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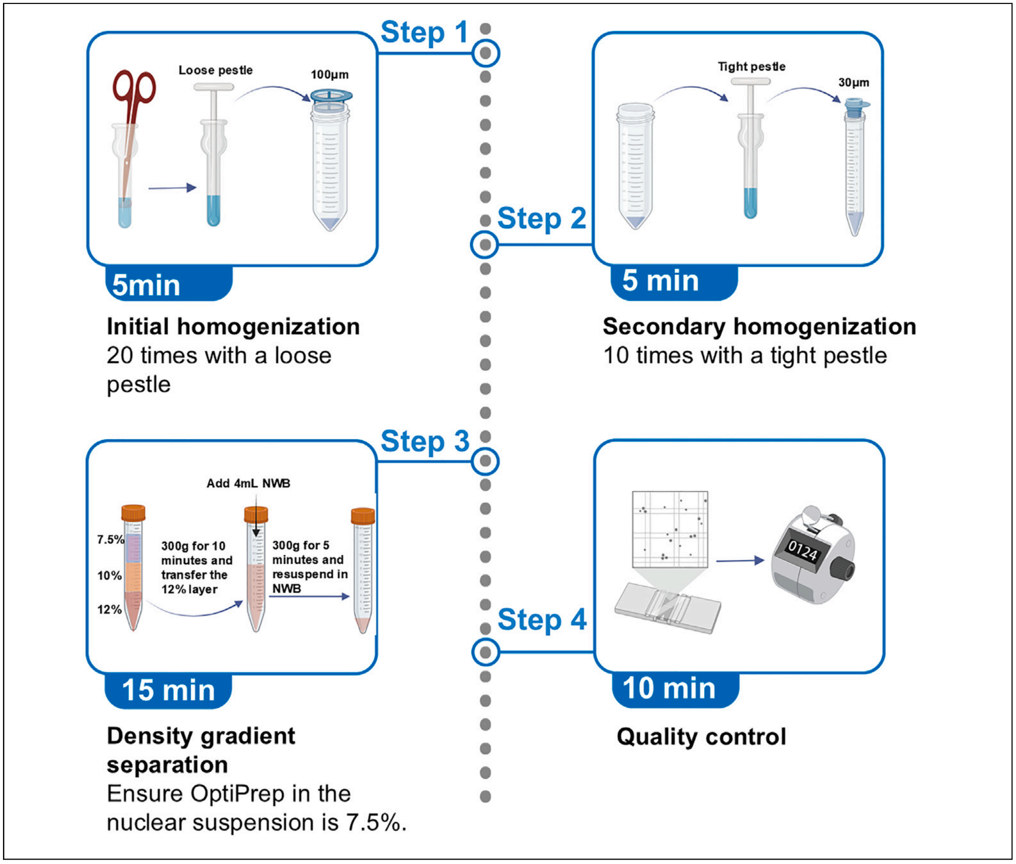
Protocol for isolating nuclei from frozen fish heart tissue for single-cell genomic assays



Chaoguang Wei, Ruonan Jia, Adelino V.M. Canário, Liangbiao Chen, Qianghua Xu, Peng Hu

qhxu@shou.edu.cn (Q.X.)
phu@shou.edu.cn (P.H.)

Highlights

Guidance on isolating single nuclei for cardiac snRNA-seq profiling

Using double filtration to reduce the amount of debris in nuclear isolation

Optimized OptiPrep gradient for removing tiny debris in the homogenate

Unbiased isolation of intact single nuclei from complex tissues, such as cryopreserved hearts, for massively parallel single-cell genomic assays is particularly challenging. Here, we present a protocol for isolating high-quality nuclei from the heart tissue of *Trematomus bernacchii*, an Antarctic fish species with notoriously challenging tissue for nuclear extraction. We describe steps for pulverizing frozen heart tissue, library construction, and sequencing. This protocol can provide a valuable reference for isolating nuclei from other cryopreserved marine fish tissues.

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Protocol for isolating nuclei from frozen fish heart tissue for single-cell genomic assays

Chaoguang Wei,[1,2,5] Ruonan Jia,[3,5] Adelino V.M. Canário,[2,4] Liangbiao Chen,[1,2] Qianghua Xu,[3,*] and Peng Hu[1,2,6,7,*]

[1]Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Ministry of Education, Shanghai Ocean University, Shanghai 201306, China

[2]International Research Center for Marine Biosciences, Ministry of Science and Technology, Shanghai Ocean University, Shanghai 201306, China

[3]Key Laboratory of Sustainable Exploitation of Oceanic Fisheries Resources, Ministry of Education, College of Marine Living Resource Sciences and Management, Shanghai Ocean University, Shanghai 201306, China

[4]Algarve Centre for Marine Sciences (CCMAR/CIMAR), Gambelas Campus, University of Algarve, 8005-139 Faro, Portugal

[5]These authors contributed equally

[6]Technical contact

[7]Lead contact

*Correspondence: qhxu@shou.edu.cn (Q.X.), phu@shou.edu.cn (P.H.)
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SUMMARY

Unbiased isolation of intact single nuclei from complex tissues, such as cryopreserved hearts, for massively parallel single-cell genomic assays is particularly challenging. Here, we present a protocol for isolating high-quality nuclei from the heart tissue of *Trematomus bernacchii*, an Antarctic fish species with notoriously challenging tissue for nuclear extraction. We describe steps for pulverizing frozen heart tissue, library construction, and sequencing. This protocol can provide a valuable reference for isolating nuclei from other cryopreserved marine fish tissues.

BEFORE YOU BEGIN

Institutional permissions

All handling of fish was conducted in accordance with the guidelines for the care and use of animals for scientific purposes, as set by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Ocean University (SHOU), Shanghai, China. This research was approved by IACUC (IACUC SHOU-DW-2012-002, SHOU-DW-20171022) of SHOU.

Preparation before experiment

Timing: ∼15–30 min

This protocol outlines an optimized strategy for isolating high-quality nuclei from frozen heart tissue of *T. bernacchii*. Prior to starting the nucleus isolation, please ensure the following:

1. Clean all equipment.
   a. Clean scissors, tweezers, and two sets of dounce homogenizers with RNaseZap.
   b. Rinse thoroughly with DEPC water to eliminate RNase contamination.

⚠ CRITICAL: Ensure all equipment is cleaned to avoid RNase contamination, which could interfere with subsequent experiments.

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2. Prepare reagents.
    a. Prepare 1 M $MgCl_2 \cdot 6H_2O$.
    b. Prepare 1 M Tricine-NaOH.
    c. Prepare 10% IGEPAL CA-630.
    d. Filter all buffers through a 0.22 μm syringe filter to ensure sterility and minimize contamination.
    e. Use RNase-free water for all reagent preparation.

    Note: Preparing 5 M NaCl and 1M $MgCl_2$ using RNase-free water can fully substitute the standard commercial reagents listed in the key resources table. IGEPAL CA-630 is a substitute for Nonidet P-40, the usage concentration of Nonidet P-40 may need to be adjusted in certain experiments.

3. Prepare tissue.
    a. Tissue should either be freshly processed or flash-frozen in liquid nitrogen for 2 min.
    b. Store the tissue at −80°C for long-term storage.
4. Process tissue.
    a. When ready to proceed, remove the tissue from the −80°C freezer.
    b. Using a surgical scalpel, cut the tissue into 40 mg sections on dry ice to maintain tissue integrity.
    c. Store the sections in an ultra-low temperature freezer or on dry ice for subsequent use.

    Note: Sample preparation for *T. bernacchii* (sample number 2017123101) includes the collection of heart tissue and storage at −80°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
T. bernacchii	Antarctica (74°55′S, 163°46′E)	Ross Sea
Critical commercial assays		
BSA	Merck	A8806-5g
Glycerol	BBI	130502
NxGen RNase inhibitor	Lucigen	30281–1
DPBS	BBI	E607009-0500
1 M Tri-HCl (pH 7.5)	Invitrogen	15567–027
Invitrogen 5 M NaCl, RNase-free	Thermo Fisher Scientific	AM9759
Invitrogen 1 M $MgCl_2$, RNase-free	Thermo Fisher Scientific	M1028-100ML
RNaseZap	Thermo Fisher Scientific	AM9780
Magnesium chloride hexahydrate	BBI	A601336-0500
Sodium chloride	BBI	A501218-0001
NaOH	Shanghai Zhanyun Chemical Co., Ltd	A6002320500
Tricine	Sigma-Aldrich	T5816-100G
60% OptiPrep	Serumwerk Bernburg AG	1893
IGEPAL CA-630	Merck	56741-50ML-F
DAPI	Thermo Fisher Scientific	62248
Other		
Fluorescence microscope	Nexcope	NIB610-FL
Centrifuge	Eppendorf	5810 R
1 mL Douncer	Wheaton	357538
Cell strainer 100 μm Nylon	Falcon	352360
MACS SmartStrainer (30 μm)	Miltenyi	130-098-458
Cell counting chamber	Countstar	C0010101

MATERIALS AND EQUIPMENT

1 M $MgCl_2 \cdot 6H_2O$ (50 mL)

Reagent	Final concentration	Amount
$MgCl_2 \cdot 6H_2O$ (Analytical grade)	1 M	8.415 g
RNase-free water		Up to 50 mL
Store at room temperature for up to 6 months.		

5 M NaCl (50 mL)

Reagent	Final concentration	Amount
NaCl (Analytical grade)	5 M	14.61 g
RNase-free water		Up to 50 mL
Store at room temperature for up to 6 months.		

3 M NaOH (50 mL)

Reagent	Final concentration	Amount
NaOH	3 M	6 g
RNase-free water		Up to 50 mL
Store at room temperature for up to 6 months.		

1 M Tricine-NaOH (50 mL, pH = 7.8)

Reagent	Final concentration	Amount
Tricine	1 M	8.9585 g
3 M NaOH		~7.5 mL
RNase-free water		Up to 50 mL
Store at room temperature for up to 6 months.		

10% IGEPAL CA-630 (10 mL)

Reagent	Final concentration	Amount
IGEPAL CA-630	10%	1 mL
RNase-free water		9 mL
Store at 4°C for up to 6 months.		

10% BSA (10 mL)

Reagent	Final concentration	Amount
BSA	10%	1 g
1× DPBS		Up to 10 mL
Store at −20°C for up to 6 months.		

Nucleus Wash buffer (5 mL)

Reagent	Final concentration	Amount
10% BSA	1%	0.5 mL
Glycerol	0.25%	0.0125 mL
Protector RNase inhibitor	40 units/mL	0.005 mL
1× DPBS		4.4825 mL
Prepare immediately before use.		

Lysis buffer (50 mL)

Reagent	Final concentration	Amount
1 M Tri-HCl (pH 7.5)	10 mM	0.5 mL
5 M NaCl	10 mM	0.1 mL
1 M $MgCl_2$ $6H_2O$	3 mM	0.15 mL
IGEPAL CA-630(10%)	0.05%	0.25 mL
DNase/RNase free H_2O		49 mL

Store at 4°C for up to 2 weeks.

Optiprep diluent buffer (50 mL)

Reagent	Final concentration	Amount
1 M NaCl	150 mM	7.5 mL
1 M $MgCl_2$ $6H_2O$	5 mM	0.25 mL
1 M Tricine-NaOH (pH7.8)	20 mM	1 mL
DNase/RNase free H_2O		41.25 mL

Store at 4°C for up to 6 months.

15% Optiprep (4 mL)

Reagent	Final concentration	Amount
60% Optiprep	15%	1 mL
Optiprep diluent buffer		3 mL

Store at 4°C for up to 2 weeks.

12% Optiprep (5 mL)

Reagent	Final concentration	Amount
60% Optiprep	12%	1 mL
Optiprep diluent buffer		4 mL

Store at 4°C for up to 2 weeks.

10% Optiprep (6 mL)

Reagent	Final concentration	Amount
60% Optiprep	10%	1 mL
Optiprep diluent buffer		5 mL

Store at 4°C for up to 2 weeks.

STEP-BY-STEP METHOD DETAILS

Pulverize frozen heart tissue of Antarctic fish *T. bernacchii*

Timing: ~40 min

To minimize RNA degradation risk, immediately proceed to the next step after each one. All equipment must be pre-cooled, and the procedure should be conducted on ice whenever possible.

1. Douncer cleaning and preparation:
   a. Clean the douncer by washing with 1 mL of 1× Lysis Buffer,[1] then remove the solution.
   b. Add 1 mL of fresh Lysis Buffer to the douncer.

2. Prepare tissue:
   a. Remove the frozen heart tissue (about 40 mg) of *T. bernacchii* from −80°C.
   b. Place the tissue into the douncer.
   c. Cut the tissue into smaller pieces (∼1 min) with straight-point medical scissors (size = 10 cm).
3. Initial homogenization: Dounce the tissue 20 times (∼2 min) with a loose pestle to begin homogenization.
4. Filtration:
   a. Filter the homogenate through a 100 μm cell strainer into a 50 mL centrifuge tube.
   b. Transfer the filtrate to a new douncer.
5. Secondary homogenization: Dounce the mixture 10 times (∼1 min) with a tight pestle.
6. Further filtration and nucleation:
   a. Filter the homogenate through a 30 μm cell strainer into a 15 mL centrifuge tube.
   b. Add 1 mL of pre-cooled Nucleus Wash Buffer (NWB) and mix gently by pipetting.
   c. Obtain approximately 2 mL of solution containing the cell nuclei.
   d. Add 2 mL 15% OptiPrep[2] to 2 mL nuclei solution and mix gently by pipetting.
7. Density gradient separation:
   a. In a new 15 mL centrifuge tube, add 4 mL of 12% OptiPrep.
   b. Slowly add 4 mL of 10% OptiPrep along the tube wall to prevent mixing.
   c. Gently add 4 mL 7.5% OptiPrep nucleated solution (from Step 6) along tube wall, on top of 10% OptiPrep layer, ensuring no mixing.

   ⚠ CRITICAL: In this protocol, the density gradient of the separation medium is crucial for the isolation of nuclei from heart tissue of *Trematomus bernacchii*, and the order of addition should be ensured during the experiment.

   Note: To better observe and absorb the bottom layer of 12% OptiPrep, phenol red (Final concentration: 15 mg/L) can be added as an indicator when preparing the 12% OptiPrep.

   Note: The density of OptiPrep in different layers can be adjusted to isolate nuclei from the heart of other species or from other types of tissue. For example, another commonly used OptiPrep density gradient is 25%, 29%, and 35%.

   d. Centrifuge at 4°C, 300 g, for 10 min using Eppendorf 5810R centrifuge with horizontal rotors A-4-81.

   Note: Swinging bucket centrifuges from other manufacturers can be used here.

8. Aspirate and dilute:
   a. Transfer the bottom layer (∼4 mL) that contains 12% OptiPrep nucleated solution to a new tube (Figure S1A).
   b. Mix 4 mL of the 12% OptiPrep nucleated solution with 4 mL of pre-cooled NWB.
   c. Centrifuge at 300 g for 5 min at 4°C.
   d. Resuspend the pellet in 500 μL of pre-cooled NWB.
9. Nuclear staining and counting:
   a. Take 20 μL of the nuclear suspension.
   b. Add 1 μL of DAPI (1:1000 dilution) and incubate in the dark for 5 min.
   c. Observe and count nuclei under a Fluorescence Microscope.

   ⚠ CRITICAL: Ensure the RNA integrity of the tissue: The integrity of the total RNA extracted from the tissue can be assessed by observing the rRNA bands through 1% agarose gel electrophoresis. For single-cell sequencing library construction using the Chromium Single Cell 3′Library Construction Kit v1, if the expected recovery is 10,000 cells, the cell concentration should be greater than 400 cells/μL, and the volume should be greater than 50 μL. It is recommended that the overall nuclear aggregation rate of nuclei be less than 10%.

Note: Cell counting can be performed using a manual mechanical counter or an automated cell counter.

Library construction

A total of 16,000 nuclei were used for library construction. SnRNA-seq libraries were prepared using the 10× Genomics Chromium Controller and the Chromium Single Cell 3′ Library Construction Kit v1 following the manufacturer's instructions. Here, we briefly describe the experimental steps.

10. GEM generation and barcoding:
    a. Suspended nuclei were loaded onto the Chromium Controller to generate single-cell Gel Beads in Emulsion (GEMs).
    b. Reverse transcription and sample indexing using a C100 Touch Thermal Cycler with a 96-Deep Well Reaction Module (Bio-Rad).
11. 3′ gene expression library construction:
    a. Purify, amplify, fragment, A-tail, and ligate adapters to cDNA according to the manufacturer's instructions.
    b. Perform sample index PCR and double sided size selection to obtain snRNA-seq libraries.

Sequencing

Sequence libraries on the Illumina NovaSeq6000 sequencing platform with PE150 kit.

Note: Browse Illumina's official website to get more technical details. (https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/novaseq/1000000019358_17_novaseq-6000-system-guide.pdf).

EXPECTED OUTCOMES

From 0.4 mg of minced tissue extracted from the frozen hearts of Antarctic fish, we successfully recovered approximately 200,000 cell nuclei after nuclear purification. The grinding solution yielded the expected homogenization (Figures 1A and 1B). The resuspended nuclear solution was free of large and small contaminants, and the nuclei were adequately stained with DAPI (Figures 1C and 1D). Quality control of the library revealed no contamination from large fragments, no dimer contamination, and no small fragment contamination (Figures 2 and S1B).

LIMITATIONS

The described nuclear isolation method requires specific laboratory conditions, such as precise centrifuge speeds and specialized grinding tools, which may limit its applicability to other settings.

TROUBLESHOOTING

Problem 1: Inadequate homogenization of heart tissue (step 5)

The homogenate contains many large tissue fragments, and the cell nuclei have not been fully released (related to Step 5).

Potential solutions

If the tissue does not achieve the desired level of homogenization (Figure 3A), consider the following strategies to optimize the process.

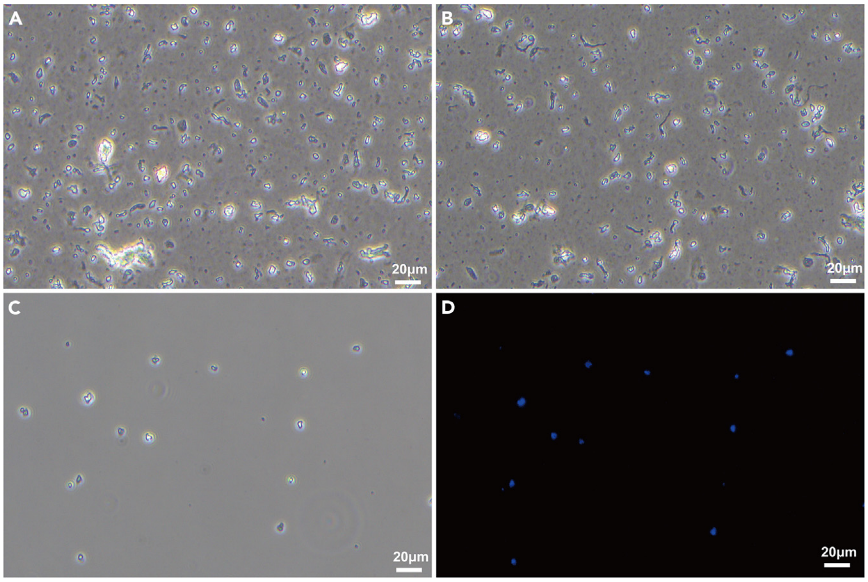

Figure 1. Homogenizations observed under a microscope during the experiment

(A) After dounce homogenization with a loose pestle and filtering through a 100 μm cell strainer, *T. bernacchii* nuclei were visualized by phase-contrast microscopy (40×).

(B) After dounce homogenization with a tight pestle and filtering through a 30 μm cell strainer, *T. bernacchii* nuclei were visualized by phase-contrast microscopy (40×).

(C) After 7.5%,10% and 12% OptiPrep gradient centrifugation, aspirate the 12% OptiPrep solution(~4 mL), then mixed with 4 mL pre-cooled NWB, centrifuge at 300 g for 5 min at 4°C, and resuspend the pellet in 500 μL pre-cooled NWB, *T. bernacchii* nuclei were visualized by phase-contrast microscopy (40×).

(D) After DAPI incubate in the dark for 5 min, *T. bernacchii* nuclei were visualized by Fluorescence Microscope. Scale bar, 20 μm.

- Initial tissue processing: Prior to homogenization, cut the tissue into small, uniform pieces with scissors. This step ensures that the tissue is sufficiently prepared for more efficient grinding during subsequent steps.
- Adjustment of grinding time: Typically, 20 strokes with a loose pestle followed by 10 strokes with a tight pestle are sufficient. For tissues with greater toughness, adjust the number of strokes based on the actual grinding conditions (e.g., The grinding times with the loose pestle can be increased to 30 times).
- Pestle model and force: Different pestle models may impact homogenization efficiency. If the pestle used differs from the recommended model, modify the number of strokes or the force applied during homogenization to achieve optimal tissue breakdown.
- Lysis buffer volume: Ensure that 1 mL of Lysis buffer is used per 40 mg of tissue. Proper volume is crucial for efficient homogenization. Insufficient lysis buffer may hinder the tissue breakdown process.
- Avoid overloading the pestle: Overloading the pestle with too much tissue can hinder effective homogenization. Always ensure that the amount of tissue is within the recommended capacity for the pestle.
- Optimization of grinding conditions: Depending on the observed results, further adjustments may be needed. These can include modifying the number of strokes, the applied force, or the amount of lysis buffer to achieve better homogenization.

Problem 2: Low nuclei yield (step 7)

Insufficient centrifugal force or time prevents cell nuclei pelleting at the bottom of the centrifuge tube (related to Step 7).

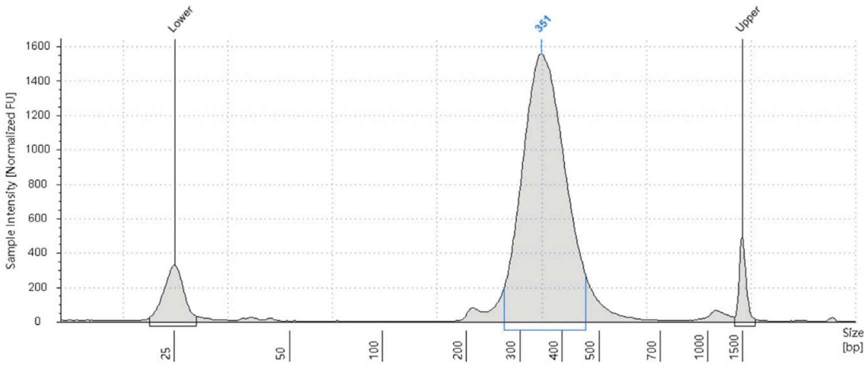

Figure 2. Following the library construction with the 10× Genomics Chromium Controller and the Chromium Single Cell 3′ Library Construction Kit v.1, the bioanalyzer electropherogram of the 3′ library displayed a peak indicating that the library was of high quality and ready for further sequencing

Potential solution

If the number of isolated nuclei is lower than expected (Figure 3B), variations in centrifuge performance may be a contributing factor. We recommend adjusting centrifuge speed based on the model and manufacturer's specifications. In our protocol, a speed of 300 g for 10 min is optimal for Antarctic fish heart tissue. However, adjusting centrifuge parameters such as speed or time may be necessary if using a different centrifuge model.

Problem 3: Nuclei aggregation during centrifugation (step 8)

Poor sample quality or excessive centrifugal force can cause nuclear fragmentation (related to Step 8).

Potential solutions

If nuclei aggregation occurs when centrifuging the mixture of 12% OptiPrep nuclear isolation solution and NWB (Figure 3C), try the following adjustments.

- Use high-quality samples: When collecting tissue samples, quench them with liquid nitrogen, and do not store them for more than 1 year (in fact, the samples in this protocol were over 5 years old). When cutting the samples, keep them on dry ice and pre-chill the tools used for cutting (forceps and scalpels).

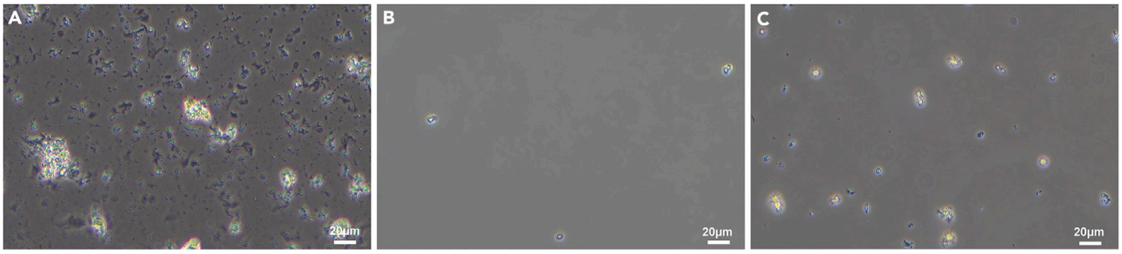

Figure 3. Problems may occur in this protocol

(A) After homogenization with a loose pestle and a tight pestle, and filtering through 100 um and 30 um cell strainers, the nuclear homogenate from frozen Antarctic fish heart tissue achieved inadequate homogenization.

(B) After separation using the OptiPrep density gradient method, the number of cell nuclei in the 12% OptiPrep nuclear fraction was found to be lower than anticipated.

(C) After centrifugation of the mixture containing 12% OptiPrep nuclear isolation solution and pre-cooled NWB, and resuspension with pre-cooled NWB, nuclear aggregation occurred. Scale bar, 20 μm.

- Adjust centrifugation conditions: Centrifugation parameters may vary depending on the brand or model of centrifuge. For Antarctic fish heart tissue, a speed of 300 g for 5 min is effective, but you may need to adjust the centrifuge speed and time depending on your equipment.
- Optimize the isolation solution: Increasing the concentration of BSA in the 12% nuclear isolation solution may reduce nuclei aggregation. However, ensure that the BSA concentration does not exceed 2% to avoid other potential complications.

RESOURCE AVAILABILITY

Lead contact

For further information or requests related to resources and reagents, please contact the lead investigator, Peng Hu (phu@shou.edu.cn), who will be responsible for fulfilling these inquiries.

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Peng Hu (phu@shou.edu.cn).

Materials availability

This study did not generate any new or unique reagents.

Data and code availability

The data generated in this study are included in the manuscript. For additional information or inquiries, please contact the corresponding author.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

P.H. and Q.X. conceived and supervised the study. C.W. and R.J. analyzed the data and wrote the initial draft. A.V.M.C. and L.C. helped with the revision and figure illustration. P.H. and Q.X. edited and finalized the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2025.103765.

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