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.