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

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Abstract

High-throughput transcriptome and epigenome profiling requires preparation of a single cell or single nuclei suspension. Preparation of the suspension with 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[1,2,3,4]. 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[8,9,10,11]. 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[18,19,20,21,22]. 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. Prepare 0.2% Tricaine solution in PBS for euthanizing the zebrafish. Chill the solution on ice.
2. Prepare a 30 mm Petri dish for mincing the tissue.
3. Prepare ice cold 1x PBS (10 mL per tissue sample).
4. Cool the centrifuge to 4 °C.
5. For nuclei isolation, use a detergent-free nuclei isolation kit (**Table of Materials**).
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.
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.
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.
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

1. For euthanizing the zebrafish, prepare a 90 mm Petri dish with 25 mL of ice-cold Tricaine solution.
2. Carefully, take the zebrafish from the tank using a fishing net and place it into the Petri dish.
3. Euthanize the fish by leaving it in Tricaine for 5 min.
4. Decapitate the animal with a sharp razor blade.
5. Using forceps, gently break open the skull and remove soft tissues, skin and bones from ventral and dorsal side of the skull.
6. Gently, transfer the brain into a fresh 30 mm dish containing ice cold PBS.
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

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.
2. Add 300 µL of cold buffer A and incubate the filter cartridge on ice with cap open for 10 min.
3. Cap the cartridge and resuspend the tissue by inverting the tube 5 times.
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.
5. Discard the filter and resuspend the pellet by vortexing vigorously for 10 s.

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.
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.
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

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.
2. Transfer the nuclei suspension to glass bottom dish for imaging.
3. Image the nuclei using a fluorescence microscope with laser excitation settings of ~405 nm (Violet) wavelength.

5. FACS based enrichment of nuclei

1. Before performing FACS, filter the nuclei using a 40 µm cell strainer into BSA coated tube.
2. Dilute the filtered suspension by adding PBS with 5% BSA to a final volume of 1,000 µL.
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.
4. Transfer 250 µL of the nuclei suspension into ‘control’ tube using BSA coated pipette tip.
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.
6. Load the unstained control sample to cell sorter. Record 5000 events.
7. Load the stained samples and record 5000 events.
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.
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.

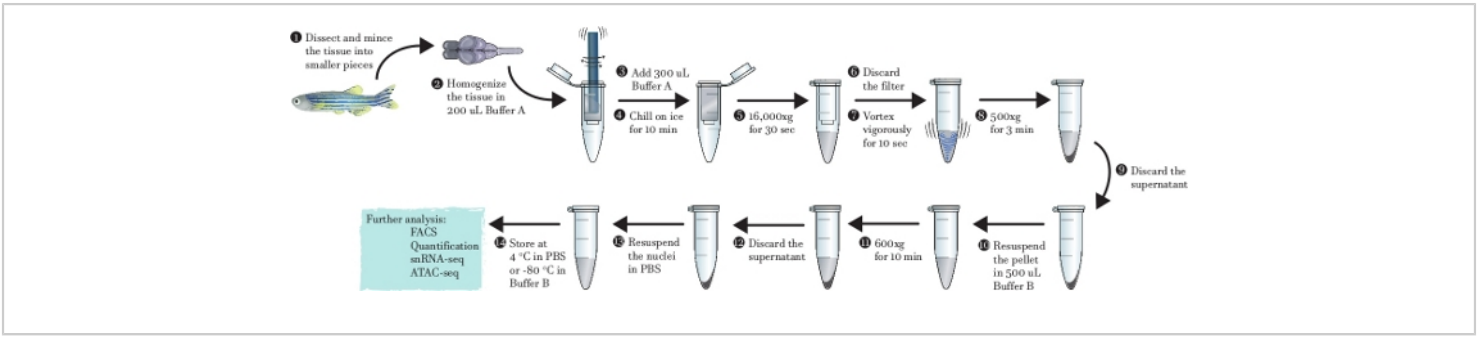


Figure 1: Schematic of detergent-free spin-column-based method for nuclei isolation.

Graphical representation of the individual steps performed during extraction of nuclei from fresh zebrafish brain tissue.

Please click here to view a larger version of this figure.

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.

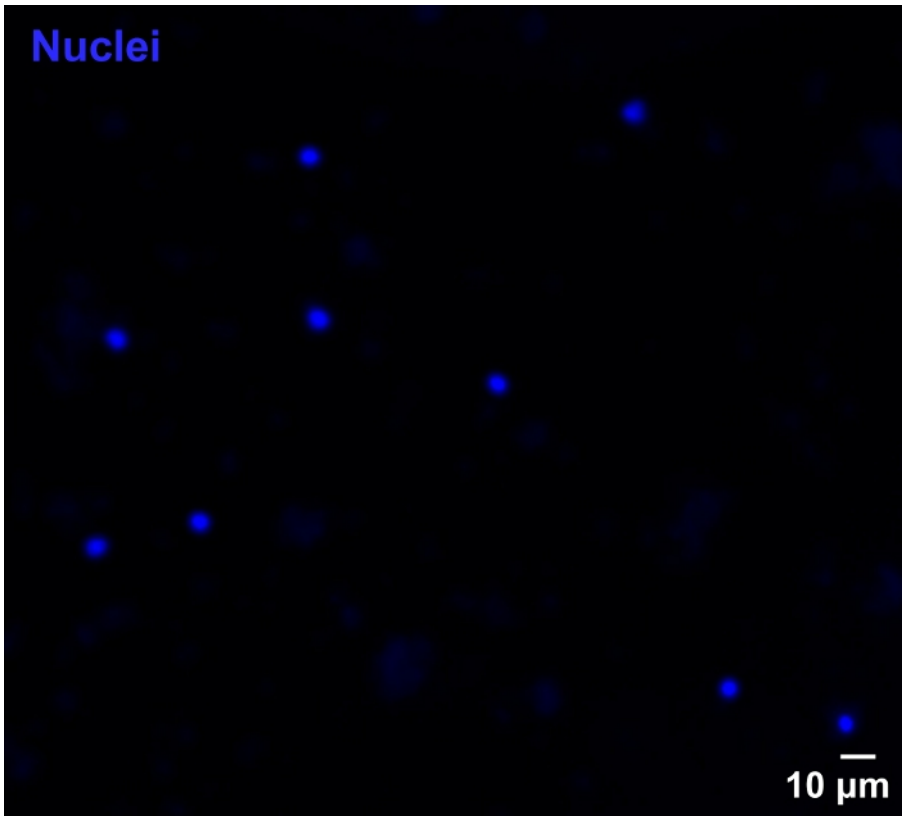


Figure 2: Single nuclei isolation from zebrafish brain.

Fluorescence microscopy image of Hoechst-stained nuclei demonstrating their intact morphology. Scale bar: 10 µm. Please click here to view a larger version of this figure.

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.

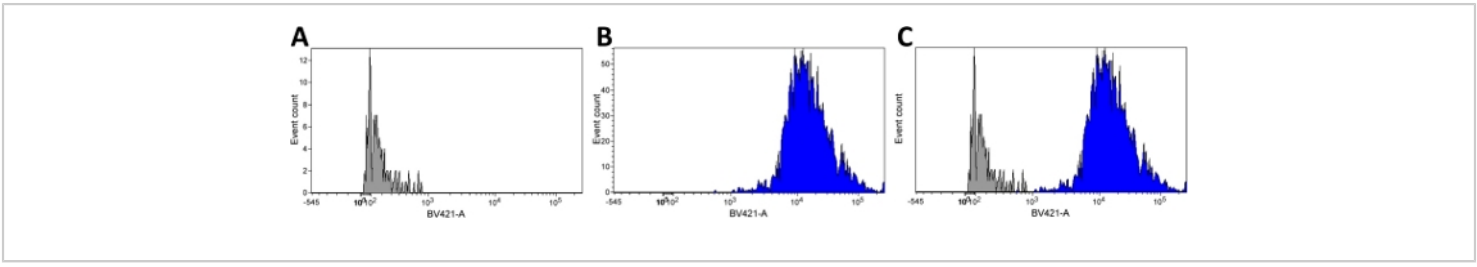
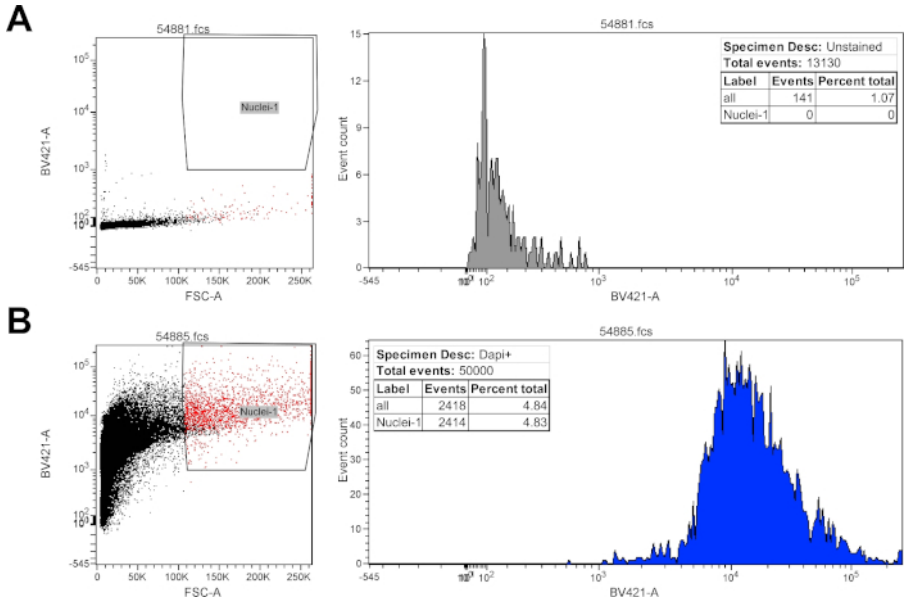


Figure 3: Isolated nuclei display strong and specific Hoechst fluorescent signal in flow cytometry.

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. Please click here to view a larger version of this figure.



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 Violet 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). Please click here to download this figure.

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.

Disclosures

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