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Highlights

Optimized protocol allows isolation of nuclei from all adipose tissue cell types

High-quality nuclei can be isolated from fresh or snap-frozen adipose tissues

Nuclei are suitable for single-nucleus sequencing-based technologies

The protocol enables adipose tissue characterization at single-nucleus resolution

Lipid-filled adipocytes are incompatible with droplet-based single-cell methods, such as 10x Genomics-based technology, thus restricting droplet-based single-cell analyses of adipose tissues to the stromal vascular fraction. To overcome this limitation and obtain cellular and molecular insight into adipose tissue composition and plasticity, single-nucleus sequencing-based technologies can be applied. Here, we provide an optimized protocol for nuclei isolation from mouse adipose tissues suitable for single-nucleus RNA sequencing. This allows for transcriptomic profiling of the entire adipose tissue at single-cell resolution.

Protocol

Isolation of nuclei from mouse white adipose tissues for single-nucleus genomics

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SUMMARY

Lipid-filled adipocytes are incompatible with droplet-based single-cell methods, such as 10x Genomics-based technology, thus restricting droplet-based single-cell analyses of adipose tissues to the stromal vascular fraction. To overcome this limitation and obtain cellular and molecular insight into adipose tissue composition and plasticity, single-nucleus sequencing-based technologies can be applied. Here, we provide an optimized protocol for nuclei isolation from mouse adipose tissues suitable for single-nucleus RNA sequencing. This allows for transcriptomic profiling of the entire adipose tissue at single-cell resolution. For complete details on the use of this protocol, please refer to Sárvári et al., 2021.

BEFORE YOU BEGIN

This protocol describes the steps to facilitate nuclei release and isolation from mouse white adipose tissues (WAT). It was primarily optimized using snap-frozen gonadal WAT isolated from lean 7–16 weeks old mice of the C57BL/6J background. However, we obtain similar yields from inguinal tissue and fresh tissues. Furthemore, the protocol can also be used for WAT from other mouse strains and from mice with HFD-induced obesity. However, tissues from very old mice (>1 year of age) might be fibrotic, which negatively impacts nuclei yield and may require adjustment of the protocol.

Note: This protocol is intended for WAT from a single mouse; however, in cases where the depots are small, pooling of tissues from two mice may be needed.

General preparation

⏱ Timing: 1 h

1. Clean Dounce homogenizers and pre-cool them on ice.

   △ CRITICAL: If nuclei are to be used for transcriptomics, rinse Dounce homogenizers with RNaseZAP and DEPC-treated water to minimize RNase activity.

2. Pre-cool centrifuges to 4°C.
3. Pre-cool tubes for nuclei isolation and Petri dishes for tissue mincing on ice.

Note: For each isolation, prepare 1× 50 mL tube, 2× 5 mL DNA low binding tubes, 2× 1.5 mL DNA low binding tubes, and 1× Petri dish.

4. Prepare nuclei isolation buffer (NIB) and nuclei resuspension buffer (NRB).
   a. Filter buffers using a 0.2 μm syringe filter and pre-cool buffers on ice.

   Note: Buffers are prepared freshly in order to minimize RNase activity.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES)	Lonza	Cat#BE17-737E; CAS: 7365-45-9
Bovine Serum Albumin	Sigma-Aldrich	Cat#B6917; CAS: 9048-46-8
Diethyl dicarbonate (DEPC)	Sigma-Aldrich	Cat#D5758; CAS: 1609-47-8
DL-Dithiothreitol (DTT)	New England Biolabs	Cat#B1034A; CAS: 3483-12-3
IGEPAL CA-630	Sigma-Aldrich	Cat#I8896; CAS: 9002-93-1
Magnesium chloride (MgCl$_2$)	Sigma-Aldrich	Cat#M1028; CAS: 7786-30-3
Phosphate buffered saline (PBS) (10×, pH 7.2)	Gibco	Cat#70013-016
Potassium chloride (KCl)	Sigma-Aldrich	Cat#P9541; CAS: 7447-40-7
RNase Inhibitor, Murine	New England Biolabs	Cat#M0314
RNaseZAP	Sigma-Aldrich	Cat#R2020
Sucrose	Sigma-Aldrich	Cat#S0389; CAS: 57-50-1
Trypan Blue	Bio-Rad	Cat#1450013
Biological samples		
Mouse white adipose tissues	N/A	N/A
Critical commercial assays		
Chromium Next GEM Chip G Single Cell Kit (48 rxns)	10x Genomics	Cat#1000120
Chromium Next GEM Single Cell 3' Kit v3.1 (16 rxns)	10x Genomics	Cat#1000268
Dual Index Kit TT Set A (for Gene Expression Libraries)	10x Genomics	Cat#1000215
Experimental models: organisms/strains		
Mus musculus C57BL/6J and C57BL/6N	N/A	N/A
Other		
0.2 μm Syringe filters	Sartorius	Cat#17845-ACK
1.5 mL DNA LoBind tubes	Eppendorf	Cat#0030108051
2 mL Dounce homogenizer	Sigma-Aldrich	Cat#D8938
5 mL DNA LoBind tubes	Eppendorf	Cat#0030122348
6 cm Petri dish	Thermo Scientific	Cat#150288
50 mL Tube	SARSTEDT	Cat#62.547.254
10× Genomics Chromium Controller	10x Genomics	Cat#PN110203; RRID:SCR_019326
BRAND counting chamber BLAUBRAND Bürker pattern	Sigma-Aldrich	Cat#BR718920
Flowmi Cell Strainer, porosity 40 μm	Sigma-Aldrich	Cat#BAH136800040
NovaSeq 6000 System	Illumina	Cat#20012850; RRID:SCR_016387
pluriStrainer 70 μm (Cell Strainer)	pluriSelect	Cat#43-50070-51
Surgical Scalpel Blade No.22	Swann-Morton	Cat#0208

MATERIALS AND EQUIPMENT

Nuclei isolation buffer (NIB)		
Reagent	Final concentration	Amount
Sucrose (0.5 M)	250 mM	500 μL
HEPES (1 M)	10 mM	10 μL

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Reagent	Final concentration	Amount
$MgCl_2$ (150 mM)	1.5 mM	10 μL
KCl (2 M)	10 mM	5 μL
IGEPAL CA-630 (1%)	0.001%	1 μL
DTT (0.1 M)	0.2 mM	2 μL
RNase inhibitor (40,000 U/mL)	0.5 U/μL	12.5 μL
DEPC-treated water	N/A	459.5 μL
Total	N/A	1 mL

Nuclei resuspension buffer (NRB)

Reagent	Final concentration	Amount
BSA in 1× PBS (5%)	1%	200 μL
$MgCl_2$ (150 mM)	2 mM	13.3 μL
RNase inhibitor (40,000 U/mL)	0.04 U/μL	1 μL
PBS (1×)	N/A	785.7 μL
Total	N/A	1 mL

Note: NIB and NRB are prepared freshly, filtered, and kept on ice until use to minimize RNase activity. Sucrose is prepared freshly in DEPC-treated water. IGEPAL CA-630 solution is diluted in DEPC-treated water and can be stored for several months at 20°C–25°C. BSA is dissolved in 1× PBS (in DEPC-treated water) and can be stored at −20°C. 1× PBS solution is prepared by diluting a 10× PBS solution in DEPC-treated water.

Alternatives: In principle, all reagents and resources listed in the key resources table can be replaced with equivalent items from other suppliers; however, the impact of alternative reagents on protocol performance has not been tested.

STEP-BY-STEP METHOD DETAILS

The following steps describe the process of isolating intact nuclei from snap-frozen mouse WAT.

Note: To minimize contamination with blood cells from the vasculature, it is recommended to do cardiac perfusion with 1× PBS in DEPC-treated water (typically 12 mL per perfusion) prior to isolating the tissue.

Tissue homogenization

⊙ Timing: 30 min

The following steps describe how to homogenize adipose tissues using a glass Dounce homogenizer.

1. Transfer 400 mg of gonadal adipose tissue (or 100 mg of inguinal adipose tissue) to a pre-cooled Petri dish on ice (Figure 1A).
2. Add 500 μL of NIB and mince the tissue thoroughly (<1 mm^3) using a scalpel (Figures 1B and 1C).

⚠ CRITICAL: Nuclei yield will be reduced if the tissue is not finely minced.

3. With the scalpel, transfer the minced adipose tissue to a pre-cooled 2 mL glass Dounce homogenizer on ice (Figure 1D).
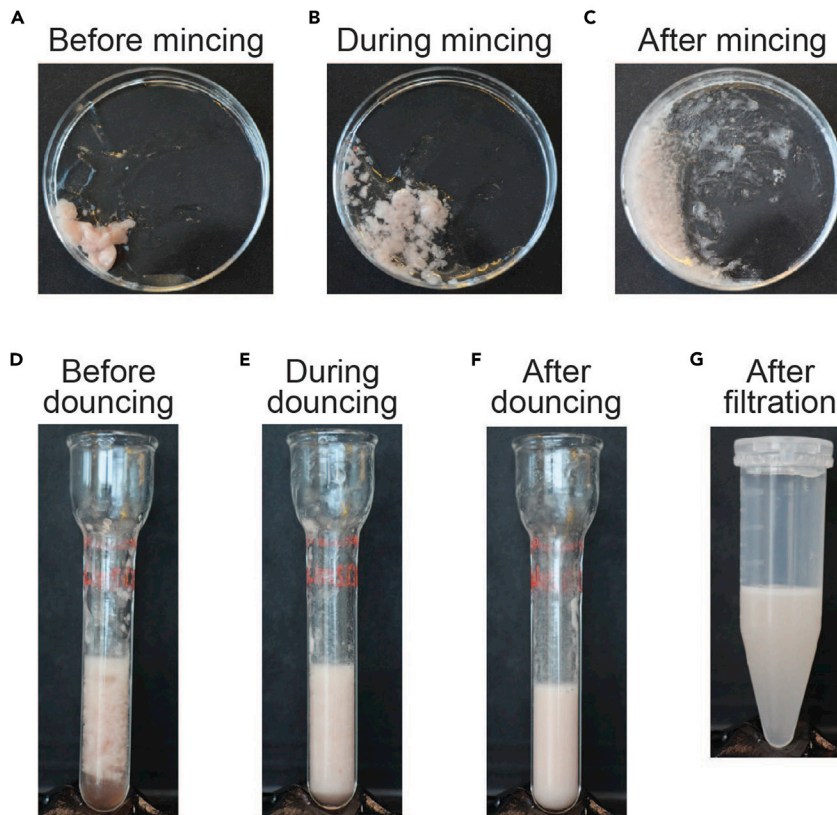
Figure 1. Adipose tissue homogenization
(A) Gonadal adipose tissue prior to mincing (step 1).
(B) Gonadal adipose tissue coarsely minced (step 2).
(C) Gonadal adipose tissue finely minced (step 2).
(D) Minced gonadal adipose tissue transferred to a 2 mL Dounce homogenizer (step 3).
(E) Gonadal adipose tissue Dounce homogenized lightly using only pestle A (step 4).
(F) Gonadal adipose tissue after Dounce homogenization using both pestle A and B (step 4).
(G) Gonadal adipose tissue homogenate after filtration (steps 5–9).

4. Homogenize the tissue to release nuclei by applying 5 strokes with pestle A (loose) followed by 5 strokes with pestle B (tight) (Figures 1E and 1F).

   ⚠ CRITICAL: Homogenize carefully to minimize generation of heat and foam, which will impact nuclei intactness.

   Note: For adipose tissues from obese mice, use the same amount of tissue as for lean tissues, but homogenize in a total volume of 1 mL NIB and apply 10 strokes with pestle A followed by 5 strokes with pestle B.

5. Pre-wet a 70 μm cell strainer with DEPC-treated water.
6. Filter the homogenate through the pre-wetted cell strainer into a pre-cooled 50 mL tube on ice.
7. Wash the Dounce homogenizer with 1 mL NIB and filter through the same cell strainer.
8. Transfer the filtered homogenate to a pre-cooled 5 mL DNA Low binding tube on ice using a P1000 pipette.
9. Wash the cell strainer with 1 mL NIB and add to the filtered homogenate in the 5 mL tube to a total volume of ~2.5 mL using a P1000 pipette (Figure 1G).
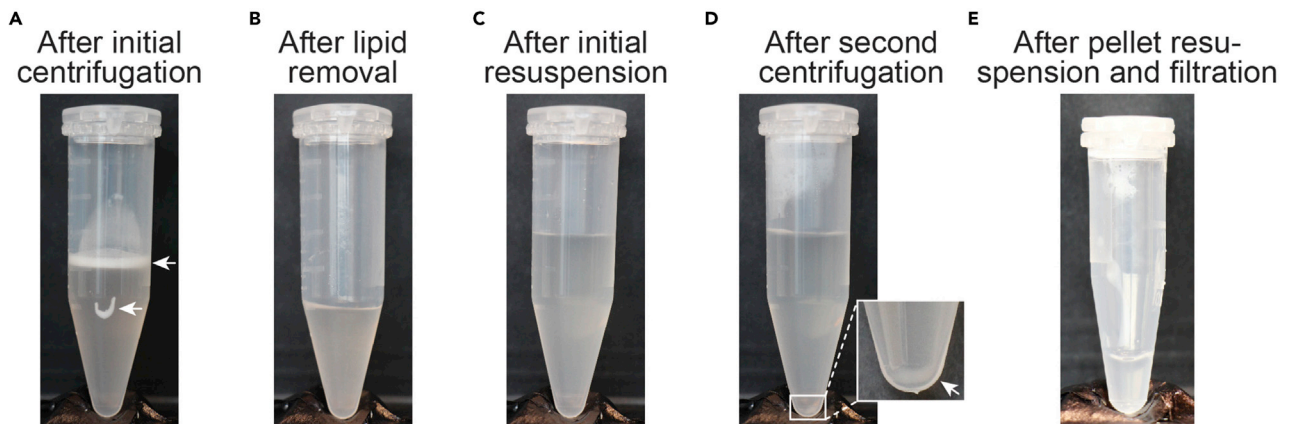
Figure 2. Isolation of nuclei

(A) Filtered gonadal adipose tissue homogenate following initial centrifugation using a fixed-angled rotor with a top lipid layer (top arrow). Nuclei are located as a smear along the side of the tube (below bottom arrow) (step 10).

(B) Adipose tissue homogenate with top lipid layer removed (step 11).

(C) Resuspension of nuclei in remaining supernatant + additional NIB (steps 12 and 13).

(D) Nuclei pellet located at the bottom of the tube (arrow) after second centrifugation using a swing-bucket rotor (step 14).

(E) Nuclei after removal of supernatant, resuspension in NRB and filtration (steps 15–18).

Isolation of nuclei

⏱ Timing: 1–2 h

The following steps describe how to use centrifugation to isolate nuclei from the adipose tissue homogenate.

10. Centrifuge the homogenate at 1000×g for 10 min at 4°C using a fixed-angled rotor (Figure 2A).

    Note: Nuclei are located as a smear along the side of the 5 mL tube.

11. Aspirate the lipid layer using a vacuum pump (Figure 2B).

    ⚠ CRITICAL: Only remove the top lipid layer. Do not remove the entire supernatant, as this will lead to loss of nuclei.

12. Resuspend the pellet in the remaining supernatant using a P1000 pipette and transfer the resuspended nuclei to a new pre-cooled 5 mL DNA Low binding tube on ice.
13. To make sure that all nuclei are transferred, wash the first 5 mL tube twice with 1 mL NIB and transfer any remaining nuclei to the new 5 mL tube containing the resuspended nuclei using a P1000 pipette (Figure 2C).
14. Pellet the nuclei by centrifuging at 500×g for 10 min at 4°C using a swing-bucket rotor (Figure 2D).
15. Aspirate the supernatant.
    a. Use vacuum aspiration to remove most of the supernatant (leave 80–100 μL supernatant) and remove remaining supernatant with a P100 pipette.

    ⚠ CRITICAL: Be careful not to disturb the nuclei pellet.

16. Resuspend the nuclei pellet in 100 μL NRB by pipetting using a regular P1000 pipette.
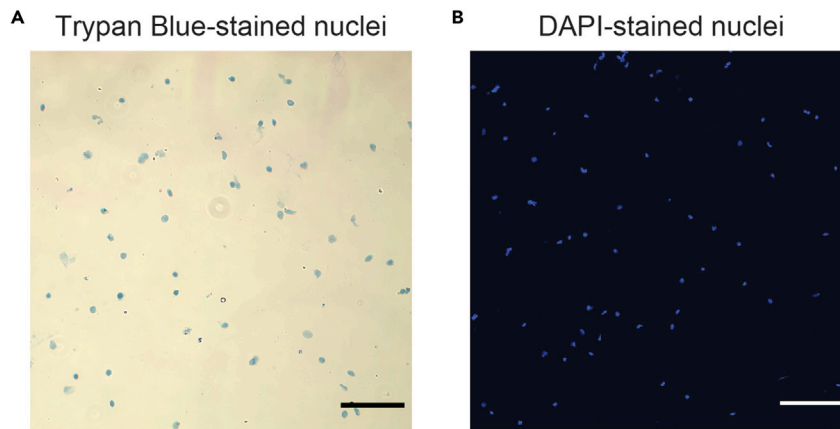17. Pre-wet a 40 μm tip strainer with NRB.

Figure 3. Nuclei isolated from epididymal adipose tissue
Nuclei isolated from epididymal adipose tissue and stained with Trypan Blue (A) or DAPI (B). Nuclei are imaged at 20× magnification. Scale bars represent 100 μm.

18. Filter the nuclei through the pre-wetted tip strainer using a P1000 pipette into a pre-cooled 1.5 mL DNA Low binding tube on ice (Figure 2E) to remove any remaining debris and avoid nuclei aggregation.

    Note: The nuclei concentration will decrease by 30%–40% following filtration.

19. Proceed to nuclei counting using a Bürker counting chamber and Trypan Blue.
    a. Mix nuclei and Trypan Blue in equal ratio in a separate tube.
    b. Transfer 10 μL of the nuclei-Trypan Blue solution to the Bürker counting chamber.
    c. Count at least 3 squares using bright-field microscopy and quantify the nuclei concentration.

$$\frac{\text{Nuclei}}{\text{mL}} = \frac{\sum \text{nuclei count in } n \text{ squares}}{n} \times 10^4 \times 2$$

    Note: If nuclei are diluted while counting, multiply the equation for calculating the concentration of nuclei with the dilution factor.

    ⚠ CRITICAL: Verify that nuclei are mostly intact and not clumping (Figures 3A and 3B). Note, that intact nuclei isolated from adipose tissues have a heterogenic morphology.

10x Genomics single-nucleus RNA sequencing

⏱ Timing: 2–3 days

The following steps describe how to partition nuclei into barcoded gel bead droplets using 10× Genomics v3.1 chemistry and library preparation for Illumina sequencing.

20. Immediately following nuclei counting, take out 12,000 nuclei and resuspend in NRB in a new pre-cooled 1.5 mL DNA low binding tube on ice to a total volume of 43.2 μL.

    Note: Here, we used 12,000 nuclei as input for 10× Genomics-based snRNA-seq aiming at an initial recovery of 7,000–8,000 nuclei. Following quality control, nuclei recovery will be further reduced, as nuclei with poor quality metrics, e.g., low number of UMIs and transcripts, are excluded. For details concerning quality control steps of the snRNA-seq data, refer to Sárvári et al., 2021.
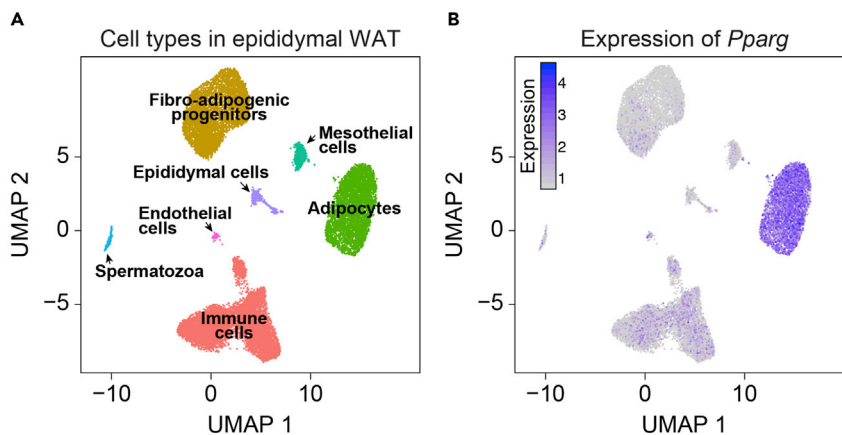
Figure 4. snRNA-seq from epididymal adipose tissue
(A) UMAP plot of cell types identified in the epididymal adipose tissue using snRNA-seq. Figure reprinted with permission from Sárvári et al., 2021.
(B) UMAP plot showing *Pparg* expression across epididymal adipose tissue cell types.

21. Load nuclei into the 10× Genomics Chromium Controller and run according to the recommendations of the manufacturer (https://support.10xgenomics.com/single-cell-gene-expression/index/doc/user-guide-chromium-single-cell-3-reagent-kits-user-guide-v31-chemistry-dual-index; steps 1 and 2).
22. Dual-indexed paired-end sequencing libraries are prepared for Illumina sequencing according to the recommendation of the manufacturer (https://support.10×genomics.com/single-cell-gene-expression/index/doc/user-guide-chromium-single-cell-3-reagent-kits-user-guide-v31-chemistry-dual-index; step 3).

Note: For indexing, it is possible to run the sample index PCR step using half sample volumes with a total reaction volume of 50 μL (25 μL Amp Mix to 15 μL sample (post ligation) and 10 μL of individual Dual Index TT Set A). The remaining sample can be stored at −20°C up to at least a month and used for another round of sample index PCR if needed, e.g., for adjusting the total number of PCR cycles prior to sequencing.

23. Each library is sequenced using an Illumina NovaSeq 6000 sequencer, aiming at a read coverage of 30,000–50,000 read pairs per droplet (250 million reads in total).

EXPECTED OUTCOMES

From 100 mg of snap-frozen inguinal adipose tissue or 400 mg of snap-frozen gonadal adipose tissue isolated from a lean mouse, the nuclei yield is typically 200,000–700,000 nuclei. From snap-frozen WAT from obese mice, the nuclei yield is approximately 25% of that from snap-frozen WAT from lean mice. This is anticipated, as adipose tissues from obese mice are heavily expanded and only a fraction of the tissue is used for nuclei isolation. However, the yield can be increased by performing multiple rounds of nuclei isolation of WAT from the same mouse using the indicated material amount for each isolation round.

By performing snRNA-seq on epididymal adipose tissues from lean and obese C57BL/6J mice using 10× Genomics-based technology, we were able to recover all major cell types in epididymal WAT (Figure 4A). The expression pattern of *Pparg*, encoding the peroxisome proliferator-activated receptor γ (PPARγ), a master regulator of adipogenesis, which is also expressed in macrophages (Lefterova et al., 2014), verifies the capture of adipocytes as well as *Pparg*-positive immune cells (Figure 4B). For details concerning processing and quality control steps of the snRNA-seq data, refer to

Sárvári et al., 2021 and best practices in single-cell/nucleus RNA-seq analyses (Luecken and Theis, 2019; Stuart et al., 2019).

LIMITATIONS

Similar to what is the case for single-cell approaches, it cannot be excluded that single-nucleus isolation introduces cell type-specific biases. Generally, however, single-nucleus approaches are considered to lead to fewer biases than single-cell approaches, as the isolation of nuclei does not rely on tissue disintegration (Denisenko et al., 2020). A trade-off for single-nucleus approaches is that the contamination with ambient RNA can be significantly higher compared to single-cell approaches and must be controlled for in the data analysis pipeline. Failure to do that may lead to spurious cell type annotations.

TROUBLESHOOTING

Problem 1

Low nuclei yield.

Potential solution

Solution 1: Insufficient tissue mincing may lead to poor nuclei recovery. To avoid this, make sure that the tissue is very finely minced before proceeding with homogenization (step 2). For small amounts of adipose tissues (<400 mg of gonadal or <100 mg of inguinal WAT), it may be necessary to decrease the volume for homogenization to increase nuclei yield.

Solution 2: Both insufficient and excessive homogenization (step 4) can lead to a lower nuclei recovery. The nuclei yield is dependent on the number of strokes that is applied while disintegrating the tissue. Nuclei release can be evaluated microscopically during the homogenization steps to ensure that a sufficient, but not excessive, number of strokes is applied.

Solution 3: After the initial centrifugation step, nuclei are located as a smear along the side of the tube. Removing too much supernatant may disturb the smeared nuclei pellet, thus leading to nuclei loss. Be sure to only remove the top fat layer (step 11). Following the second centrifugation step, nuclei are pelleted at the bottom of the tube. Remove as much supernatant as possible without disturbing the nuclei pellet (step 15).

Solution 4 (steps 1–6): If the tissue is sufficiently large, it can be divided in adequate portions (400 mg of gonadal or 100 mg of inguinal WAT), which are homogenized separately but are filtered (step 6) into the same 50 mL tube after which the pooled homogenate is processed as one sample.

Problem 2

Low nuclei concentration.

Potential solution

For 10× Genomics single-nucleus studies, lowly concentrated nuclei samples may not allow for loading of the desirable number of nuclei onto the 10× chip. The nuclei concentration can be increased by resuspending the final nuclei pellet in a lower volume (steps 16–18) (we have successfully resuspended and filtered nuclei in a volume down to 60 μL).

Problem 3

Poor nuclei morphology (Figure 5).

Potential solution

Maintaining nuclei intactness is especially important for single-nucleus transcriptomics, as nuclei with a poor morphology are likely to leak transcripts that will contribute to ambient RNA.

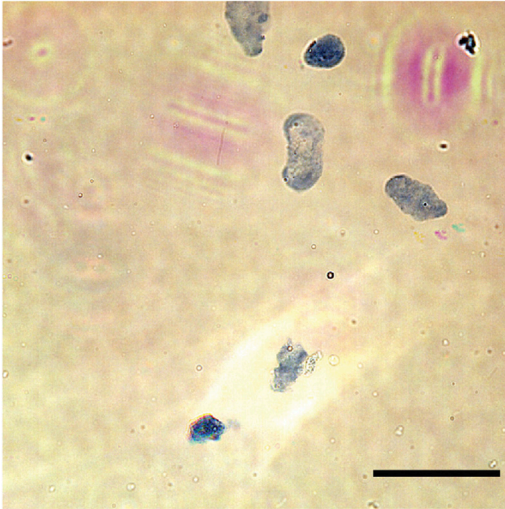## Nuclei with a poor morphology

Figure 5. Poor nuclei morphology
Trypan Blue-stained nuclei isolated from epididymal adipose tissue with poor intactness. Nuclei are imaged at 60× magnification. The scale bar represents 50 μm.

Solution 1: Excessive homogenization may impact the integrity of the nuclei negatively. To avoid this, Dounce homogenize gently on ice and/or reduce the number of strokes (step 4).

Solution 2: Harsh pipetting may impact the integrity of the nuclei. To avoid this, pipette gently on ice and/or use wide-bore tips (steps 12 and 13).

Solution 3: As an alternative method, flow cytometry techniques may be used to enrich for intact nuclei (Sun et al., 2020).

Problem 4
Nuclei are aggregating (Figure 6A).

Potential solution
This problem can be caused by inappropriate handling of the nuclei during several steps of the nuclei isolation procedure.

Solution 1: Highly concentrated nuclei (above 5,000 nuclei/μL according to 10× Genomics), and nuclei that are not intact (troubleshooting, problem 3), may lead to clumping. To avoid this, resuspend nuclei in a larger volume and resuspend thoroughly followed by filtration (steps 16–18).

Solution 2: Insufficient tissue lysis may cause nuclei to aggregate with remaining tissue or cell debris. To avoid this, increase the number of strokes while homogenizing (step 4) and evaluate lysis microscopically to ensure sufficient tissue disintegration.

Problem 5
Presence of debris (Figure 6B).

Potential solution
The first filtration step (steps 5 and 6) removes most of the debris. However, smaller debris material might be present following filtration. The final filtration step (steps 17 and 18) serves to remove any remaining debris that otherwise can lead to clogging of the microfluidics system of the 10×
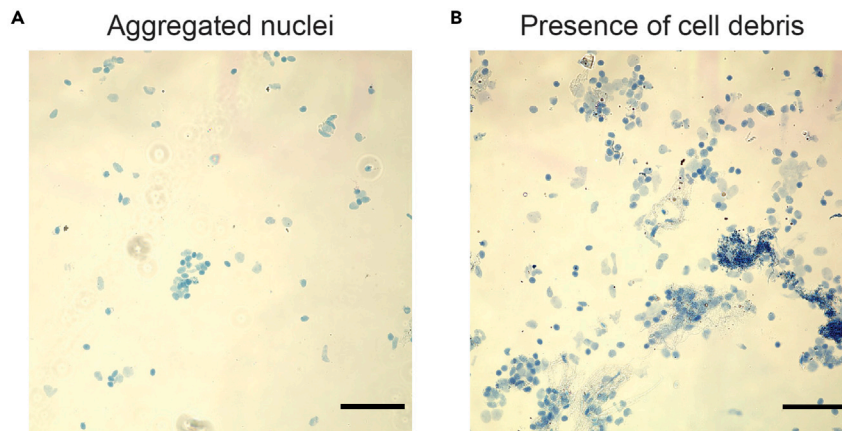
Figure 6. Aggregated nuclei
Trypan Blue-stained nuclei isolated from epididymal adipose tissue that are clumping to other nuclei (A) or remaining cell debris (B). Nuclei are imaged at 20× magnification. Scale bars represent 100 μm.

Genomics Chromium Controller. However, if debris is still present, it is possible to exchange the 70 μm cell strainer (steps 5 and 6) with a cell strainer having a smaller mesh size (<70 μm). However, this may negatively affect the nuclei yield.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Susanne Mandrup (s.mandrup@bmb.sdu.dk).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate any unique dataset or code.

AUTHOR CONTRIBUTIONS

Methodology, E.L.V.H., E.G., and L.L.; investigation, E.L.V.H., E.G., A.K.S., L.L., and R.N.; validation, E.L.V.H., E.G., L.L.; writing – original draft, E.L.V.H. and S.M.; writing – review & editing, S.M., J.G.S.M., E.L.V.H., E.G., and A.K.S.; visualization, E.L.V.H. and E.G.; supervision, S.M. and J.G.S.M.; project administration, S.M. and J.G.S.M.; funding acquisition, S.M. and E.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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