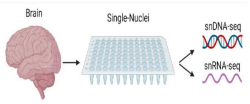
Sep 23, 2024

Human post-mortem nuclei preparation and single-nucleus multiome DNA/RNA sequencing by ResolveOME



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Abstract

Here we describe a protocol for nuclei isolation, immunostaining and sorting (FANS: Fluorescence-activated nuclear sorting) for single-nucleus DNA/RNA-seq using ResolveOME (BioSkryB) method from human post-mortem brain samples. We have used it to isolate nuclei from human post-mortem substantia nigra tissue, but it can be adapted for nuclei from different body areas.

The ResolveOME protocol is generally designed to work with single live mammalian cells and here we have done minor modifications in order to use it for human post-mortem brain samples.

Attachments

PDF 2024_06_13_ResolveOM... 3.1MB

PDF 2024_06_14_Best Prac... 587KB

PDF BN-020_Single Nucleu... 182KB

Image Attribution

Title image was created with BioRender.com (RRID:SCR_018361, agreement LR277KLRDJ).

Guidelines

Critical note: Unless otherwise indicated, keep all the reagents and steps at 4 °C to protect DNA and RNA integrity.

Materials

Table 1. Kits and reagents:

	A	B	C	D	E
	Item	Supplier	Catalogue Number	Preparation before use	Storage
	Minute™ 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
	PI (Propidium iodide)	Thermo Fisher Scientific	P3566	Prepare aliquots	Freezer
	ResolveDNA™ Cell Buffer for FACS Kit -	BioSkryB	PN100183	-	Freezer
	ResolveOME™ Box 1, 96 Reactions	BioSkryB	PN100768	-	Freezer
	ResolveOME™ Box 2, 96 Reactions	BioSkryB	PN100973	-	Freezer
	Single Use Library Adapter Set A, 96 Reactions	BioSkryB	PN100940	-	Freezer
	Single Use Library Adapter Set B, 96 Reactions	BioSkryB	PN100941	-	Freezer
	ResolveOME™ Bead Purification Kit	BioSkryB	PN100772	-	Fridge
	High Sensitivity dsDNA Assay kit	ThermoFisher Scientific	Q32854	-	RT & Fridge
	HS D5000 Reagent	Agilent	5067-5593	-	Fridge
	HS D1000 Reagent	Agilent	5067-5585	-	Fridge
	Ethanol, for molecular biology	Merck	51976-500ML-F	Prepare 80% in the same day needed for bead purification wash	RT
	Optional: Horseradish Peroxidase (HRP)	ThermoFisher Scientific	31490	-	Freezer
	Optional: TMB substrate	BioLegend	421501	-	Fridge

1. Minute™ Single Nucleus Isolation Kit for Neuronal Tissues/Cells **Invent Biotechnologies inc Catalog #**BN-020
2. UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Catalog #**10977049
3. PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Invitrogen - Thermo Fisher Catalog #**AM9625
4. UltraPure™ BSA (50 mg/mL) **Thermo Fisher Scientific Catalog #** AM2616
5. Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T9284
6. RNasin(R) Plus RNase Inhibitor, 10,000u **Promega Catalog #**N2615
7. Propidium Iodide - 1.0 mg/mL Solution in Water **Thermo Fisher Catalog #**P3566
8. Qubit™ dsDNA Quantification Assay Kits **Thermo Fisher Scientific Catalog #**Q32854
9. High Sensitivity D5000 Reagents **Agilent Technologies Catalog #**5067-5593
10. High Sensitivity D1000 Reagents **Agilent Technologies Catalog #**5067-5585
11. Ethanol for molecular biology **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**51976-500ML-F
12. Optional: Pierce™ Horseradish Peroxidase **Thermo Fisher Catalog #**31490
13. Optional: TMB High Sensitivity Substrate Solution **BioLegend Catalog #**421501

Table 2. Antibodies for immunodetection:

	A	B	C	D	E	F
	Antibody	Type	Supplier	Catalogue Number	Stock Concentration	Working Dilution
	Anti-Olig2-AF 647	Conjugated	Abcam	ab225100	0.5 mg/ml	1/1000
	IgG-AF647	Isotype Contr ol	Abcam	ab199093		

1. Recombinant Alexa Fluor® 647 Anti-Olig2 antibody [EPR2673] (ab225100) **Abcam Catalog #**ab225100
2. Recombinant Alexa Fluor® 647 Rabbit IgG, monoclonal [EPR25A] - Isotype Control (ab199093) **Abcam Catalog #**ab199093

Equipment:

General lab pipettes and low bind filtered pipette tips.

- 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. e.g. Sigma Aldrich 1 - 14K Refrigerated Micro Centrifuge).

- Gentle plate shaker in a fridge or similar instrument that can allow antibody incubation @ 4 °C with gentle mixing by placing the tubes covered in parafilm, inside falcon tubes on the plate shaker. Alternatively, a tube roller in the fridge can be used.
- Lab Fridge and Freezers (-20 °C & -80 °C).
- Plate centrifuge.
- BD FACS Aria Fusion sorter (BD Biosciences).
- PCR hood for single cell sample handling.
- PCR-Cooler & PCR-Rack (Eppendorf 10192281).
- Thermal Cycler, e.g. Multigene Optimax Gradient Thermal cycler with 96 well block (Labnet MB052).
- DynaMang-96 Side Magnet (Invitrogen 12331D).
- Qubit Fluorometer.
- Agilent TapeStation.
- PCR Plate Mixer.
- PCR Plate Spinner.
- Benchtop PCR 0.2 ml Strip & 1.5 ml Tube Centrifuge, e.g. Fisherbrand™ Mini-Centrifuge (Fisher Scientific 16617645).
- Vortex Mixer, e.g. iSwix VT Digital Vortex Mixer (Appleton Woods ST6000).
- Optional: 16-Tube SureBeads™ Magnetic Rack (Bio-Rad Laboratories 1614916).
- Optional: Magnet compatible with 0.2 ml strips, e.g. Dual Volume Strip Tube Magnet (BioSkryb 100226).

Consumables:

Low Bind 96-Well PCR Plates HS 39269099 (BioSkryB PN100149) or other low bind 96-well plates compatible with lab PCR.

- PCR Plate Sealing Film, e.g. BioSkryB PN100152 or ThermoFisher Scientific AB0558 .
- Alternatively other DNase/RNase-free caps compatible with the plates to be used.
  VersiCap Mat, 96-well, domed cap strips **Thermo Fisher Catalog #**AB1810
- 1.5 ml Low Bind Microcentrifuge Tubes, e.g. X500 Eppendorf DNA LoBind Tubes, 1.5ml, PCR clean (Fisher Scientific 16628742).
- Parafilm (general supplier).
- 50 ml falcon tubes (Appleton Woods AB036).
- Accudrop beads (Becton Dickinson, 345249).
- PBS, pH 7.4 (flow cytometry grade) **Thermo Fisher Catalog #**A1286301
- Qubit™ Assay Tubes **Fisher Scientific Catalog #**Q32856
- High Sensitivity D5000 ScreenTape **Agilent Technologies Catalog #**5067-5592
- High Sensitivity D1000 ScreenTape **Agilent Technologies Catalog #**5067-5584
- Ice & dry-ice.

Table 3. Optical Filters used for sorting using BD FACS Aria Fusion:

	A	B	C
	Laser	Diva Parameter Name	Fluorophore(s)
	560 nm Yellow - Green	YG 610/20	PI
	633 nm Red	R 670/30	Olig2-AF647, IgG-AF-647

Surface/pestle cleaning materials:

RNase AWAY™ Decontamination Reagent **Thermo Fisher Catalog #**10328011

- RNaseZap® **Thermo Scientific Catalog #**AM9780
- DNA AWAY Surface Decontaminant (Thermo Fisher Scientific 1022347)
- NaOH 0.2M (any supplier)
- 70% EtOH in dH_2O
- dH_2O
- Conti Washcloth Patient Cleansing Wipes 75 Wipes **Brosch Direct Catalog #**PH5959

Personal protective equipment: lab coats, googles, gloves

Safety warnings

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

Nuclei extraction from human post-mortem brain tissue

50m

1 **Experimental steps:**

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 in a faster and more consistent manner compared to the manual method that requires hands-on preparation of the buffers to be used.
Due to our technical needs for cell-type selection and the difficulty to weigh very small amounts of frozen tissue, we use as minimum 10 mg of frozen post-mortem brain tissue.
Furthermore, as our downstream aim is to do single-cell multiome DNA & RNA sequencing of selected nuclei, the use of this kit is followed by nuclei immune-staining and sorting.
- are optional according to Invent Biotech team, but we use them to prepare nuclei population that are as clean possible.

Note

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

Day 1

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

1.2 Incubate the pestles in a 50 mL falcon tube containing RNase AWAY O/N @ Room temperature.

2 **Day 2**

2.1 Wash pestles with MiliQ-H_2O and then with dH_2O (RNase-free), air-dry with a dry wipe and pre-cool at 4 °C .

> **Note**
>
> The pestles should be cooled @ 4 °C for at least 00:30:00 before use.

2.2 Clean carefully the PCR hood with 70% EtOH, dH_2O, DNA AWAY and RNase Zap and place not opened sterile filtered pipette tips.

2.3 Wipe the RNA lab pipettes with DNA AWAY and RNase Zap and place in the PCR hood.

2.4 Place in the PCR hood Low Bind 96-well plates to be used for nuclei sorting.

2.5 UV-treat the Low Bind 96-well plates and pipettes to be used for 00:20:00 - 00:30:00 before use. 50m

2.6 Pre-weight 1.5 ml tubes need for tissue scaling.

2.7 Prepare PBS (1x, RNase-free).

2.8 Clean the human tissue handling hood with [M] 0.2 Molarity (M) NaOH, 10% Presept, 70% EtOH and dH_2O, DNA AWAY and RNase Zap.

2.9 Transfer the pipettes, filtered tips in the human tisssue handling hood.

2.10 Clean a pair of forceps with [M] 0.2 Molarity (M) NaOH, 10% Presept, 70% EtOH and dH_2O, DNA AWAY and RNase Zap and transfer it to the human tissue handling hood.

2.11 Place a small beaker with [M] 0.2 Molarity (M) NaOH in the human tissue handling hood in order to use it to decontaminate the forceps and filtered tips after use.

2.12 In the human tissue handling hood Prepare 5% BSA in PBS (1x) and store On ice until use, e.g. for 1 donor:

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

2.13 Add RNasin Plus Ribonuclease Inhibitor to Buffer A and B prior to use to final 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

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

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

2.16 Weigh the tissue in a scale aiming for ~ 19 mg (aim for 15 mg - 30 mg of each tissue/donor).

2.17 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 not otherwise stated.

2.18 Homogenize the tissue using a pre-chilled pestle by grinding gently with twisting force for 50-60 times.

> **Note**
>
> Keep the tubes on wet ice while doing this step.

2.19 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.

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

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

> **Note**
>
> Incubation time can vary between 00:05:00 - 00:10:00 .

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

2.23 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.
> - If there is a liquid retention in the filter reduce the amount of starting material by half.

2.24 Centrifuge at 600 x g, 4°C, 00:05:00 . 5m

2.25 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 obvious as these are isolated nuclei.

2.26 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.

2.27 Carefully overlay the 200 µL nuclear suspension from on top of Buffer B by slowly expelling the nuclear suspension against the wall of the tube.

2.28 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.
> - The nuclei pellet may not be visible. This depends on the brain region used.
> - Extending the centrifugation time to a total of 00:15:00 - 00:20:00 may be beneficial to increase nuclei yield.

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

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

Nuclei immunostaining

30m

3 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 and store it on ice until use.

Note

Critical Notes:

- When discarding the supernatants after each centrifugation step, always leave the last 50 µL at the bottom.
- After each centrifugation of this part of the protocol, resuspend in the same volume 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 immunostaining control nuclei in 200 µL .

4 Resuspend the pellet in 500 µL (or volume of choice depending on the initial tissue input) of cold Blocking Buffer by pipetting up and down gently 5 times.

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

5 Separate the samples into different tubes for nuclei with antibodies and negative control samples, e.g.:

A. Nuclei with PI and Olig2-AF647s: 500 µL .
B. Nuclei with PI and Isotype Control (IgG-AF647): 50 µL . Then supplement with Blocking Buffer to have final volume 200 µL .
C. Nuclei with PI, but no AF647: 50 µL . Then supplement with Blocking Buffer to have final volume 200 µL .
D. Nuclei without PI or AF647: 50 µL . Then supplement with Blocking Buffer to have final volume 200 µL .

6 Incubate the nuclei in Blocking Buffer for 00:30:00 @ 4 °C on a plate shaker (tubes coved in parafilm, placed inside a falcon tube and the falcon tube on a plate shaker in the fridge).

30m

7 Add antibodies directly to the nuclei in Blocking Buffer.

8 Incubate all samples for at least 00:30:00 @ 4 °C on a plate shaker (tubes coved in parafilm and foil, placed inside a falcon tube and the falcon tube on a plate shaker in the fridge).

30m

> **Note**
>
> During this time, place the bottle containing Cell Buffer from BioSkryB kit at @ 4 °C for 00:30:00 - 01:00:00 .

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

Note

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

10 Carefully discard supernatant leaving ~ 50 µL of buffer above the pellet.

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

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

13 Wash nuclei again with Blocking Buffer.

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

Note

Critical Notes:

- During this time prepare ice-cold Blocking Buffer with PI (final 1 µg/ml, e.g. 1998 µL Blocking Buffer + 2 µL PI = 2000 µl
- Prepare 96-well low bind plates for sorting by placing 3 µL of the thawed Cell Buffer in each well of the plate, seal the plates carefully and keep at 4 °C until use.

15 Resuspend nuclei to sort in 800 µL and the control nuclei in 200 µL Blocking Buffer with PI (or volume of interest depending on the input tissue).

Note

- BioSkryB recommends using PI instead of DAPI for nuclear staining, as DAPI interferes with the ResolveOME chemistry in the downstream steps.
- Do not wash the nuclei after the addition of the PI staining solution.

16 Re-label clean 1.5 ml low bind Eppendorf tubes.

17 Filter the nuclei using Flowmi Cell Strainers and place the filtered nuclei in the pre-labelled clean 1.5 mL low binding Eppendorf tubes.

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

Note

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

Nuclei sorting (FANS) 16m

19 Before single-nucleus 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.

20 **Optional**: As an extra layer of assessment for accurate sorting, assess plate positioning with colorimetric method.

Adapted from: Rodrigues OR, Monard S. A rapid method to verify single-cell deposition setup for cell sorters. Cytometry A. 2016 Jun;89(6):594-600. doi: **10.1002/cyto.a.22865**. PubMed PMID: 27144818

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

20.2 Make a working solution (1 mg/ml), e.g. For 2 mL : 200µl stock HRP (10 mg/ml) + 1800 µL diH_2O + 2 drops of Accudrop beads. Store in the fridge.

20.3 To run the test:

16m

1. Aliquot 2 µL of TMB into each well of the test plate.
2. Sort a single bead into a whole plate (or wells needed).
3. Once the sort is completed, immediately seal the plate and centrifuge 500 x g, 00:01:00 .
4. Wait 00:05:00 - 00:10:00 and count the number of wells that have turned blue.

Note

Critical Note:

We aim for >90% success.

4. If successful deposition is achieved, proceed with sorting cells. If not successful, recalibrate the alignment and try again!

21 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

Use negative control samples as the threshold references.

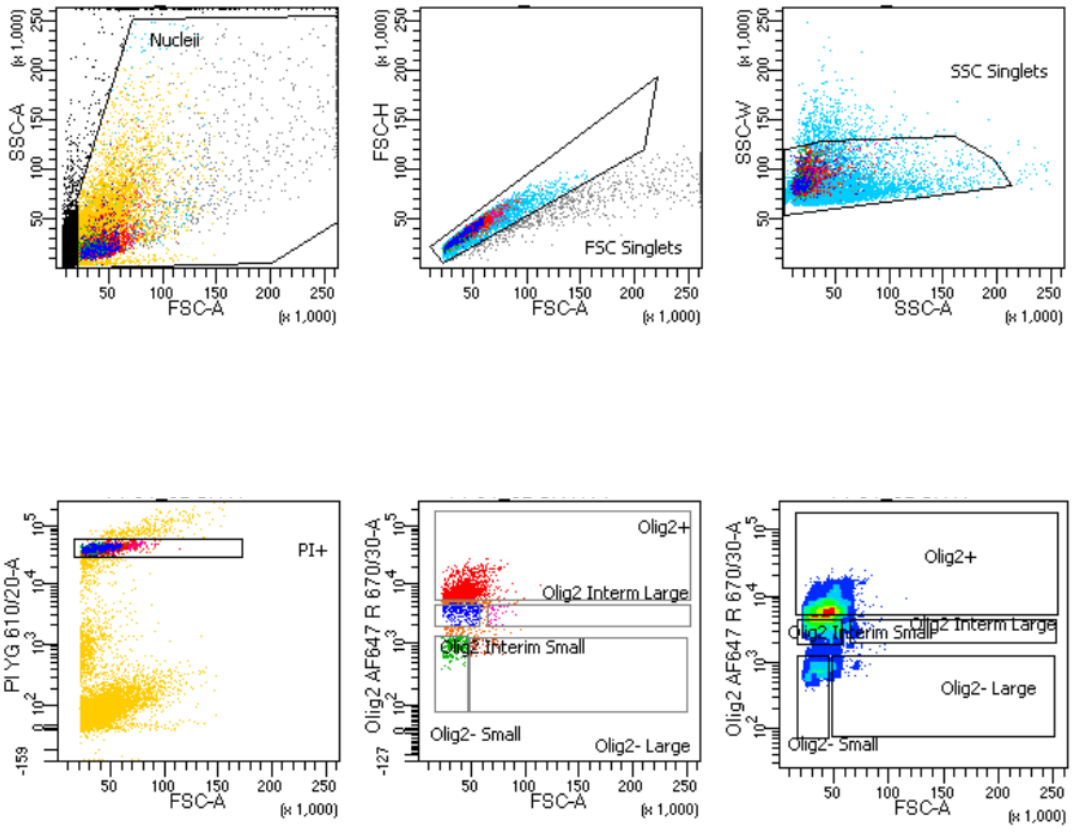
22 Select gating parameters to isolate the singlets from the overall detected particles by selecting forward (FSC) and side scatter (SSC), FCS single cell gate and SSC single cell gate.

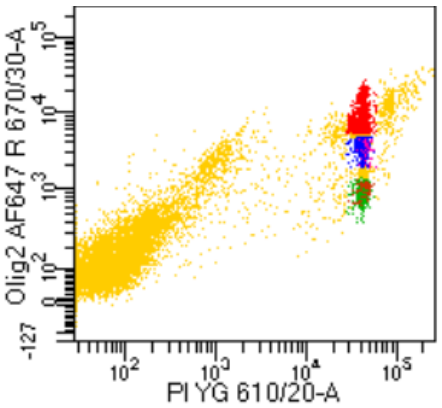
23 Select the human post-mortem nuclei by their PI expression.

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

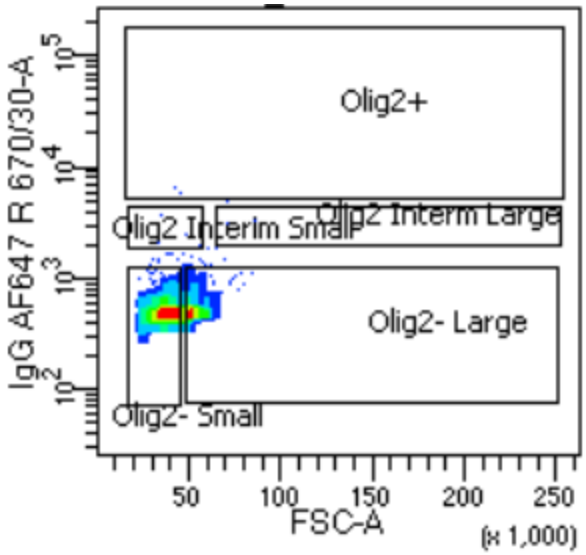
Figure 1. Example of the gating strategy of human post-mortem samples.

A. Nuclei with Olig2-AF647 and PI

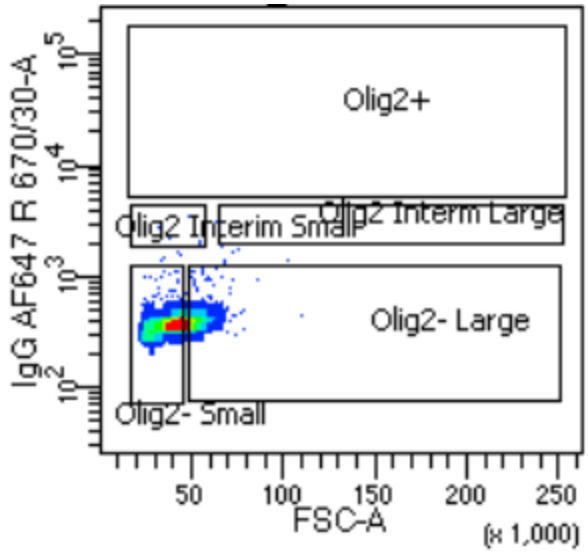




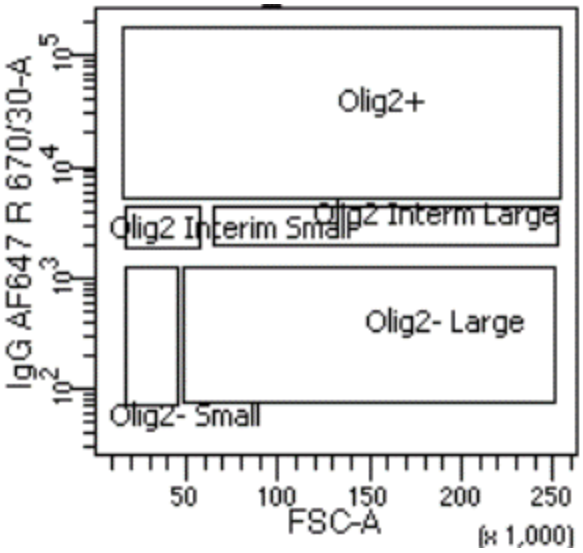
B. Nuclei with isotype AF647 control and PI



C. Nuclei with PI but no AF647



D. Nuclei without PI (no nuclei selection) or AF647



25 Centrifuge for 00:00:10 at low speed the collection plates to ensure reagent at bottom and place them On ice . 10s

26 Sort single-nuclei of interest into the centrifuged 96-well collection plates that are placed in a pre-cooled plate holder.

27 After sorting, seal immediately seal the plates with VersiCap Mat cap strips and pre-labelled plate sealers.

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

29 Place each plate in individually sealed bag.

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

31 Stored the plate(s) -70 °C until further use.

Single-nucleus DNA/RNA multiome by ResolveOME kit

32 Follow the ResolveOME kit according to BioSkryB guidelines (BioSkryB TAS-074.1).

Note

As the input material here are nuclei from human post-mortem brain samples instead of cells from cell culture, we suggest the following modifications:

- Dilute the DNA fraction in 30 µL Elution Buffer.
- Dilute the RNA fraction in 5 µL Elution Buffer.

Protocol references

This protocol was adapted from:

Protocols.io

- Ester Kalef-Ezra, Lucia Friscioni, Dominic Horner, Caoimhe Morley, George Morrow, Yanping Guo, Christos Proukakis 2024. Fluorescence-activated nuclei sorting (FANS) for single-cell Whole Genome Sequencing (scWGS). protocols.io https://dx.doi.org/10.17504/protocols.io.81wgbzybygpk/v1.
- Ester Kalef-Ezra, Dominic Horner, George Morrow, Vanda Knitlhoffer, Andy Goldson, Iain Macaulay, Yanping Guo, Christos Proukakis 2024. Nuclei isolation, immunostaining, and Fluorescence-activated nuclei sorting (FANS) for Smart-Seq2. protocols.io https://dx.doi.org/10.17504/protocols.io.j8nlk8wjxl5r/v1.

Manufacturer protocols

- Nuclei isolation using Invent Bioscience kit BN-020 (Invent Biosciences).
- ResolveOME™ Whole Genome and Transcriptome Single-Cell Core Kit, TAS-074.1 | 06/2024 (BioSkryB)
- Best Practices: FACS Plate Alignment Considerations (BioSkryB).

Publication

- Perez-Rodriguez D, Kalyva M, Santucci C, Proukakis C. Somatic CNV Detection by Single-Cell Whole-Genome Sequencing in Postmortem Human Brain. Methods Mol Biol. 2023;2561:205-230. doi: **10.1007/978-1-0716-2655-9_11**. PMID: 36399272.
- Rodrigues OR, Monard S. A rapid method to verify single-cell deposition setup for cell sorters. Cytometry A. 2016 Jun;89(6):594-600. doi: **10.1002/cyto.a.22865**. PubMed PMID: 27144818

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