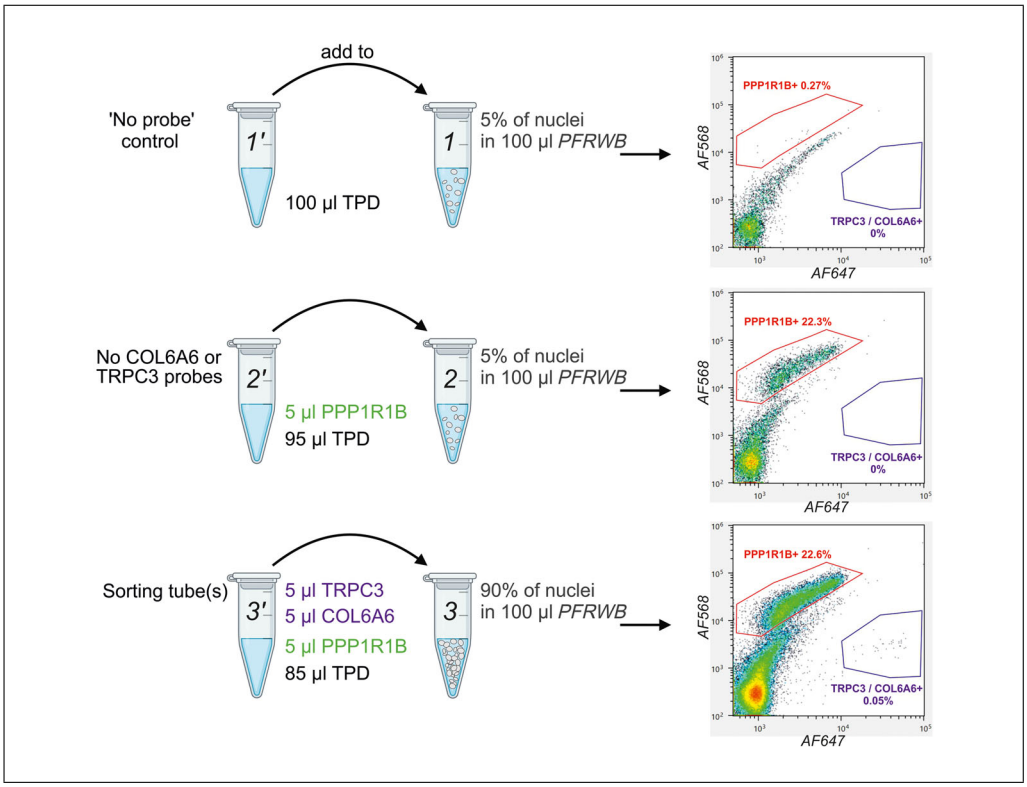

Figure 9 Probe combinations used in a FANS experiment to isolate the nuclei of striatal cholinergic interneurons and MSNs. PrimeFlow probes are mixed with PrimeFlow RNA Target Probe Diluent (TPD) in tubes 1'–3'. After these mixes are warmed to 40°C, they are added to nuclei divided into tubes 1–3. FANS is performed on nuclei in tube 3. Tubes 1–2 are used as controls that help to position the sorting gates. PFRWB - PrimeFlow RNA Wash Buffer.

library preparation, the genomic DNA extracted from sorted populations of cell nuclei is used as template DNA in PCR amplification with primers that contain sequencing adapters and sample-specific barcodes for demultiplexing of sequencing data.

Materials

1× PBS containing DAPI (see recipe)
Lysis Buffer (chilled on ice, see recipe)
Illumina Tagment DNA TDE1 Enzyme and Buffer Small Kit (Illumina, cat. no. 20034197)
2×TD Buffer (chilled on ice, see recipe)
SUPERase-In RNase Inhibitor (Thermo Fisher Scientific, cat. no. AM2696)
Recombinant RNasin Ribonuclease Inhibitor (Promega, cat. no. N2515)
DNase/RNase-Free water
Buffer DEF (prepare right before use)
Reverse Crosslink Solution (see recipe)
MinElute Reaction Cleanup Kit (Qiagen cat. no. 28204)
Barcoded PCR amplification primers (Supplementary Table 1 in (Buenrostro et al., 2013))
Q5 2× High Fidelity DNA Polymerase (New England Biolabs, cat. no. M0492L)
AMPure XP Reagent (Beckman Coulter, cat. no. A63881)
80% ethanol (see recipe)

Bucket with ice
Countess 3 Automated Cell Counter (Thermo Fisher Scientific or regular hemocytometer)

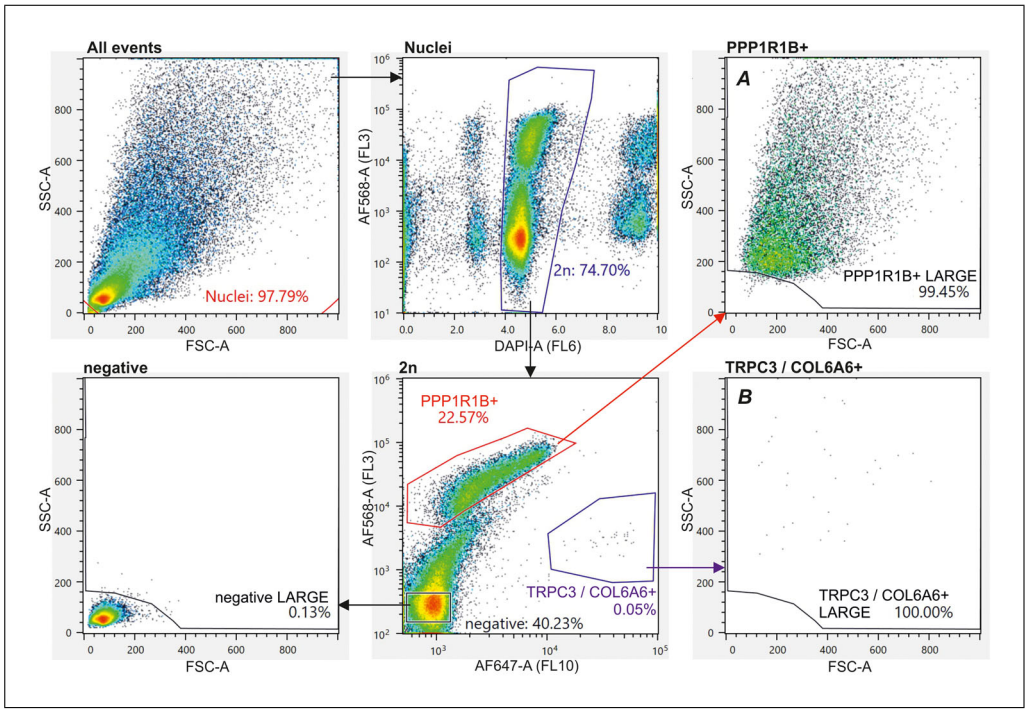

Figure 10 FANS strategy for isolating nuclei of striatal cholinergic interneurons and MSNs. After selecting nuclei from 'SSC vsFSC' plot, gate on euploid DNA-containing objects (2n nuclei, usually the major population) so that possible aggregates of nuclei with higher DAPI signal intensity are left out. To collect MSN nuclei, select PPP1R1B+ (i.e., AF568+) nuclei and display these on a plot showing SSC and FSC (red arrow). Select nuclei that have higher SSC and FSC signals, as MSN nuclei appear larger and more granular than glial cell nuclei or most striatal interneuron nuclei (sorting **gate A**). To collect cholinergic interneuron nuclei, follow a similar strategy by selecting TRPC3/COL6A6+ (i.e., AF647+) nuclei that appear large and more granular (sorting **gate B**) (blue arrow). All gates on labeled populations should be drawn so that they contain as few events as possible in samples lacking the respective TRPC3/COL6A6-AF647 and PPP1R1B-AF568 probes. **Gates A** and **B** should be positioned so that nuclei with low autofluorescence (from gate 'negative', mostly non-neuronal nuclei) would be excluded from these gates (bottom left plot).

Countess™ Cell Counting Chamber Slides (Thermo Fisher Scientific, cat. no. C10228)
Heat block
PCR strip tubes (Millipore, cat. no. 11667009001)
PCR thermocycler
High Sensitivity D1000 ScreenTape (Agilent, cat. no. 5067-5584)
High Sensitivity D1000 Reagents (Agilent, cat. no. 5067-5585)
Agilent 4200 TapeStation System
Qubit™ 1× dsDNA HS Assay Kit (Thermo Fisher Scientific, cat. no. Q33231)
Qubit Fluorometer

Treatment of Unfixed Nuclei with Tn5 Transposase

1. Take a small aliquot of the cell nuclei isolated by ultracentrifugation and resuspended in buffer D (step 11 in Basic Protocol 1) and dilute it 10× with 1× PBS containing DAPI (0.5 µg/ml). Load 10 µl of the diluted suspension onto a Countess™ Cell Counting Chamber Slide and count the DAPI-stained nuclei using the DAPI channel of a Countess 3 Automated Cell Counter. Calculate the concentration of nuclei (nuclei/ml) in the original undiluted suspension.

2. Transfer 800,000 nuclei to a new tube and centrifuge for 5 min at 950 × *g*, 4°C.

   The number of nuclei used here depends on the abundance of the cell population from which nuclei are going to be collected by FANS. To analyze each of the two MSN subtypes

with a typical abundance of 5% to 10% of all nuclei, start with 800,000 nuclei, which is expected to result in 20,000 to 40,000 sorted nuclei (assuming 50% recovery). Our experience has shown that high-quality ATAC-seq libraries require a genomic DNA input from a minimum of ∼5000 isolated nuclei. The generation of ATAC-seq libraries from low-abundance cell types thus requires the initial treatment of many more nuclei.

3. Carefully remove the supernatant by pipetting, resuspend pellet in 200 µl Lysis Buffer, and centrifuge for 10 min at 500 × *g*, 4°C.

4. Carefully remove supernatant by pipetting, and resuspend nuclei in Transposition Mix (200 µl per 800 000 nuclei):

Transposition Mix (per 200 µl rxn)

2×TD Buffer	100 µl
Tagment DNA TDE1 Enzyme (Tn5)	10 µl
SUPERase-In RNase Inhibitor	1 µl
Recombinant RNasin Ribonuclease Inhibitor	1 µl
DNase/RNase-Free water	88 µl

Prepare Transposition Mix immediately before using it to resuspend the nuclei.

5. Incubate on a heat block at 37°C for 30 min.

6. Remove sample from the heat block and add 1 ml Buffer DEF. Mix by gentle pipetting and keep on a shaker for 8 min (covered with foil).

7. Quench formaldehyde by adding 100 µl of 2 M glycine. Incubate at room temperature on a shaker for 5 min (covered with foil).

8. Centrifuge for 5 min at 1000 × *g* at 4°C to pellet nuclei. Remove supernatant and resuspend nuclei in 1 ml Buffer D.

9. Centrifuge for 5 min at 1000 × *g* at 4°C to pellet nuclei. Remove supernatant and resuspend nuclei in 1 ml Permeabilization buffer.

10. Centrifuge for 5 min at 800 × *g* at 4°C to pellet nuclei. Remove supernatant and resuspend nuclei in 1 ml Permeabilization buffer.

11. For permeabilization, incubate at room temperature on a shaker for 30 min.

12. Proceed to Basic Protocol 3 (or Alternate Protocol 1) for labeling and FANS.

Extraction of Genomic DNA and Generation of ATAC-seq Sequencing Libraries

The following steps are performed after the nuclei have been collected by sorting.

13. After the Tn5-treated nuclei of interest have been isolated by FANS (step 29 of Basic Protocol 3, no PKD added), centrifuge the nuclei for 10 min at 1600 × *g*, 4°C. Remove supernatant and resuspend nuclei in 200 µl Reverse Crosslink solution. Incubate overnight on a heat block at 55°C.

14. Isolate DNA using Qiagen MinElute Reaction Cleanup Kit according to MinElute® Handbook (Protocol: MinElute Reaction Cleanup Kit using a Microcentrifuge), except add 500 µl Buffer ERC for loading into the MinElute column and elute in 20 µl Buffer EB.

The samples can be kept warm (55°C) before loading onto the column to avoid precipitate formation.

15. Proceed with PCR amplification by mixing the following reagents in a clean PCR strip tube (on ice):

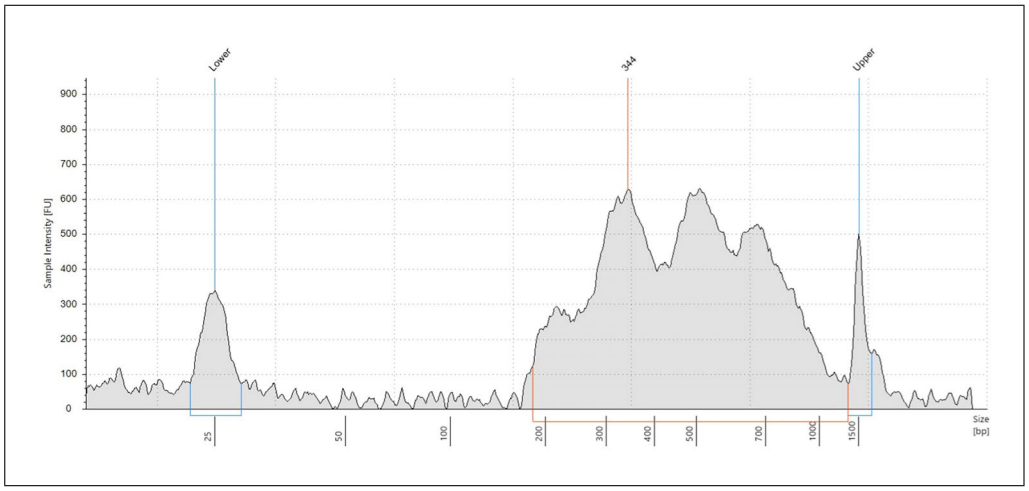

Figure 11 Representative TapeStation trace showing the size-distribution of a purified ATAC-seq library generated from 19,000 nuclei of DRD2-expressing MSNs. High-quality libraries exhibit a nucleosomal banding pattern.

Purified gDNA	10 µl (i.e., half of the purified DNA)
Nuclease-free water	10 µl
Ad1_noMX universal primer	2.5 µl (of 25 µM stock)
Ad2.* indexing primer	2.5 µl (of 25 µM stock)
Q5 2× High Fidelity DNA Polymerase	25 µl

Use unique Ad2. indexing primer for each sample in a sequencing library pool.*

Run the following program on a PCR thermocycler (72°C for 5 min, 98°C for 30 s, 12×[98°C for 10 s, 63°C for 30 s, 72°C for 1 min]). Use 12 cycles of amplification but increase to 13–14 cycles if the number of isolated Tn5-treated nuclei is less than 10,000.

16. Double-sided bead purification of PCR product:
    a. Add 0.5× (25 µl) AMPure XP Reagent beads (warmed to room temperature) to the PCR sample and pipette up and down 10× to mix thoroughly
    b. Incubate at room temperature for 10 min
    c. Place on magnetic rack for 5 min
    d. Transfer supernatant to a new PCR strip tube
    e. Add 1.3× original volume (65 µl) AMPure XP Reagent Beads and pipette up and down 10× to mix thoroughly.
    f. Incubate at room temperature 10 min.
    g. Place on magnetic rack for 5 min
    h. Discard supernatant.
    i. Wash beads with 200 µl freshly prepared 80% EtOH, pipetting over the beads 10×, then discard as completely as possible.
    j. Allow beads to air dry with cap open for 5 min on magnet
    k. Resuspend beads in 20 µl nuclease-free H_2O and pipette up and down 10× to mix thoroughly.
    l. Incubate on magnet for 5 min
    m. Transfer 19 µl of eluted library to a clean tube.
17. Analyze the size distribution of purified DNA fragments, e.g., on an Agilent TapeStation using High Sensitivity D1000 ScreenTape (Fig. 11).
18. Measure double-stranded DNA concentration with Qubit™ 1× dsDNA HS Assay Kit. Use Qubit-measurement results for pooling of libraries for sequencing.

19. Assessment of Illumina-sequencing data quality from these ATAC-seq libraries is described in Basic Protocol 5.

EXTRACTION OF GENOMIC DNA AND NUCLEAR RNA AND PREPARATION OF SEQUENCING LIBRARIES

BASIC PROTOCOL 4

Basic Protocol 4 describes the extraction of nuclear RNA and genomic DNA (gDNA) from populations of nuclei isolated with FANS according to Basic Protocols 2 and 3. We also briefly describe our standard procedure of generating FANS-seq libraries with the Revelo™ RNA-Seq High Sensitivity library preparation kit and pooling these libraries for next-generation sequencing. However, there are different RNA-seq library-preparation kits available and many of these can likely be used in the workflow just as successfully.

Materials

Bucket with ice
AllPrep DNA/RNA FFPE Kit (Qiagen, cat. no. 80234)
MinElute Reaction Cleanup Kit (Qiagen, cat. no. 28204)
Revelo™ RNA-Seq High Sensitivity library preparation kit (Tecan, cat. no. 30201359)
High Sensitivity D1000 ScreenTape (Agilent, cat. no. 5067-5584)
High Sensitivity D1000 Reagents (Agilent, cat. no. 5067-5585)
Agilent 4200 TapeStation System
Qubit™ 1× dsDNA HS Assay Kit (Thermo Fisher Scientific, cat. no. Q33231)
Qubit Fluorometer
3 heat blocks
Nuclease-free 1.5-ml microcentrifuge tubes (e.g., USA Scientific, cat. no. 1615-5500)

The RNA isolation protocol follows the procedure described for RNA isolation in Qiagen's AllPrep® DNA/RNA FFPE Handbook for AllPrep DNA/RNA FFPE Kit.

RNA Extraction

1. Set heat blocks to 56°C, 65°C, and 70°C. Take the RNeasy MinElute spin columns (for RNA) and QIAmp MinElute spin columns (for gDNA) out to equilibrate to room temp.
2. Thaw nuclei (from step 31 of Basic Protocol 3, steps 37 and 58 of Basic Protocol 2) at room temperature, add 10 µl Proteinase K (included in the kit), mix by flicking the tube, and place on 56°C heat block for 15 min.
3. Transfer samples directly from heat block to ice for a few minutes and centrifuge at 20,000 × *g* for 15 min (room temperature).
4. Transfer most of the RNA-containing supernatant to a new tube, leaving 10-15 µl in the original tube. Directions for isolating gDNA from the pellet can be found at the end of this section.
5. Place tubes with the supernatant on a 65°C heat block. Incubate for 30 min.
6. Move samples to 70°C heat block and incubate for an additional 30 min. Adjust the heat block previously used to 80°C.
7. Move samples to 80°C for 15 min and follow the 'Purification of total RNA' procedure described in AllPrep® DNA/RNA FFPE Handbook. For small numbers of nuclei, elute RNA in the minimal elution volume recommended (14 µl). Store RNA samples at −80°C.

Genomic DNA Isolation from Pellet

8. For larger numbers of collected nuclei (>100,000), follow the 'Purification of genomic DNA' procedure described in AllPrep® DNA/RNA FFPE Handbook.
9. For smaller numbers of nuclei (especially those with <10,000), resuspend the genomic DNA pellet from in 90 µl Buffer ATL, add 20 µl proteinase K (both from AllPrep DNA/RNA FFPE Kit), mix by vortexing, and spin down.
10. Incubate at 56°C, 1 hr.
11. Incubate at 90°C for 2 hr, preferably in a heat block that prevents condensate formation, as a precipitate may form in the solution remaining at the bottom.
12. Isolate DNA using Qiagen's MinElute Reaction Cleanup Kit following the 'MinElute Reaction Cleanup Kit using a Microcentrifuge protocol in MinElute Handbook, except elute DNA in 15 µl.

The expected amount of genomic DNA recovered ranges typically from 1 to 2 pg per nucleus. Note that gDNA samples can always be vacuum-concentrated if needed.

Preparation and Pooling of RNA-seq Libraries

13. To prepare RNA-seq libraries, use the Revelo™ RNA-Seq High Sensitivity library preparation kit and follow the procedure specified in the user guide. We recommend including the 'Library Amplification Optimization with qPCR' step for selecting an appropriate number of amplification cycles in the 'Library Amplification' step. The number of amplification cycles required ranges typically from 7 to 8 cycles, depending on the quality of RNA in the tissue used and the number of nuclei from which RNA was isolated.
14. Pooling can be done based on dsDNA concentrations of individual libraries measured with Qubit™ 1× dsDNA HS Assay Kit. While unwanted adapter dimers are typically not present in these RNA-seq libraries, we do recommend analyzing the size-distribution of DNA fragments in the final pooled library, e.g., on an Agilent TapeStation machine using High Sensitivity D1000 ScreenTape.

BASIC PROTOCOL 5

PROCESSING AND QUALITY CONTROL OF FANS-seq AND ATAC-seq DATA

The quality control and quantification of RNA-seq and ATAC-seq data from human samples is well established, although a range of bioinformatics tools and methodologies has been applied to this work. In this protocol, we describe the bioinformatics processing of both RNA-seq and ATAC-seq data in the context of FANS samples using tools tested and validated within our laboratory for hundreds of samples. We will also highlight standards by which we assess the quality of these data but do not recommend an automatic exclusion of datasets for which some of the specified quality threshold values are not attained (Table 3). Data failing to meet quality control standards should be further evaluated prior to exclusion from any downstream analyses.

We also highlight the use of cell type-specific ATAC-seq data to evaluate gene activity to complement cell type-specific FANS-seq data. By identifying open chromatin regions within gene promoters, downstream analyses of FANS-seq data can be focused on those genes for which expression is supported by ATAC-seq data (Matlik et al., 2024; Pressl et al., 2024). This helps minimize the number of possible false-positive differences in gene expression that can result from contaminating ambient transcripts or contamination with genomic DNA. It will also improve functional enrichment analysis of cell type-enriched genes or genes with disease-associated expression changes and will enhance downstream correlation with putative cis-regulatory element regions.

Table 3 FANS-seq and ATAC-seq Quality Metric Thresholds

FANS-seq quality metric	Threshold value
Read duplication rate	<15%
% of high-quality reads aligned	>60% to 70%
% of reads aligned to intergenic regions	<20% to 25%
Number of genes with >10 TPM	8000
ATAC-seq quality metric	Threshold value
Non-Redundant Fraction (NRF)	>0.5
PCR Bottlenecking Coefficient 1 (PBC1)	>0.8
PCR Bottlenecking Coefficient 2 (PBC2)	>3
Transcription Start Site (TSS) Enrichment Score	>7
Fraction of reads in peaks (FRIP)	>0.2

Materials

Software
- Salmon - *https://combine-lab.github.io/salmon/*
- Samtools - *http://www.htslib.org*
- IGV - *https://igv.org*
- MACS3 - *https://github.com/macs3-project/MACS*
- R - *https://www.r-project.org*

R packages
- BSgenome.Hsapiens.UCSC.hg38 - *https://www.bioconductor.org/packages/BSgenome.Hsapiens.UCSC.hg38/*
- TxDb.Hsapiens.UCSC.hg38.knownGene - *https://www.bioconductor.org/packages/TxDb.Hsapiens.UCSC.hg38.knownGene/*
- ChIPQC - *https://bioconductor.org/packages/ChIPQC/*
- ATACseqQC - *https://bioconductor.org/packages/ATACseqQC/*
- rtracklayer - *https://bioconductor.org/packages/rtracklayer/*
- GenomicAlignments - *https://bioconductor.org/packages/GenomicAlignments/*
- tximport - *https://bioconductor.org/packages/tximport/*
- Rsubread - *https://bioconductor.org/packages/Rsubread/*

Prepare Reference Data

Genomic reference data, including the full human genome sequence and associated gene models, are required to process and analyze FANS-seq and ATAC-seq data. Genome assemblies and gene models for the human genome in FASTA and GTF formats, respectively, can be retrieved from resources such as NCBI (*https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000001405.40/*) and Ensembl (*http://useast.ensembl.org/Homo_sapiens/Info/Index*).

Within our pipelines, we make use of the prepackaged genome sequence and gene models contained within the *Bioconductor* annotation packages repository. For human data, we use the *BSgenome.Hsapiens.UCSC.hg38* package for the full genome sequence and the *TxDb.Hsapiens.UCSC.hg38.knownGene* package for gene models. Alongside the reproducibility benefits gained from *Bioconductor*'s explicit versioning of this reference data, the use of *Bioconductor* packages allows for extensive interoperability with other tools within the *Bioconductor* package ecosystem in our downstream analysis.

Processing and Quality Control of FANS-seq Data

1. FASTQ files containing raw sequence data are assessed for quality using the *Rfastp Bioconductor* package (Wang & Carroll, 2024). In contrast to *FastQC, which can*

handle only single-end read data and subsampling reads for quality control, Rfastp uses the whole FASTQ file in assessment and, importantly, correctly handles paired-end data in evaluating duplicated reads. In addition, *Rfastp* can provide QC-filtered and clipped FASTQ files for further downstream analysis without the use of secondary tools.

Library complexity is initially evaluated through *Rfastp*'s calculated duplication rate. A high 'duplication rate' (e.g., >15%) points to low library complexity, typically due to degraded starting material in the library preparation phase. *Rfastp* can also be used to identify potential adaptor contamination across sequencing reads and to recognize problems within sequencing itself through the evaluation of PHRED scores - across sequencing cycles and spatially, across the sequencing flow cell.

2. Aligning FANS-seq data to the genome can be performed by various splice-aware aligners, including Rsubread, STAR, and *Hisat2*. In our protocols, we use the Rsubread aligner, which has been shown to be faster, better, and more efficient for RNA-seq analysis than other splice-aware aligners (Liao et al., 2019).

   First, we create a FASTA file from our *BSgenome.Hsapiens.UCSC.hg38* package using the *writeXstringSet* method from the *Bioconductor BSgenome* package. This is then used to create our genome index for later alignment of FANS-seq data using the *Rsubread* buildindex command with default parameters.

   For alignment of FANS-seq reads, we use the *Rsubread* subjunc command with default settings and provide our gene models to capture annotated, non-canonical splice junctions (lacking typical lariat structure sequence). The resulting read name-sorted BAM file is used later for gene-level read-counting, and a second, coordinate-sorted, indexed BAM file is produced using the *samtools'* sort command.

   The resulting coordinate-sorted BAM file is used to produce a library size-normalized signal graph in the *bigWig* format using the *rtracklayer* and *GenomicAlignments Bioconductor* package This allows visualization of the position of aligned reads along the linear genome in genome browsers such as *Integrated Genomics Viewer* (IGV). For both FANS-seq and ATAC-seq datasets, we recommend a step of manual curation where the distribution of aligned reads is inspected visually in IGV to verify the presence of reads over marker genes specific for the collected population, as well as the absence of reads over marker genes that are not expressed in the cell type. This can help identify possible sample contamination with the nuclei of these cells.

3. Following alignment, the *Picard tools*' *collectRNAseqMetrics* command is run with the coordinate-sorted BAM file alongside our gene models. This produces a broad set of RNA-seq-specific quality metrics, including the proportion of reads mapping to exons, CDSs, UTRs, and intergenic regions, 5' to 3' bias through the average distribution of reads over gene lengths, and the proportion of properly paired mapped reads with high mapping qualities. In high-quality FANS-seq libraries, the fraction of reads aligned is large (>60% to 70%), and the fraction of intergenic reads is minor (<20% to 25%).

4. Quantifying gene expression from RNA-seq data can be performed using standard read counting algorithms such as *HTSeq-Count* and *featureCounts* and pseudo-alignment-based tools such as *Kallisto* and *Salmon*. For FANS-seq, we first quantify unspliced and spliced read counts in genes using *Rsubreads' featureCounts* method with our gene models, allowing for the counting of reads overlapping multiple genes (Liao et al., 2014). Transcript expression is also performed using *Salmon* (Patro et al., 2017), and gene expression measure is summarized from transcript expression using the *tximport Bioconductor* package. Once the number of reads per gene is normalized to transcript length, the resulting transcripts per million (TPM) values serve as an

important measure of library diversity. In high-quality libraries, the number of genes with a TPM value >10 typically exceeds 8000 (based on reads aligned to annotated gene exons).

Processing and Quality Control of ATAC-seq Data

1. Quality control of ATAC-seq FASTQ files is performed with *Rfastp* (as with FANS-seq data).

2. Alignment of ATAC-seq data to the genome does not require a splice-aware aligner, and popular choices include *Bowtie2*, *BWA*, and *Rsubread*. We use the *Rsubread* package to share our alignment index across FANS-seq and ATAC-seq data processing.

   We use the *Rsubread* align command with the minFragLength and maxFragLength parameters set to 0 bp and 5000 bp, respectively. Following alignment, a coordinate-sorted and indexed BAM file is created using the *samtools'* sort command.

   As with FANS-seq, a file in *bigWig* format is produced using the *rtracklayer Bioconductor* package. Manual inspection of *bigWig* files in IGV should be also performed for ATAC-seq data to identify the presence or absence of signal within the transcription start sites of marker genes of expected or potential contaminating cell populations.

3. The identification of open chromatin regions from ATAC-seq data is typically performed using peak-calling software. The use of *MACS* peak-caller is well established for ATAC-seq data, and we use this with standard parameters in paired-end mode to generate lists of open regions in *BED* and *narrowPeak* formats (Zhang et al., 2008). Only properly paired and uniquely mapped reads are used for the input to *MACS* peak calling. Peak calls overlapping known human-specific artifact blacklists (*https://github.com/Boyle-Lab/Blacklist/raw/master/lists/hg38-blacklist.v2.bed.gz*) are removed prior to downstream analysis.

4. ATAC-seq-specific quality metrics listed in Table 3 are generated using both the *ChIPQC* (Carroll et al., 2014) and *ATACseqQC* packages (Ou et al., 2018). The fraction of reads in peaks is calculated using the *ChIPQC* package's *ChIPQCsample* command using the coordinate-sorted BAM file and MACS peaks-calls as inputs. The *ATACseqQC* package's *bamQC* and *TSSEscore* commands are used to calculate non-redundant fractions, PCR Bottleneck coefficients, and TSS enrichment scores listed in Table 3. We recommend further inspection of datasets for which the calculated value of one or more of these metrics falls below the thresholds marked in Table 3.

Exclusion of Genes without Chromatin Accessibility at Transcriptional Start Site

1. We recommend generating a list of high-confidence consensus peaks by creating a nonredundant peak set for each cell type and then filtering down to peaks present in most samples (i.e., >50% of samples).

2. A peak annotation tool (e.g., *ChIPseeker* (Wang et al., 2022)) can then be used to calculate the distance to the closest consensus peak for each of the transcriptional start sites (TSS) in the annotated human genome (e.g., NCBI RefSeq *hg38* gene annotation).

3. Genes for which none of their annotated transcriptional start sites overlap with a high-confidence consensus peak (distance >0 bp) are then filtered out before further analyses of gene expression changes.

   While the distribution of FANS-seq and ATAC-seq reads can always be inspected visually in IGV for genes of interest, we consider the exclusion of genes without accessible TSS particularly useful before unbiased analysis where such inspection is not feasible (e.g., Gene Ontology analysis, Gene Set Enrichment Analysis or Gene regulatory network inference).

REAGENTS AND SOLUTIONS

Buffer B (50 ml)

22.5 ml 2 M Potassium Chloride (900 mM final, Thermo Fisher Scientific, cat. no. AM9640G)
1.5 ml 1 M Magnesium Chloride (30 mM final, Thermo Fisher Scientific, cat. no. AM9530G)
12 ml 0.5 M Tricine-KOH, pH 7.8 (120 mM final)
Bring volume to 50 ml with DNase/RNase-Free water
Store up to 6 months at 4°C

Buffer C (30 ml)

5 ml Buffer B
Dissolve 3 EDTA-free Protease Inhibitor Cocktail tablets (Roche, cat. no. 04693159001) and chill solution on ice
Add 25 ml OptiPrep Density Gradient Medium (Sigma Aldrich, cat. no. D1556)
30 µl 0.5 M spermidine solution (0.5 mM final) (Sigma Aldrich, cat. no. S2501)
45 µl 0.1 M spermine solution (0.15 mM final) (Sigma Aldrich, cat. no. S2876)
30 µl 1 M dithiothreitol (1 mM final) Thermo Fisher Scientific, cat. no. 165685000)
30 µl SUPERase-In RNase Inhibitor (Thermo Fisher Scientific, cat. no. AM2696)
30 µl Recombinant RNasin Ribonuclease Inhibitor (Promega, cat. no. N2515)
Prepare fresh for each experiment, keep on ice

Buffer D (50 ml)

31.7 ml DNase/RNase-Free water
8.3 ml Buffer B
dissolve 4 EDTA-free Protease Inhibitor Cocktail tablets (Roche, cat. no. 04693159001) and chill solution on ice
Add 10 ml 1.25 M sucrose solution (final 0.25 M)
50 µl 0.5 M spermidine solution (0.5 mM final) (Sigma Aldrich, cat. no. S2501)
75 µl 0.1 M spermine solution (0.15 mM final) (Sigma Aldrich, cat. no. S2876)
50 µl 1 M dithiothreitol (1 mM final) Thermo Fisher Scientific, cat. no. 165685000)
50 µl SUPERase-In RNase Inhibitor (Thermo Fisher Scientific, cat. no. AM2696)
50 µl Recombinant RNasin Ribonuclease Inhibitor (Promega, cat. no. N2515)
Prepare fresh for each experiment, keep on ice

Buffer DEF (prepare right before use)

935 µl Buffer D
2 µl EDTA (0.5 M), pH 8.0 (Thermo Fisher Scientific. cat. no. AM9260G)
62.5 µl Paraformaldehyde 16% Aqueous Solution (Electron Microscopy Sciences, cat. no. 15710)

Buffer E (6.5 ml)

3.1 ml Buffer D
3.4 ml Buffer C
Prepare fresh for each experiment, keep on ice

DAPI Buffer (10 ml)

1 ml 10× PBS (Thermo Fisher Scientific, cat. no. AM9624)
8.5 ml DNase/RNase-Free water
Add 1 µl DAPI and vortex before chilling on ice (from 5 mg/ml stock, e.g., Sigma Aldrich cat. no. D9542)
500 µl 10% Bovine Serum Albumin, Nuclease-Free (0.5% final, Sigma Aldrich, cat. no. 126615)

10 µl Recombinant RNasin Ribonuclease Inhibitor (Promega, cat. no. N2515)
Store up to 48 hr at 4°C, keep on ice

DTT, 1 M (10 ml)

1.54 g 1,4-Dithiothreitol (Sigma Aldrich, cat. no. 10197777001)
Dissolve in DNase/RNase-Free water (bring volume to 10 ml)
Filter-sterilize (through a 0.2 µm filter)
Store up to 12 months at -20°C

Ethanol, 80%

2 ml DNase/RNase-Free water
8 ml 100% Ethanol (Americanbio, cat. no. AB00515-00500)
Prepare fresh for each experiment

Glycine, 2 M (10 ml)

1.5 g Glycine (2M final, Sigma Aldrich, cat. no. G7126)
Dissolve in DNase/RNase-Free water (bring volume to 10 ml)
Filter-sterilize (through a 0.2 µm filter)
Store up to 12 months at 4°C

Lysis buffer (10 ml)

9.85 ml DNase/RNase-Free water
20 µl 5 M NaCl solution (10 mM final, Thermo Fisher Scientific, cat. no. AM9760G)
30 µl 1 M $MgCl_2$ solution (3 mM final, Thermo Fisher Scientific, cat. no. AM9530G)
100 µl 1 M UltraPure Tris-HCl Buffer, pH 7.5 (10 mM final, Thermo Fisher Scientific, cat. no. 15567027)
1 µl IGEPAL CA-630 (0.01% final, Sigma Aldrich cat. no. I8896)
Mix well
Store up to 6 months at 4°C

PBS containing DAPI, 1×

1 ml 10× PBS (Thermo Fisher Scientific, cat. no. AM9624)
9 ml distilled water
1 µl of DAPI (from 5 mg/ml stock, e.g., Sigma Aldrich cat. no. D9542)
Prepare fresh for each experiment

Permeabilization buffer (10 ml)

10 ml ice-cold Wash Buffer
Add 5 µl 100% Triton X-100 (0.05% final, Sigma Aldrich, cat. no. T8787)
Store up to 48 hr at 4°C

Reverse Crosslink Solution (10 ml)

8.1 ml DNase/RNase-Free water
400 µl 5 M NaCl solution (10 mM final, Thermo Fisher Scientific, cat. no. AM9760G)
20 µl 0.5 M EDTA (Thermo Fisher Scientific, cat. no. AM9260G)
500 µl 1 M UltraPure Tris-HCl Buffer, pH 7.5 (10 mM final, Thermo Fisher Scientific, cat. no. 15567027)
1 ml UltraPure™ SDS Solution, 10% (1% final, Thermo Fisher Scientific, cat. no. 15553027)
Mix well
Store up to 6 months at room temperature

Add 5 µl Proteinase K (10 µg/ml final, Thermo Fisher Scientific, cat. no. EO0491) right before use

Spermidine, 0.5 M (10 ml)

1.27 g Spermidine trihydrochloride (Sigma Aldrich, cat. no. S2501)
Dissolve in DNase/RNase-Free water (bring volume to 10 ml)
Filter-sterilize (through a 0.2-µm filter)
Store up to 12 months at −20°C

Spermine, 0.1 M (10 ml)

0.35 g Spermine tetrahydrochloride (Sigma Aldrich, cat. no. S2876)
Dissolve in DNase/RNase-Free water (bring volume to 10 ml)
Filter-sterilize (through a 0.2-µm filter)
Store up to 12 months at −20°C

Sucrose, 1.25 M (200 ml)

Dissolve 85.5 g sucrose (Thermo Fisher Scientific, cat. no. BP220-1) in 120 ml DNase/RNase-Free water
Bring volume to 200 ml with DNase/RNase-Free water
Filter-sterilize (through a 0.2-µm filter)
Store up to 6 months at 4°C

TD buffer, 2× (10 ml)

100 µl 1 M $MgCl_2$ solution (10 mM final, Thermo Fisher Scientific, cat. no. AM9530G)
200 µl 1 M UltraPure Tris-HCl Buffer, pH 7.5 (20 mM final, Thermo Fisher Scientific, cat. no. 15567027)
*2 ml Dimethyl formamide (20% final, Sigma Aldrich cat. no. D4254)
Bring up to 10 ml with DNase/RNase-Free water
*Before adding dimethyl formamide, adjust pH to 7.5 with 100% acetic acid
Mix well
Store up to 6 months at 4°C

Tricine-KOH buffer, 0.5 M, pH 7.8 (500 ml)

Dissolve 44.8 g of tricine (Sigma Aldrich, cat. no. T0377) in 450 ml of DNase/RNase-Free water on a magnetic stirrer
Adjust pH to 7.8 by adding KOH (Sigma Aldrich cat. no. 417661)
Bring volume to 500 ml with DNase/RNase-Free water
Filter-sterilize (through a 0.2 µm filter)
Store up to 12 months at 4°C

Wash Buffer (50 ml)

5 ml 10× PBS (Thermo Fisher Scientific, cat. no. AM9624)
42.5 ml DNase/RNase-Free water
50 µl 1 M DTT
2.5 ml 10% Bovine Serum Albumin, Nuclease-Free (0.5% final, Sigma Aldrich, cat. no. 126615)
Chill on ice
50 µl SUPERase-In RNase Inhibitor (Thermo Fisher Scientific, cat. no. AM2696)
50 µl Recombinant RNasin Ribonuclease Inhibitor (Promega, cat. no. N2515)
Store up to 48 hr at 4°C

Table 4 Troubleshooting

Problem	Possible cause	Solution
Basic Protocol 1		
Low yield of nuclei	Incorrect concentration of Optiprep in ultracentrifugation buffers	Check whether the Optiprep concentration used matches the concentrations specified in protocol 1.
Basic Protocols 2 and 3 (Alternate Protocol 1)		
Most of the nuclei are in aggregates	Nuclei have aggregated during sample preparation	1) Make sure the pellet of nuclei is completely resuspended during the formaldehyde fixation step: mix the suspension of nuclei by pipetting right before and after the addition of formaldehyde. 2) Make sure pellets are resuspended completely during washing steps.
Low number of singlet nuclei	DAPI concentration is too low	When most events fall around zero on the DAPI axis, it is likely that nuclei have not been exposed to a high enough DAPI concentration. In this case, add more of your DAPI-containing wash buffer to your nuclei and incubate at 4°C on gentle rotation for about 15 min before you check for better singlet separation.
Poor staining or no staining with antibodies	Poor tissue quality, protein integrity is compromised	If tissue sample quality is poor, there is no other solution but to omit the sample. Samples with poor protein integrity will also not be usable in downstream assays.
Poor staining or no staining with PrimeFlow probes	Nuclear RNA was degraded before tissue was frozen	If nuclear RNA has degraded during the time between death and tissue harvesting/freezing, or because of the way tissue was handled, there is no other solution but to use material from other tissue donors, if possible. With new tissue material that has previously not been used for nuclear RNA-seq experiments, this is the most probable cause for failure in staining nuclei.
Poor staining or no staining with PrimeFlow probes	Nuclear RNA has degraded during sample preparation	This is likely the cause when previous experiments with tissue from the same donor suggest that nuclear RNA had not degraded prior to the experiment. We recommend preparing fresh buffers used in Basic Protocol 1.
Data suggest contamination of collected populations with other cell types	FACS malfunction	A small fraction of the collected nuclei can be run on the FACS or on flow cytometer right after collection to assess the purity of collected population.
Data suggest contamination of collected populations with other cell types	Insufficient separation of stained population	When selecting which population of nuclei to collect, be more conservative and select only those nuclei that show strongest staining. Always analyze FMO control samples and position sorting gates accordingly.
Support Protocols 1 and 2		
Low concentration of ATAC-seq library	Too few Tn5-treated nuclei were collected	1) Repeat the experiment and attempt to collect more nuclei. 2) Increase the number of PCR amplification cycles during ATAC-seq library preparation.

(*Continued*)

Table 4 Troubleshooting, *continued*

Problem	Possible cause	Solution
Basic Protocol 5		
Low quality of RNA-seq data according to QC metrics (see problem 'Poor staining or no staining with PrimeFlow probes')	Nuclear RNA has degraded during sample preparation or before tissue was frozen	
Low quality of RNA-seq data according to QC metrics	Too few nuclei were collected	Repeat the experiment with a larger piece of tissue and attempt to collect more nuclei.
Low quality of ATAC-seq data according to QC metrics	Too few Tn5-treated nuclei were collected	1) Repeat the experiment and attempt to collect more nuclei. 2) Make sure the recommended nuclei-to-Tn5 ratio is used during transposase-treatment step.

COMMENTARY

Critical Parameters

All resuspension steps should be carried out gently, as too-vigorous pipetting can lead to loss of integrity of nuclei and chromatin release, which could cause clogging of the pipette tip orifice and further disintegration of nuclei. Also, although in our experience, contamination with RNases happens very rarely, one should take precautions to avoid contamination with RNases as loss of RNA integrity would not only prevent the successful generation of RNA-seq data but would also prevent the labeling of nuclei with PrimeFlow probes (see Troubleshooting Table).

Troubleshooting Table

For a list of possible problems encountered when performing these protocols and proposed solutions, see **Table 4**.

Understanding Results

The labeling and FANS procedures described in Basic Protocols 1–3 can be used to isolate nuclei of specific cell types for analysis with different methodologies (RNA-seq, whole genome sequencing, etc.) to address different biological questions (disease-associated changes, aging-related changes, etc.) or to collect nuclei of rare cell types for further analysis with single-nucleus approaches (e.g., snRNA-seq). We have used these procedures to generate cell type-specific FANS-seq data and ATAC-seq data and to analyze cell type-specific somatic mutations at specific loci (Matlik et al., 2024; Pressl et al., 2024) and DNA methylation (Fig. 12). Whether the procedures are used to generate these or other types of data (e.g., mass spectrometry data), we recommend performing a pilot experiment to assess the purity of isolated populations. Impurities and contamination with the nuclei of other cell types can be assessed most sensitively from RNA-seq data, so we recommend that FANS-seq datasets are generated in a pilot experiment, even if the overall goal does not involve the analysis of nuclear transcriptomes. Notably, our published RNA-seq datasets from these cell types (NCBI GEO accessions GSE227729 and GSE233386) can serve as a benchmark for assessing the purity of isolated populations of nuclei. Regardless of what type of omics data are generated and used to assess sample purity, cell type-driven clustering of datasets in principal component analyses and expression/accessibility of cell type-specific marker genes serve as important indicators to watch out for in data quality control steps.

The protocols we have described here are also valuable for those who wish to develop a labeling strategy for collecting nuclei from other cell types not mentioned in our protocols. For this, the first goal is to identify a target protein or RNA that is specific to and highly abundant in the nuclei of the cell type of interest. A good starting point would be the analysis of publicly available snRNA-seq datasets (e.g., the Allen Brain Atlas resources, *https://portal.brain-map.org/help-and-community/guide-cell-types*), even if these are generated from a different species. Once such targets of labeling have been identified, we recommend testing multiple antibodies or PrimeFlow probes to find those that give the best labeling in FANS (i.e., best separation from an unlabeled population of nuclei) and generating FANS-seq data from both the labeled population, unlabeled populations and

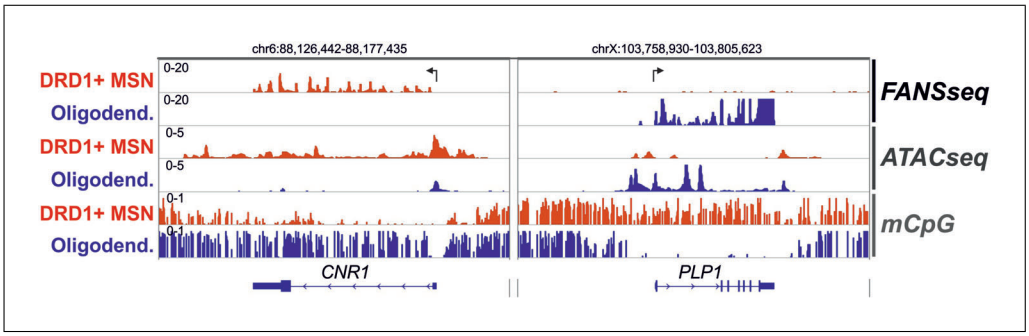

Figure 12 Representative distribution nuclear transcripts, accessible chromatin and 5-methyl-CpG dinucleotides around *CNR1* and *PLP1* genes with enriched expression in striatal DRD1-expressing projection neurons (dMSN) and oligodendrocytes, respectively. These features were determined by generating FANS-seq and ATAC-seq data from striatal nuclei with the procedures described above. The position of 5-methyl-CpG dinucleotides was mapped from oxidative bisulfite sequencing data and represents the distribution of true 5-methyl-CpG dinucleotides, not confounded by the presence of 5-hydroxymethyl-CpG dinucleotides. Arrows mark the position of annotated transcriptional start sites.

from unsorted (input) nuclei. The gene expression profiles of these populations can then be assessed for the enrichment of marker genes in the labeled population and depletion of these transcripts in the unlabeled population (both in comparison to marker gene expression in unsorted nuclei). If data suggest that the collected population is not sufficiently clean, additional probes/antibodies can be added to label and exclude unwanted contaminating nuclei.

Time Considerations

Completion of Basic Protocols 1 and 2 takes 2 consecutive days. Completion of Basic Protocols 1 and 3 takes 2–3 consecutive days. The generation of ATAC-seq libraries according to Support Protocols 1 or 2 adds an additional day to each procedure. The extraction of RNA and gDNA described in Basic Protocol 4 takes half a day. See section *Strategic planning*.

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Author Contributions

Christina Pressl: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; writing original draft. **Matthew Baffuto:** Data curation; investigation; methodology; writing review and editing. **Paul Darnell:** Data curation, investigation; methodology; writing review and editing. **Cuidong Wang:** Investigation; methodology. **Thomas S. Carroll:** Formal analysis; methodology, writing review and editing. **Nathaniel Heintz:** Conceptualization; funding acquisition; project administration; resources; supervision; writing review and editing. **Kert Mätlik:** Conceptualization; data curation; formal analysis; investigation; methodology; project administration; writing original draft.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data supporting the protocol are available in NCBI GEO at *https://www.ncbi.nlm.nih.gov/geo/*, reference numbers GSE227729 and GSE233386.

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