Journal of Visualized Experiments

Nuclei isolation from fresh frozen brain tumors for single-nucleus RNA-seq and ATAC-seq

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61542R1
Full Title:	Nuclei isolation from fresh frozen brain tumors for single-nucleus RNA-seq and ATAC-seq
Section/Category:	JoVE Cancer Research
Keywords:	Nuclei isolation, snRNA-seq, snATAC-seq, 10x Genomics, Glioma
Corresponding Author:	Sevin Turcan Universitatsklinikum Heidelberg Heidelberg, BW GERMANY
Corresponding Author's Institution:	Universitatsklinikum Heidelberg
Corresponding Author E-Mail:	s.turcan@dkfz-heidelberg.de;sevin.turcan@med.uni-heidelberg.de
Order of Authors:	Sevin Turcan
	Ashwin Narayanan
	Enrique Blanco-Carmona
	Engin Demirdizen
	Xueyuan Sun
	Christel Herold-Mende
	Matthias Schlesner
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Heidelberg, Baden-Württemberg, Germany

Cover Letter

HEIDELBERG
UNIVERSITY
HOSPITAL

UniversitätsKlinikum Heidelberg

July 02, 2020

Dear Dr. DSouza,

We are happy to submit our revised manuscript (JoVE61542) titled "*Nuclei isolation from fresh frozen brain tumors for single-nucleus RNA-seq and ATAC-seq*" by Narayanan *et al.*

We thank the reviewers and the editor for their insightful suggestions. We have addressed all the editorial and reviewer comments. This involved addition of new analysis (Figure 4) that was requested. We believe the changes have substantially improved the manuscript. We thank you for your enthusiasm and for your interest in our work.

In the revised version of the manuscript, we incorporated the following major edits:

1. We included a computational comparison of snRNA-seq data obtained using the protocol described in the manuscript to four publicly available snRNA-seq datasets (Figure 4). These results are now added to the Representative Results section and show that our protocol generates high-quality data when compared to recently published snRNA-seq datasets.

2. We extended the Discussion section to include further details about the protocol, along with potential issues and considerations (e.g. clumping, amount/quality of starting material, and processing/storage times).

3. We updated the Protocol section to include additional quality control steps (Steps 1.1.7 and 3.1.3) and notes.

We hope the manuscript is now suitable for publication and look forward to hearing from you.

Sincerely,

Sevin Turcan, PhD

Max-Eder Junior research group leader
Department of Neurology
Heidelberg University Hospital
sevin.turcan@med.uni-heidelberg.de
+49 (0) 6221 56 5929

TITLE:

Nuclei Isolation from Fresh Frozen Brain Tumors for Single-Nucleus RNA-seq and ATAC-seq

AUTHORS AND AFFILIATIONS:

Ashwin Narayanan[1], Enrique Blanco-Carmona[2], Engin Demirdizen[1], Xueyuan Sun[1], Christel Herold-Mende[3], Matthias Schlesner[2], Sevin Turcan[1]

[1]Neurology Clinic and National Center for Tumor Diseases, University Hospital Heidelberg, Heidelberg, Germany.
[2]Bioinformatics and Omics Data Analytics, German Cancer Research Center (DKFZ), Heidelberg, , Germany.
[3]Division of Experimental Neurosurgery, Department of Neurosurgery, University of Heidelberg, INF 400, , Heidelberg, Germany.

Corresponding Author:
Sevin Turcan
sevin.turcan@med.uni-heidelberg.de

Email Addresses of Co-authors**:**
Ashwin Narayanan (a.narayanan@dkfz.de)
Enrique Blanco-Carmona (e.blancocarmona@dkfz-heidelberg.de)
Engin Demirdizen (e.demirdizen@dkfz.de)
Xueyuan Sun (xueyuan.sun@dkfz-heidelberg.de)
Christel Herold-Mende (Christel.Herold-Mende@med.uni-heidelberg.de)
Matthias Schlesner (m.schlesner@dkfz-heidelberg.de)

KEYWORDS:

Nuclei isolation, snRNA-seq, snATAC-seq, 10x Genomics, Glioma

SUMMARY:

Intra-tumoral heterogeneity is an inherent feature of tumors, including gliomas. We developed a simple and efficient protocol that utilizes a combination of buffers and gradient centrifugation to isolate single nuclei from fresh frozen glioma tissues for single nucleus RNA and ATAC sequencing studies.

ABSTRACT:

Adult diffuse gliomas exhibit inter- and intra-tumor heterogeneity. Until recently, the majority of large-scale molecular profiling efforts have focused on bulk approaches that led to the molecular classification of brain tumors. Over the last five years, single cell sequencing approaches have highlighted several important features of gliomas. The majority of these studies have utilized fresh brain tumor specimens to isolate single cells using flow cytometry or antibody-based separation methods. Moving forward, the use of fresh-frozen tissue samples from biobanks will provide greater flexibility to single cell applications. Furthermore, as the single-cell field advances, the next challenge will be to generate multi-omics data from either a single cell or the

same sample preparation to better unravel tumor complexity. Therefore, simple and flexible protocols that allow data generation for various methods such as single-nucleus RNA sequencing (snRNA-seq) and single nucleus Assay for Transposase-Accessible Chromatin with high-throughput sequencing (snATAC-seq) will be important for the field.

Recent advances in the single cell field coupled with accessible microfluidic instruments such as the 10x genomics platform have facilitated single cell applications in research laboratories. To study brain tumor heterogeneity, we developed an enhanced protocol for the isolation of single nuclei from fresh frozen gliomas. This protocol merges existing single cell protocols and combines a homogenization step followed by filtration and buffer mediated gradient centrifugation. The resulting samples are pure single nuclei suspensions that can be used to generate single nucleus gene expression and chromatin accessibility data from the same nuclei preparation.

INTRODUCTION:

Diffuse lower grade gliomas (LGG), the most common primary brain tumor in adults, are infiltrative neoplasms that often arise in the cerebral hemisphere. LGGs exhibit both inter- and intra-tumor heterogeneity, which is not only driven by the tumor population but also by the non-malignant cells intricately involved in tumor development and progression[1-5].

Over the last decade, there has been an avalanche of genomic data gathered in the field of gliomas. These data mainly come from bulk tumor sequencing studies and have contributed immensely to the molecular characterization, and the current classification of brain tumors[5-11]. However, even though these studies revealed the broad molecular landscape associated with gliomas, there is still a disappointing lack of progress regarding therapeutic intervention. One of the obstacles to treatment resistance in brain tumors is intra-tumor heterogeneity. To address this issue, various studies have been focusing on the genomic, transcriptomic, proteomic, and epigenetic heterogeneity present within a tumor at a single cell level[12-17].

Although there have been remarkable technological advancements in the single cell field over the last couple of years, one of the major limiting factors is the availability of fresh specimens needed to isolate the cells and perform these experiments. To overcome this limitation, there have been several successful attempts at performing assays, such as snRNA-seq and snATAC-seq from frozen tissues, using nuclei rather than cells[18,19]. The majority of these methods rely on either fluorescence-activated cell sorting (FACS) or filtration strategies. Both single cell and single nuclei approaches have their strengths and drawbacks. Single cell approaches maintain mitochondrial transcripts, which although may be informative, can also reduce transcriptome coverage due to their high abundance. Single nuclei isolation approaches eliminate a high percentage of the mitochondrial fraction, thereby allowing a more in-depth coverage of the nuclear transcripts[20].

There are various commercially available platforms that have been used over the recent years to assay single cell genomics data, including RNA-seq and ATAC-seq. One of the most prominent platforms is the 10x Genomics Chromium platform for single cell gene expression and single cell ATAC profiling. As the platform works with the help of microfluidic chambers, any debris or

aggregates can clog the system leading to loss of data, reagents, and valuable clinical samples. Therefore, the success of single cell studies depends largely on the accurate isolation of single cells/nuclei.

The protocol that we will demonstrate herein is a slightly modified combination of the DroNc-seq and Omni-ATAC-seq protocols, and utilizes a similar approach to recent studies that utilize snRNA-seq to understand neurological disorders and neuronal cell types in the human brain[18,19,21-24]. The protocol uses a combination of enzymatic/mechanical dissociation of frozen samples followed by filtration and gradient centrifugation and allows for fast and accurate isolation of single nuclei from fresh frozen glioma tissues. We have successfully used this protocol to generate snRNA-seq and snATAC-seq data from the same nuclei preparation from brain tumor specimens.

PROTOCOL:

Fresh frozen glioma samples were obtained from the National Center for Tumor diseases (NCT)-tissue bank in Heidelberg, Germany. The use of patient material was approved by the Institutional Review Board at the Medical Faculty of Heidelberg, and informed consent was obtained from all patients included in the study.

1. Experimental preparation

1.1. Perform all steps on ice or at 4 °C.

1.2. Pre-chill tubes, dishes, razor blades, Douncers and pestles to 4 °C.

1.3. Prepare all buffers in advance. These buffers are stable at room temperature. Sterile filtration is recommended, especially for sucrose. The stock preparations are modified from Corces et al.[19]. See **Tables 1-7**.

1.4. Remove samples from liquid nitrogen or -80 °C freezer storage and keep on dry ice until Step 2.1.

2. Tissue dissection and dissociation

2.1. Transfer fresh frozen tissue sample (10-60 mg) to a pre-chilled Petri dish. Mince/chop fresh frozen tissue with a razor blade to small pieces on ice.

2.2. Add 500 µL of chilled nuclei lysis buffer to a pre-chilled 1.5 mL tube. Place the tissue pieces in the 1.5 mL tube containing the nuclei lysis buffer, and transfer to a Douncer (**Table of Materials**).

NOTE: There are two Dounce homogenizer pestles: “loose” or “A” pestle for initial sample reduction and “tight” or “B” pestle for complete sample homogenization.

2.3. Dounce the tissue pieces with the “loose” pestle for about 20 strokes, until friction is reduced. If debris is present, the sample may be pre-cleared by filtration with a 100 mm strainer.

2.4. Dounce with the “tight” pestle for 20 strokes to achieve complete tissue homogenization.

2.5. Transfer the homogenate (about 500 µL) into a pre-chilled 2 mL tube. Add 1 mL of chilled lysis buffer. Mix gently and incubate on ice for 5 min. Gently mix with a wide-bore pipette tip and repeat 1-2 times during the incubation.

2.6. Filter the entire homogenate using a 30 µm strainer mesh, collect into a polystyrene round bottom FACS tube and transfer back into a new pre-chilled 2 mL tube. A single strainer is typically sufficient for the entire homogenate.

2.7. Check under a light microscope to verify the removal of large debris and the intactness of the nuclear membrane. Nuclei need to be round and the nuclear membrane should not be distorted. If debris is present, nuclei can be re-filtered.

2.8. Centrifuge the nuclei at 500 x *g* for 5 min at 4 °C on a bench top centrifuge. Remove the supernatant, leaving behind ~50 µL with pellet containing the nuclei. Gently resuspend the pellet in another 1 mL of nuclei lysis buffer and incubate for 5 min on ice.

2.9. Centrifuge the nuclei at 500 x *g* for 5 min at 4°C. Remove the supernatant without disturbing pellet, add 500 mL of 1x homogenization buffer (HB) (**Table 4**) and incubate for 5 min without resuspending. Then, resuspend the nuclei in another 1.0 mL of 1x HB.

2.10. Centrifuge the nuclei at 500 x *g* for 5 min at 4 °C. Remove the supernatant and gently resuspend the nuclei in 200 µL of 1x HB into a new 1.5 mL tube.

3. Gradient centrifugation

3.1. Add 200 µL of 50% iodixanol solution (**Table 5**) to give a final concentration of 25% iodixanol. Mix well 10 times with pipette set on 300 mL.

3.2. Add 300 µL of 29% iodixanol solution (**Table 6**) under the 25% mixture. Use a P1000 fine tip to avoid mixture of the layers.

3.3. Add 300 µl of 35% Iodixanol solution (**Table 7**) under the 29% mixture. Use a P1000 fine tip to avoid mixture of the layers.

Caution: This step requires gradual removal of the pipette tip during pipetting to avoid excessive volume displacement.

3.4. Place the samples in a swinging bucket centrifuge, spin for 20 min at 3,500 x *g* at 4°C with

the brake off.

3.5. Gently remove the samples without shaking and observe under light. A clear white band of 95% pure nuclei should be visible between the second and third layer (**Figure 1**).

4. Isolation of nuclei

4.1. Aspirate the top layers until the white nuclei band at the interphase of 29%-35%.

4.2. Collect the nuclei band in a 200 µL volume, transfer to a fresh tube and filter with a 20 µm filter (**Table of Materials**).

NOTE: The nuclei do not need to be resuspended prior to filtration.

4.3. Check under a light microscope to verify the removal of large debris and the intactness of the nuclear membrane. Nuclei need to be round and the nuclear membrane should not be distorted.

4.4. Count nuclei using Trypan blue staining on a hemocytometer and aliquot nuclei for snRNA-seq/snATAC-seq.

5. Single nuclei RNA and ATAC seq

5.1. Immediately process the nuclei using the single cell gene expression and single cell ATAC reagent kits (**Table of Materials**).

NOTE: The nuclei sample concentrations for the 10x Genomics system are 1500-3000 nuclei per µL for snRNA-seq, and 3500-7000 nuclei per µ µL for snATAC-seq. The nuclei can be diluted using 1x PBS.

5.2. Sequence the resulting libraries at the Genomics Core Facility.

5.3. Perform quality control analysis of the data. Nuclei are included for further analysis if they contain Unique Molecular Identifier (UMI) >1000, number of genes >500 and percent of mitochondrial genes <5%, and are within mean + three standard deviations of the UMIs and genes.

REPRESENTATIVE RESULTS:

Single nuclei genomics is an evolving field with limited data and protocols. A critical factor that influences the outcome of single nuclei assays is the isolation of pure and intact nuclei. We combined two published protocols (DroNc-seq and Omni-ATAC-seq protocols) to isolate high-quality and pure nuclei from fresh frozen glioma tissue blocks in a relatively short time thereby maintaining the stability of the transcripts (**Figure 1**).

The use of various filtration steps along with the gradient centrifugation using iodixanol/sucrose gradient allows for the isolation of pure nuclei with the majority of debris discarded (**Figure 2**). The same isolated nuclei preparation can be used for both snRNA-seq and snATAC-seq. Importantly, since the nuclei used are from the same sample, the data generated can be co-embedded using algorithms such as Seurat to generate clusters and to provide a multi-omics perspective[25] (**Figure 3**).

To determine whether the protocol is comparable to published snRNA-seq datasets, we compared data obtained using the procedure with four publicly available snRNA-seq studies related to the central nervous system (CNS): Slyper et al.[20], Lake et al.[26], Jakel et al.[24] and Habib et al.[18]. To compare the quality control metrics, we downloaded the following count matrices from the Gene Expression Omnibus (GEO): GSE104525 (Habib dataset, 2017), GSE97930 (Lake dataset, 2018), GSE118257 (Jakel dataset, 2019) and GSE140819 (Slyper dataset, 2020). For the Lake dataset, a common raw count matrix was created by merging the individual matrices for cerebellar hemisphere, frontal cortex and visual cortex. For the Slyper dataset, raw count for the sample HTAPP-443-SMP-5491 (high-grade pediatric glioma) was selected.

To perform an unbiased comparison, all samples except the Jakel dataset were processed using a common standardized protocol. First, we used the Seurat R-statistical package to create a Seurat object of each raw matrix[27]. This was followed by two steps: 1) to remove potential droplets, the following cutoffs were used - nuclei containing less than 1000 UMI, less than 500 genes or more than 5% of mitochondrial RNA were excluded from the analysis and 2) to exclude doublets, nuclei that fell outside of the mean plus three standard deviations for the distribution of UMIs and genes were removed. For the Jakel dataset, this second step could not be performed, as the publicly available dataset was preprocessed with a less stringent quality control step.

To compare the distribution of UMIs and genes across samples, we merged all the datasets and visualized the distribution of the number of UMIs and genes using a violin plot (**Figure 4**). This result indicated that the method is comparable to the latest snRNA-seq protocol described in Slyper et al.[20].

The protocol illustrated here deals with glioma samples but the same approach can feasibly be applied for non-CNS tumors and tissues. Nevertheless, this will require optimization of lysis buffer compositions and incubation times.

FIGURE AND TABLE LEGENDS:

Figure 1: Flow chart for nuclei isolation. The flow chart provides a brief outlook on the steps involved in the isolation of single nuclei from a fresh frozen glioma tissue. Representative images for the tumor sample and the nuclear band after Iodixanol/sucrose gradient (circled with red dotted line) are shown.

Figure 2: Examples of nuclei before and after gradient centrifugation. (**A**) The image of the sample before performing gradient centrifugation and filtration shows large amounts of debris

(B) The image of the sample after gradient centrifugation and filtration step shows intact nuclei with minimal amount of debris.

Figure 3: Example of co-embedded data from snRNA-seq and snATAC-seq using the same nuclei preparation. The *Seurat* R-statistical package was used to integrate the snRNA-seq and snATAC-seq data[27]. (**A**) Co-embedded image of snRNA-seq and snATAC-seq data (**B**) Clusters produced by co-embedding of snRNA-seq and snATAC-seq data.

Figure 4: Quality control parameters of different human brain snRNA-seq datasets showing the individual number of UMI (A) and number of genes (B) per nuclei. The number of nuclei that passed quality filters were as follows: 3527 nuclei from the Slyper dataset, 14636 nuclei from the Narayanan dataset generated using the described protocol, 7369 nuclei from the Jakel dataset, 16494 nuclei from the Lake dataset, and 4652 nuclei from the Habib dataset.

Table 1: Preparation of 6x Homogenization Buffer Stable Master Mix.

Table 2: Preparation of 1 M sucrose.

Table 3: Preparation of 6x Homogenization Buffer Unstable Solution (650 µL per sample).

Table 4: Preparation of 1x Homogenization Buffer Unstable Solution (2 mL per sample).

Table 5: Preparation of 50% Iodixanol Solution (200 µl per sample).

Table 6: Preparation of 29% Iodixanol Solution (300 µl per sample).

Table 7: Preparation of 35% Iodixanol Solution (300 µl per sample).

DISCUSSION:

The field of intra-tumoral heterogeneity is at an exciting stage, with novel assays and platforms being developed to challenge and expand the existing knowledge. Intra-tumoral heterogeneity is a crucial factor that contributes to disease progression and resistance to current treatment modalities in gliomas[28]. Recent studies on brain tumors have focused on this important aspect by using single cell transcriptomic and epigenomic assays to better characterize the cellular heterogeneity within the same tumor[29-32]. One of the current bottlenecks with the single cell assays in brain tumors, and other solid tumors, is the availability of fresh clinical specimens. To overcome this problem, various studies have shown that using nuclei from fresh frozen tissues can be an alternative to fresh specimens and can be successfully used to interrogate cellular heterogeneity[18,33].

Here, we improve the previous single nuclei isolation methods in terms of simplicity, length, abundance, and quality of nuclei. This approach further brings the advantage of profiling snRNA-seq and snATAC-seq from the same nuclei preparation, allowing for multi-omics studies. In this procedure, we have modified existing protocols by adding an extra process of gradient separation

using a non-ionic iodixanol-based medium. This approach allows for the isolation of pure nuclei without the need for FACS sorting, and the quality of the nuclei is assessed under a light microscope. A good quality suspension of nuclei should have no clumping, minimal debris, and intact nuclei. Although infrequent, clumping of nuclei can occur during the isolation step, which can be resolved by light pipetting using wide-bore P200 pipette tips or by straining the nuclei through a 20 μm filter. The most critical factor in single nuclei genomics is the ability to obtain pure and intact single nuclei. The presence of debris or aggregates can lead to a block of the microfluidic chambers within the single cell platforms, thereby leading to either low-quality data or to a possible failure of the experiment. The purpose of using filters at various steps followed by observation under a light microscope is to prevent such occurrences.

The amount and quality of starting material (e.g., fresh frozen tumor) are also important considerations. We have successfully used the protocol described here with brain tumor tissue blocks ranging from 10 mg to 60 mg. For these specimens, 500 μL of nuclei lysis buffer (Step 1) is sufficient to obtain sufficient good quality nuclei for single nuclei sequencing. Significantly larger tumor blocks that contain greater than 60-70% tumor content can be chopped into smaller pieces, and pieces can either be immediately placed back into liquid nitrogen or -80 °C storage or divided into tubes for single nuclei isolation and mixed once the lysis step is completed. In addition, tissue quality and tumor content should be assessed by Hematoxylin and Eosin (H&E) staining followed by confirmation from a pathologist.

The next important factors to consider are temperature and time of processing. Improper tissue storage, sample handling, and lengthy protocols can negatively impact the quality of the final genomics data. The samples need to be kept on ice, and the time for sample processing needs to be quick to prevent degradation of nuclei. The average processing for one sample with this protocol is 45-60 minutes, and it takes approximately 2 hours to process four samples. Improper tissue storage can also affect nuclei isolation and data quality. We assessed whether length of storage time at -80 °C impacted the quality of the data generated using the described protocol. The comparison of sequencing results obtained from the frozen samples banked between 1 to 14 years (2006-2019) showed no differences in data quality with respect to storage time. Therefore, properly frozen and stored fresh frozen tissue samples can be used for retrospective studies using this protocol.

There are several limitations with the single nuclei protocols. For example, different cell types cannot be sorted based on cell surface markers, and cytoplasmic transcripts are not detectable. Also, we omit the mitochondrial transcripts hence losing out on certain biological information related to tumor metabolism. Furthermore, different tissue types may require different lysis buffers thus requiring optimization of the protocol with each new experimental setup.

Overall, the protocol described here is simple, reduces sample processing time, and yields high-quality nuclei. The lack of a sorting step reduces stress on the nuclei and eliminates the need for a cell sorter. The absence of mitochondrial RNA increases the sequencing depth of nuclear transcripts. Importantly, similar to other single nuclei isolation protocols, this enhanced protocol facilitates the use of archived frozen specimens for retrospective analysis. We have successfully

applied this workflow to perform snRNA-seq and snATAC-seq using the same nuclei preparation from brain tumors without the need to change buffers. Therefore, this approach allows for multi-omics studies from the same specimen.

In conclusion, the single nuclei isolation method described here is a highly effective, accurate and rapid technique which can be applied to perform single-nucleus sequencing studies.

ACKNOWLEDGMENTS:

We thank the Single Cell Open Lab (scOpenLab) at the German Cancer Center (DKFZ) for helpful discussions. This research was supported by the German Cancer Aid, Max-Eder Program grant number 70111964 (S.T.).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Huse, J. T., Holland, E. C. Targeting brain cancer: advances in the molecular pathology of malignant glioma and medulloblastoma. *Nature Reviews Cancer.* **10** (5), 319-331 (2010).

2 Kreso, A., Dick, J. E. Evolution of the cancer stem cell model. *Cell Stem Cell.* **14** (3), 275-291 (2014).

3 Filbin, M. G., Suva, M. L. Gliomas Genomics and Epigenomics: Arriving at the Start and Knowing It for the First Time. *Annual Review of Pathology.* **11**, 497-521 (2016).

4 Ferris, S. P., Hofmann, J. W., Solomon, D. A., Perry, A. Characterization of gliomas: from morphology to molecules. *Virchows Archive.* **471** (2), 257-269 (2017).

5 Louis, D. N. et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathologica.* **131** (6), 803-820 (2016).

6 Eckel-Passow, J. E. et al. Glioma Groups Based on 1p/19q, IDH, and TERT Promoter Mutations in Tumors. *The New England Journal of Medicine.* **372** (26), 2499-2508 (2015).

7 Suzuki, H. et al. Mutational landscape and clonal architecture in grade II and III gliomas. *Nature Genetics.* **47** (5), 458-468 (2015).

8 Cancer Genome Atlas Research, N. et al. Comprehensive, Integrative Genomic Analysis of Diffuse Lower-Grade Gliomas. *The New England Journal of Medicine.* **372** (26), 2481-2498 (2015).

9 Noushmehr, H. et al. Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell.* **17** (5), 510-522 (2010).

10 Yan, H. et al. IDH1 and IDH2 mutations in gliomas. *The New England Journal of Medicine.* **360** (8), 765-773 (2009).

11 Yan, H., Bigner, D. D., Velculescu, V., Parsons, D. W. Mutant metabolic enzymes are at the origin of gliomas. *Cancer Research.* **69** (24), 9157-9159 (2009).

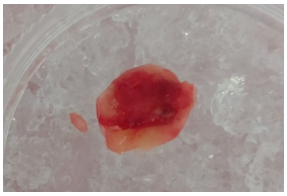
12 Gawad, C., Koh, W., Quake, S. R. Single-cell genome sequencing: current state of the science. *Nature Reviews Genetics.* **17** (3), 175-188 (2016).

13 Tanay, A., Regev, A. Scaling single-cell genomics from phenomenology to mechanism. *Nature.* **541** (7637), 331-338 (2017).

14 Wu, M., Singh, A. K. Microfluidic Flow Cytometry for Single-Cell Protein Analysis. *Methods in Molecular Biology.* **1346**, 69-83 (2015).

15 Schwartzman, O., Tanay, A. Single-cell epigenomics: techniques and emerging applications. *Nature Reviews Genetics.* **16** (12), 716-726 (2015).
16 Macaulay, I. C., Ponting, C. P., Voet, T. Single-Cell Multiomics: Multiple Measurements from Single Cells. *Trends in Genetics.* **33** (2), 155-168 (2017).
17 Buenrostro, J. D. et al. Single-cell chromatin accessibility reveals principles of regulatory variation. *Nature.* **523** (7561), 486-490 (2015).
18 Habib, N. et al. Massively parallel single-nucleus RNA-seq with DroNc-seq. *Nature Methods.* **14** (10), 955-958 (2017).
19 Corces, M. R. et al. An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues. *Nature Methods.* **14** (10), 959-962 (2017).
20 Slyper, M. et al. A single-cell and single-nucleus RNA-Seq toolbox for fresh and frozen human tumors. *Nature Medicine.* **26** (5), 792-802 (2020).
21 Mathys, H. et al. Single-cell transcriptomic analysis of Alzheimer's disease. *Nature.* **570** (7761), 332-337 (2019).
22 Krishnaswami, S. R. et al. Using single nuclei for RNA-seq to capture the transcriptome of postmortem neurons. *Nature Protocols.* **11** (3), 499-524 (2016).
23 Al-Dalahmah, O. et al. Single-nucleus RNA-seq identifies Huntington disease astrocyte states. *Acta Neuropathologica Communications.* **8** (1), 19 (2020).
24 Jakel, S. et al. Altered human oligodendrocyte heterogeneity in multiple sclerosis. *Nature.* **566** (7745), 543-547 (2019).
25 Butler, A., Hoffman, P., Smibert, P., Papalexi, E., Satija, R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nature Biotechnology.* 10.1038/nbt.4096 (2018).
26 Lake, B. B. et al. Integrative single-cell analysis of transcriptional and epigenetic states in the human adult brain. *Nature Biotechnology.* **36** (1), 70-80 (2018).
27 Stuart, T. et al. Comprehensive Integration of Single-Cell Data. *Cell.* **177** (7), 1888-1902 e1821 (2019).
28 Bready, D., Placantonakis, D. G. Molecular Pathogenesis of Low-Grade Glioma. *Neurosurgery Clinics of North America.* **30** (1), 17-25 (2019).
29 Venteicher, A. S. et al. Decoupling genetics, lineages, and microenvironment in IDH-mutant gliomas by single-cell RNA-seq. *Science.* **355** (6332) (2017).
30 Tirosh, I. et al. Single-cell RNA-seq supports a developmental hierarchy in human oligodendroglioma. *Nature.* **539** (7628), 309-313 (2016).
31 Weng, Q. et al. Single-Cell Transcriptomics Uncovers Glial Progenitor Diversity and Cell Fate Determinants during Development and Gliomagenesis. *Cell Stem Cell.* **24** (5), 707-723 e708 (2019).
32 Neftel, C. et al. An Integrative Model of Cellular States, Plasticity, and Genetics for Glioblastoma. *Cell.* **178** (4), 835-849 e821 (2019).
33 Al-Ali, R. et al. Single-nucleus chromatin accessibility reveals intratumoral epigenetic heterogeneity in IDH1 mutant gliomas. *Acta Neuropathologica Communications.* **7** (1), 201 (2019).

Figure 1



Fresh frozen tissue

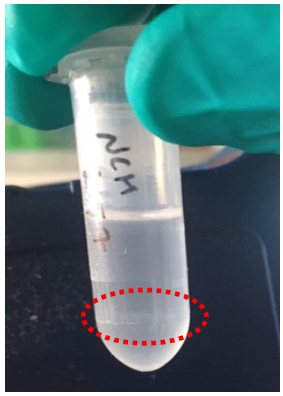
Chop, Dounce homogenize in EZ lysis buffer (20-30 times)

Wash & filter (30 μm filter) Resuspend pellet in Homogenization Buffer

Tissue lysis and clearing of large debris

Quality control using light microscopy

Separate nuclei using Iodixanol/sucrose density gradient



Nuclear band

Spin 3500 x *g* for 20 min at 4°C

Filter to remove debris and aggregates (20 μm filter)

Quality control using light microscopy

Nuclei isolation

snRNA-seq

snATAC-seq

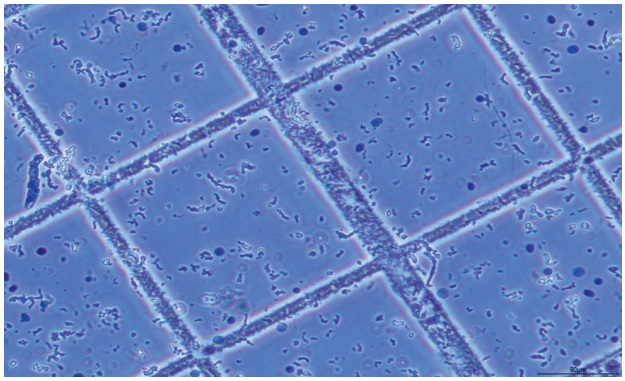
10x Genomics

Sequencing

Figure 2

A

Before gradient separation



B

After gradient separation

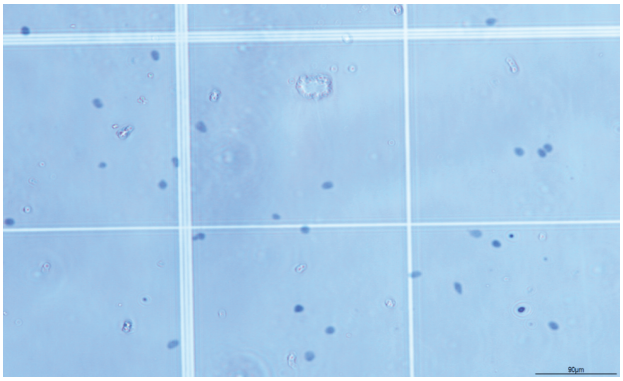


Figure 3

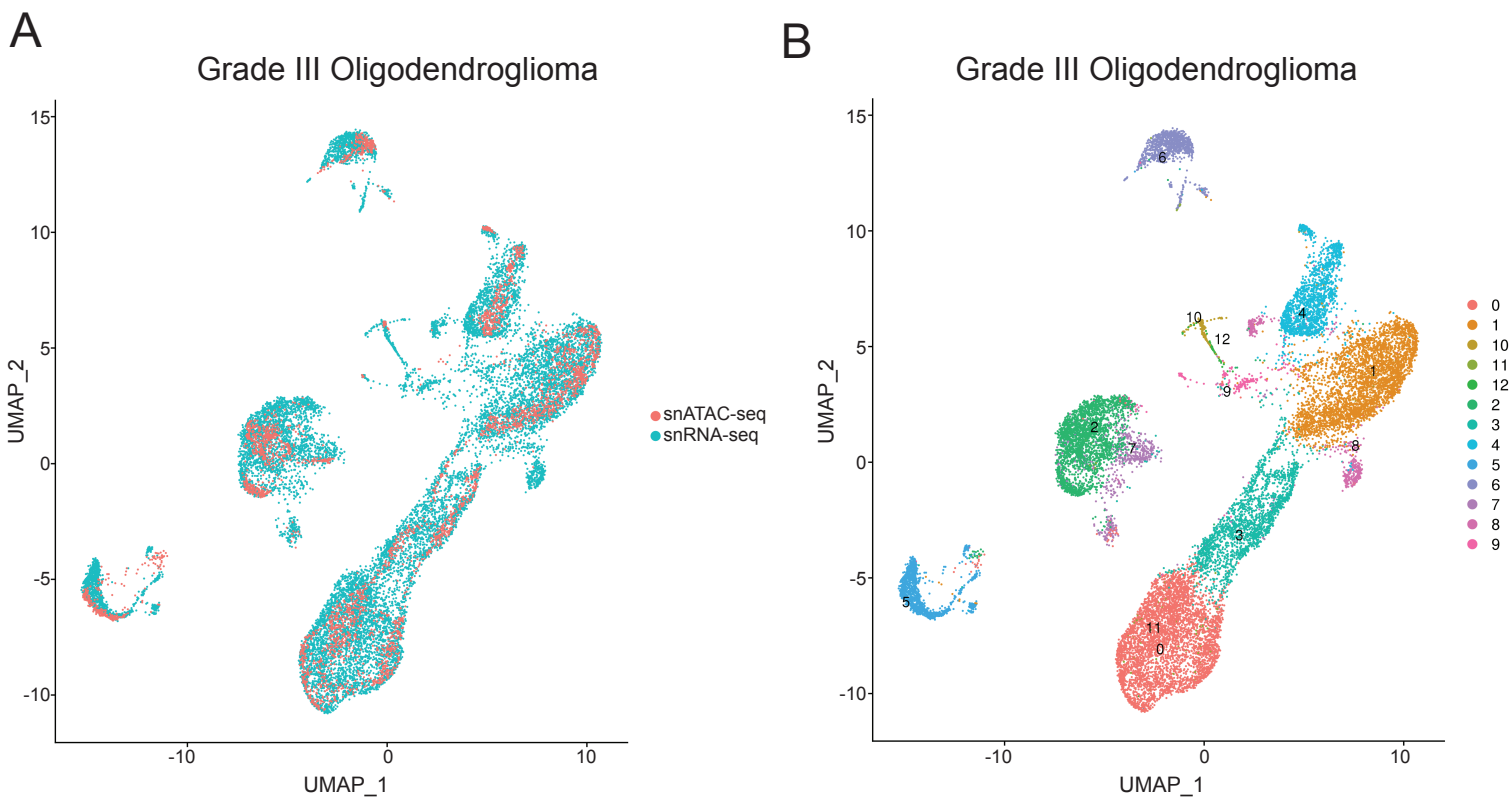
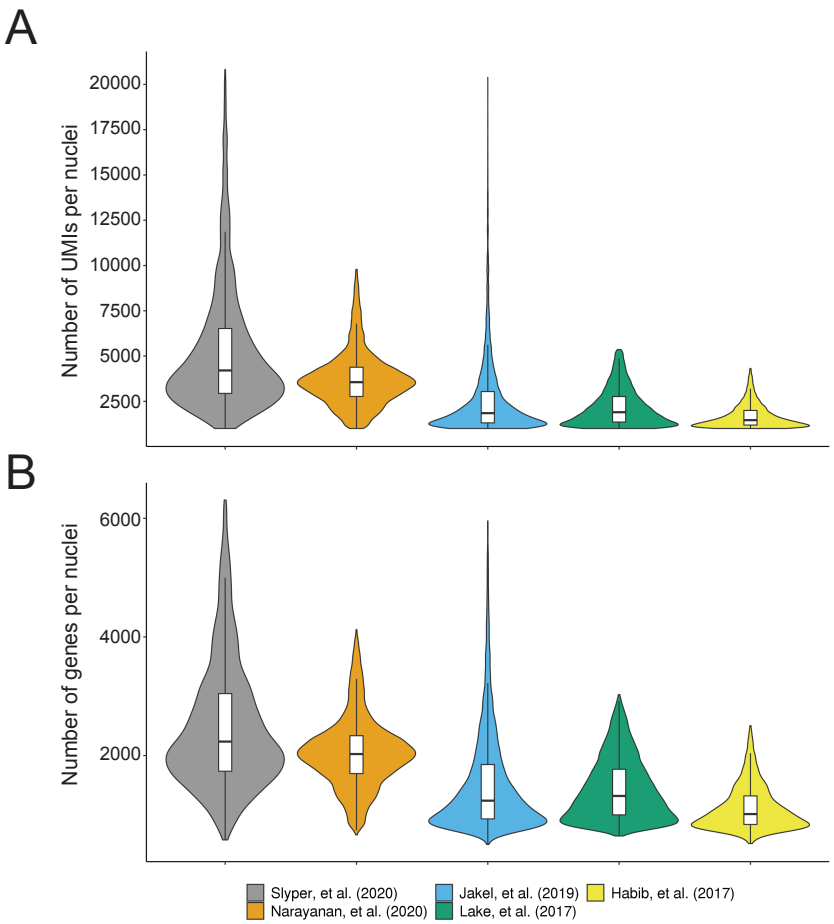


Figure 4



6x Homogenization Buffer Stable Master Mix

Reagent	Final Conc.	Vol for 100 (mL)
1 M $CaCl_2$	30 mM	3.0
1 M $Mg(Ac)_2$	18 mM	1.8
1 M Tris pH 7.8	60 mM	6.0
H_2O		89.2

Keep at room temperature, avoid direct exposure to light

1 M Sucrose
34.23 g of sucrose
Dissolve in 78.5 mL of water
Fill up to 100 mL with water

Table 3

6x Homogenization Buffer Unstable Solution (650 mL per sample)

Reagent	Final Conc.	Vol per sample (µL)
6x Homogenization Buffer Stable	6x	648.84
100 mM PMSF (Phenylmethylsulfonyl fluoride)	0.1 mM	1.08
14.3 M β-mercaptoethanol	1 mM	0.08

Table 4 Click here to access/download;Table;Table 4.xlsx

1x Homogenization Buffer Unstable Solution (2 mL per sample)

Reagent	Final Conc.	Vol per sample (µL)
6x Homogenization Buffer Unstable	1x	333.33
1 M Sucrose	320 mM	640.00
50 mM EDTA	0,1 mM	4.00
10% NP40	0.1%	20.00
H_2O		1006.27

Table 5

50% Iodixanol Solution (200 µL per sample)

Reagent	Final Conc.	Vol per sample (µL)
6x Homogenization Buffer Unstable	1x	66.67
60% Iodixanol Solution	50%	333.33

29% Iodixanol Solution (300 µL per sample)

Reagent	Final Conc.	Vol per sample (µL)
6x Homogenization Buffer Unstable	1x	100
1 M Sucrose	160 mM	96
60% Iodixanol Solution	29%	290
H_2O		114

35% iodixanol Solution (300 µL per sample)

Reagent	Final Conc.	Vol per sample (µL)
6x Homogenization Buffer Unstable	1x	100
1 M Sucrose	160 mM	96
60% Iodixanol Solution	35%	350
H_2O		54

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2-Mercaptoethanol	Sigma	M6250	
$CaCl_2$	Sigma	21115-100ML	
Dounce Homogenizer	Active motif	40401	
EDTA (0.5 M)	Thermo Scientific	R1021	
Falcon 5 mL Round Bottom Polystyrene Test Tube	Corning	352235	
Iodixanol (aka Optiprep)	Stem cell technologies	07820	
MACs Smart Strainers (30 µm)	Miltenyi Biotec	130-098-458	
MACS SmartStrainers (100 µm)	Miltenyi Biotec	130-098-463	
$Mg(Ac)_2$	Sigma	63052-100ML	
NP-40	Abcam	ab142227	
Nuclei Isolation Kit: Nuclei EZ Prep	Sigma	NUC101-1KT	
Phenylmethanesulfonyl fluoride (PMSF)	Sigma	P7626	
Pre-Separation Filters (20 µm)	Miltenyi Biotec	130-101-812	
Safe lock tubes 1.5 mL	Eppendorf	0030120086	
Safe lock tubes 2.0 mL	Eppendorf	0030120094	
Single Cell ATAC	10x Genomics		
Single Cell Gene Expression	10x Genomics		
Sucrose	Sigma	S0389	
Wide Bore pipette tips (1000 µL)	Themo Fisher Scientific	2079GPK	
Wide Bore pipette tips (200 µL)	Themo Fisher Scientific	2069GPK	

We thank the editor and the peer reviewers for their constructive suggestions and thoughtful comments, which has substantially improved the manuscript. Please find below our point-by-point responses to the editorial and reviewer comments:

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

>> We have now thoroughly proofread the manuscript.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

>> The discussion is now extended to cover these points in more detail.

• **Figures:** Add spaces between numeral and units; e.g., 30μM should be 30 μM.

>> This is now revised.

• **Tables:** Please remove the embedded Tables from the manuscript. All tables should be uploaded to the Editorial Manager site in the form of Excel files. A description of the table should be included with the Figure legends.

>> All tables are now removed.

• **References:**Please spell out journal names.

>> We have now corrected this error.

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Eppendorf, illumina NovaSeq 6000, Nuclei EZ,
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

>> This is now revised.

2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.
>> This is now removed.

• **Table of Materials:**Please sort alphabetically.

>> This is now revised.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

>> Not applicable.

Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
The authors present a unique method to isolate nuclei from astrocytomas. The major concern is there is not a sufficient comparison to any other isolation methodology (with respect to RNA-seq results)

>> We thank the reviewer for this comment. We have now revised the manuscript to include a comparison of our snRNA-seq results to four publicly available snRNA-seq datasets generated from central nervous system tissues. We utilized a stringent cut-off to exclude nuclei containing < 1000 Unique Molecular Identifiers (UMIs), < 500 genes or > 5% mitochondrial RNA. We compared the number of UMIs and genes per nuclei obtained using our method to these published datasets. This analysis indicated that our isolation method is similar to Slyper *et al (*Nature Medicine, 2020). We added these results to the 'Representative Results' section and in the new Figure (Figure 4) in the revised manuscript (Lines 209-231, page 6).

Reviewer #2:
Manuscript Summary:
The authors present a well-written and coherent protocol for isolation of nuclei from fresh frozen tissue. Disseminating this protocol is very important as the authors note in their manuscript. Therefore, it is of essence to fully and clearly characterize it. The protocol presented here is similar to what many groups are currently using for snRNAseq. The authors ought to refer to these papers [1-4] among others. This would impart additional validity to the methods presented in this submission. This protocol is significantly different from other protocols that use FACS and even other snRNAseq protocols [5], and that should also be highlighted. Overall, the steps are clear, and the use of figure 1 facilitates following the detailed steps.

Major Concerns:
A major concern is the lack of use of any RNAse and/or protease inhibitors. Have the authors determined that these reagents are not necessary? Empirical data is needed to justify omitting such reagents used in most published protocols. For example, a comparison between data output from experiments with and without use of RNAse/protease inhibitors is

important to show here. Specifically, the fastQC results from the fastq files, 10x cell-ranger report, average nUMI per nucleus, library saturation, and number of detected genes. This is a significant point especially that the aforementioned reagents can be costly for larger scale experiments.

>> We thank the reviewer for the comments and the references. We have now incorporated the referenced papers in our manuscript (Lines 99-100, page 3). In our protocol, we use phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor as a reagent for the 6x homogenization buffer unstable solution described in Table 3. We do not use an RNAse inhibitor; however, we did not observe loss of data quality when compared to published datasets. We compared our snRNA-seq results to four publicly available snRNA-seq generated from the central nervous system (either normal or disease states). We utilized a stringent cut-off to exclude nuclei containing < 1000 UMIs, < 500 genes or > 5% mitochondrial RNA. We compared nUMIs and nGenes per nuclei obtained using our method to published data. Overall, quality of data obtained using our isolation method is similar to Slyper *et al* (Nature Medicine, 2020). We added these results to the 'Representative Results' section and in the new Figure (Figure 4) in the revised manuscript (Lines 209-231, page 6).

Minor Concerns:
Specific points that need to be addressed in the protocol are the following:
1. The authors should provide additional details about the starting material. Specifically: A. What is the maximum volume of tissue that can be initially used in step 1? What is the minimum volume of tissue that can be used? B. What are important tissue quality requirement that need to be met for good quality data, specifically; RNA integrity number. Can any sample be analyzed regardless of RIN values?

>> All primary tissues that we processed were between 10 – 60 mg. For these samples, 500 µl of Nuclei EZ lysis buffer (Step 1) is sufficient. However, the reviewer raises an important point regarding sample size. We recommend chopping larger samples into smaller pieces, and either a) placing back into liquid nitrogen or -80°C storage or b) dividing into different tubes for processing and mixing once the lysis step is completed. We do not utilize RIN for tissue integrity; we assess tissue quality based on tumor content (H&E staining) and consultation with a pathologist. We added these points to the revised Protocol (Line 119, page 3) and Discussion section (Lines 302-320, page 8). The quality of libraries is determined after running on TapeStation and decision to proceed to sequencing is based on library quality and quantity.

2. Step 1.6: What volume do you pass through the strainer? How many strainers are used?

>> We use only one strainer per sample, and we pass the entire sample in Nuclei EZ lysis buffer through it. We now added this point to Step 1.1.6 (Lines 134-135, page 4)

3. Step 1.7: Is EZ lysis buffer the same as Nuclei EZ lysis buffer?

>> Yes, this is correct. We have now added the commercial name to the Materials section.

4. Please explain the steps needed to verify the intactness of nuclear membranes. The authors note that in the discussion section (last page) and in figure 1, but do not incorporate into the detailed protocol.

>> We have now incorporated this QC step into the protocol (Step 1.1.7 and Step 3.1.3) (Lines 136-138, page 4, and Lines 165-167, page 5).

5. Step 3.2: Are the nuclei resuspended before being passed into the filter? If so, what is the buffer used to resuspend the collected nuclei?

>> The nuclei do not need to be resuspended. We revised the protocol to include this note (Line 164, page 5).

6. How do the authors avoid nuclei aggregates and clumping?

>> We have not encountered this problem with our tissue type, but should clumping and aggregates occur, we suggest using a) wide-bore P200 pipette tips to dissociate the clumps/aggregates to prevent stressing the nuclei, and b) passing the nuclei through 20 µm filters. We revised the Discussion to include these recommendations (Lines 291- 300, page 8).

7. What volume and buffer are used to dilute nuclei prior to processing on the 10X instrument?

>> We aim to have between 1500-3000 nuclei per µl (snRNA-seq), and 3500-7000 nuclei per µl (snATAC-seq), before loading the sample on 10x. If the concentration is too high, we recommend diluting the samples with 1X PBS. We revised the manuscript to add this information as a Note (lines 176-178, page 5).

8. Since details are not provided on step 4, it should be noted that these steps are done in a genomics core facility (if that is the case). Otherwise, details should be provided.

>> We revised the manuscript to note that sequencing is performed at the genomics core (Line 180, page 5).

9. What are the most common problems that a user of this protocol might face, and how to resolve them? A good example might be nuclear pellet dislodgment. How frequently does that happen in this protocol?

>> We have not observed nuclear pellet dislodgment; however, the most common issue can be clumping of the nuclei. This can be avoided by pipetting using a wide-bore pipette tip before plating the nuclei. We now revised the Discussion section accordingly (Lines 291-300, page 8) and included several other considerations that are important for the protocol.

Minor points:
1. The manuscript page numbers shown in the provided pdf file all show either no number or (6 of 6), I know this is an editorial matter, but having properly numbered pages facilitates making comments on specific pages.

>> We apologize for this error – we corrected the page numbering in the revised version.

2. The materials noted in the last page cite MACS smart strainers at 30um. The text notes a 35um strainer. This should be addressed.

>> We thank the reviewer for noticing this typo - It is 30 µm and it has been corrected in the revised manuscript (Materials section).

3. Buffers section: PMSF abbreviation should be spelled out. Nowhere in the text is it clarified.

>> This is now corrected (revised Table 3 and Materials section).

4. The FACS tubes and Eppendorf tubes catalogue numbers need to be provided.

>> The catalogue numbers are now added to the Materials section.

References

1. Mathys, H., et al., Single-cell transcriptomic analysis of Alzheimer's disease. Nature, 2019. 570(7761): p. 332-337.
2. Krishnaswami, S.R., et al., Using single nuclei for RNA-seq to capture the transcriptome of postmortem neurons. Nat Protoc, 2016. 11(3): p. 499-524.
3. Al-Dalahmah, O., et al., Single-nucleus RNA-seq identifies Huntington disease astrocyte states. Acta Neuropathol Commun, 2020. 8(1): p. 19.
4. Jakel, S., et al., Altered human oligodendrocyte heterogeneity in multiple sclerosis. Nature, 2019. 566(7745): p. 543-547.
5. Velmeshev, D., et al., Single-cell genomics identifies cell type-specific molecular changes in autism. Science, 2019. 364(6441): p. 685-689.

>> We thank the reviewer for these references. We incorporated these references into the revised manuscript (Lines 99-100, page 3).

Reviewer #3:

Manuscript Summary:

In the paper entitled 'Nuclei isolation from fresh frozen brain tumors for single-nucleus RNA-seq and ATAC-seq' Narayanan et al. have explained a novel way for isolating nuclei from fresh frozen brain tumor samples. The protocol combines existing protocols for single cell isolation and introduces additional steps of density gradient centrifugation and filtration to isolate nuclei while minimizing debris. As explained by the authors the major advantage of the protocol is surpassing the need of fresh glioma samples. This protocol can be performed on freshly frozen glioma samples which increases the availability of the samples. However the sample needs to be handled extremely carefully and the steps should be performed in quick succession. This protocol also reduces the need of a FACS sorter reducing additional sample processing steps and making the protocol more accessible. The authors have performed experiments like RNA-seq and ATAC-seq and successfully collected data to ensure the efficacy of the protocol.

While the protocol explained by the authors is helpful in isolating nuclei from freshly frozen glioma samples, a better explanation of certain steps with additional details are required for successful execution of the protocol.

Major Concerns:

1. The protocol mentions the use of freshly frozen samples but fails to clarify how much old frozen sample can be used. The use of freshly frozen glioma samples is one of the major advantages of the protocol, so the maximum time limit for a frozen sample which can be used for the protocol must be clarified.

>> We thank the reviewer for this comment. We used samples dating back as far as 2006 and as recent as 2019 (stored in the freezer at -80C). We compared data quality of these samples and did not observe a negative impact of storage length on nuclei or data quality. In our experience, as long as the samples are stored properly, they can be used as starting material for this protocol. We now added this as a discussion point in the revised manuscript (lines 312-322 page 8).

2. The chopped/minced tissue is transferred to 500µl of chilled Nuclei EZ Lysis Buffer in step 1.2. The adequate amount of tissue, either in terms of weight or dimension, to be chopped for mixing in 500µl buffer must be mentioned. This will allow the users to calculate whether the available sample is enough for the protocol and will also reduce the wastage of precious sample. It will be very helpful if a standardized figure mentioning the amount of tissue per 500µl of chilled Nuclei EZ Lysis Buffer is mentioned.

>> This is an important point – however, we did not exhaustively test the amount of tissue per 500µl lysis buffer, therefore have not included a standardized tissue weight per volume. In the revised manuscript, we included this issue as a discussion topic. Briefly, majority of our tissues were within 10 – 60 mg. For these starting amounts of tissue, 500 µl of Nuclei EZ lysis buffer (Step 1) yields sufficient number of nuclei for the 10x genomics platform. However, we recommend chopping significantly larger samples into smaller pieces, and either a) placing pieces back into liquid nitrogen or -80C storage or b) dividing into tubes for processing and mixing once the lysis step is completed. We added these points to the revised Protocol (Line 119, page 3) and Discussion section (Lines 302-320, page 8).

3. The steps at which quality control can be done by using microscope shall also be mentioned in the steps along with the note whether the sample should be filtered at the particular step or not in the presence of debris.

>> We have now revised the manuscript to include the quality control steps (Step 1.1.7 and Step 3.1.3; Lines 136-138, page 4, and Lines 165-167, page 5).

4. In step 1.6 protocol mentions the use of 35 µm-strainer mesh while the flow chart (Figure 1) mentions the use of 30 µm-strainer mesh.

>> Thank you for noticing this error; it should have been a 30 µm-strainer mesh, we have corrected the protocol accordingly.

5. In step 2.4 the protocol mentions centrifugation at 3000 x g at 4°C while the flow chart (Figure 1) mentions the centrifugation speed as 3500 x g.

>> It is 3500 x *g* and it has now been corrected.

6. It must be clarified that after the isolation how quickly the nuclei should be processed for further experiments. If the authors recommend that the isolated nuclei can be preserved then the conditions and adequate preserving duration must be mentioned.

>> We have not tried preserving the nuclei; however, would expect sample loss during recovery (thawing). Therefore, we recommend processing the nuclei as soon as it is isolated.

7. Figure 3 is not explained properly and it is hard to comprehend what the figure is trying to project. A better explanation of the figure is needed and will be helpful for the readers.

>> We modified the text to further explain Figure 3 (Lines 205-207, page 6; Lines 249-252, page 7).

Minor Concerns:
1. The sequence of authors on the title page and on the second page below the title is different, this needs to be corrected.

>> This is now corrected.

2. The average yield of the nuclei with reference to the amount of sample used should be mentioned.

>> We re-checked whether there was a correlation between sample amount and nuclei yield. However, we do not see a direct correlation with sample size and nuclei yield. We suspect that there will be a correlation between tumor cellularity (for ex. a small amount of high grade tumor tissue can be hypercellular compared to a larger chunk of a lower grade tumor) and number of nuclei.

3. It will be helpful to mention the total time taken to complete the protocol (on average) .

>> We revised the Discussion section to include processing time (lines 314 – 318, page 8). Specifically, each sample on average takes 45 minutes to 1 hour, and the average processing time for 4 samples is approximately 2 hours.

4. If there is any step on which the protocol can be halted for some time in case of any emergency, then that step and the time duration of the halt should be mentioned.

>> We have not tried freezing the isolated nuclei; we directly proceed with subsequent steps. However, we do not recommend halting the protocol at any step and suggest processing the samples directly with the 10x single cell platform (Step 4.1.1).

5. Step 2.5 mentions the white band with purified nuclei between second and third gradient layer. An image of the same will be helpful for reference.

>> We now incorporated an image of the white band in the revised Figure 1.

6. In the figure 1, at step where the centrifugation at 3500 x g at 4°C is mentioned the degree symbol (°) is missing.

>> This is now corrected.

7. The letter 's' in the figure 1 in second last step mentioning snRNA-seq and snATAC-seq is capital. It should be small.

>> This is now corrected.

8. The two steps in figure 1 where the size of filters is mentioned, μM is used which denotes micro molar. 'μm' must be used in place of 'μM'.

>> This now corrected. We thank the reviewer for noticing these errors.