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Nuclei isolation from whole tissue using a detergent and enzyme-free method

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TITLE:

Nuclei Isolation from Whole Tissue using a Detergent and Enzyme-Free Method

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KEYWORDS:

nucleus isolation, zebrafish, brain, neuron, fresh tissue, detergent-free, enzyme-free, spin-column, single-nucleus RNA-seq, ATAC-seq, FACS

SUMMARY:

Single nuclei isolation relies on dissociation and detergent-based permeabilization of the cell membrane, steps that need optimization and are prone to introducing technical artifacts. We demonstrate a detergent and enzyme-free protocol for rapid isolation of intact nuclei directly from whole tissue, yielding nuclei suitable for single-nucleus RNA-seq (snRNA-Seq) or ATAC-seq.

ABSTRACT:

High-throughput transcriptome and epigenome profiling requires preparation of a single cell or single nuclei suspension. Preparation of the suspension with an intact cell or nuclei involves dissociation and permeabilization, steps that can introduce unwanted noise and undesirable damage. Particularly, certain cell-types such as neurons are challenging to dissociate into individual cells. Additionally, permeabilization of the cellular membrane to release nuclei requires optimization by trial-and-error, which can be time consuming, labor intensive and financially nonviable. To enhance the robustness and reproducibility of sample preparation for high-throughput sequencing, we describe a rapid enzyme and detergent-free column-based nuclei isolation method. The protocol enables efficient isolation of nuclei from the entire zebrafish brain within 20 minutes. The isolated nuclei display intact nuclear morphology and low propensity to aggregate. Further, flow cytometry allows nuclei enrichment and clearance of cellular debris for downstream application. The protocol, which should work on soft tissues and cultured cells, provides a simple and accessible method for sample preparation that can be utilized for high-throughput profiling, simplifying the steps required for successful single-nuclei RNA-seq and ATAC-seq experiments.

INTRODUCTION:

Single-cell RNA-seq (scRNA-Seq) and ATAC-seq are versatile tools to study complex biological systems at single-cell resolution. They are widely utilized to define cell subtypes and states, gene networks and to assess cellular heterogeneity. A prerequisite for performing scRNA-seq is the preparation of a single cell suspension by tissue dissociation. Due to the variation in the extracellular matrix composition and mechanical properties, individual tissues require optimization of the dissociation protocol for preparation of single cell suspension.

Dissociation of tissues into single cells typically involves treatment with digestive enzymes, including collagenase, dispase or trypsin, at 37 °C[1–4]. As transcriptional machinery remains active at 37 °C, enzymatic dissociation can introduce mRNA expression artifacts and noise[5,6]. Notably, prolonged incubation can induce stress responsive genes and heat-shock response in a non-uniform manner – leading to technical variability in the experiment[7].

Another drawback of generating a single cell suspension is the difficulty in obtaining viable and intact cell-types with complex morphologies. In particular, neurons, adipocytes and podocytes are challenging to isolate[8–11]. For instance, Wu and colleagues demonstrated the absence of glomerular podocytes in scRNA profiles from an adult mouse kidney[12]. Similar nonoptimal observations have been made regarding the recovery of interconnected neurons from brain tissue[8,13,14]. In sum, dissociation protocols can introduce detection bias towards easier to dissociate cell-types, leading to a misrepresentation of the cellular architecture of the organ.

To overcome the technical noise and bias introduced during sample preparation in scRNA-Seq., isolation and profiling the nucleus provides an attractive alternative. As nuclear morphology is similar between different cell-types, isolation of the nuclei circumvents the issue of isolating intact and viable cells with complex morphologies. For instance, Wu and colleagues demonstrated successful profiling of glomerular podocytes with the single-nucleus RNA-Seq. (snRNA-Seq.) of an adult mouse kidney, which was missing from scRNA-Seq[12]. Intriguingly, comparative studies between single-cell and single-nucleus RNA-seq have suggested a decrease in induction of stress and heat-shock response genes with snRNA-Seq[12]. The studies further suggest a high correlation between the genes detected by the two methods. However, a recent study on human microglia failed to detect genetic activation in Alzheimer's disease[15]. Thus in certain contexts, snRNA-Seq is a suitable alternative for scRNA-Seq[16, 17]. Additionally, the nuclear isolation can be utilized for single-cell ATAC-Seq., providing information about the regions of open-chromatin within individual cells.

The protocol for nuclei isolation involves three major steps: i) detergent-based lysis of cell membrane to release the nucleus; ii) tissue homogenization using a Dounce homogenizer; and iii) enrichment of nuclei and removal of cell debris using gradient centrifugation or flow cytometry[18–22]. Among this, the first two steps depend on the tissue type and need to be empirically optimized. Mild detergent leads to partial rupture of cell membrane and inefficient retrieval of nuclei from the tissue[23]. On the other hand, high level of detergent and harsh homogenization leads to rupture of the nuclear membrane and their loss[24,25]. Ruptured nuclei further tend to clump together and form aggregates, which if not removed can lead to artifacts in the downstream profiling experiment.

To circumvent the issues related to detergent optimization for nuclei isolation, we introduce a protocol to isolate intact nuclei from fresh samples using a detergent-free and spin-column-based method. The protocol yields nuclei from whole organ within 20 minutes, limiting the induction of artifactual transcription. The isolated nuclei can be enriched with FACS for single-nuclei RNA-Seq. and ATAC-seq, providing a simple and universal method that enables robust and reproducible high-throughput profiling.

PROTOCOL:

All the procedures presented below were performed in accordance with institutional (Université Libre de Bruxelles (ULB)) and national ethical and animal welfare guidelines and regulation, which were approved by the ethical committee for animal welfare (CEBEA) from the Université Libre de Bruxelles (protocols 578N-579N).

1. Preparation before tissue dissection

1.1. Prepare 0.2% Tricaine solution in PBS for euthanizing the zebrafish. Chill the solution on ice.

1.2. Prepare a 30 mm Petri dish for mincing the tissue.

1.3. Prepare ice cold 1x PBS (10 mL per tissue sample).

1.4. Cool the centrifuge to 4 °C.

1.5. For nuclei isolation, use a detergent-free nuclei isolation kit (**Table of Materials**).

1.6. Before starting the protocol, pre-chill buffer A and B provided in the kit by placing them on ice for at least 30 min prior to nuclei isolation.

1.7. For handling the isolated nuclei, coat the plastic reagents such as tubes and pipette tips with 5% BSA solution. For this, prepare the solution by dissolving 2 g of BSA in 40 mL of PBS. Coating plastic items with 5% BSA reduces nuclei sticking to plastic. This step enhances the recovery of isolated nuclei.

1.8. Coat the pipette tips by pipetting 5% BSA solution 2-3 times. Air-dry the tips for 2 hours. Prepare 10 plastics tips per sample.

1.9. Coat the tubes for collection of nuclei by filling them with 5% BSA. Invert the tubes 3 times to ensure an efficient coating. Remove the solution and air-dry the tubes upside down on a clean tissue paper for 2 hours. Per sample, coat one collection tube provided in the kit. Additionally, prepare coated 1.5 mL tubes for nuclei collection after FACS.

NOTE: Glass tips are highly recommended alternative to the plastic pipette tips to minimize the sticking.

2. Dissection of zebrafish brain

2.1. For euthanizing the zebrafish, prepare a 90 mm Petri dish with 25 mL of ice-cold Tricaine solution.

2.2. Carefully, take the zebrafish from the tank using a fishing net and place it into the Petri dish.

2.3. Euthanize the fish by leaving it in Tricaine for 5 min.

2.4. Decapitate the animal with a sharp razor blade.

2.5. Using the forceps, gently break open the skull and remove soft tissues, skin and bones from ventral and dorsal side of the skull.

2.6. Gently, transfer the brain into a fresh 30 mm dish containing ice cold PBS.

2.7. Mince the brain into small pieces using a razor blade to ease the loading of the sample on the spin column.

3. Single nuclei isolation

3.1. Transfer the minced tissue to the filter cartridge provided in the nuclei isolation kit and add 200 µL of cold buffer A to sensitize the tissue. Grind the tissue using the plastic rod provided by the kit for 2 min.

3.2. Add 300 µL of cold buffer A and incubate the filter cartridge on ice with cap open for 10 min.

3.3. Cap the cartridge and resuspend the tissue by inverting the tube 5 times.

3.4. Centrifuge at 16,000 x *g* for 30 s. In this step, cells are ruptured when passing through the filter and high-speed centrifugal force is applied. The flow through contains intact nuclei, which pellet at the bottom of the tube. Note that the nuclei pellet obtained at this stage is colorless.

3.5. Discard the filter and resuspend the pellet by vortexing vigorously for 10 s.

3.6. Pellet the nuclei by centrifuging the solution at 500 x *g* for 3 min. Discard the supernatant carefully as the nuclei pellet is colorless.

3.7. Resuspend the pellet in 0.8 mL of cold buffer B and centrifuge at 600 x *g* for 10 min. In this step, nuclei are separated from membrane debris. The colorless pellet obtained contains isolated nuclei.

3.8. Resuspend the isolated nuclei in 500 µL of PBS with 5% BSA. Keep the nuclei suspension on ice to perform FACS after the quantification.

4. Visualization of nuclei morphology

4.1. Confirm nuclear morphology by Hoechst staining. For this, remove 100 µL of single nuclei suspension in a new tube using BSA coated tips. To stain the nuclei, add 0.1 µL of Hoechst (1 mg/mL). Gently vortex the tube.

4.2. Transfer the nuclei suspension to glass bottom dish for imaging.

4.3. Image the nuclei using a fluorescence microscope with laser excitation settings of ~405 nm (UV) wavelength.

5. FACS based enrichment of nuclei

5.1. Before performing FACS, filter the nuclei using a 40 µm cell strainer into BSA coated tube.

5.2. Dilute the filtered suspension by adding PBS with 5% BSA to a final volume of 1000 mL.

5.3. Label two round bottom FACS tube as 'control' and 'stained'. The 'control' tube will contain un-stained nuclei, while the 'stained' tube will have Hoechst stained nuclei.

5.4. Transfer 250 µL of the nuclei suspension into 'control' tube using BSA coated pipette tip.

5.5. Transfer the remaining 750 µL solution to FACS tube labeled as 'stained' and add 1 µL of Hoechst dye to stain the nuclei. Mix by slow vortexing.

5.6. Load the unstained control sample to cell sorter. Record 5000 events.

5.7. Load the stained samples and record 5000 events.

5.8. Draw FACS gates that allows identification of single nuclei. Nuclei can be selected by comparing the Hoechst fluorescence signal between control and stained sample.

5.9. Sort Hoechst-positive nuclei from the stained tube into new 1.5 mL tube containing 50 µL of PBS with 5% BSA.

NOTE: Isolated nuclei can be collected into a desired medium according to requirements of the downstream application.

REPRESENTATIVE RESULTS:

The protocol described above was used to generate single nucleus suspension directly from

zebrafish brain tissue. The isolation typically requires 20 minutes and avoid the use of detergent or digestive enzyme. A schematic summarizing the individual steps of the protocol is provided in **Figure 1**, which can be printed to be used for guidance.

[Place **Figure 1** here]

Visualization of nuclear morphology

For qualitative confirmation of the nuclear morphology, the isolated nuclei were stained with Hoechst and visualized using fluorescence microscopy. The nuclei appeared intact, round and well-separated (**Figure 2**). Importantly, nuclear aggregation, a sign of nuclear membrane rupture, was absent.

[Place **Figure 2** here]

FACS-based enrichment of the intact nuclei

Enrichment of isolated nuclei and removal of cellular debris was performed by flow cytometry by gating on the presence of a Hoechst fluorescence signal. The Hoechst signal was detected upon excitation with violet, 405 nm, laser (Brilliant Violet 421 – BV421). Unstained nuclei displayed background fluorescence (**Figure 3A, Supplementary Figure 1A**), while stained nuclei exhibited strong fluorescent signal (**Figure 3B, Supplementary Figure 1B**). As illustrated in **Figure 3C**, the unstained and Hoechst stained nuclei were well segregated in the violet channel.

[Place **Figure 3** here]

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic of detergent-free spin-column-based method for nuclei isolation. Graphical representation of the individual steps performed during extraction of nuclei from fresh zebrafish brain tissue.

Figure 2. Single nuclei isolation from zebrafish brain. Fluorescence microscopy image of Hoechst-stained nuclei demonstrating their intact morphology. Scale bar: 10 µm.

Figure 3. Isolated nuclei display strong and specific Hoechst fluorescent signal in flow cytometry. Histogram plots for single nuclei suspension displaying the distribution of Hoechst staining. Hoechst is excited by violet, 405 nm, laser (Brilliant Violet 421 – BV421). The unstained sample (**A**) displays signal in the range of 10^0-10^3, while Hoechst stained nuclei (**B**) emit signal in the 10^3-10^5 range. An overlay of fluorescence intensity emitted by unstained (grey) and stained (blue) samples (**C**) demonstrates clear separation between the two populations.

Supplementary Figure 1: Flow cytometry gating strategy for isolated nuclei. Representative flow plots for isolated nuclei suspension. Isolated nuclei were analyzed using forward scatter and UV laser BV421 which excites Hoechst at 405 nm. The unstained sample (**A**) displayed BV421 signal in the 10^0-10^3 range. Out of 13130 events, 141 events were detected as single nuclei based on

FSC-A (1.07% of total), and 0 events for unstained nuclei based on BV421 signal (0% of total). The Hoechst stained nuclei (**B**) displayed BV421 signal in the 10^3-10^5 range. Out of 50000 events, 2418 events were detected as single nuclei based on FSC-A (4.84% of total), and 2414 events for Hoechst-positive nuclei based on BV421 signal (4.83% of total).

DISCUSSION:

Profiling the transcriptome and epigenome at a single-cell resolution has revolutionized the study of biological systems. Studies at the resolution of a single cell for a solid tissue depend on dissociation of the organ into individual cells or nuclei. Dissociation is a destructive procedure that can introduce technical artifacts, which can prevent development of an accurate representation of the system[5, 6]. For instance, enzymatic dissociation can harm cells with complex morphologies, such as neurons or podocytes, and can induce expression of stress and heat-shock response genes[7,12]. Additionally, use of detergent during dissociation can rupture the nuclear membrane and lead to aggregation[23,25]. Thus, optimizing the dissociation to obtain a single cell or nuclei suspension of the highest quality is paramount to the success of high-throughput profiling experiments.

Here, we demonstrate a detergent and enzyme-free nuclei isolation method that allows extraction of intact nuclei from zebrafish brain in less than 20 minutes. The protocol yields nuclei with typical morphology and robust integrity (**Figure 2**). From a single zebrafish brain weighing 6 mg, the protocol yields a total of 60,000 nuclei determined by a hemocytometer count. The isolated nuclei can be utilized for multiple downstream applications, including snRNA-seq, ATAC-seq and immunostaining. The isolated nuclei may include cross-contamination from cytoplasmic fractions, particularly from components of endoplasmic reticulum and mitochondria. For high-throughput profiling experiments, clearance of cellular debris, particularly mitochondria, is strongly recommended. Flow cytometry (**Figure 3**) provides a viable option for purification of nuclei. Alternatively, the sucrose gradient can also be utilized for removal of debris.

The protocol has been tested on mouse thyroid gland (data not shown) and provides results similar to zebrafish brain tissue. Overall, the protocol provides a robust, reproducible, and universal method for preparation of single nucleus suspension, helping to simplify logistics for high-throughput profiling experiments.

ACKNOWLEDGMENTS:

We thank members of the Dr. Sabine Costagliola and Singh lab for comments on the manuscript. This work was supported by the Fonds de la Recherche Scientifique-FNRS under Grant number 34772792 – MISU to S.P.S.

DISCLOSURES:

The authors have no conflict of interest. The payment for making the article open access was made by Invent Biotechnologies Inc., USA.

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Figure 1

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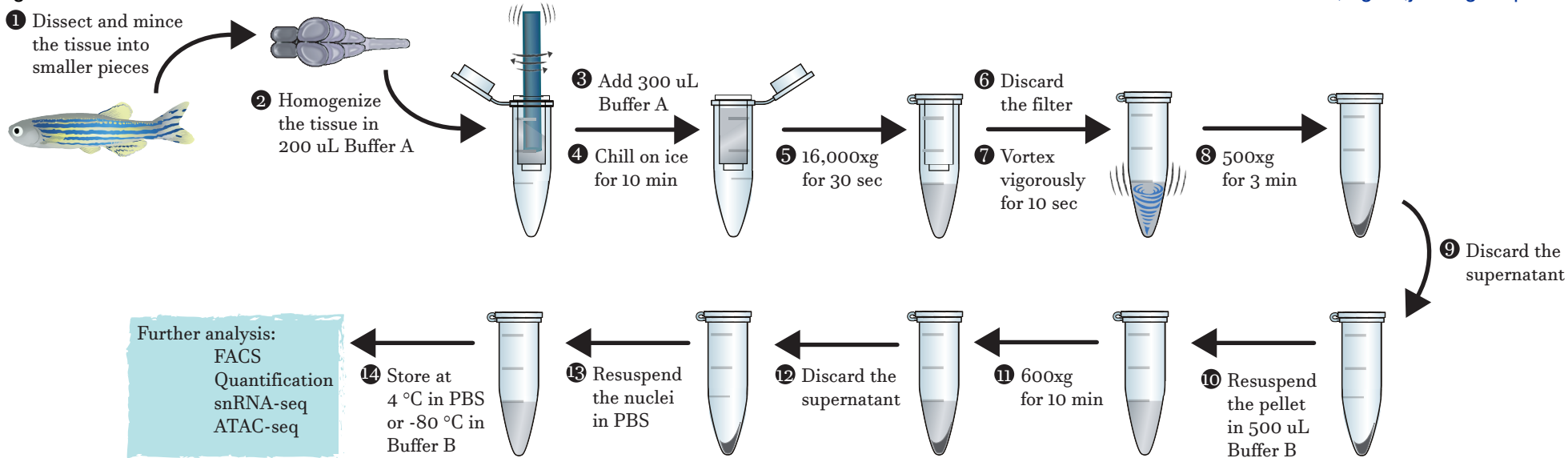

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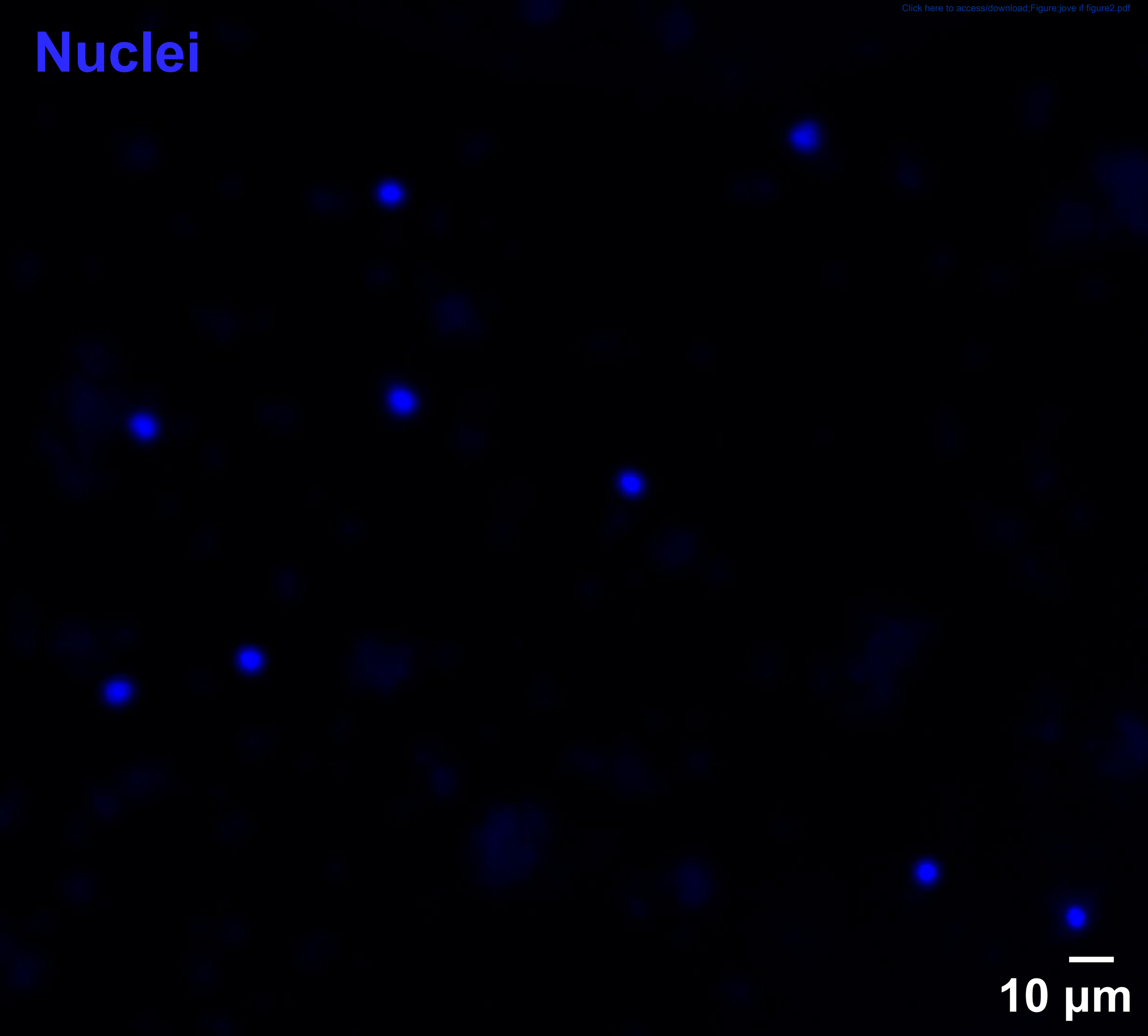

Figure 3

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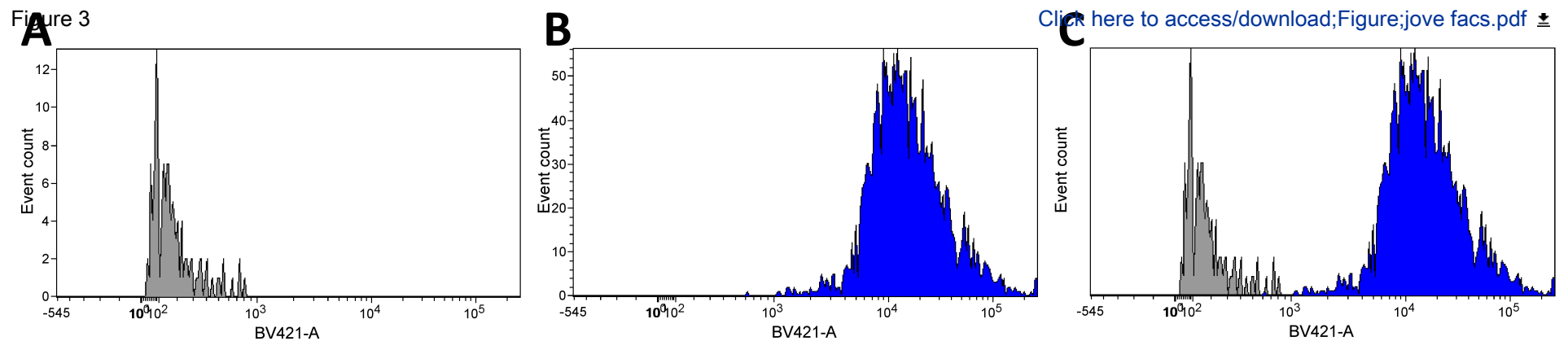

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
Bovine serum albumin (BSA)	Carl Roth	90604-29-8	Albumin fraction V
Cell sorter	BD Biosciences		FACSAria III
Centrifuge	Sartorius	A-14C	
Eppendorf tubes (1.5 mL)	Eppendorf	22363204	
Falcon (15 mL)	Corning	352096	Polypropylene centrifuge tubes
Falcon (5 mL)	Corning	352052	Polystyrene round bottom test tubes
Fine forceps	Fine Science Tools	11295-10	
Flowmi cell strainer (40 μm)	Sigma	BAH136800040	
Fluorescence microscope	Leica	DMI6000 B	
Glass bottle (250 mL)	VWR	215-1593	
Glass bottomed dish	World Precision Instruments	FD3510-100	Fluorodish 35 mm
Glass Pasteur pipettes	VWR	612-1701	
Glass pipette socket	Carl Roth	388.1	Pipetting aid pi-pump 2500
Hoechst staining dye solution	Abcam	ab228551	Hoechst 33342
Minute Detergent-Free Nuclei Isolation Kit	Invent Biotechnologies	NI-024	
PBS (10X)	ThermoFisher	70011069	
Petri dish (30 mm)	FisherScientific	11333704	Pyrex
Petri dish (90 mm)	Corning	758-10178-CS	Gosselin
Pipette tips	VWR	89079	10 µL, 200 µL, 1000 µL
Pipettes	Gilson	F167380	Pipetman
Razor blade	Swann-Morton	7981809	
Tricaine methane sulfonate	Sigma	E10521	
Vortex machine	Scientific Industries	SI-0236	Vortex-Genie 2

Rebuttal Letter
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Response to Reviewers:

We would like to thank the reviewers for taking the time to provide us with insightful and constructive comments, which helped us in improving the manuscript. We have performed an additional experiment and improved the text that we hope satisfies the reviewers.

Please find below our point-by-point response (in green).

Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
The manuscript entitled "NUCLEI ISOLATION FROM WHOLE TISSUE USING A DETERGENT AND ENZYME-FREE METHOD" by Sema Elif Eski et al. describes a detergent and enzyme-free protocol for rapid isolation of intact nuclei directly from whole tissue. They provide a stepwise protocol that can provide intact nuclei. The manuscript written clear and it is valuable for the field. However, the differences between frozen vs fresh tissue is not clear. It would be helpful to mention differences between frozen vs fresh tissue by comparing number of detected genes in read depth normalized single nuclei transcriptomes similar to Slyper M. et al 2020.

The authors thank the reviewer #1 for acknowledging the clarity of the manuscript and its contribution to the field. We agree that we did not provide information or comparison related to the sequencing data obtained from the protocol. We restricted our description to the protocol for isolation of nuclei, as a visual description of this process is central to the JoVE video. Detailed description of RNA-Seq. dataset and comparison(s) would be the scope of a separate manuscript.

Major Concerns:
none

Minor Concerns:
1- Gating strategy is not clear. Please show each gate in a step wise manner.

We have utilized Forward scatter and UV laser as gating strategy. This information is now included as Supplementary Fig. 1. (Please note that the utilization of side scatter and forward scatter in isolation did not clearly segregate nuclei from debris. Thus, we needed to gate using Hoechst signal in the stained samples, and back-track to draw the corresponding gate in the control samples).

2- Please take out the sentence "Isolated nuclei can be stored at 4 °C for 2 days without change in the morphology. However, the authors have not tested the impact of prolonged storage on RNA or chromatin quality."
Our experience suggests that time between dissociation and sorting is inversely correlated with number of detected genes. If not backed up by data this sentence is not helpful.

We agree with the reviewer that RNA quality is reduced with time. We have removed the line.

3- Please provide more details on which dounce homogenizer and the procedure.

Our protocol does not utilize dounce homogenizer. Instead, it uses a pestle to grind the tissue to small size to improve its passage through the spin column. The pestle is part of the kit and works to reduce the tissue size within the spin-column itself. We have edited this in protocol 2.7 to remove the word 'homogenization' to avoid the confusion.

4- Without detailed benchmarking authors should avoid mentioning applicability of the protocol in mouse or any fresh or frozen soft tissue. This comment should either backed up with data or should be removed

We completely agree with the reviewer and have removed the line 'The performance is expected to be comparable for any fresh or frozen soft tissue' from the discussion section. We have tested the performance of the kit on mouse thyroid gland. However, as the protocol is exactly similar to zebrafish brain in terms of nuclei isolation and sample preparation for 10x sequencing, we did not include the repeated information in the text. As such we have mentioned 'mouse thyroid gland (data not shown)'. A manuscript with the data is currently under preparation.

Reviewer #2:
Manuscript Summary:
The research article titled "NUCLEI ISOLATION FROM WHOLE TISSUE USING A DETERGENT AND ENZYME-FREE METHOD" talks about the isolation of intact nuclei from various tissues to further use these for single cell high throughput transcriptome profiling.

Major Concerns:
1. My biggest concern is about the focus on a single kit "Minute 115 TM Detergent-Free Nuclei Isolation Kit (Invent 116 Biotechnologies, Plymouth, USA)" already available in the market and known to successfully isolate intact nuclei. I do not see any refinement of the steps suggested by the manufacturer or a method tweaked and optimized by the authors. Also, nowhere in the manuscript, it mentions the mechanism by which the kit works. Thus, the reader may gain only incremental value from this work.

We understand the reviewer's concern. However, the main aim of the manuscript is to make the scientific community aware of the possibility to extract nuclei without detergent or homogenization. Such protocol is not currently published in JoVE library and is also not used in single-nuclei benchmarking studies. Thus, we believe the possibility to extract nuclei without the use of detergent provides a useful alternative.

We are unable to provide details on the synthesis of the column as this information is not available to us. However, in our hands the column-based method has provided reproducible results. Thus, we focused on this method in the current manuscript.

2. The authors mention that enrichment of nuclei and removal of debris has been done by using flow cytometer and intact nuclei are shown with Hoechst stain. However, to show the removal of debris and other cytoplasmic contamination from the nuclei, the author should use one of the biochemical techniques i.e, western blotting for nuclei and cytoplasm specific proteins. Cross contamination by cytoplasmic proteins is the nuclear fraction and vice versa is often seen. This manuscript does not check for this explicitly.

We completely agree with the concern raised by the reviewer. There is a possibility for cross-contamination by the cytoplasmic fraction. In-fact, mRNA present within (or bound to) endoplasmic reticulum could be captured. We are unable to test this explicitly as our lab does not have the expertise on the topic. Hence, we have added the following line in the discussion section, 'The isolated nuclei may include cross-contamination from cytoplasmic fractions, particularly from components of endoplasmic reticulum and mitochondria.'.

3. The authors have not mentioned the number of nuclei obtained from the total number of cells taken as a starting material. It is a very useful information required to calculate the initial amount when a certain number of nuclei are required and should be mentioned in the manuscript.

We agree with the reviewer that this is a critical piece of information. We conducted an additional experiment in which we weighed the mass of isolated zebrafish brain and prepared nuclei isolate from the same, which was quantified. From a single zebrafish brain weighing 6 mg, we could isolate 60,000 nuclei. This information has been added to the text as 'From a single zebrafish brain weighing 6 mg, the protocol yields a total of 60,000 nuclei determined by hemocytometer count.'.

Minor Concerns:
snRNAseq is suggested as a "substitute' for scRNAseq. This statement needs to be qualified, in certain conditions, snRNAseq can act as an acceptable alternative.

We completely agree with the reviewer. In-fact, after the submission of the manuscript a pre-print was posted on bioRxiv demonstrating that snRNAseq could not detect microglial activation in human tissues (Thrupp et al., bioRxiv, 10.1101/2020.04.13.035386). We have cited this pre-print and edited the following text,
"Intriguingly, comparative studies between single-cell and single-nucleus RNA-seq have suggested a decrease in induction of stress and heat-shock response genes with snRNA-Seq[12]. The studies further suggest a high correlation between the genes detected by the two methods. However, a recent study on human microglia failed to detect genetic activation in Alzheimer's disease[15]. Thus, in certain contexts, snRNA-Seq. is a suitable alternative for scRNA-Seq[16,17]."

Supplemental File (Figures, Permissions, etc.)

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