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

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EDITORIAL SUMMARY Here, the authors describe an optimised workflow for isolating single nuclei from archived post-mortem tissues that does not require sorting or ultracentrifugation and can be used in single nucleus RNA and ATAC sequencing pipelines.

PROPOSED TWEET A new protocol describing a simple and reliable workflow for nuclei extraction from archived post-mortem tissues suitable for single nucleus RNA or ATAC sequencing.

PROPOSED TEASER Single nuclei isolation from post-mortem tissues

Related links

Key references using this protocol

Jessa, S. et al. Nat Genet 51, 1702–1713 (2019): https://doi.org/10.1038/s41588-019-0531-7

Reiner, B. C., et al. bioRxiv, 2020.2007.2029.227355 (2020): https://doi.org/10.1101/2020.07.29.227355

Nagy, C. et al. Nat Neurosci 23, 771–781 (2020): https://doi.org/10.1038/s41593-020-0621-y

Abstract

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 post-mortem and archived tissue types using standard lab equipment. The stages of the protocol include tissue preparation and dissociation, nuclei extraction, and nuclei concentration assessment and capture. The protocol is comparable to other published protocols but does not require fluorescence assisted nuclei sorting 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 RNA-seq and ATAC-seq using the 10X Genomics' Chromium system. The protocol reliably results in efficient capture of single nuclei for high-quality single-nucleus RNA-seq libraries.

Keywords: nuclei extraction, single nucleus suspension, snRNA-seq, snATAC-seq

Introduction

Advancements in technology have allowed researchers to preform large-scale transcriptomic studies at the level of a single-cell. Droplet-based cell isolation has become a favorite in the field for its scalability and simplicity of use with either in-house fluidic set-ups[1,2], or commercially available equipment (10x Genomics)[3]. This technique is particularly interesting for tissues with highly heterogeneous cellular compositions like intestine[4], lung[5], spinal cord[6], and brain[7,8]. There has been particular interest in deconvoluting brain architecture and function, which at its base, starts by accurately identifying all the cells types present[9-11]. However, truly harnessing the power of individual cellular transcriptomes comes with 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 homogenates[12]. The chemical dissociation of tightly interconnected brain cells and other cell-types has been found to alter transcription profiles[13,14]. Given that nuclear transcriptomes closely reflect the cell's cytosolic profile[15,16], isolating the nuclei from brain tissue has proven to be an excellent strategy for single-cell level studies. Likewise, other tissues that have either been frozen for long term storage or that are formed by syncytium, such as in skeletal muscles[17,18], could benefit from this approach.

Development of the Protocol

Numerous protocols for isolating nuclei from brain cells have been published[10,11,19-23]; some rely on the additional purification by fluorescence assisted cell sorting (FACS)[20,23], which is costly, time consuming and not readily available for all researchers, while others have made adjustments to the microfluidics component used to isolate and capture single nuclei[10], which can

also be limiting to labs. 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 post-mortem tissue. Our preparation produces stable and easily quantifiable nuclear suspension even when using archived brain tissue. We have used this approach to successfully compare the gene expression differences in the post-mortem prefrontal cortex of depressed patients who died by suicide compared to 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 post-mortem brain in schizoprenia[26]. Thus, gene expression and chromatin accessibility can be measured from post-mortem brain tissue using this protocol. We anticipate that newer techniques from 10x Genomics such as those that combine scATAC-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 nuclei aggregation. The suspension is repeatedly filtered to remove large debris. Most centrifugation steps are performed at low speeds 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 nuclei concentration by fluorescence microscopy. The concentration of the nuclei 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 500-1000 nuclei per microliter is sufficient for nuclei capture and should not result in excessive aggregation.

Comparison with other methods

As previously mentioned, existing single-nucleus RNA sequencing (snRNA-seq) protocols either rely on FACS[20,23] which is harder to scale, or on droplet-based approaches which use in house set-ups[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[9-11]. Moreover, when studying specific phenotypes for which it is harder to obtain tissues, it is not always possible to select for short post-mortem intervals (PMIs) and archival times. Early protocols were also limited to high-quality tissue which may not be an option for answering certain types of research questions.

As with several more recently published potocols[19-22], we have been able to adapt our nuclear prep 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 post-nuclei capture applications such as whole genome sequencing for the study of somatic mutations or single-nucleus Assay for Transposase-Accessible Chromatin (snATAC-seq) as supported by preliminary results from our lab.

Each of the more recently developed protocols have their own strengths and weaknesses and, in some cases, adaptations for specific tissue types such as macro-dissections for white matter regions[21]. The strength of our protocol is that it is mostly unaffected by variations in post mortem interval (PMI) or archival times of the samples **(Fig 2)**. Some of these protocols also incorporate ultra-centrifugation[22,27], which is time consuming and 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 than can either interfere with droplet formation or be integrated into droplets, increasing background sequencing noise. Here, we show that brain tissue which has been stored at -80ºC for as long as 22 years, can produce high quality single-nuclei 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 post-mortem brain tissue (**Fig. 3**), although other labs have been able to successfully use this protocol for nuclei extraction for snRNA-seq. Moreover, our attempts to use nuclei isolated by fluorescent assisted nuclei sorting (FANS) as input to the 10X Genomics protocol did not yield acceptable results, although this approach has been adopted successfully by other groups[20]. The modifications made here are primarily for use with post-mortem brain tissue that has been archived for long periods of time, but can also be applied to any frozen post-mortem sample. Similar to previous studies[10,11], we applied a few modifications to the standard bioinformatic analysis with the CellRanger pipeline from 10X Genomics to address a number of issues which arise with droplet-based single-nucleus sequencing. First, we assembled a pre-mRNA reference to account for unprocessed transcripts found in the nucleus[28]. Second, given that previous studies have consistently shown fewer identifiable transcripts in glial cells[10,11] we performed customized barcode filtering to include cells with a wider range of unique molecular identifiers (UMIs) while removing noise. With these minor modifications to the analysis[24] our isolation approach for our tissue type, i.e. archived post-mortem brain, produced much improved data compared to the available 10X Genomics nuclei preparation protocols in our hands.

Experimental design

The most important factor to take into consideration while designing single-cell or single-nucleus RNA-seq experiments is the potential batch effects. Given that the Chromium system only allows for the capture of 8 samples at a time and that for many experiments that total number of samples to be analyzed may be 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 co-variates to take into consideration include age, PMI, and sex. It may be possible to account for the effects of these variables by matching samples by these parameters within each batch.

In cases where cell-type specific gene-expression data has been previously published, 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 fluorescence assisted nuclei sorting (FANS) purified populations of expected cell-types based on known genetic markers to validate the cell-type identification from the single-nucleus transcriptomic data[29]. High-throughput *in situ* hybridization (ISH)[30] and ISH based nuclei sorting[29] 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[11,21].

Another strategy which has been recently applied 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*[31], or the chromosome accessibility ratios for sex-chromosome versus autosomes[32] to separate the cells from each sample. Since both samples are captured on the same lane of the microfluidic chip, it may be possible to account for lane to lane variability using this approach. Moreover, the use of cryosections of histological grade tissue blocks may be a good strategy to account for uniform input from a micro-anatomically heterogeneous regions such as the cerebral cortex[11,22].

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 for determining 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 get information about cytoplasmic transcription which may be limiting for obtaining data for some cell types[33]. Some tissue types, such as spinal cord or intestine, may require additional processing, such as through FANS or collagenase treatment. Representation of all cell types may 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 may result in damage to fragile tissue or unacceptable levels of loss of material if starting with small amounts of precious tissue. Moreover, we cannot rule out the possibility that use of the iodixanol gradient may cause biases in the types of nuclei recovered and this may 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 post-mortem prefrontal cortex tissue obtained from the Douglas Bell Canada Brain Bank, post-mortem intestinal tissue (with modifications such as collagenase treatment), and surgical samples of tumor tissue[25]. CAUTION All experiments involving the use of human samples must be performed in accordance with the relevant institutional and national regulations. Use of post-mortem tissues was approved by the Institutional Review Board of the Douglas Hospital.

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)
- $MgCl_2.6H_2O$ (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 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 RNAse Inhibitor (Millipore Sigma, 3335399001)

CRITICAL: Other RNAse inhibitors may not be compatible with the protocol and may result in low yield of nuclei.

- Optiprep™ Density Gradient Medium, 60% weight/volume iodixanol (Millipore Sigma, D1556-250)
- Gibco™ PBS, pH 7.4 (1X), (Thermo Fisher Scientific, cat. no. 100100203)
- 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 & Gel Bead Kit v2 or newer (10X Genomics Inc, cat. no. 120237)
- Chromium Single Cell A Chip Kit (10X Genomics Inc, cat. no. 120236)
- SPRIselect Reagent Kit (Beckman Coulter, cat. no. B23318)
- Tween 20 (Bio-Rad, cat. no. 1610781)
- Buffer EB (250mL) (Qiagen, cat. no. 19086)
- Glycerin (glycerol), 50% (v/v) Aqueous Solution (Ricca Chemical Company (or other), cat. no. 3290-32)
- DynaBeads MyOne™ Silane Beads (5mL) (Thermo Fisher, 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, 100mL) (Thermo Fisher, cat. no. 12090-015)
- Nuclease-Free Water (Thermo Fisher, cat. no. AM9937)
- TapeStation High Sensitivity D1000 Sample Buffer (Agilent, cat. no. 5067-5603) or Tape Station High Sensitivity D5000 Sample Buffer & Ladder (Agilent, cat. no. 5067-5593)
- TapeStation High Sensitivity D1000 ScreenTape (Agilent, cat. no. 5067-5584) or High Sensitivity D5000 ScreenTapes (Agilent, cat. not. 5067-5592)

EQUIPMENT:

Lab equipment

- Scalpel
- Spatula
- Weighing boat
- Weighing scale
- Refrigerated bench-top centrifuge for 5mL tubes (Eppendorf, model 5430R)
- Refrigerated bench-top centrifuge for 15 mL tubes (Beckman Coulter, model Allegra X-14R)
- Countess® II FL Automated Cell Counter (Thermo Fisher Scientific, cat. no. AMAQAF1000)
- 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)
- 7 ml Tissue Grinder, Dounce (Wheaton, cat. no. 357542)

- 15 ml centrifuge tubes (Corning, cat.no. 430791)
- Centrifuge tube, 50 mL screw cap (Sarstedt, 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. 30108310)
- INCYTO C-Chip Disposable Hemocytometers (SKC Films Inc., cat. no. DHCN012 or DHCN015)
- TempAssure PCR 8-tube strip (USA Scientific, cat. no. 1402-4700)
- 10mL serological pipette
- Invitrogen EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific)
- Chromium Controller (10X Genomics)
- Divided Polystyrene Reservoirs (25mL, 50) (VWR, cat. no. 41428-960)
- 200UL Filter Tips (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 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[34]
- mixtools (1.1.0)[35]
- R, version 3.4 or higher[36]

Software for analysis of snATAC-seq

- scATAC-pro version 1.1.4[37]

REAGENT SETUP

Stock solutions

CRITICAL The following reagents should be prepared ahead of time:

- Prepare 1M NaCl, 100mM $MgCl_2.6H_2O$, 1M $MgCl_2.6H_2O$, 1M KOH solutions in ddH_2O in separate 50 mL centrifuge tubes and store at room temperature (21-22°C for our laboratory) for up to 6 months..

- Prepare 250 mL of 10% (weight/volume) BSA solution in a glass bottle by dissolving crystalline BSA in ddH_2O at room temperature. Store at 4°C for up to 1 week. Keep crystalline BSA at -20°C.
- Prepare 250 mL of 1M Tris HCl buffer in a glass bottle by dissolving Tris in ddH_2O. Adjust pH to 7.4 by adding HCl dropwise. Store at room temperature for up to 6 months. This is a time-consuming step.
- Prepare 250 mL of 0.5M Tricine KOH buffer in a glass bottle by dissolving Tricine in ddH_2O and adjusting pH to 7.8 by adding 1M KOH dropwise. This is a time-consuming step. Store at room temperature for up to 6 months.

CRITICAL Buffer recipes provided are calculated assuming preparation of 8 samples for capture using a full Chromium chip.

Optiprep™ diluent (altered as per Kriaucionis et al., 2009)[38]: Combine the following in a 250 mL glass bottle. Store at room temperature.

Component	Volume (in mL)	Final concentration
1M KCl	15	150 mM
1M $MgCl_2.6H_2O$	0.5	5 mM
0.5M Tricine-KOH (pH 7.8)	4	20 mM
Deionized water	80.5	–
Total volume	100	–

Optiprep™ solutions: Using Optiprep™ diluent solution dilute the Optiprep™ reagent to make 50% weight/volume iodixanol and 29% weight/volume iodixanol solutions from Optiprep™ solution in separate 50 mL centrifuge tubes. Protect from light and store at room temperature 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 (in µL)	Final concentration
1M Tris-HCl pH 7.4	200	10 mM
1M NaCl	200	10 mM
100 mM $MgCl_2.6H_2O$	600	3 mM
NP-40 (10%)	100	0.05% (v/v)
Deionized water	18900	–
Total volume	20000	–

Nuclei 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 (in mL)	Final concentration
10% BSA	100	5% (w/v)
Glycerol	0.5	0.25% (v/v)
Protector RNAse inhibitor	0.2	40 units/mL

1X PBS	to 200 (~100)	0.5X
Total volume	200	–

EQUIPMENT SETUP

- Precool both centrifuges to 4ºC.
- Set up EVOS FL Auto microscope with 10X magnification, bright field and DAPI channels.

Procedure

Tissue preparation (Timing 1-4 hours)

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 1.5mL microcentrifuge tube using a spatula and place back on dry ice. Clean scalpel and spatula with 70% ethanol (v/v) 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 between 30-50 mg.

   CAUTION: Post-mortem human tissue can contain pathogens. Take precautions including wearing PPE and seek medical attention if the scalpel breaks your skin.

Nuclei extraction (Timing 2-3 hours)

2. Transfer tissue using spatula to douncing tube on ice. Add 3mL of ice-cold lysis buffer and dounce with loose pestle 10 times and 5 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 15mL centrifuge tube by pouring and add 2 mL of chilled lysis buffer. Incubate on ice for 5 minutes, gently swirling to mix 2 times 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-14X centrifuge, spin down the lysed tissue suspensions at 500g for 5 minutes at 4°C.
7. Decant supernatant into a waste beaker without disrupting the nuclei 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 10mL of nuclei wash buffer to the pelleted nuclei and gently pipet 8-10 times to mix.
9. Repeat step 5-7 using the resuspended nuclei.
10. Using a 10 mL serological pipette add 5mL of nuclei wash buffer to the pelleted nuclei and gently pipet 8-10 times to mix.
11. Repeat steps 6-7 using the resuspended nuclei.
12. Using a 1000 µL pipette tip, add 1 mL of nuclei wash buffer to pelleted nuclei and gently pipet 8-10 times to mix.
13. Add 1 mL of 50% (w/v) working solution of iodixanol (Optiprep™) to the nuclei and mix well to obtain 2 mL of 25% (w/v) iodixanol solution containing nuclei.
14. Prepare an iodixanol cushion of 2 ml of 29% (w/v) iodixanol solution in a 5mL Eppendorf centrifuge tube.
15. Gently add the 2 mL nuclei 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 minutes 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 1000 µL pipette tip, resuspend the nuclei pellets in 500 µL or less of nuclei wash buffer. Gently pipette 8-10 times or until nuclei are resuspended.
19. For a quick estimate of nuclei concentration, mix 10 µL of the nuclei 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 post-mortem cortical tissue we have observed that the average diameter is around 10 µm. However, nuclei may have a range of sizes and it is only concerning if a long tail of particles of more than 30 µm are detected at this may 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 around 500,000 cells/ mL or 500 cells/ µL by adding an appropriate volume of nuclei wash buffer. It may 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 which will prevent efficient capture of single nuclei in subsequent steps. We have achieved good suspensions and capture with up to 1000 nuclei/ µL, but if aggregation is observed, lower concentrations (as low as 500 nuclei/ µL) may be better.

21. Add Hoechst stain to the resuspended nuclei at a 1:2000 dilution to obtain counts using fluorescence microscopy.

Nuclei concentration assessment and capture (Timing 45 mins)

CRITICAL Immediately prior to loading the Hoechst stained nuclei on the Chromium system, check to make sure the nuclei are well segregated (nuclei may clump) and recheck sample concentration using a fluorescent microscope like the Evos FL Auto (Thermofisher)

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

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

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

24. Set the Evos to 10X 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 may 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 nuclei counting when processing many samples for capture. User's 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 aliquot 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. D or later. **?TROUBLESHOOTING**

    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 empirically determined that choosing the loading volume by using a count that is 30% less than the observed count worked best for our samples. This adjustment may vary from tissue type to tissue type. 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) in order 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.

26. Load the Chromium Chip and harvest the nuclei captured in droplets (i.e. GEMs) according to the Chromium protocol CG00052 Rev. D

Library preparation and sequencing (Timing as per the Chromium protocol, ~8 h split over 2 days)

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 3000 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.

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

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 refdata-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, counted the UMIs corresponding to genes using the cellranger count command and default parameters. These steps may 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[39], SC3[40], Monocle3[41], etc., we used the Seurat R package (version 2.2.0, 2.3.0)[42]. 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[30,34] which can be used for combining the data from individual subjects if inter-individual 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 cut-offs 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 which are expected to be heterogenous 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 which biologically contain fewer molecules . For our dataset, given the known trend for higher number of RNA molecules

in neuronal nuclei compared to glial nulcei[10,11,43], the distribution of number of UMIs was fit with three normal distributions using the normalmixEM function from the mixtools[35] 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 non-neuronal cells that have an intermediate numbersof 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 "non-neuronal" 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 non-neuronal cells as evidenced by and almost 6 fold increase in their population[24].

Of note, in our experience with the newest version of Cell Ranger (3 and above) which incorporates the EmptyDrops algorithm for cell calling, it may 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.

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 may indicate presence of excessive debris rather than high nuclei yield and absence of a visible pellet is not necessarily cause for concern. However, in certain cases it may 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 nuclei yield under the microscope. If very low nuclei 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 (9-10 in protocol) 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	The capture rate for nuclei may not be the same as that for cells.	Empirically determine the difference between the capture rate expected and observed and

	table provided by the 10x loading guidelines		adjust loading volume accordingly.

TIMING

Step 1, Tissue preparation: 1 hour for 8 samples if cutting pieces using a scalpel, up to 4 hours if collecting cryosections

Step 2-21, Nuclei extraction: 2-3 hours

Step 22-26, Nuclei concentration assessment and capture: 45 minutes

Step 27, Library preparation and sequencing, can be split into two 4 hours blocks on 2 days

Anticipated Results

We expect our single-nuclei extraction protocol to produce high quality single-nucleus suspensions **(Fig. 3)** from frozen archived post-mortem tissues. The nuclear suspensions are relatively free from debris and do not show substantial aggregation of nuclei even after 16 hours of refrigeration, upon visual inspection **(Fig. 3-f)**. 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 RIN did not affect most of the quality metrics of snRNA-seq results with this nuclei extraction protocol (**Fig. 2**). The samples for which data are presented in Figure 2 were processed using two different gradients of iodixanol – a weaker gradient using 29% and 25% volume/volume dilutions of Optiprep™ reagent (majority of samples) and a stronger gradient using the 29% and 25% weight/volume dilutions of iodixanol as described in this protocol and previously[38]. We subsequently found that the stronger gradient produces cleaner nuclei preparations and yields better sequencing quality control metrics, such as 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. Thus, the protocol published

herein utilizes the 29% and 25% weight/volume dilutions of iodixanol for the gradient. Finally, we have produced preliminary results using nuclei extracted from post-mortem brain with our extraction protocol as input to the single-nucleus ATAC-seq approach employing 10X Genomics Chromium for single-cell capture. Using MACS2[44] for peak calling and the scATAC-pro pipeline[37] for cell calling, we achieved about 83% of total fragments uniquely mapped to genome assembly GRCh38, fraction of reads in peak (FRiP) scores of up to 23%, median fragments mapping per cell in the range of 12,000 – 15,000, and TSS (transcription start site) enrichment, according to ENCODE definition, indicating a signal-to-noise ratio of more than 3.9 (Fig 4e-f).

Data Availability

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

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and Related Disorders. The present study used the services of the Molecular and Cellular Microscopy Platform (MCMP) at the Douglas Institute.

Author Contributions

MM and C. Nagy developed nuclei extraction protocol, prepared nuclei, and wrote the manuscript. YCW preformed 10X snRNA-seq protocol. C. Nascimento performed 10X snATAC-seq protocol. AC performed snATAC-seq data analysis. MS and JFT guided bioinformatic analysis. NM contributed to tissue processing and data interpretation. JR provided technical single-cell expertise and experimental support. GT provided general oversight, including in experimental design. All authors contributed to manuscript preparation.

Competing Interests

No, I declare the authors have no competing interests as defined by Nature Research, or other interests that might be perceived to influence the interpretation of the article.

Figures Legends

Figure 1: Schematic representation of the steps of the protocol. Frozen tissue is dissected, homogenized by douncing, lysed, and then washed, filtered, and centrifuged several times until a single-nucleus suspension is obtained.

Figure 2: Effect of sample quality parameters on single-nucleus capture and sequencing metrics. The archival time, post mortem interval (PMI), age, and pH of the brains accounted for less than 10% of the variation in number of cells (nuclei) retained after filtering, median number of genes per nucleus, and median no of UMIs (unique molecular identifiers) per nucleus. The RIN of the samples had had a significant negative effect ($p = 3.4x10^{-6}$) 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 Nagy et al. (2020)[24].

Figure 3: Images of extracted nuclei. (a-d) Before optimization, using the 10X Genomics demonstrated protocol, the extracted nuclei from two different samples (a and b) show large amounts of debris and the size distribution is skewed towards larger sizes (> 10 μm). After optimization, representative images of nuclei extracted from two samples (c and d) show much less debris and size-distributions are centered around 10 μm, within the expected range for human brain nuclei. Images were acquired with the Countess Cell Counter using Trypan blue for staining. Note that extracted nuclei should be marked as dead cells, as seen. (e-f) Extracted nuclei do not tend to aggregate even after (e) 2.5 hours or (f) 16 hours of storage at 4°C. Note that the size distribution after 16 hours is still centered around 10 μm, indicating an absence of aggregation. (g) Representative images of extracted nuclei stained with Hoechst (1:2000) acquired at 10X magnification on the Evos microscope. Figures (c), (d), and (g) correspond to samples used in Nagy et al. (2020)[24]. All scale bars represent 500 μm.

Figure 4: cDNA traces and quality metrics for snRNA-seq libraries before and after optimization of nuclei extraction. (a) A FANS based nuclei isolation (using Millipore anti NeuN-PE FCMAB317PE antibody and DRAQ5 both at 1:300 dilution) of single-nucleus suspensions prepared as per Lutz et al. (2017)[45] resulted in very low yield cDNA libraries whereas (b) the optimized nuclei extraction protocol resulted in good yield of cDNA in the expected size range. Samples in both (a) and (b) are derived from archived post-mortem brain tissue. Perkin Elmer

Caliper traces are shown for snRNA-seq cDNA libraries at a dilution of 1:6. The expected library size is between 200-9000 bp and here we performed quantification in the 300-6000 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 nuclei 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 nuclei extraction protocol is compatible with 10X Genomics' commercial snATAC-seq protocol, as can be seen from the Tapestation trace and fragment-size distribution (163-700bp) of a successfully prepared snATAC-seq library prepared from archived post-mortem human brain tissue. (e) 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 and the transcription start site (TSS) enrichment score across captured nuclei for a post-mortem human brain sample. Samples 118 and 215 in (b) and sample 215 in (c) are from the Nagy et al. (2020)[24] dataset.

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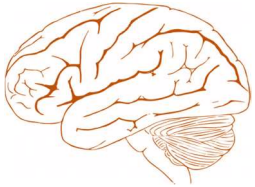
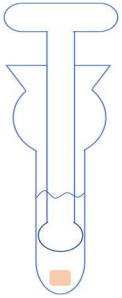
Step 1



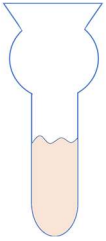
Dissect 30-50 mg of frozen tissue

Step 2



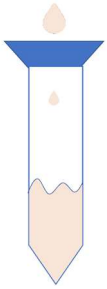
Dounce in cold lysis buffer

Steps 3-4

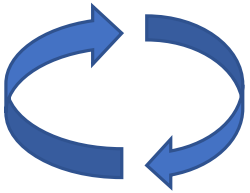


Lyse for 5 mins on ice, add wash buffer

Steps 5-11 (3 repeats of centrifugation & wash)



Resuspend in wash buffer & filter if directed

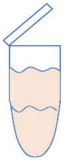


Centrifuge at 500g & 4°C for 5 mins

Step 12

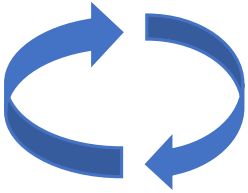
Resuspend in 1 mL wash buffer

Steps 13-15



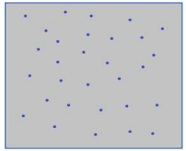
Mix with 50% iodixanol (w/v), layer on 29%(w/v) cushion

Step 16

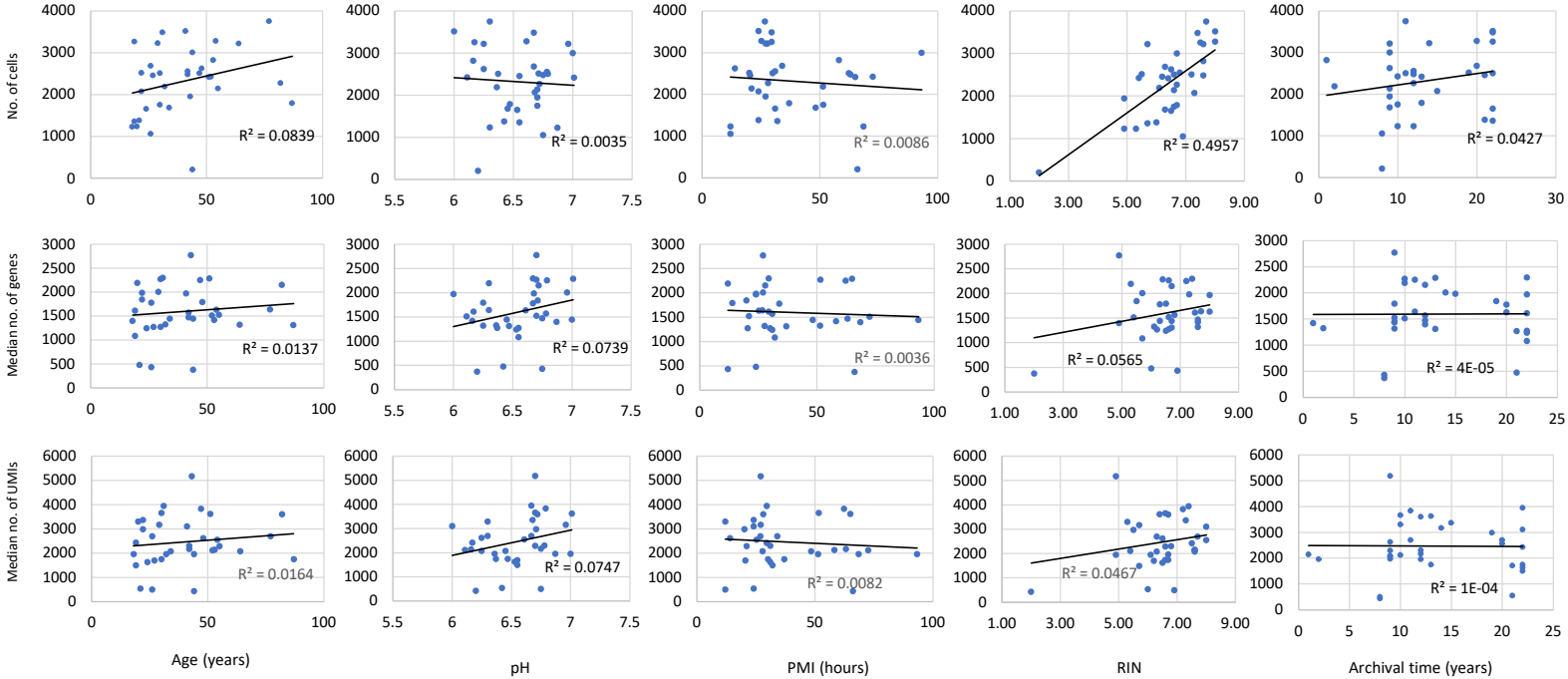


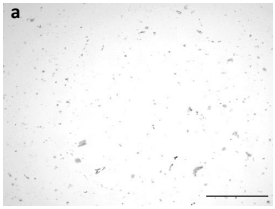
Centrifuge at 10000g & 4°C for 30 mins

Steps 17-24



Resuspend in wash buffer, count, capture


No. of cells
Median no. of genes
Median no. of UMIs
R² = 0.0839
R² = 0.0035
R² = 0.0086
R² = 0.4957
R² = 0.0427
R² = 0.0137
R² = 0.0739
R² = 0.0036
R² = 0.0565
R² = 4E-05
R² = 0.0164
R² = 0.0747
R² = 0.0082
R² = 0.0467
R² = 1E-04
Age (years)
pH
PMI (hours)
RIN
Archival time (years)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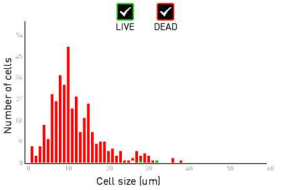
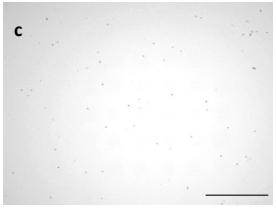

LIVE
DEAD
Number of cells
Cell size [um]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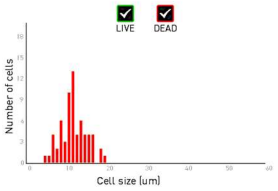
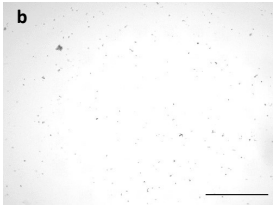

LIVE
DEAD
Number of cells
Cell size [um]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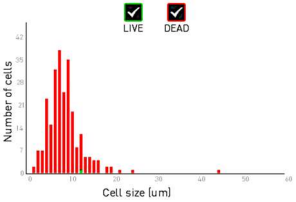
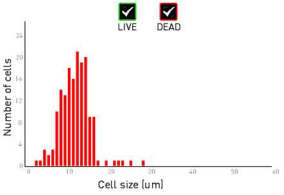
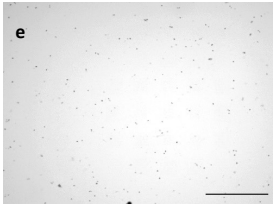
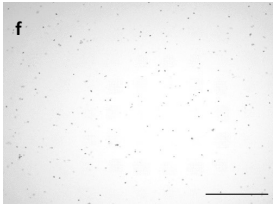

LIVE
DEAD
Number of cells
Cell size [um]


d


LIVE
DEAD
Number of cells
Cell size [um]


e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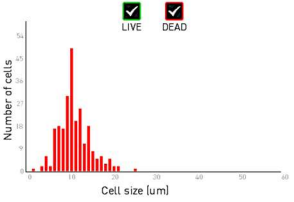
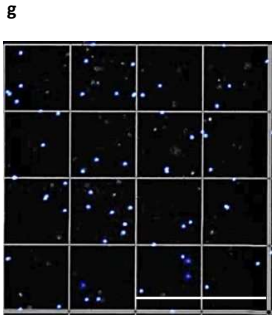

LIVE
DEAD
Number of cells
Cell size [um]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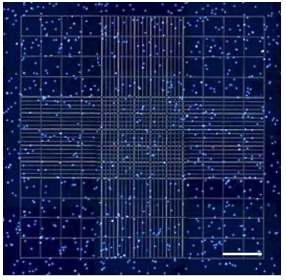
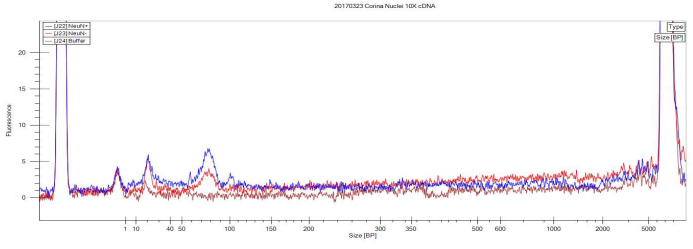
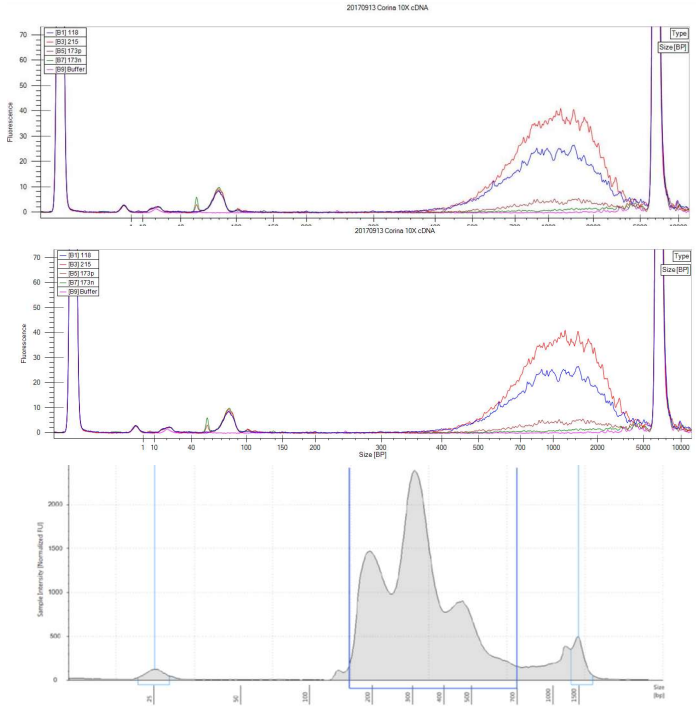


a



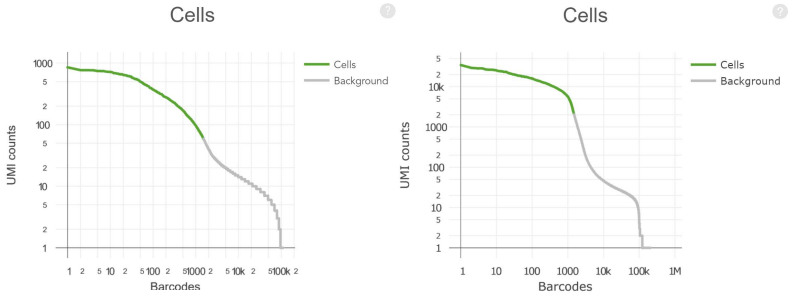
Sample Name	cDNA [300-6000bp] (ng/ul)	cDNA Yield (ng)
NeuN+	0.06	2.22
NeuN-	0.12	4.44

b



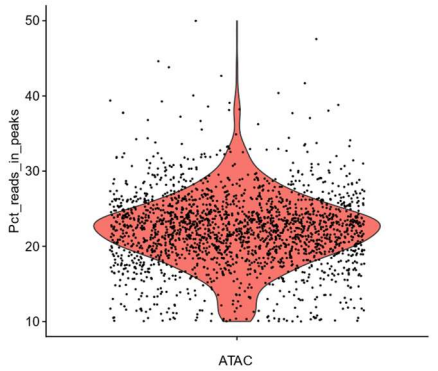
Sample Name	cDNA [163-700bp] (pg/ul)	cDNA Yield (ng)
84	6140	

c



Sample Name	Number of reads	Estimated no. of cells	Mean reads per cell	Median genes per cell	Median UMI count per cell
NeuN+	165,749,748	1,507	109,986	80	124
215	144,125,115	1,486	96,988	3,501	7,287

e



f