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Nuclei isolation, immunostaining, and Fluorescence-activated nuclei sorting (FANS) for Smart-Seq2

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Ester Kalef-Ezra[1,2], Dominic Horner[1], George Morrow[3], Vanda Knitlhoffer[4], Andy Goldson[4], Iain Macaulay[4], Yanping Guo[3], Christos Proukakis[1,2]

[1]Department of Clinical and Movement Neurosciences, UCL Queen Square Institute of Neurology, London, UK;
[2]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20815;
[3]The Flow Cytometry Translational Technology Platform, UCL Cancer Institute, London, UK;
[4]Earlham Institute, Norwich Research Park, Norwich, UK

ASAP Collaborative Rese…

University College London

Ester Kalef-Ezra
University College London

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Abstract

This protocol describes the steps for extracting nuclei from human postmortem brain samples, immunofluorescence, and nuclei sorting (FANS) for single-nucleus RNA-seq using the Smart-Seq2 method.
We have used it to isolate nuclei from human brain samples (such as the cingulate cortex), but it can be adapted for nuclei from different body areas, cell culture materials, and/or different species.

Guidelines

Unless otherwise indicated, all the reagents must be kept at 4 °C and all the steps in the protocol must be performed at 4 °C.

Materials

Materials for sections 1 and 2

Commercial Reagents:

Table 1. Reagents and kit for nuclei isolation, immunodetection, and nuclei lysis.

	A	B	C	D	E
	Item	Supplier	Catalogue Number	Preparation prior use	Storage
	RNase AWAY ™ Decontamination Reagent	Thermo Fisher Scientific (Life Technologies)	10328011	-	RT
	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
	10x PBS, RNase free	Invitrogen	AM9625.	Dilute in dH2O to make 1x PBS	Fridge
	UltraPure™ BSA (50 mg/mL), tested for DNase, RNase, endonuclease, and protease activity	Thermo Fisher Scientific	AM2616, UltraPure™ BSA (50 mg/mL, 5%)		Fridge
	Triton-X100	Merck Life Science Limited	T9284-100M	Prepare 10% solution in dH2O	RT
	RNasin Plus RNase inhibitor	Promega	N2615		Freezer (-20°C)
	DAPI (4',6-diamidino-2-phenylindole	Sigma-Aldrich	D9542	Prepare 1 mg/mL aliquots of 5 µl	Freezer (-20°C)

- RNase AWAY™ Decontamination Reagent **Thermo Fisher Catalog #**10328011
- Minute™ Single Nucleus Isolation Kit for Neuronal Tissues/Cells **Invent Biotechnologies inc Catalog #**BN-020
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Catalog #**10977049
- PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Invitrogen - Thermo Fisher Catalog #**AM9625

- UltraPure™ BSA (50 mg/mL) **Thermo Fisher Scientific Catalog #** AM2616
- Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T9284
- RNasin(R) Plus RNase Inhibitor, 10,000u **Promega Catalog #**N2615

Table 2. Antibodies for immunodetection.

	A	B	C	D	E	F
	Antibody	Type	Supplier	Catalogue Number	Stock Concentration	Working Dilution
	Anti-NeuN-AF488*	Conjugated	Millipore	MAB377X	1 mg/mL	1/100
	Anti-Olig2-AF647	Conjugated	Abcam	ab225100	0.5 mg/ml	1/1000
	IgG1-AF488	Isotype Control	Merck Life Science Ltd	FCMAB310A4		
	IgG-AF647	Isotype Control	Abcam	ab199093		

- 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
- IgG1-AF488 Isotype Control Merck Life Science Ltd FCMAB310A4
- Recombinant Alexa Fluor® 647 Rabbit IgG, monoclonal [EPR25A] - Isotype Control (ab199093) **Abcam Catalog #**ab199093

General consumables:

- Gloves
- RNaseZap (Thermo Scientific) for surface cleaning
- NaOH 0.2M, Precept, 70% EtOH in dH2O for surface cleaning
- Cleaning wipes (e.g., Conti Washcloth Dry Brosch Direct PH5959)

Equipment for nuclei isolation and sorting:

- General lab pipettes
- Pair of forceps for tissue transfer

- 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)
- Gentle plate shaker or tube roller in a fridge or similar instrument that can allow antibody incubation @ 4 °C with gentle mixing
- Freezer (@ -20 °C) that can fit a tube holder rack
- Plate centrifuge
- BD FACS Aria Fusion sorter (BD Biosciences)

Consumables for nuclei sorting:

- PCR1232, Azenta Life Sciences Framestar 96 Well Skirted PCR Plate Low Profile 0.1mL Working Volume Wells 2-Component Clear Wells Rigid Clear Frame
- Azenta Life Sciences Adhesive Plate Seals Clear Film Sheets Suitable for PCR Peel-Able (Scientific Laboratory Supplies, PCR0516)

Azenta Life Sciences Adhesive Plate Seals Clear Film Sheets Suitable For PCR Peel-Able **Azenta Life Sciences Catalog #**PCR0516

- AlumaSeal 96 Sealing Foils for PCR Plates with Narrow Sealing Surface, Aluminum Foil, 38µm Thick, Pierceable (Thames Restek UK Limited, F-96-100)
- 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
- Ice & dry-ice

Table 3: Lysis solution for Smart-Seq2.

	A	B	C	D	E
		Final concentration	1 well (µl)	3 x 96-well plates	6 x 96-well plates
	Triton-X100 (10%)	12.0	0.04	12.0	24.0
	RNasin Plus inhibitor (40 U/ul)	15.0	0.05	15.0	30.0
	dH2O (nuclease-free)	573.0	1.91	573.0	1146.0
	Total	**600 µl**	**2 µl**	**600 µl**	**1200 µl**

- Pierce™ Horseradish Peroxidase **Thermo Fisher Catalog #**31490

- TMB substrate (TMB, BioLegend 421501)
- dH2O (or miliQ H2O or diH2O)

Materials for section 3

Horseradish Peroxidase (HRP, Life Technologies 31490)
TMB substrate (TMB, BioLegend 421501)
dH2O (or miliQ H_2O or diH_2O)

Table 4. Properties of the Sorter and fluorophores used.

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

Table 5. 5% BSA in PBS (1x) preparation.

	A	B
	PBS (1x, RNase-free)	2.083.34 µl
	BSA (stock 30%, Merck 126625)	416.66 µl
	Total:	2.500,00 µl

Table 6. Prepare Blocking Buffer: 0.8% BSA + 0.2 U/µl RNasin in 10X PBS, pH 7.4 (1x). E.g. for 2 ml:

	A	B	C
	Solution	Volume	Final
	dH2O (DNase/RNase-free)	1.470 µ	
	10X PBS (RNase-free) pH 7.4	200 µl	1x
	5% BSA (freshly made)	320 µl	
	Vortex briefly to mix		
	RNasin Plus RNase inhibitor (40 U/µl)	10 µl	
	Mix gently by inverting the tube 5-10 times, store on ice until use		

Safety warnings

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

Nuclei isolation from human post-mortem brain tissue

1 **Preparation:**

UV-treat 96-well plates prior to use.

Note

The plates may appear discolored, but this is normal, and no cause for concern.

Note

- Critical Note: The Invent Biotech BN-020 kit was initially created for customers to avoid sorting and according to the manufacturer can work 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 10 mg of frozen post-mortem brain tissue. Furthermore, as our downstream aim is to do single-cell RNA sequencing of selected nuclei, the use of this kit is followed by nuclei immune-staining and sorting. Steps 12-17 are optional according to Invent Biotech team, but we use them to prepare nuclei populations that are as clean as possible.

Note

All centrifugation steps can be performed at Room temperature, but here we did them at 4 °C to increase RNA preservation.

Note

Spray down all work surfaces to be used with RNaseZap.

2 Pre-weight tubes are needed for tissue scaling.

3 Clean pestles the day before with [M] 0.2 Molarity (M) NAOH, 10% Presept and place them in a falcon tube containing RNase AWAY Decontamination Reagent.

4 Incubate the pestles with the RNase AWAY O/N @ Room temperature .

5 Prepare PBS (1x, RNase-free) and, Sorting Buffer (1x PBS + [M] 5 millimolar (mM) EDTA).

Experimental steps:

27m 30s

6 Clean Human Tissue handling hood with [M] 0.2 Molarity (M) NaOH, 10% Presept, 70% EtOH and dH_2O (nuclease-free).

7 Prepare the Lysis Buffer in the materials section.

8 Distribute 2 µL Lysis Buffer in each well of the plates needed for sorting.

9 Wash the pestles thoroughly with MiliQ-H_2O and then with dH_2O (RNase-free) and let them air-dry on a clean wipe tissue before use. Once dried, place them in a clean wipe tissue in a clean container (e.g., falcon tube or sealed bag) and put them in the fridge to pre-cool.

> **Note**
>
> Pestles should be cooled for at least **30 min** before use.

10 Clean human handling hood with [M] 0.2 Molarity (M) NaOH, 10% Precept, 70% EtOH, RNaseZap.

11 Transfer all materials needed, carefully handle human brain samples in a human handling hood, and wash them with RNaseZap.

12 Prepare 5% BSA in PBS (1x) and store On ice until use, e.g., for 1 sample.

13 Add RNasin Plus Ribonuclease Inhibitor to Buffer A and B prior touse to afinal concentration of 0.2 U/µl , e.g., for 1 sample:

- Buffer A: 5 µL RNasin Plus Ribonuclease Inhibitor in 995 µL Buffer A
- Buffer B: 5 µL RNasin Plus Ribonuclease Inhibitor in 995 mL Buffer B

14 Remove tissue from -80 °C freezer and transfer it to the human tissue handling hood on dry-ice.

15 Cut small brain pieces of tissue of interest in pre-weighed 1.5 ml Eppendorf tubes.

16 Weigh the tissue in a scale aiming for 15 mg - 30 mg of each tissue/donor depending on the downstream needs.

Note

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, isolating the nuclei, and pooling the nuclei together in one tube prior to immunostaining.

17 Add 200 µL cold Buffer A (containing 0.2 U/µl RNasin Plus Ribonuclease Inhibitor) in each tube containing tissue and place it on wet ice.

Note

Critical Note: From now on, keep the samples on wet ice if otherwise stated.

18 Homogenize the tissue using the pestle (pre-chilled) provided by grinding gently with twisting force for 50-60 times.

Note

Keep the tubes on wet ice while doing this step.

Note

Clean used pestles with [M] 0.2 Molarity (M) NaOH, 10% Precept, 70% EtOH, RNase AWAY Decontamination Reagent, miliQ-dH_2O, dH_2O (RNase-free), let them air-dry and pre-chill them @ 4 °C before re-using them.

19 Add 500 µL cold Buffer A (containing 0.2 U/µl RNasin Plus Ribonuclease Inhibitor) to the tube and continue to grind for 20-30 times.

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

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

Note

Incubation time can vary between 00:05:00 - 00:10:00.

22 Cap the filter and immediately centrifuge at 13000 x g, 4°C, 00:00:30. 30s

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

Note

Try to avoid lipids that attach to the wall of the tube.

Note

If there is liquid retention in the filter, reduce the amount of starting material by half.

Figure 1. Sample after centrifugation.

24 Centrifuge at 600 x g, 4°C, 00:05:00 .

5m

25 Pour out the supernatant and resuspend the pellet in 200 µL PBS with 5% BSA that will be overlaid on top of Buffer B (containing 0.2 U/µl RNasin Plus Ribonuclease Inhibitor) in the next step.

> **Note**
>
> The pellet may not be evident as these are isolated nuclei.

26 Add 1 mL cold Buffer B (containing 0.2 U/µl RNasin Plus Ribonuclease Inhibitor) to a 1.5 ml Eppendorf tube.

> **Note**
>
> Remove bubbles if present.

27 Carefully overlay the 200 µl nuclear suspension from step 17 on top of Buffer B by slowly expelling the nuclear suspension against the wall of the tube.

28 Centrifuge the tube at 1000 x g, 4°C, 00:10:00 . 10m

> **Note**
>
> After centrifugation, cellular debris, oil, and myelin will stay on the top (white-milky layer). The purified nuclei are found in the pellet.

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

> **Note**
>
> Extending the centrifugation time to a total of 00:20:00 may be beneficial to increase nuclei yield.

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

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

Section 2: Nuclei immunostaining 1h 30m

31 Prepare Blocking Buffer: 0.8% BSA + 0.2 U/µl RNasin in 10X PBS, pH 7.4 (1x), e.g. for 2 ml:

> **Note**
>
> When discarding the supernatants after each centrifugation step, always leave the last 50 µl at the bottom.

Note

After each centrifugation of this part of the protocol, resuspend in thesame volumes as in the 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. As an example, after the final centrifugation resuspend, the nuclei treated with the conjugated antibodies in 500 µL and the nuclei treated with the isotype control in 200 µL.

32 Resuspend the pellet in 450 µl (or a volume of choice depending on the initial tissue input) of cold Blocking Buffer and resuspend by pipetting up and down gently 5 times.

Note

Be sure to rinse the wall of the tube to collect all nuclei.

33 Separate the samples into 2 tubes each:

a. With Abs: 500 µl
b. Negative Control (with isotype control and/or without antibodies): remaining nuclei supplemented with Blocking Buffer to have a final volume 200 µl.

Note

Critical Note: Prepare nuclei staining negative control tubes, e.g., DAPI only and/or DAPI + isotype controls. For this purpose, remove 50 µl from each nuclei isolation, transfer it to another tube, and add 50-150 µl Blocking Buffer.

34 Incubate the nuclei in Blocking Buffer for 00:30:00 @ 4 °C in a rotating wheel or in falcon tubes in a tube roller.

30m

35 Add antibodies directly to the Blocking Buffer.

36 Add DAPI (the 1/10.000, e.g. add 5 µl of DAPI diluted 1/100 in PBS 1x) to all nuclei.

Note

DAPI concentration may need to be optimized based on sample and sorter used.

37 Incubate all samples for at least 00:30:00 at 4 °C in a rotating wheel or falcon tubes in a tube roller. 30m

38 Pellet nuclei at 800 x g, 4°C, 00:10:00 . 10m

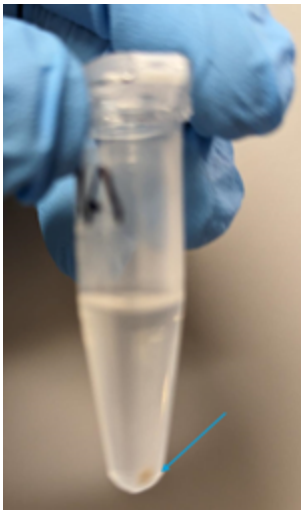

Figure 2. Example of the nuclei pellet (arrowed) after centrifugation.

Note

The pellet may not be clearly visible, and this depends on the brain region and the quantity of the nuclei.

39 Carefully discard the supernatant, leaving ~50 µl of buffer above the pellet.

40 Resuspended in pre-chilled Blocking Buffer (same volume as before) by gently pipetting up and down 5 times.

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

42 Wash nuclei again with Blocking Buffer.

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

44 Resuspend nuclei in 600 µl (or volume of interest depending on the input tissue) pre-chilled Blocking Buffer and proceed to filtering using Flowmi Cell Strainers in clean 1.5 mL Eppendorf tubes.

45 Transfer the nuclei On ice to sorting facility on ice and perform the sorting as soon as possible.

Section 3. Nuclei sorting (FANS) 22m 10s

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

47 As an extra layer of assessment for accurate sorting, assess plate positioning with colorimetric method.

47.1 Add 1 mL of dH_2O into the vial of powder HRP, and dissolve (this stock is 10x concentrated as compared to working solution).

47.2 Make a working solution ([M] 1 mg/mL) by:

To get 2 mL :

- 200 µL stock HRP ([M] 10 mg/mL)
- 1800 µL diH2O
- 2 drops of Accudrop beads

Keep in the fridge.

47.3 **To run the test:**

Aliquot 2 µL of TMB substrate (fridge in the FACS room) into each well of the test plate.

47.4 Sort a single bead into a whole plate (or wells needed).

47.5 Once the sort is completed, immediately seal the plate and centrifuge (500 x g, 00:01:00) and wait 00:05:00 - 00:10:00 and count the number of wells that have turned blue. 11m

> **Note**
>
> We aim for >90% success.

> **Note**
>
> If successful deposition is achieved, proceed with sorting cells – if not, recalibrate the alignment and try again!

48 Spin the sorting plates and arrange plate orientation.

> **Note**
>
> Use negative control samples as threshold references.

> **Note**
>
> If possible, keep a consistent gating position across the samples from different donors.

- 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.
- From the nuclei population (DAPI+), apply further gating parameters based on the antibodies used.

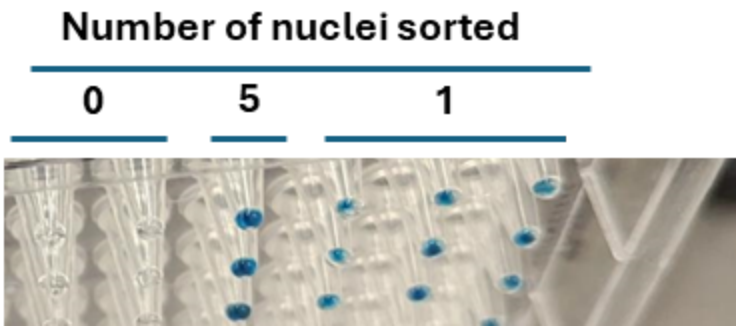

Figure 3. Example of the colour change on TMB plates after the colorimetric method: when not sorted nuclei, sorted 5 nuclei or single-nuclei in each well.

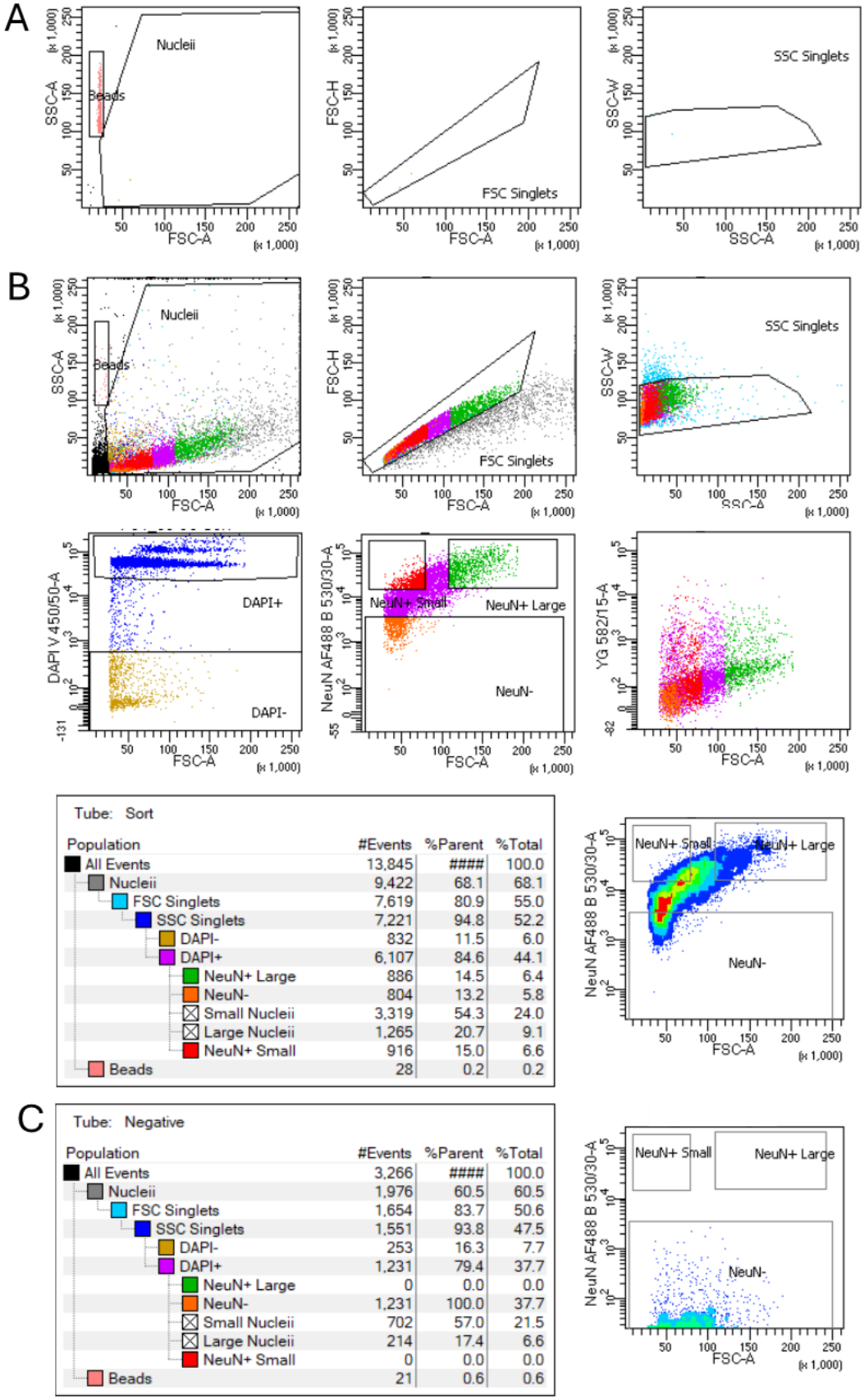

Figure 4. Example of gating parameters for (A) the colorimetric test using beads and (B-C)

human post-mortem cingulate cortex stained with (B). NeuN-AF647 and DAPI or (C) DAPI only as negative control.

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

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

51 After sorting, seal carefully the plates with adhesive plate foil and a seal and place On ice .

52 Immediately centrifuge briefly at 500 x g, 4°C, 00:01:00 . 1m

53 Place each plate in an individually sealed bag (dry-ice resistant).

54 Place immediately on dry-ice and transfer to the lab if needed.

55 Store the plate(s) -70 °C until further use and transfer the plates on dry-ice, if necessary, until single-cell Smart-Seq2.

Section 4. Single-nucleus Smart-Seq2

56 Smart-Seq2 is performed according to Picelli et al. 2014 (Nature Protocols) with the following modifications:

- cDNA is amplified with 25 cycles of PCR.
- oligo-dT30VN, template-switching oligonucleotide (TSO), and IS PCR primers are modified by 5' biotinylation (Zeisel et al. 2015, Science) (all ordered from IDT).

Note

Smart-Seq2, Nextera library preparation and Illumina sequencing have been performed at Earlham Institute facilities and the related steps are summarised here.

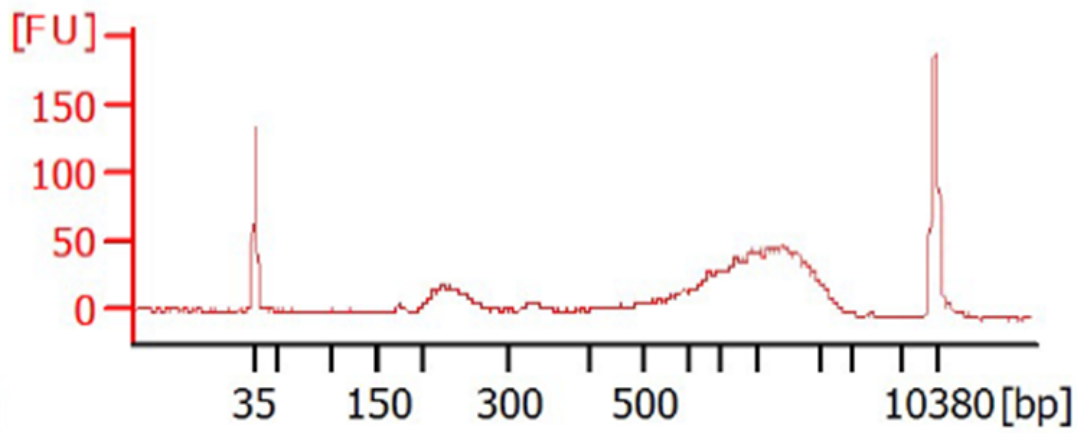

Figure 5. Example of a single-nucleus cDNA profile, which was analysed by using a High Sensitivity DNA Bioanalyzer chip.

57 0.6X SPRI-bead clean-up is performed on the cDNA to minimize the presence of short fragments (e.g., primer-dimers) using Biomek NX (Beckman Coulter) liquid handling robot.

58 cDNA samples are normalized to 0.2 ng/µL based on an average concentration of 11 samples ran on the bioanalyzer.

59 Library construction is performed using the Nextera XT sample prep kit (Illumina), a miniaturized protocol involving 12 cycles of PCR.

60 Libraries are prepared in 384-well PCR plates. The I.DOT (Dispendix) instrument is used to array reagents and indices.

61 Libraries were pooled prior to library clean-up using the Mosquito (SPT Labtech), and 0.8X SPRI-bead clean-up was performed on the 96-plex “plate pools” by hand. “Plate pools” were pooled equimolarly after QC using Bioanalyzer and qPCR.

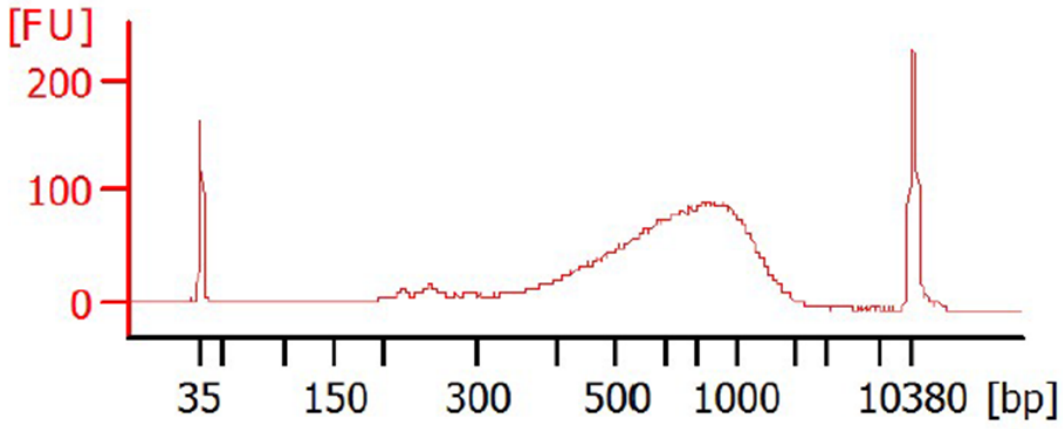

Figure 6. Example of the profile of a pooled library profile.

62 The pooled library is sequenced in an Illumina sequencer.

Note

We used 1 lane of the NovaSeq X Plus 10B flow cell with 150bp PE reads.

Protocol references

This protocol was adapted from:

- Nuclei isolation using Invent Bioscience kit BN-020 (Invent Biosciences protocol)
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