Extraction of nuclei from archived postmortem tissues for single-nucleus sequencing applications

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Single-cell and single-nucleus sequencing techniques are a burgeoning field with various biological, biomedical and clinical applications. Numerous high- and low-throughput methods have been developed for sequencing the RNA and DNA content of single cells. However, for all these methods, the key requirement is high-quality input of a single-cell or single-nucleus suspension. Preparing such a suspension is the limiting step when working with fragile, archived tissues of variable quality. This hurdle can prevent such tissues from being extensively investigated with single-cell technologies. We describe a protocol for preparing single-nucleus suspensions within the span of a few hours that reliably works for multiple postmortem and archived tissue types using standard laboratory equipment. The stages of the protocol include tissue preparation and dissociation, nuclei extraction, and nuclei concentration assessment and capture. The protocol comparable to other published protocols but does not require fluorescence-assisted nuclei sorting (FANS) or ultracentrifugation. The protocol can be carried out by a competent graduate student familiar with basic laboratory techniques and equipment. Moreover, these preparations are compatible with single-nucleus (sn)RNA-seq and assay for transposase-accessible chromatin (ATAC)-seq using the 10X Genomics Chromium system. The protocol reliably results in efficient capture of single nuclei for high-quality snRNA-seq libraries.

Introduction

Advancements in technology have allowed researchers to perform large-scale transcriptomic studies at the level of a single cell. Droplet-based cell isolation has become a favorite method in the field for its scalability and simplicity of use with either in-house fluidic setups1,2 or commercially available equipment (10X Genomics)3. This technique is particularly interesting for tissues with highly heterogeneous cellular compositions such as intestine4, lung5, spinal cord6 and brain7,8. There has been particular interest in deconvoluting brain architecture and function, which at its base starts by accurately identifying all the cells types present9–11. However, truly harnessing the power of individual cellular transcriptomes requires assessing differences between those transcriptomes in different physiological states. This is of particular value for complex diseases where multiple genes contribute with additive effects, making it difficult to identify changes in tissues homogenates12. The chemical dissociation of tightly interconnected brain cells and other cell types has been found to alter transcription profiles13,14. Given that nuclear transcriptomes closely reflect the cell’s cytosolic profile15,16, isolating the nuclei from brain tissue has proven to be an excellent strategy for studies at the single-cell level. Likewise, other tissues that have either been frozen for long-term storage or that are formed by syncytium, such as in skeletal muscles17,18, could benefit from this approach.

Development of the protocol

Numerous protocols for isolating nuclei from brain cells have been published10,11,19–23; some rely on additional purification by fluorescence-assisted cell sorting (FACS)20,23, which is costly, time-consuming and technically demanding. Our protocol is designed to address some of the limitations of previous methods by reducing the amount of equipment required and by simplifying the overall procedure.
consuming and not readily available for all researchers, while others have made adjustments to the microfluidics component used to isolate and capture single nuclei\(^8\), which can also be limiting to laboratories. Our protocol has been developed for use with the commercially available Chromium Single Cell Controller. This is a highly optimized system that allows scalable single-cell capture. We have adapted our protocol to allow the Chromium system to efficiently capture nuclei from archived postmortem tissue. Our preparation produces stable and easily quantifiable nuclear suspension even when using archived brain tissue. We have used this approach to successfully compare gene expression differences in the postmortem prefrontal cortex of depressed patients who died by suicide with that in psychiatrically healthy controls\(^24\). The protocol has also been successfully applied, with minor modifications, to collect single-nucleus transcriptomic data from surgical samples of glioblastoma\(^25\) and in a recent study of postmortem brain in schizophrenia\(^26\). Thus, gene expression and chromatin accessibility can be measured from postmortem brain tissue using this protocol. We anticipate that newer techniques from 10X Genomics such as those that combine single-cell (sc) ATAC-seq and snRNA-seq to study them simultaneously in frozen tissues could also take advantage of this protocol.

**Overview of the procedure**

The experimental workflow (Fig. 1) begins with cellular lysis by dounce homogenization in low-concentration detergent. Integral to the protocol are numerous wash steps to reduce ambient nucleic acid contamination, in a buffer containing a high percentage of bovine serum albumin to prevent nucleus aggregation. The suspension is repeatedly filtered to remove large debris. Most centrifugation steps are performed at low speed to prevent damage to the nuclei. Finally, an iodixanol cushion is used to purify the nuclei. The nuclei numbers and concentration are assessed by a cell counter or hemocytometer. In addition, Hoechst or DAPI can be used to stain DNA for assessing the nucleus concentration by fluorescence microscopy. The concentration of the nucleus suspension is important to reduce aggregation, particularly in tissues that have undergone long-term storage and are thus more likely to be damaged, fragile and inclined to aggregate. Generally, a concentration of 500–1,000 nuclei/µL is sufficient for nucleus capture and should not result in excessive aggregation.

**Comparison with other methods**

As previously mentioned, existing snRNA-seq protocols either rely on FACS\(^20,23\), which is harder to scale, or on droplet-based approaches, which use in-house setups\(^10\). Early protocols used relatively fresh frozen tissue, which is not available in most tissue banks where samples are likely to have
undergone long-term storage\textsuperscript{9–11}. Moreover, when studying specific phenotypes for which it is harder to obtain tissues, it is not always possible to select for short postmortem interval (PMI) and archival times. Early protocols were also limited to high-quality tissue, which might not be an option for answering certain types of research questions.

As with several more recently published protocols\textsuperscript{19–22}, we have been able to adapt our nuclear preparation to be compatible with the 10X Chromium system, which is becoming increasingly available as a service platform. Furthermore, the wet-lab aspect of the protocol will produce nuclei suitable for multiple postnucleus capture applications, such as whole-genome sequencing for the study of somatic mutations or snATAC-seq, as supported by preliminary results from our laboratory.

Each of the more recently developed protocols has its strengths and weaknesses and, in some cases, adaptations for specific tissue types such as macrodissections for white matter regions\textsuperscript{21}. The strength of our protocol is that it is mostly unaffected by variations in PMI or archival times of the samples (Fig. 2). Some of these protocols also incorporate ultracentrifugation\textsuperscript{22,27}, which is time consuming, requires specialized equipment and could be damaging to fragile nuclei. We are able to circumvent the additional challenges that arise with archived tissue such as the fragility of the cells and organelles upon freeze-thawing, which typically results in large amounts of debris and ambient RNA that can either interfere with droplet formation or be integrated into droplets, increasing background sequencing noise. Here, we show that brain tissue that has been stored at \textdegree{C} for as long as 22 years can produce high-quality single-nucleus suspensions.

Directly applying either the cell preparation protocol or the demonstration protocol for nuclei developed from 10X Genomics did not produce useable results in our hands with archived postmortem brain tissue (Fig. 3), although other laboratories have been able to successfully use this protocol for nucleus extraction for snRNA-seq. Moreover, our attempts to use nuclei isolated by FANS as input to the 10X Genomics protocol did not yield acceptable results, although this approach has been adopted successfully by other groups\textsuperscript{20}. The modifications made here are primarily for use with postmortem brain tissue that has been archived for long periods of time, but can also be applied to any frozen postmortem sample. Similar to previous studies\textsuperscript{10,11}, we applied a few modifications to the standard bioinformatic analysis with the Cell Ranger pipeline from 10X Genomics to address a number of issues that arise with droplet-based single-nucleus sequencing. First, we assembled a pre-mRNA reference to account for unprocessed transcripts found in the nucleus\textsuperscript{28}. Second, given that previous studies have consistently shown fewer identifiable transcripts in glial cells\textsuperscript{29–31}, we performed customized barcode filtering to include cells with a wider range of unique molecular identifiers.

Fig. 2 | Effect of sample quality parameters on single-nucleus capture and sequencing metrics. The archival time, PMI, age and pH of the brains accounted for <10% of the variation in number of cells (nuclei) retained after filtering, median number of genes per nucleus and median number of UMIs per nucleus. The RIN of the samples had had a significant negative effect (\(P = 3.4 \times 10^{-6}\)) on the number of nuclei captured, especially for RIN < 4, but did not have a large effect on the median numbers of genes or UMIs. The \(R^2\) values based on Pearson correlations and \(P\) values (\(n = 34\) samples) were calculated using the cor.test function in R. Linear trendlines were added using Microsoft Excel. For two of the data points, the median UMIs, median genes and number of cells are the aggregated values of two runs for those samples. All data in this figure are from the dataset published in ref. 24.
(UMIs) while removing noise. With these minor modifications to the analysis\textsuperscript{24}, our isolation approach for our tissue type, that is, archived postmortem brain, produced much improved data compared with the available 10X Genomics nucleus preparation protocols in our hands.

**Experimental design**

The most important factor to take into consideration while designing single-cell or snRNA-seq experiments is the potential for batch effects. Given that the Chromium system only allows for the capture of eight samples at a time and that for many experiments the total number of samples to be analyzed is greater than eight, it may be preferable to create a balanced experimental design, if possible. This will help limit the effects of batch-to-batch variability. For example, if two phenotypic or treatment groups are to be compared, it would be ideal to include equal numbers of samples from each group in every batch. Moreover, other potential covariates to take into consideration include age, PMI and sex. It might be possible to account for the effects of these variables by matching samples by these parameters within each batch.

**Fig. 3 | Images of extracted nuclei.** a–d, Before optimization, using the 10X Genomics demonstrated protocol, the extracted nuclei from two different samples (a,b) show large amounts of debris, and the size distribution is skewed towards larger sizes (>10 \( \mu \)m). After optimization, representative images of nuclei extracted from two samples (c,d) show much less debris, and size distributions are centered around 10 \( \mu \)m, within the expected range for human brain nuclei. Images were acquired with the Countess cell counter using Trypan blue for staining. e,f, Note that extracted nuclei should be marked as dead cells. Extracted nuclei do not tend to aggregate even after 2.5 h (e) or 16 h (f) of storage at 4 °C. Note that the size distribution after 16 h is still centered around 10 \( \mu \)m, indicating an absence of aggregation. g, Representative images of extracted nuclei stained with Hoechst (1:2,000) acquired at 10× magnification on the Evos microscope. c,d and g correspond to samples used in ref.\textsuperscript{24}. Scale bars, 500 \( \mu \)m.
In cases where cell-type-specific gene expression data have been published previously, or single-cell or nucleus gene expression datasets are available, these data can be used for comparison to help determine whether the cell types identified and single-nucleus transcriptomic profiles detected are comparable to previously published literature. In cases where such datasets are not available, it may be informative to prepare bulk tissue samples in parallel or to perform sequencing of FANS-purified populations of expected cell types on the basis of known genetic markers to validate the cell-type identification from the single-nucleus transcriptomic data. High-throughput in situ hybridization (ISH) and ISH-based nucleus sorting have also been used to confirm experimentally determined cell types from snRNA-seq. In the case of complex tissues, it can be useful to perform careful dissection and even to cryosection the tissue before preparing nuclei to ensure that the approximate cell-type composition for each sample will be comparable.

Another strategy that has been applied recently to increase cost-effectiveness as well as to aid in batch effect correction is combining male and female samples in a single capture followed by using the expression of sex-specific X-chromosome genes such as XIST and Y-chromosome genes such as SRY, or the chromosome accessibility ratios for sex chromosomes versus autosomes, to separate the cells from each sample. Since both samples are captured on the same lane of the microfluidic chip, it might be possible to account for lane-to-lane variability using this approach. Moreover, the use of cryosections of histological grade tissue blocks might be a good strategy to account for uniform input from microanatomically heterogeneous regions such as the cerebral cortex.

Expertise needed to implement the protocol
This protocol requires access to a 10X Genomics Chromium system and corresponding reagents, or an in-house droplet-based single-nucleus sequencing system. A hemocytometer or cell-counting microscope will be required to determine proper loading concentration. Wet-lab work will require familiarity with standard molecular biology approaches such as cDNA synthesis and sequencing library preparation.

Advantages and limitations
We are unable to obtain information about cytoplasmic transcription, which might be limiting for obtaining data for some cell types. Some tissue types, such as spinal cord or intestine, might require additional processing, such as through FANS or collagenase treatment. Representation of all cell types might not be uniform, as different cell types are differentially susceptible to lysis during the isolation procedure.

We cannot rule out the possibility that the multiple rounds of washing and centrifugation incorporated into our protocol might result in damage to fragile tissue or unacceptable levels of material loss if starting with small amounts of precious tissue. Moreover, we cannot rule out the possibility that use of the iodixanol gradient might cause biases in the types of nuclei recovered, and this might require empirical assessment for different tissue types. We have not systematically assessed this bias.

Materials

**Biological materials**
- Tissue samples: this protocol was successfully applied for processing frozen archived postmortem prefrontal cortex tissue obtained from the Douglas Bell Canada Brain Bank, postmortem intestinal tissue (with modifications such as collagenase treatment) and surgical samples of tumor tissue.

**Reagents**
- NP-40 detergent at 10% (vol/vol) concentration (Abcam, cat. no. ab142227)
- Bovine serum albumin Fraction V (BioShop, cat. no. ALB001.25)
- Tris (BioShop, cat. no. TRS003.5)
- NaCl (BioShop, cat. no. SOD001.1)
- MgCl₂·6H₂O (BioShop, cat. no. MAG510)
- HCl (BioShop, cat. no. HCL333) **CAUTION** Concentrated HCl is highly corrosive and should be handled inside a fume hood while wearing personal protective equipment (PPE).
KCl (BioShop, cat. no. POC308)
KOH (BioShop, cat. no. PHY202) **CAUTION** Concentrated KOH is highly corrosive and should be handed inside a fume hood while wearing PPE.
Tricine (BioShop, cat. no. TRI001)
Glycerol (BioShop, cat. no. GLY001)
Protector RNAs inhibitor (Millipore Sigma, cat. no. 3335399001) **CRITICAL** Other RNase inhibitors might not be compatible with the protocol and could result in low yield of nuclei.
Optiprep density gradient medium, 60% (wt/vol) iodixanol (Millipore Sigma, D1556-250)
Gibco PBS, pH 7.4 (1×; Thermo Fisher Scientific, cat. no. 10010023)
Deionized water
Ethanol 100% (Sigma, cat. no. 459836-500ML)
Trypan blue stain (0.4%; Thermo Fisher Scientific, cat. no. T10282)
Hoechst stain (Invitrogen, cat. no. H1399)
Chromium Single Cell 3’ Library and Gel Bead Kit v2 or newer (10X Genomics Inc, cat. no. 120237)
Chromium Single Cell A Chip Kit (10X Genomics Inc, cat. no. 120236)
SPRSelect Reagent Kit (Beckman Coulter, cat. no. B23318)
Twee 20 (Bio-Rad, cat. no. 1610781)
Buffer EB (250 mL; Qiagen, cat. no. 19086)
Glycerin (glycerol), 50% (vol/vol) aqueous solution (Ricca Chemical Company [or other], cat. no. 3290-32)
Dynabeads MyOne Silane beads (5 mL; Thermo Fisher Scientific, cat. no. 37002D), may be included in newer 10X Genomics snRNA-seq kits
Low TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 100 mL; Thermo Fisher Scientific, cat. no. 12090-015)
Nuclease-free water (Thermo Fisher Scientific, cat. no. AM9937)
TapeStation High Sensitivity D1000 sample buffer (Agilent, cat. no. 5067-5603) or TapeStation High Sensitivity D5000 sample buffer and ladder (Agilent, cat. no. 5067-5593)
TapeStation High Sensitivity D1000 ScreenTape (Agilent, cat. no. 5067-5584) or High Sensitivity D5000 ScreenTape (Agilent, cat. no. 5067-5592)

**Equipment**

**Lab equipment**

- Scalpel
- Spatula
- Weighing boat
- Weighing scale
- Refrigerated benchtop centrifuge for 5 mL tubes (Eppendorf, model 5430R)
- Refrigerated benchtop centrifuge for 15 mL tubes (Beckman Coulter, model Allegra X-14R)
- Countess II FL automated cell counter (Thermo Fisher Scientific, cat. no. AM410000)
- Countess II FL automated cell counter Chamber Slides (Thermo Fisher Scientific, cat. no. C10228)
- Flowmi cell strainer, 40 μm (Bel-Art, cat. no. H13680-0040)
- MACS SmartStrainers, 30 μm (Miltenyi Biotec, cat. no. 130-098-458)
- Dounce tissue grinder, 7 mL (Wheaton, cat. no. 357542)
- Centrifuge tubes, 15 mL (Corning, cat. no. 430791)
- Centrifuge tube, 50 mL, with screw cap (Sarstedt, cat. no. SAR62547205)
- DNA LoBind microcentrifuge tubes, 1.5 mL (Eppendorf, cat. no. 022431021)
- 250 mL glass bottles
- DNA LoBind microcentrifuge tubes, 5.0 mL (Eppendorf, cat. no. 301083010)
- INCYTO C-Chip disposable hemocytometers (SKC Films, cat. no. DHCN012 or DHCN015)
- TempAssure PCR 8-tube strip (USA Scientific, cat. no. 1402-4700)
- Serological pipette, 10 mL
- Invitrogen EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific)
- Chromium controller (10X Genomics)
- Divided polystyrene reservoirs, 25 mL, 50 (VWR, cat. no. 41428-960)
- Filter tip, 200 μL (Rainin, cat. no. 17007961)
- Pipet-Lite Multi Pipette L8-200XLS (Rainin, cat. no. 17013805)
- TapeStation 2200 (Agilent) or equivalent equipment
Software for sequence alignment and gene-barcode counting

- CellRanger, version 2.1.0 (https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest) **CRITICAL** Linux OS must meet the minimum requirements for running CellRanger as described on the 10X Genomics webpage (https://support.10xgenomics.com/single-cell-gene-expression/software/overview/system-requirements).

- bcl2fastq2, version 2.19 (https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html)

Software for secondary analysis in R

- Seurat, version 2.3.0 or higher (https://satijalab.org/seurat/articles/install.html)
- mixtools (1.1.0) (https://cran.r-project.org/web/packages/mixtools/index.html)
- R, version 3.4 or higher (https://www.r-project.org/)

Software for analysis of snATAC-seq

- scATAC-pro, version 1.1.4 (https://github.com/tanlabcode/scATAC-pro)

Reagent setup

**Stock solutions**

**CRITICAL** The following reagents should be prepared ahead of time.

- Prepare 1 M NaCl, 100 mM MgCl₂·6H₂O, 1 M MgCl₂·6H₂O and 1 M KOH solutions in ddH₂O in separate 50 mL centrifuge tubes and store at room temperature (RT; 21–22 °C for our laboratory) for up to 6 months.
- Prepare 250 mL of 10% (wt/vol) BSA solution in a glass bottle by dissolving crystalline BSA in ddH₂O at RT. Store at 4 °C for up to 1 week. Keep crystalline BSA at −20 °C.
- Prepare 250 mL of 1 M Tris HCl buffer in a glass bottle by dissolving Tris in ddH₂O. Adjust pH to 7.4 by adding HCl dropwise. This is a time-consuming step. Store at RT for up to 6 months.
- Prepare 250 mL of 0.5 M Tricine KOH buffer in a glass bottle by dissolving Tricine in ddH₂O and adjusting pH to 7.8 by adding 1 M KOH dropwise. This is a time-consuming step. Store at RT for up to 6 months **CRITICAL** Buffer recipes provided are calculated assuming preparation of eight samples for capture using a full Chromium chip.

**Optiprep diluent (altered per ref. 38)**

Combine the following in a 250 mL glass bottle. Store at RT.

| Component | Volume (mL) | Final concentration (mM) |
|-----------|------------|--------------------------|
| 1 M KCl   | 15         | 150                      |
| 1 M MgCl₂·6H₂O | 0.5             | 5                        |
| 0.5 M Tricine-KOH (pH 7.8) | 4                | 20                       |
| Deionized water | 80.5          | –                        |
| Total volume | 100      | –                        |

**Optiprep solutions**

Using Optiprep diluent solution, dilute the Optiprep reagent to make 50% (wt/vol) iodixanol and 29% (wt/vol) iodixanol solutions from Optiprep solution in separate 50 mL centrifuge tubes. Protect from light and store at RT for up to 6 months.

**Lysis buffer (LB)**

Combine the listed components in a 50 mL centrifuge tube. This buffer should be made fresh and kept at 4 °C or on ice.

| Component | Volume (μL) | Final concentration |
|-----------|------------|---------------------|
| 1 M Tris-HCl pH 7.4 | 200 | 10 mM               |
| 1 M NaCl | 200 | 10 mM               |
| 100 mM MgCl₂·6H₂O | 600 | 3 mM                 |
| NP-40 (10%) | 100 | 0.05% (vol/vol)     |
| Deionized water | 18,900 | –                    |
| Total volume | 20,000 | –                    |
Nucleus wash buffer (NWB)
Combine the listed components in a 250 mL glass bottle. This buffer should be made fresh and kept at 4 °C or on ice.

| Component                        | Volume (mL) | Final concentration |
|----------------------------------|-------------|---------------------|
| 10% BSA                          | 100         | 5% (wt/vol)         |
| Glycerol                         | 0.5         | 0.25% (vol/vol)     |
| Protector RNase inhibitor        | 0.2         | 40 units/mL         |
| 1× PBS to 200 (~100)             | to 200      | 0.5×                |
| Total volume                     | 200         | 200                 |

Equipment setup
• Precool both centrifuges to 4 °C
• Set up EVOS FL Auto microscope with 10× magnification, bright field and DAPI channels

Procedure

**Tissue preparation ● Timing 1–4 h**
1. Cut tissue using a scalpel and weigh 30–50 mg of frozen tissue per sample. Keep tissue on dry ice while cutting to minimize degradation. Transfer to a 1.5 mL microcentrifuge tube using a spatula, and place back on dry ice. Clean scalpel and spatula with 70% ethanol (vol/vol) between samples. Use a fresh weigh boat for each sample. Alternatively, this step could be replaced by cryosectioning a fresh frozen histology grade dissection of tissue and collecting several sections such that the total weight is 30–50 mg.
   **! CAUTION** Postmortem human tissue can contain pathogens. Take precautions, including wearing PPE, and seek medical attention if the scalpel breaks your skin.

**Nucleus extraction ● Timing 2–3 h**
2. Transfer tissue using spatula to douncing tube on ice. Add 3 mL of ice-cold lysis buffer and dounce with loose pestle ten times and five more times with the tight pestle.
   **▲ CRITICAL STEP** Use proper douncing technique to ensure proper mechanical breakdown of tissue. Proceed slowly and avoid bubbles. Grind tissue against the bottom of the tube using the douncer with each stroke.
3. Transfer homogenized tissue to a 15 mL centrifuge tube by pouring, and add 2 mL of chilled lysis buffer. Incubate on ice for 5 min, gently swirling to mix twice during incubation.
4. Add 5 mL of chilled wash buffer to lysed tissue to quench lysis. Swirl to mix.
5. Place 30 μm MACs SmartStrainer on a 15 mL centrifuge tube. Pipette lysed tissue suspension on top of filter to remove cell debris and large clumps. In case of blocked flow through the filter, tap filter gently to encourage the suspension to flow through.
6. In the precooled Allegra X-14R centrifuge, spin down the lysed tissue suspensions at 500g for 5 min at 4 °C.
7. Decant supernatant into a waste beaker without disrupting the nucleus pellet.
   **! CAUTION** The supernatant should be treated as biohazardous waste and treated with bleach before disposal.
   **▲ CRITICAL STEP** Pour out supernatant in a single motion, as repeated pouring motions can dislodge the pellet. If the pellet dislodges during decanting, slowly remove the supernatant using a pipette.
8. Using a 10 mL serological pipette, add 10 mL of NWB to the pelleted nuclei and gently pipette 8–10 times to mix.
9. Repeat Steps 5–7 using the resuspended nuclei.
10. Using a 10 mL serological pipette, add 5 mL of NWB to the pelleted nuclei and gently pipette 8–10 times to mix.
11. Repeat Steps 6 and 7 using the resuspended nuclei.
12. Using a 1,000 μL pipette tip, add 1 mL of NWB to pelleted nuclei and gently pipette 8–10 times to mix.
13. Add 1 mL of 50% (wt/vol) working solution of iodixanol (Optiprep) to the nuclei, and mix well to obtain 2 mL of 25% (wt/vol) iodixanol solution containing nuclei.
14 Prepare an iodixanol cushion of 2 mL of 29% (wt/vol) iodixanol solution in a 5 mL Eppendorf centrifuge tube.
15 Gently add the 2 mL nucleus suspension on top of the iodixanol cushion by pipetting slowly against the wall of the tube to avoid mixing.
16 In the precooled Eppendorf centrifuge, spin the tubes containing nuclei layered over iodixanol cushion at 10,000 g for 30 min at 4 °C.
17 Carefully pour out the supernatant, leaving the least possible amount of volume in the tube without disrupting the pellet.

**TROUBLESHOOTING**

18 Using a 1,000 μL pipette tip, resuspend the nucleus pellets in ≤500 μL of NWB. Gently pipette 8–10 times or until nuclei are resuspended.

For a quick estimate of nucleus concentration, mix 10 μL of the nucleus suspension with 10 μL of Trypan blue in a separate tube. Load 10 μL of the mixture onto a Countess hemocytometer slide. Count nuclei on the Countess hemocytometer, and measure range of sizes. For human nuclei from archived postmortem cortical tissue we have observed that the average diameter is ~10 μm. However, nuclei can have a range of sizes, and it is only concerning if a long tail of particles of more than 30 μm is detected, as this might indicate debris and aggregation. Trypan blue is a live–dead stain, and properly isolated nuclei should be marked as dead cells.

**TROUBLESHOOTING**

20 Using the estimated count from the Countess, dilute nuclei to ~500,000 cells/mL or 500 cells/μL by adding an appropriate volume of NWB. It might be possible to increase these concentrations for capturing more nuclei.

▲ **CRITICAL STEP** If the concentration of nuclei is too high, it can result in aggregation that will prevent efficient capture of single nuclei in subsequent steps. We have achieved good suspensions and capture with up to 1,000 nuclei/μL, but if aggregation is observed, lower concentrations (as low as 500 nuclei/μL) might be better.

21 Add Hoechst stain to the resuspended nuclei at a 1:2,000 dilution to obtain counts using fluorescence microscopy.

**Nucleus concentration assessment and capture ● Timing 45 min**

▲ **CRITICAL** Immediately prior to loading the Hoechst stained nuclei on the Chromium system, check to make sure the nuclei are well segregated (nuclei might clump) and recheck sample concentration using a fluorescent microscope such as the Evos FL Auto (Thermo Fisher Scientific).

22 OPTIONAL: use a 1 mL pipette to take a minimum of 200 μL of the sample, and filter it through a 40 μm Flowmi pipette tip filter before counting and loading. This will remove clumped nuclei and large debris that can clog the microfluidics of Chromium chips.

23 Load 10 μL of sample onto a disposable hemocytometer slide and into the Evos.

▲ **CRITICAL STEP** Make sure to pipette the full volume of the nucleus suspension up and down several times to avoid settling of the pellet at the bottom of the tube before loading for accurate counts.

24 Set the Evos to 10× magnification, and image the entire hemocytometer grid field of view in DAPI and bright field. This image makes counting nuclei easier and serves as a record as well (Fig. 3g). If a fluorescence microscope is not available, it might be sufficient to use the counts based on Trypan blue staining, but unfortunately it will not be possible to distinguish between debris and nuclei using this approach. On the other hand, using a fluorescence microscopy may be more time consuming for nucleus counting when processing many samples for capture. User discretion and considering the state and type of tissue are important parameters when determining cell counting strategy.

25 In parallel to Steps 22–24, make the RT Master Mix and prepare aliquots of appropriate volumes of master mix and water into PCR tubes according to the number of nuclei to be targeted for capture, referring to the Chromium protocol CG00052 Rev. F or later (https://assets.ctfassets.net/ant8imn79xiti/RT8DCvH8zTr8BMv8YjCmVxd6a0ed8015d89bf9e02128a4c9fb8962cf/C00052_SingleCell3_ReagentKtv2UserGuide_RevF.pdf).

26 Load the Chromium chip and harvest the nuclei captured in droplets (i.e., gel bead-in emulsions) according to the Chromium protocol CG00052 Rev. D

**TROUBLESHOOTING**
Box 1 | Downstream data analysis

### Alignment, demultiplexing and generation of counts matrix

(Time: variable)

Since our experiments utilized human nuclei, we built a pre-mRNA reference using the cellranger mkref (CellRanger, version 2.0.1) command. Default parameters were used, starting with the rfsdata-cellranger-GRCh38-1.2.0 transcriptome and as per the instructions provided on the 10X Genomics website. For mouse tissue, the corresponding pre-mRNA reference would need to be created for the mouse genome. We demultiplexed reads by sample index using the cellranger mkfastq command (CellRanger v2.1.0), aligned FASTQ files to the custom transcriptome, demultiplexed cell barcodes and counted the UMIs corresponding to genes using the cellranger count command and default parameters. These steps can be performed with custom code if desired.

### Custom filtering to recover low-transcript-number cell types

(Time: variable)

While there are many options for software to be used for downstream analysis of snRNA-seq data, such as scater, SC3 and Monocle3, we used the Seurat R package (version 2.2.0, 2.3.0). Unfiltered gene barcode matrices for each sample were loaded into R using the Read10X function. At this step, cell names can be modified such that the subject name, batch and biological condition are appended to them. Seurat objects were created corresponding to each sample using the CreateSeuratObject function with the imported unfiltered gene-barcode matrices provided as the raw data. Individual Seurat objects for each sample were combined sequentially using the MergeSeurat function. No filtering or normalization was performed up to this step. Since we were working with a single nucleus dataset, all mitochondrial genes that are transcribed from the mitochondrial genome were removed, along with genes not detected in any cell. More recently, several methods have been developed to align multiple datasets of snRNA-seq and other single-cell level data that can be used for combining the data from individual subjects if interindividual variability or batch effects are deemed to have a large influence on the results.

For preliminary filtering, some nuclei with very low number of genes detected (<110) and nuclei with very high numbers of UMIs detected (in the top 0.5%) were removed as low-quality nuclei and potential multiplets, respectively. These cutoffs are arbitrary but can be based upon the distribution of the data. For example, in our dataset there was a sharp increase in the number of UMIs from 16,393 at the 99.5th percentile to 102,583 at the maximum, which probably represents the multiplets in the dataset. If the dataset contains multiple cell types that are expected to be heterogeneous in terms of the number of molecules of RNA present per nucleus (such as when the nuclei of different cell types are known to be of very different size), the following approach can be used for removing low-quality cells without unduly biasing the filtering against nuclei that biologically contain fewer molecules. For our dataset, given the known trend for higher number of RNA molecules in neuronal nuclei compared with glial nuclei, the distribution of number of UMIs was fit with three normal distributions using the normalmixEM function from the mixtools package. The rationale is that the filtered barcodes contain a population of low-quality ‘noise’ barcodes that have a very low number of UMIs on average, a population of nonneuronal cells that have an intermediate numbers of UMIs and a population of neuronal cells that have a high number of UMIs. After fitting the normal distributions, only the barcodes with a high probability (>0.95) of belonging to either the putative ‘nonneuronal’ or putative ‘neuronal’ distributions, and a low probability (<0.05) of belonging to the ‘noise’ distribution were retained for further analysis. As an example, for a subset of 20 subjects, applying our custom filtering approximately doubled the total number of cells, as numerous cells previously discarded as empty barcodes could now be included, most of which represented nonneuronal cells as evidenced by and almost sixfold increase in their population.

Of note, in our experience with the newest version of Cell Ranger (3 and above), which incorporates the EmptyDrops algorithm for cell calling, it might not be necessary to customize the process of calling cells to account for biases in number of genes expressed and RNA molecules across cell types.

▲ CRITICAL STEP  The Chromium capture rate for nuclei from archived tissue is lower (~20% less) than the capture rate for cells. To account for this, it is necessary to adjust the count used to determine loading volume. We determined empirically that choosing the loading volume by using a count that is 30% less than the observed count worked best for our samples. This adjustment might vary across tissue types. For example, if the sample has a concentration of 500 nuclei/μL, the sample volume should be loaded as if it has 350 cells/μL (70% of 500 cells) to recover the targeted number of nuclei.

▲ CRITICAL STEP  Resuspend nuclei by pipette, mixing the full volume several times immediately before loading to prevent aggregation of nuclei.

**Library preparation and sequencing**  ● **Timing** as per the Chromium protocol, ~8 h split over 2 d

27  Perform reverse transcription, cDNA amplification and library preparation according to the Chromium protocol CG00052 Rev. D. Libraries can be sequenced on an Illumina sequencer. Sequencing two samples per lane of a HiSeq 4000 machine can yield 150,000,000 reads per sample. This can translate to ~50,000 reads per nucleus if capturing 3,000 nuclei per sample based on default CellRanger parameters and provides sufficient information for cell-type identification and differential expression analysis. However, the exact number of reads per cell will depend on how many nuclei are loaded and on the algorithm used to call cells.

28  Downstream analysis options for the sequencing results are described in Box 1.
Troubleshooting

Troubleshooting advice can be found in Table 1.

### Table 1 | Troubleshooting table

| Step | Problem | Possible reason | Solution |
|------|---------|----------------|----------|
| 17   | Absence of visible pellet after Optiprep cushion centrifugation | A large pellet might indicate presence of excessive debris rather than high nucleus yield, and absence of a visible pellet is not necessarily cause for concern. However, in certain cases it might indicate very low yield of nuclei | Continue with downstream steps, assuming the location of the nuclei based on the direction in which the tube is placed within the centrifuge, and assess nucleus yield under the microscope. If very low nucleus yield is observed, consider increasing the amount of input material |
| 19   | Low yield of nuclei | Too little starting material (<30 mg) | Consider eliminating one of the wash steps (Step 9 or 10) and resuspending in less volume (5 mL instead of 10 mL) in Step 8 |
| 24   | Number of nuclei captured does not meet the expected number based on the table provided by the 10X loading guidelines | The capture rate for nuclei might not be the same as that for cells | Empirically determine the difference between the capture rate expected and observed, and adjust loading volume accordingly |

### Timing

- **Step 1**, Tissue preparation: 1 h for eight samples if cutting pieces using a scalpel, up to 4 h if collecting cryosections
- **Steps 2–21**, Nucleus extraction: 2–3 h
- **Steps 22–26**, Nucleus concentration assessment and capture: 45 min
- **Step 27**, Library preparation and sequencing: can be split into two 4 h blocks on 2 d

### Anticipated results

We expect our single-nucleus extraction protocol to produce high-quality single-nucleus suspensions (Fig. 3) from frozen archived postmortem tissues. The nuclear suspensions are relatively free of debris and do not show substantial aggregation of nuclei even after 16 h of refrigeration, upon visual inspection (Fig. 3). Capture of single-nuclei using these nuclear suspensions on a microfluidic device for droplet-based snRNA-seq reproducibly produces high-quality libraries with sufficient cDNA yield for sequencing (Fig. 4). The variability in sample parameters such as PMI, archival time, pH and RNA integrity number (RIN) did not affect most of the quality metrics of snRNA-seq results with this nucleus extraction protocol (Fig. 2). The samples for which data are presented in Fig. 2 were processed using two different gradients of iodixanol—a weaker gradient using 17.5% and 15% (wt/vol) dilutions of Optiprep reagent (majority of samples) and a stronger gradient using the 29% and 25% (wt/vol) dilutions of iodixanol, as described in this protocol and previously. We subsequently found that the stronger gradient produces cleaner nucleus preparations and yields better sequencing quality control metrics, such as a higher fraction of reads in cell and higher numbers of UMIs and genes detected, especially using the updated Cell Ranger 3 pipeline and 10X Genomics v3 single-cell sequencing chemistry (C.N. and M.M., unpublished results). Thus, the protocol published herein utilizes the 29% and 25% (wt/vol) dilutions of iodixanol for the gradient. Finally, we have produced preliminary results using nuclei extracted from postmortem brain with our extraction protocol as input to the single-nucleus ATAC-seq approach employing 10X Genomics Chromium for single-cell capture. Using MACS2 for peak calling and the scATAC-pro pipeline for cell calling, we achieved ~83% of total fragments uniquely mapped to genome assembly GRCh38, median fraction of reads in peak scores of 23%, median fragments mapping per cell in the range of 12,000–15,000, and transcription start site enrichment, according to ENCODE definition, indicating a signal-to-noise ratio of >3.9 (Fig. 4e,f).
**Fig. 4 | cDNA traces and quality metrics for snRNA-seq libraries before and after optimization of nucleus extraction.** a, b, FANS-based nucleus isolation (using Millipore anti NeuN-PE FCMB317PE antibody and DRAQ5, both at 1:300 dilution) of single-nucleus suspensions prepared as per Lutz et al. (2017) resulted in very low yield cDNA libraries (a), whereas the optimized nucleus extraction protocol resulted in good yield of cDNA in the expected size range (b). Samples in both a and b are derived from archived postmortem brain tissue. PerkinElmer Caliper traces are shown for snRNA-seq cDNA libraries at a dilution of 1:6. The expected library size is 200–9,000 bp, and here we performed quantification in the 300–6,000 bp range. The minimum yield of cDNA should be 2 ng, and as can be seen, the yield was much improved (>90 ng) after protocol optimization. c, With similar numbers of sequencing reads and median reads per cell, the libraries produced using the optimized nucleus extraction protocol have much higher median numbers of genes and UMIs per cell, as can be seen from the elbow plot produced by Cell Ranger as well as the tabulated summary metrics. The NeuN+ sample (cDNA trace shown in a) was processed with Cell Ranger 1.3.1 and the hg19 transcriptome, while sample 215 (cDNA trace shown in b) was processed with Cell Ranger 2.1.0 and the GRCh38 1.2.0 pre-mRNA reference. d, Our nucleus extraction protocol is compatible with 10X Genomics commercial snATAC-seq protocol, as can be seen from the TapeStation trace and fragment-size distribution (163–700 bp) of a successfully prepared snATAC-seq library prepared from archived postmortem human brain tissue. e, f, Preliminary processing of snATAC-seq data aligned to the hg38 genome, showing the distribution of the percentage of reads in peaks (pct_reads_in_peaks) across captured nuclei (e) and the transcription start site enrichment score across captured nuclei (f) for a postmortem human brain sample. Samples 118 and 215 in (b) and sample 215 in (c) are from the ref. 24 dataset.

**Reporting Summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Raw sequencing data are accessible on GEO using the accession number GSE144136.

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Author contributions
M.M. and C. Nagy developed nucleus extraction protocol, prepared nuclei and wrote the manuscript. Y.C.W. preformed 10X snRNA-seq protocol. C. Nascimento performed 10X snATAC-seq protocol. A.C. performed snATAC-seq data analysis. M.S. and J.F.T. guided bioinformatic analysis. N.M. contributed to tissue processing and data interpretation. J.R. provided technical single-cell expertise and experimental support. G.T. provided general oversight, including in experimental design. All authors contributed to manuscript preparation.

Competing interests
The authors declare no competing interests.

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Data collection

We used the following softwares for data collection: Cellranger version 2.0.1, Cellranger version 2.1.0, bcl2fastq2, version 2.19.

Data analysis

We used the following R packages for secondary data analysis: Seurat (version 2.2.0, 2.3.0), mixtools (1.1.0), scATAC-pro version 1.1.4.

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| Sample size     | No statistical methods were used to predetermine sample size. Sample size was determined based on sample sizes used in previous similar studies. |
|-----------------|---------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data have been excluded.                                                                                                                                                             |
| Replication     | Nuclei extraction was performed more than 34 times using the protocol with comparable results.                                                                                      |
| Randomization   | No randomization was performed.                                                                                                                                                         |
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- Population characteristics: Age (18-87 years), post-mortem interval (12-93 hours), archival time (1-22 years).
- Recruitment: Brains are recruited by the Douglas-Bell Canada Brain Bank in collaboration with the Quebec coroner’s office.
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