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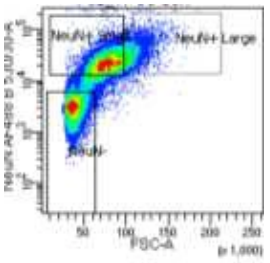
Fluorescence-activated nuclei sorting (FANS) for single-cell Whole Genome Sequencing (scWGS)



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Abstract

This protocol describes the isolation steps of nuclei from human post-mortem brain samples, immunofluorescence, and nuclei sorting (FANS) for low coverage (<1x) single-cell Whole Genome Sequencing (scWGS) to detect mega-base somatic Copy Number Variations (CNVs).

We have used this protocol to isolate nuclei from the frontal cortex, cingulate cortex, and substantia nigra tissues. However, it can be adapted for nuclei from different body areas, cell culture materials, and/or samples from other species.

Guidelines

Unless otherwise indicated, all the reagents must be kept at 4ºC, and perform the steps in the protocol at 4ºC. To preserve nuclear integrity, all the solutions are supplemented with complete cOmplete EDTA-free Protease Inhibitor Cocktail.

Materials

1. Commercial Reagents:

- **Table 1. Reagents and kit for nuclei isolation using Single Nucleus Isolation Kit (Option 1).**

	A	B	C	D	E
	Item	Supplier	Catalogue Number	Preparation prior use	Storage
	MinuteTM Single Nucleus Isolation Kit for Neuronal Tissues/Cells	Invent Biotechnologies	BN-020	Read kit guidelines	Fridge
	UltraPure DNase/RNase-Free Distilled Water	Thermo Fisher Scientific	10977049	Aliquot in 7 ml or 50 ml tubes	RT
	PBS (Phosphate Buffered Saline) 10X Solution (pH 7.4)	Fisher Scientific	15815418	Make 1x with dH2O	Fridge
	Bovine Serum Albumin (BSA)	Sigma-Aldrich	A7030	-	Fridge
	1X PBS with 5% BSA	-	-	500 mg of BSA in ~10ml 1X PBS	Fridge

Minute™ Single Nucleus Isolation Kit for Neuronal Tissues/Cells **Invent Biotechnologies inc Catalog #**BN-020

PBS (Phosphate Buffered Saline) 10X Solution (pH 7.4) **Fisher Scientific Catalog #**15815418

UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Catalog #**10977049

Bovine serum albumin (BSA) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A7030

- **Table 2. Reagents for manual nuclei isolation (Option 2).**

	A	B	C	D	E
	Item	Supplier	Catalogue Number	Preparation prior use	Storage
	UltraPure DNase/RNase-Free Distilled Water	Thermo Fisher Scientific	10977049	Aliquot in 7 ml or 50 ml tubes	RT
	PBS (Phosphate Buffered Saline) 10X Solution (pH 7.4)	Fisher Scientific	15815418	Make 1x with dH2O	Fridge
	50x complete Protease Inhibitor Cocktail EDTA-free (PIC)	Roche via Sigma Aldrich	4693159001	Use 1 tablet in 1 ml dH2O, Aliquot 0.5 ml each	Freezer (20oC)
	Triton-X100	Sigma Aldrich	T9287	Prepare 10% aliquot	RT
	ODGM (Optiprep Density Gradient Medium)	Sigma Aldrich	D1556	Aliquot 10 ml each	Fridge

UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Catalog #**10977049

PBS (Phosphate Buffered Saline) 10X Solution (pH 7.4) **Fisher Scientific Catalog #**15815418

OptiPrep™ Density Gradient Medium **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D1556

- **Table 3. Home-Made Reagents for manual nuclei isolation (Option 2).**

	A	B	C
	Item	Sterilization Method	Storage
	1 M MgCl2	Autoclave	RT
	1 M Tris/HCl pH 8.8	Autoclave	RT
	1 M Sucrose: Filtered	Filtration	Freezer (-20oC)
	1 M KCl	Autoclave	RT
	1 mg/ml DAPI (4',6-diamidino-2-phenylindole)	No	Freezer (-20oC)
	70% EtOH in dH2O	No	RT

- **Table 4. Antibodies for immunodetection.**

	A	B	C	D	E	F
	Antibody	Type	Supplier	Catalogue Number	Stock Concentration	Working Dilution
	anti-NeuN (mouse)*	1ry	Millipore	MAB377	1 mg/mL	1/100
	anti-Sox6 (mouse)	1ry	Protein Tech	14010-1-A	600 µg/mL	1/400
	anti-NeuN-AF488 (mouse)	Conjugated	Millipore	MAB377X	1 mg/mL	1/100
	Anti-Olig2	Conjugated	Abcam	ab225100	0.5 mg/ml	1/1000
	anti-Nurr1-AF488	Conjugated	Santa Cruz Biotechnologies	sc-376984 AF488	200 µg/ml	Jan-25
	AF568 goat anti-mouse	2ry	Life Technologies	A11004	2 µg/ml	1/500
	AF568 goat anti-rabbit	2ry	Life Technologies	A11011	2 µg/ml	1/500
	AF488 goat anti-mouse	2ry	Life Technologies	A11001	2 µg/ml	1/500
	AF488 goat anti-rabbit	2ry	Life Technologies	A11008	2 µg/ml	1/500
	IgG1-AF488	Isotype control	Merck Life Science Ltd	FCMAB310A4		
	IgG-AF647	Isotype control	Abcam	ab199093		

Anti-NeuN Antibody **Merck Millipore (EMD Millipore) Catalog #**MAB377

- Anti-NeuN Antibody, clone A60, Alexa Fluor®488 conjugated **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**MAB377X
- Recombinant Alexa Fluor® 647 Anti-Olig2 antibody [EPR2673] (ab225100) **Abcam Catalog #**ab225100
- Nurr1 Antibody (F-5) **Santa Cruz Biotechnology Catalog #**376984
- Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568 **Life Technologies Catalog #**A-11004
- Goat anti-Rabbit IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 **Thermo Fisher Scientific Catalog #**A11011
- Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 **Life Technologies Catalog #** A-11001
- Goat anti-rabbit alexaFluor-488 antibody **Thermofisher Catalog #**A11008
- Milli-Mark™ Mouse IgG1-k, clone MOPC-21, Alexa Fluor® 488 conjugate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**FCMAB310A4
- Recombinant Alexa Fluor® 647 Rabbit IgG, monoclonal [EPR25A] - Isotype Control (ab199093) **Abcam Catalog #**ab199093

Note

According to MAB377X description, this antibody works for most neuronal cell types throughout the adult nervous system. However, some neurons fail to be recognized by NeuN at all ages: INL retinal cells, Cajal-Retzius cells, Purkinje cells, inferior olivary and dentate nucleus neurons, and sympathetic ganglion cells and dopaminergic neurons (Mullen et al., 1992; Wolf et al., 1996., Cannon et al., 2009).

2. Reagents for immunodetection:

- DAPI (4',6-diamidino-2-phenylindole (stock 1 mg/ml): prepare aliquots of 5 ul each and store at -20°C
- Goat Serum (Sigma Aldrich G9023): prepare aliquots of 500 ul each and store at -20°C
- Blocking Buffer: 10% goat serum + 2% PIC in PBS 1x.

Note

For RNA as downstream application, 5% BSA + 0.2 U/µl RNasin in PBS (1x) is suggested as a Blocking Buffer.

- Optional: Nuclei Storage Buffer: 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.8), 166 mM sucrose and 1 mM dithiothreitol (DTT), 1x complete EDTA-free Protease Inhibitor Cocktail.
- Sorting Buffer: EDTA (final 5 mM) in PBS (1x)

3. General consumables:

- Flowmi Cell Strainers for 1000uL Pipette Tips, Mesh Size: 70um, Sterile (Fisher Scientific Ltd 15346248)

- Low binding filtered tips (sterile)
- Low-binding 1.5ml and 0.2 ml tubes (sterile)
- Gloves
- DNA AWAY™ Surface Decontaminant, Surface decontaminant; 8.5 oz. (250mL) **Thermo Fisher Catalog #**7010PK
- 70% EtOH in dH_2O,
- Cleaning wipes (e.g., Conti Washcloth Dry Brosch Direct PH5959)

4. Additional general consumables and equipment for manual nuclei isolation (Option 2):

- 1ml syringe without needle (sterile) – Only for manual nuclei isolation (Option 2)
- PES Syringe filter, 0.2 µm (sterile)
- PCR cabinet (Here, we used Air Science, Lydiate, UK)
- PCR cabinet (Here, we used Air Science, Lydiate, UK)
- KIMBLE 2mL Glass Dounce Tissue Grinder Set **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D8938

5. Equipment:

- General lab pipettes
- Pair of forceps and scissors
- Tissue culture hood for human sample handling
- Refrigerated centrifuge for 1.5ml tubes that can achieve at least 13000 x g (here used Sigma Aldrich 1 - 14K Refrigerated Micro Centrifuge)
- Rotator Disk or Tube Roller place in a fridge or cold-room or similar instrument that can allow antibody incubation @4°C with gentle mixing
- Optional: Haemocytometer to measure/assess nuclei staining
- Optional: Microscope to measure/assess nuclei staining. Here we used Nikon Eclipse TE300 inverted microscope coupled to a CCD camera - KERN optics)

6. Consumables for nuclei sorting:

- 96-Well PCR Plate, Non-Skirted (Cuttable), Natural **StarLab Catalog #**E1403-0100
- VersiCap Mat, 96-well, domed cap strips **Thermo Scientific Catalog #**15234574
- BD FACS™ Accudrop Beads **Becton Dickinson (BD) Catalog #**345249
- PBS, pH 7.4 (flow cytometry grade) **Thermo Fisher Catalog #**A1286301
- Antibiotic-Antimycotic (100X) **Thermo Fisher Scientific Catalog #**15240062

7. Equipment for nuclei sorting:

- BD FACS Aria Fusion sorter (BD Biosciences)

- Plate centrifuge

- **Table 5. Optical Filters used for sorting using BD FACS Aria Fusion.**

	A	B	C
	Laser	Diva Parameter Name	Fluorophore(s)
	405nm Violet	V 450/50	DAPI
	488nm Blue	B 530/30	NeuN-AF488, IgG-AF488, Nurr1-AF488
	633nm Red	R 670/30	Olig2-AF647, IgG-AF-647
	560nm Yellow-Green	YG 582/15	IgG-AF568

8. Reagents for Single-Cell Whole Genome Sequencing and library preparation for Illumina sequencing:

- Single-cell Whole Genome Amplification. Here we used Takara PicoPLEX® Single Cell WGA Kit R300672)
- Low TE Buffer **Invitrogen - Thermo Fisher Catalog #**12090-015
- DNA Library preparation kit. Here we used Agilent SureSelect Enzymatic Fragmentation Kit (5191-6764) and SureSelect XT HS2 DNA Library Preparation Kit (G9985A) with automation using Agilent Bravo. We have also used Illumina DNA PCR-Free Prep, Tagmentation, 96 Samples (20041795) in combination with IDT for Illumina DNA/RNA UD Indexes, such as Set A, Tagmentation (96 Indexes, 96 Samples (20027213). However, other methods can also be used.

Safety warnings

Please follow the Safety Data Sheets (SDS) for all reagents for safe handling and safety hazards.

Before start

In both cases before use: Clean Human Tissue handling hood with [M] 0.2 Molarity (M) NaOH, 10% Presept, 70% EtOH, and dH_2O.

Nuclei isolation from human post-mortem brain tissue

28m

1 In both cases prior use: Clean Human Tissue handling hood with [M] 0.2 Molarity (M) NaOH, 10% Presept, 70% EtOH and dH_2O.

2 Transfer all materials needed and handle human brain samples carefully in ahuman handling hood.

Note

Critical Notes:
- The Invent Biotech BN-020 kit was initially created for customers to avoid sorting. According to the manufacturer, the kit works with as little as 1 mg of neuronal tissue or cells. However, in our case, we adapted it to isolate nuclei faster and more consistently than the manual method, which requires hands-on preparation of the buffers to be used. Due to our technical needs for cell-type selection and the difficulty of weighing very small amounts of frozen tissue, we use a minimum of 10 mg (usually 10-30mg) of frozen post-mortem brain tissue.
Furthermore, as our downstream aim is to do single-cell whole genome amplification of selected nuclei, the use of this kit is followed by nuclei immunostaining and sorting. Due to that, we use the BN-020 kit according to manufacturer instructions with minor modifications.
- According to the Invent Biotech protocol, the Buffer B steps (here 6.9 - 6.13) are optional, but we use them to prepare the cleanest nuclei population possible.
- All centrifugation steps in this part of the protocol can be performed at Room temperature ..

STEP CASE

Single Nucleus Isolation Kit 41 steps

3 Clean pestles before, between, and after use with [M] 0.2 Molarity (M) NAOH, 10% Presept and dH_2O and let them air-dry before use.

4 Prepare 5% BSA in PBS (1x) and store at 4 °C .

5 Prepare PIC (1x complete EDTA-free Protease Inhibitor Cocktail) and store in the freezer or remove an aliquot from the freezer and place it On ice .

6 Use the Invent Biotech BN-020 kit according to manufacturer instructions with minor modifications as follows:

6.1 Cut small brain pieces of tissue of interest in pre-weighed tubes and weigh the tissue on a scale aiming for 10 mg - 30 mg of each tissue/donor depending on the downstream needs.

Note

- The kit can be used for smaller starting material, but we used aminimum 10 mg in order to be able to scale it properly and have enough nuclei for the downstream application.
- If a larger volume of starting material is needed to avoid potential liquid retention in the filter, we suggest splitting the brain pieces into different tubes for nuclei isolation. Then, pool the nuclei together in the same tube before immunostaining.

6.2 Add 200 µL of cold buffer A in each tube containing a tissue and place it On ice .

Note

From now on, place tubes On ice , if not stated differently.

6.3 Homogenize the tissue using the pestle provided by grinding gently with twisting force 50-60 times. Then, add 500 µL cold buffer A to the tube and continue to grind 20-30 times.

6.4 Incubate the tube On ice for 00:05:00 and carefully transfer homogenate to a filter (column) in the collection tube (avoid larger debris that sink to the bottom of the tube). 5m

6.5 Incubate the tube with the cap open at -20 °C for 00:07:00 . 7m

Note

According to the manufacturer protocol, the incubation time can vary between 00:05:00 - 00:10:00 .

6.6 Cap the filter and immediately centrifuge at 13000 x g, 00:00:30 . 30s

6.7 Discard the filter (column) and resuspend the pellet by pipetting up and down gently ,10-20 times.

Note

- Try to avoid lipids that attach to the wall of the tube.
- If there is a liquid retention in the filter reduce the amount of starting material by half.

Figure 1. Sample after centrifugation with the filter column.

6.8 Centrifuge at 600 x g, 00:05:00 . 5m

Note

The pellet may not be obvious as these are isolated nuclei and it may be in the side close to the bottom of the tube.

6.9 Pour out the supernatant and resuspend the pellet in 200 µL PBS with 5% BSA that will be overlaid on top of buffer B in the next step.

Add 1 mL cold buffer B to a 1.5 ml eppendorf tube.

Note

Remove bubbles if present.

6.10 Carefully overlay the 200 µL nuclear suspension on top of buffer B by slowly expelling the nuclear suspension against the wall of the tube.

6.11 Centrifuge the tube at 1000 x g, 00:10:00. After centrifugation, cellular debris, oil, and myelin will stay on the top (white-milky layer). The purified nuclei are found in the pellet. 10m

Note

The nuclei pellet may not be visible. This depends on the brain region used.

6.12 Carefully remove the milky layer by withdrawing it into a 1 ml pipette tip and discard the rest of the supernatant.

6.13 Pour out the remaining buffer B, leaving 50 µL in the bottom of the ultracentrifuge tube (as it contains the nuclear fraction).

6.14 Resuspend the pellet in 50 µL - 200 µL PBS containing Blocking Buffer.

Note

Ensure to rinse the wall of the tube to collect all nuclei.

Nuclei immunostaining 2h

7

Note

1. When discarding the supernatants after each centrifugation step, leave the last 50 µL at the bottom.
2. Resuspend in same volumes as in prior step to keep the final volume consistent between the steps, but on the last centrifugation step of this section increase the volume to have at least 100 µL in each tube.

8 Incubate nuclei in blocking buffer for 00:30:00 at 4 °C in a rotating wheel or in falcon tubes in a tube roller. 30m

9 Add 1ry antibodies or conjugated antibodies directly to the blocking buffer.

10 Incubate the samples for at least 00:30:00 at 4 °C with gentle shaking. 30m

Note

1. Alternatively, incubation can be done for 01:00:00 or over-night.
2. Prepare negative control for the immunostaining, e.g., split the sample to 150 µL with 1ry antibodies and 50 µL without 1ry antibodies as negative control.

11 Pellet nuclei at 800 x g, 4°C, 00:10:00 and carefully discard supernatant. 10m

12 Resuspended in pre-chilled blocking buffer for a gentle wash.

13 Re-pellet nuclei at 800 x g, 4°C, 00:10:00 and discard supernatant. 10m

14 Add 2ry antibodies (e.g., AF488 1:500) and DAPI (0.1 µg/ml) in blocking buffer and incubate for at least 00:30:00 at 4 °C with gentle shaking. 30m

Note

1. If conjugated antibodies were used in step 10, skip this step.
2. DAPI concentration may need to be optimized based on sample and sorter used.

15 Wash nuclei again with blocking buffer.

16 Pellet nuclei at 800 x g, 4°C, 00:10:00 and carefully discard the supernatant. Resuspend nuclei in sorting buffer and proceed to filtering using Flowmi Cell Strainers in clean 1.5 mL eppendorf tubes. 10m

Note

If the sorting is scheduled for another day, resuspend nuclei in Nuclei Storage Buffer and store in the fridge for up to 2 weeks. The day of sorting, re-stain the nuclei with DAPI and filter sterilize.

Nuclei sorting (FANS)

17 Prepare 96-well collection that are compatible with your lab PCR machine by placing 5 µL of TE Buffer in each well and seal very carefully using VersiCap Mat strips.

Note

We use 96-well cuttable plates (Starlabs E1403-0100), but other plates such as low bind 96-well plates are recommended.

18 Transfer nuclei and collection plates on an ice-bucket to the sorting facility.

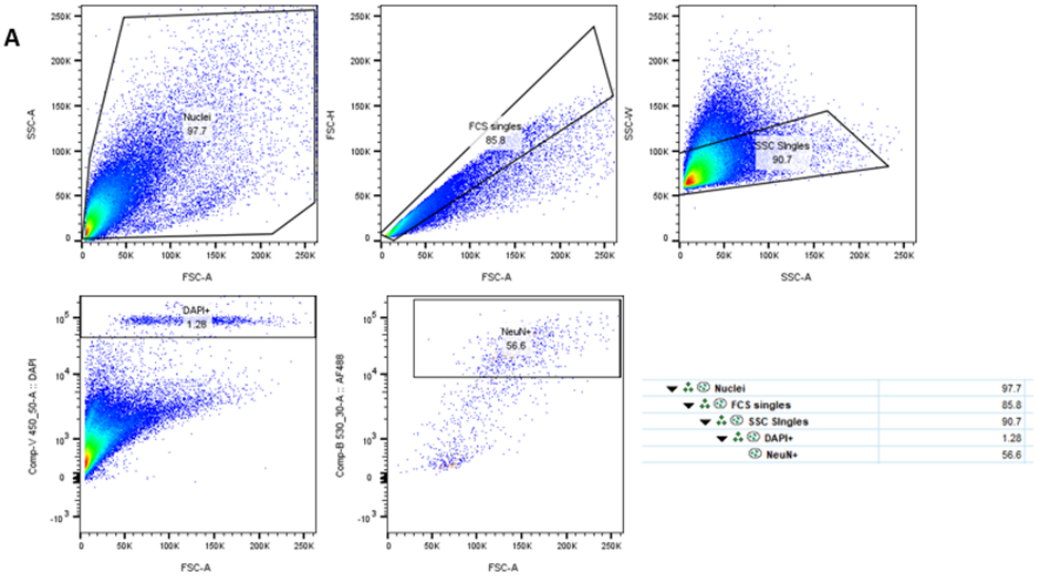
Note

In our case, we use BD FACSAria Fusion Cell Sorter instrument at the UCL Cancer Institute Flow Cytometry facility.

19 Acquire small amounts of nuclei from all samples and perform a batch analysis to assess the sample quality and identify the targeted populations for sorting.

Note

1. Use negative control samples as threshold references.
2. If possible, keep a consistent gating position across the samples from different donors.
3. Select gating parameters to isolate the singlets from the overall detected particles by selecting forward (FSC) and side scatter (SSC), FCS single cell gate and SSC single cell gate. Then select the nuclei by their DAPI expression.
4. From the nuclei population (DAPI+), apply further gating parameters based on the antibodies used.



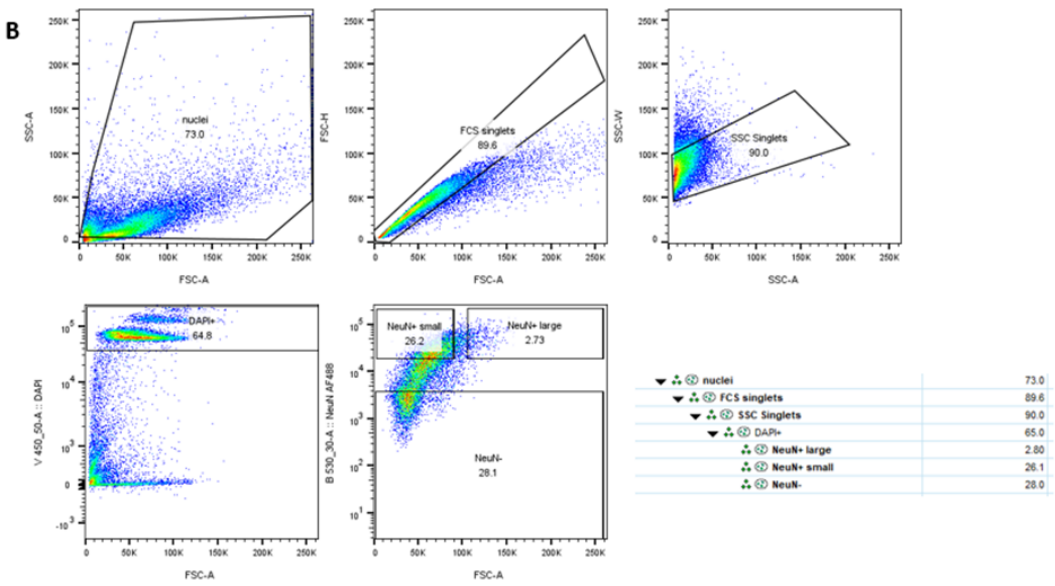


Figure 3. Examples of the gating profiles when sorting human post-mortem nuclei from cingulate cortex stained with NeuN antibodies (A) isolated manually or (B) by using the (B) BN-020 kit. The FCS files were analysed on FlowJo.

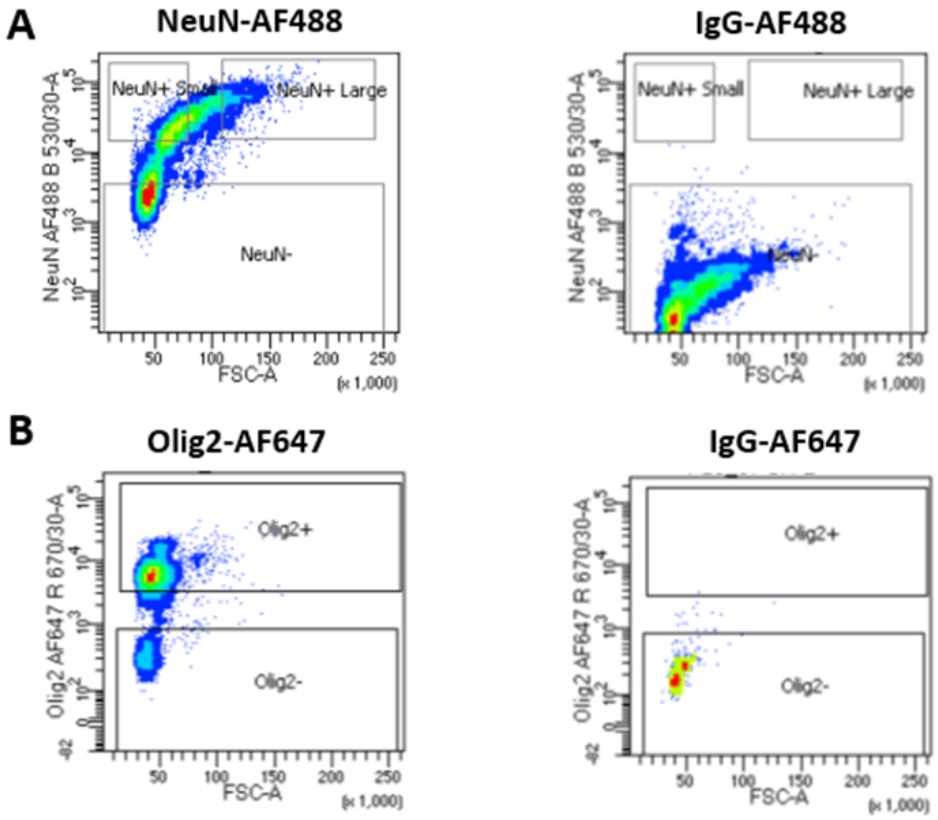


Figure 4. Examples human post-mortem nuclei (A) Cingulate cortex and (B) substantia nigra tissues stained with the conjugated antibodies (A) NeuN-AF488 or (B) Olig2-AF647 (left panel) or their isotype controls (right panel).

20 Centrifuge the collection plates for 00:00:10 at low speed to ensure reagent at bottom. 10s

21 Sort the single-nuclei of interest into the centrifuged 96-well collection plates.

Note

Before single cell sorting, use Accudrop beads for a test sort to evaluate the position of the plate and to ensure the sorted cells will be deposited into each well accurately in the middle.

22 Cap the collection plates carefully to ensure all sorted cells are merged in the reagent and not attached to the side of tube.

23 Centrifuge the collection plates and place them On ice for transportation to the laboratory.

> **Note**
>
> You can directly proceed to Section 4 or store the plates at -70 °C.

Single-nucleus Whole Genome Amplification and library preparation

24 Thaw collection plates On ice.

25 Centrifuge the collection plates briefly prior use.

26 Perform single-nucleus Whole Genome Amplification according to Takara's guidelines.

> **Note**
>
> We observe approximately 85-100% amplification success rate depending on the sample/donor used.

27 Prepare compatible libraries for sequencing, such as Agilent SureSelect XT HS2, Illumina DNA PCR-Free prep or other DNA library preparation kit.

28 Perform library pooling and sequence the libraries.

Protocol references

This protocol was adapted from:

Option A. Single Nucleus Isolation Kit:
Adapted from Invent Bioscience kit BN-020 (Invent Biosciences protocol) manual.

Option B. Manual nuclei isolation and immunostaining:
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