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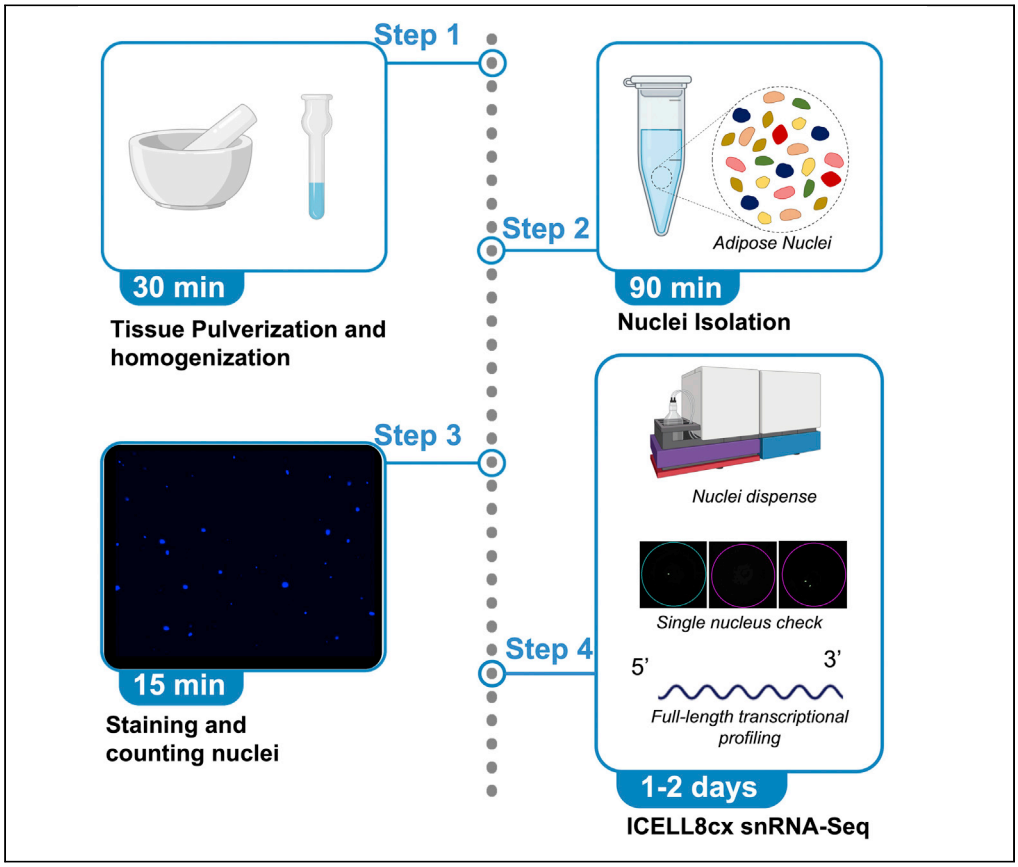
Protocol

Isolation of nuclei from frozen human subcutaneous adipose tissue for full-length single-nuclei transcriptional profiling



Automated single-cell dispensing is incompatible with white adipose tissue (WAT) due to lipid-laden adipocytes. Single-nuclei RNA-Seq permits transcriptional profiling of all cells from WAT. Human WAT faces unique technical challenges in isolating nuclei compared to rodent tissue due to greater extra-cellular matrix content and larger lipid droplets. In this protocol, we detail how to isolate nuclei from frozen subcutaneous human WAT for single-nuclei RNA-Seq.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for isolating nuclei from frozen human white adipose tissue

Optimized parameters for single-nuclei dispense using ICELL8 platform

Nuclei are suitable for full-length sequencing-based technologies

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Isolation of nuclei from frozen human subcutaneous adipose tissue for full-length single-nuclei transcriptional profiling

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SUMMARY

Automated single-cell dispensing is incompatible with white adipose tissue (WAT) due to lipid-laden adipocytes. Single-nuclei RNA-Seq permits transcriptional profiling of all cells from WAT. Human WAT faces unique technical challenges in isolating nuclei compared to rodent tissue due to greater extra-cellular matrix content and larger lipid droplets. In this protocol, we detail how to isolate nuclei from frozen subcutaneous human WAT for single-nuclei RNA-Seq.
For complete information on the generation and use of this protocol, please refer to Whytock et al. (2022).[1]

BEFORE YOU BEGIN

The protocol describes the steps to isolate nuclei from 100mg of snap-frozen human subcutaneous WAT. This protocol has been validated in frozen abdominal subcutaneous WAT from humans with leanness, overweight and obesity (range: 20.9–39.1 kg/m^2), from male and female participants and with an age range of 20–89 years. We have obtained higher nuclei yields from human visceral adipose tissue of the same weight. We have obtained similar nuclei yields from gluteal-femoral subcutaneous WAT of the same weight in females only. Due to the lack of substantial subcutaneous gluteal-femoral adipose tissue in males, we have not yet validated this protocol in this depot in males. Biopsies are obtained from a tumesced biopsy using a liposuction technique (NCT04034706) and immediately cleaned with Dulbecco's phosphate-buffered saline (DPBS) before being snap-frozen in liquid nitrogen (LN_2). Currently this protocol has only been validated for snap-frozen human WAT and not in rodent adipose tissues. For nuclei isolation from murine WAT, the reader is encouraged to read Van Hauwert et al.,[2] Every step of this protocol has been optimized for 100mg of human WAT. If more tissue is available, it is advised to run 2 × 100mg samples in parallel and combine nuclei at the end rather than increasing the starting material for one protocol execution. Having too much tissue may lead to more nuclei aggregation and lipid accumulation.

Institutional permissions

Samples from this study were approved by AdventHealth Institutional Review Board and carried out in accordance with the Declaration of Helsinki. Participants provided written informed consent for their study participation. The clinical trial was registered at (NCT04034706).

General preparation

Timing: 1 h

1. Prepare homogenization workstation.
   a. Pre-cool centrifuges to 4°C.
   b. Fill canister with LN_2.
   c. Set up automated homogenizer and place glass vessel on ice.
   d. Place 1.7 mL DNA LoBind tubes on ice.

Note: Each isolation procedure requires 2 × 50mL conical tubes, 5 × 1.7mL LoBind Tubes, 1 x mortar, 1 x pestle, 1 x spatula, 1 x Tissue Grinder, 1 × 2mL glass vessel, 40 μm Cell Strainer, 1 × 100 μm Cell Strainer, 1 × 1mL TB syringe and 1 × 25*g* needle.

2. Prepare reagents used in the buffers.
   a. Dilute 1mg of DTT in 2.82mL of nuclease free water to get 1mM DTT, aliquot and store in −20°C.
   b. Dissolve 20.54*g* sucrose in 40mL nuclease free water to get 1.5M Sucrose, aliquot and store in −20°C.
   c. Dissolve 0.5*g* BSA in 50mL nuclease free water and filter through 0.2μm syringe filter to get 1% BSA- nuclease free water.
   d. Dilute 100μL of 100% Triton X-100 in 900μL of nuclease-free water to get 10% triton x-100.

Note: To pipette 100μl of 100% Triton X-100, use a P1000 pipette tip with the tip cut off or a wide orifice pipette tip. Pipette the 100μL of 100% Triton X-100 into the 900μL of nuclease-free water to assist in mixing the solution.

3. Prepare homogenization buffer and nuclei resuspension buffer.
   a. Keep on ice and protected from light.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
BSA	Sigma-Aldrich	820452
ReadyProbes cell viability imaging kit	Thermo Fisher Scientific	R37610
MgCl2	Ambion	AM9530G
Tris buffer pH 8.0	Thermo Fisher Scientific	AM9855G
KCL	Thermo Fisher Scientific	AM9640G
Sucrose	Sigma-Aldrich	S0389
DTT	Thermo Fisher Scientific	R0861
100x protease inhibitor	Thermo Fisher Scientific	78437
SUPERaseIn RNase Inhibitor	Thermo Fisher Scientific	AM2695
Triton X-100	Fisher Scientific	AC327372500
Ribolock RNAse inhibitor	Thermo Fisher Scientific	EO0382
UltraPure™ 0.5M EDTA, pH 8.0	Gibco	15575020
Tagment DNA enzyme 1	Illumina	20034198
Beckman Coulter AMPURE XP KIT	Fisher Healthcare	NC9959336
Nuclease Free Water (not DEPC treated)	Thermo Fisher Scientific	AM9930
DPBS no calcium no magnesium	Thermo Fisher Scientific	14190144
Critical commercial assays		
SMART-Seq® ICELL8® Application Kit – 5 Chip	TakaraBio USA	640221

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Qubit™ 1X dsDNA Assay Kits, high sensitivity (HS) and broad range (BR)	Invitrogen	Q33230
High sensitivity DNA kit	Agilent	5067–4626
Biological samples		
Human subcutaneous abdominal white adipose tissue from premenopausal females	N/A	N/A
Other		
1.7 mL DNA LoBind tubes	Eppendorf	022431021
Falcon® 100 μm Cell Strainer	Corning Life Sciences	352360
Falcon® 40 μm Cell Strainer	Corning Life Science	352340
50 mL conical tubes	Sarstedt	62.547.254
Countess cell counting chamber slides	Thermo Fisher Scientific	C10228
0.2 μm corning syringe filters	Millipore Sigma	CLS431229
Countess 3 Automated Cell Counter	Thermo Fisher Scientific	AMQAX2000
2100 Bioanalyzer	Agilent	G2939BA
ICELL8 cx	TakaraBio USA	640188
Mortar & pestle	Health Care Logistics	14075
Tissue homogenizer	Glass-Col	099C K54
Electron Microscopy Sciences Potter-Elvehjem Tissue Grinder, 2 mL	Fisher Scientific	50-190-0304
Cole-Parmer Essentials Spatula/Micro Spoon, Stainless Steel, 210mm; 3/PK	Cole-Parmer	# UX-06287-18
Glass Vessel – 2mL	Cole-Parmer	# UX-44468-13
BD™ Needle 25 G	Becton Dickinson	30382903051251
1 mL TB syringe	Becton Dickinson	309659

MATERIALS AND EQUIPMENT

Homogenization Buffer

Reagent	Final concentration	Amount (μL)
$MgCl_2$ (1 M)	5 mM	15
Tris Buffer, pH 8.0 (1 M)	10 mM	30
KCl (2 M)	25 mM	37.5
Sucrose (1.5 M)	250 mM	500
DTT (1 mM)	1 μm	3
Protease Inhibitor (100x)	1x	30
Superasin (20 U/μL)	0.2 U/μL	30
Nuclease-free water	-	2354.5
Total Volume	-	3000

Nuclei Resuspension Buffer

Reagent	Final concentration	Amount (μL)
1 M $MgCl_2$ (1 M)	5 mM	10
1 M Tris Buffer, pH 8.0 (1 M)	10 mM	20
2 M KCl (2 M)	25 mM	25
EDTA 0.5M	1 mM	4
Ribolock RNAse inhibitor (40U/ul)	0.2U/μL	20
1% BSA-nuclease free water (−20°C)	-	1921
Total Volume	-	2000

Note: Buffers are prepared for immediate use and then discarded. Do not re-use.

Alternatives: Reagents and resources may be replaced with alternative items from other supplies; however, the impact of alterative reagents has not been tested on this protocol.

STEP-BY-STEP METHOD DETAILS

The following steps describe how to isolate nuclei from snap-frozen human subcutaneous white adipose tissue (WAT) for subsequent full-length single nuclei transcriptional profiling.

Tissue pulverization and homogenization

Timing: 30 min

The following steps describe how to pulverize and homogenize snap-frozen human subcutaneous WAT.

1. Add 2mL of homogenization buffer into glass vessel and place on ice in a beaker.

2. Pulverize 100mg of frozen human subcutaneous WAT in liquid nitrogen (LN_2) using a mortar and pestle (Figures 1B–1D).

   Note: This step does not need to be performed in a safety hood. It is advised to wear cryo-gloves to assist with handling the mortar and pestle when using LN_2. A face shield should be worn during this process to protect the face from LN_2. Care should be taken not to splash the LN_2 when performing the pulverization.

   Note: We have previously isolated nuclei from 50mg of WAT and 50mg of VAT using this technique. Reducing the starting material of WAT to 50mg poses the risk of not isolating enough nuclei for subsequent ICELL8cx dispense and therefore 100mg starting material is recommended. If using 50mg of WAT, it is recommended to scale down the homogenization and nuclei resuspension buffers to reduce loss of nuclei.

   a. To cool the mortar and pestle with LN_2, first pour LN_2 in the mortar with pestle until full, let it evaporate and repeat once for a total of two times.
   b. In a separate mortar place spatula and pour LN_2. Continue to top up LN_2 during pulverization process to keep spatula cool.

   Note: It is important to keep all pulverization equipment cool during the entire pulverization step.

   c. Re-fill mortar with LN_2 and then place 100mg of WAT in the mortar.

   Note: Keep sample on dry ice until it is in the mortar.

   d. Pulverize tissue by firmly pressing down on WAT with pestle in the mortar.
   e. Continue strokes until WAT has completely powdered (approximately 50 strokes).

   Note: During this time, you may need to top up LN_2 (approximately twice) to ensure equipment and tissue stays cold.

   f. Tilt the mortar towards the lip and let LN_2 evaporate. This will leave a pile of pulverized WAT.

   Note: When LN_2 is evaporating, pulverized tissue may pop into the air. Wearing laboratory glasses is highly recommended.

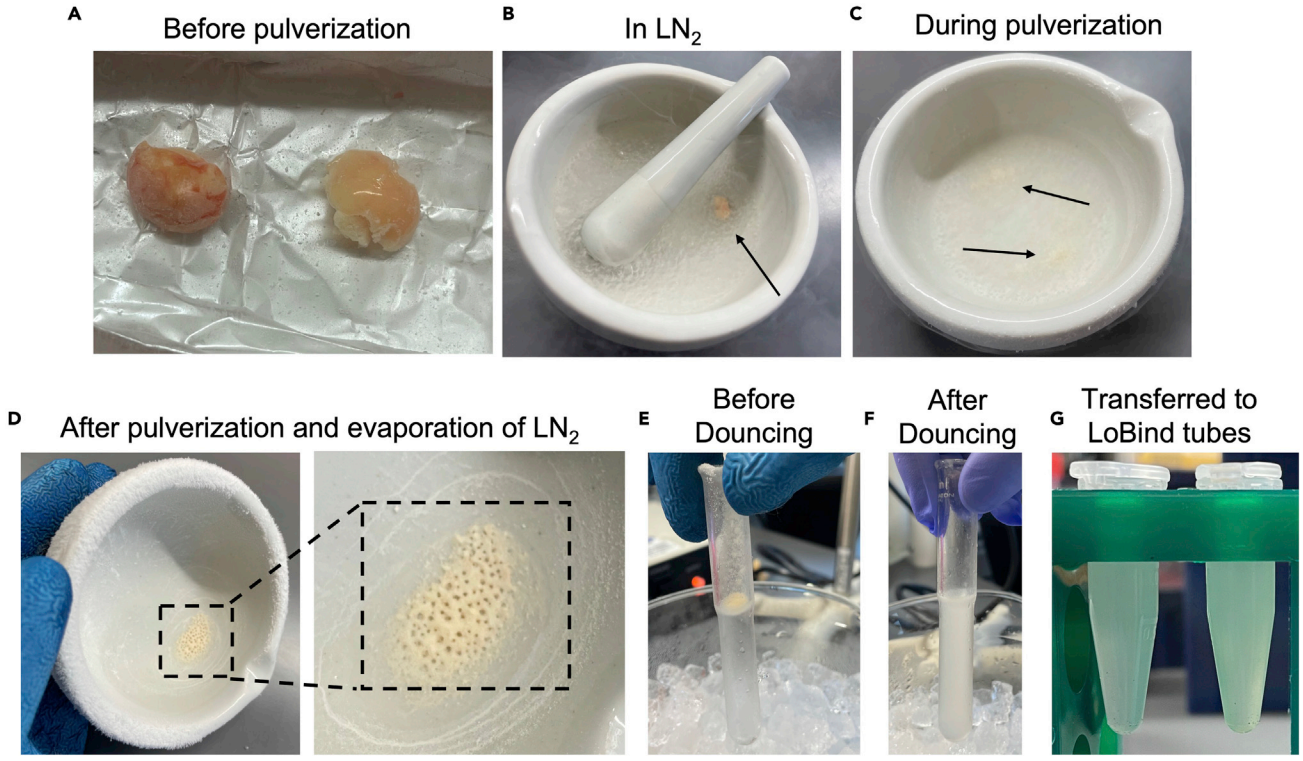

Figure 1. White Adipose Tissue pulverization and homogenization

(A) Human WAT prior to pulverization. WAT on the right has had a cleaner biopsy and is ideal starting material.
(B) Human WAT in LN_2 prior to pulverization (step 2b).
(C) Human WAT during pulverization (step 2c).
(D) Human WAT powdered after LN_2 has evaporated (step 2e).
(E) Powdered WAT in glass vessel prior to homogenization (step 2f).
(F) WAT after homogenization (step 3).
(G) WAT homogenate in 1.7mL LoBind Tubes.

g. Use LN_2 cooled spatula to scrape the pulverized WAT into the glass vessel containing 2mL of homogenization buffer (Figure 1E).

⚠ CRITICAL: Omitting the pulverization step significantly reduces nuclei yield from human WAT.

3. Homogenize 12 strokes forward and 12 strokes backward with automated dounce homogenizer (Figures 1E and 1F).

Note: If an automated dounce homogenizer is not available, manual hand douncing should be sufficient with 10 strokes of pestle A and 10 strokes of pestle B, although this has not been validated.

⚠ CRITICAL: There should be no visible lumps after this process.

4. Transfer homogenate equally to 2 × 1.7mL LoBind tubes (Figure 1G).
   a. Use 400µL of homogenization buffer to clean glass vessel and add to the LoBind tubes containing the homogenate.

Note: Each tube should contain 1200µL of homogenate.

Nuclei isolation

Timing: 90 min

The following steps describe nuclei isolation, filtration, and cleaning steps to achieve a clean nuclei preparation .All steps should be performed on ice, unless otherwise stated.

5. Add 12 μL of 10% Triton X-100 to each 1.7mL LoBind Tubes (to achieve 0.1% Triton X-100 (v/v)) and pulse vortex.
6. Leave on ice for 30 min, protect from light and pulse vortex every 5 min.
7. Place a 100μm filter over a 50mL conical tube and a 40μm filter over a 50mL conical tube and pre-wet each filter with 100μL PBS (−/−).
8. Filter homogenate solution through 100μm and 40μm filters (Figure 2A). This step can be completed on a flat bench lab and does not need to be completed on ice.

   Note: It is advised to work quickly because it is not done on ice.

   a. Pipette homogenate through 100μm filter into the 50mL conical tube.
   b. Use 400μL homogenization buffer to wash any remaining homogenate solution from the 1.7mL LoBind tubes and transfer through 100μm filter.
   c. Pipette homogenate solution through the 40μm filter into the 50mL conical tube.
   d. Dispense filtered homogenate solution equally into pre-chilled 2 × 1.7mL LoBind Tubes.
9. Centrifuge the homogenates at 2700g for 10 min at 4°C (Figure 2B).

   Note: Following centrifugation, nuclei should be visible at the bottom of the Eppendorf as a pellet. There is usually a layer of lipid on the top of homogenate solution. When isolating nuclei from human WAT we found 1000g is insufficient to pellet down from the lipid.

10. Remove lipid layer by carefully pipetting off using a P1000 tip.
11. Remove remaining supernatant and leave ~50μL (Figure 2C).

    Note: If you have a larger pellet, remove supernatant up until you reach the pellet.

12. Repeat for 2nd tube containing homogenate solution.Keep 1st tube on ice during the process.

    ⚠ CRITICAL: It is important to perform steps 10–12 in an quick manner to prevent nuclei re-suspending in solution and being removed with supernatant.

13. Re-suspend 50μL of homogenate solution in each 1.7mL LoBind tube and combine to one 1.7mL LoBind tube.
    a. Resuspend first 50μL pellet thoroughly with pipetting up and down (approximately 30 times).
    b. Transfer homogenate solution to pre-chilled 1.7mL LoBind tube.
    c. Resuspend second 50μL pellet solution thoroughly with pipetting up and down (approximately 30 times).
    d. Transfer homogenate solution to pre-chilled 1.7mL LoBind used in step 13b.

    Note: The homogenate solution will now be in the same 1.7mL LoBind tube.

14. Add 100μL homogenization buffer to homogenate solution and mix by pipetting.
15. Centrifuge the homogenate at 2700g for 10 min at 4°C (Figure 2D).

    Note: Following centrifugation step there should be a visible pellet and no lipid at the top.

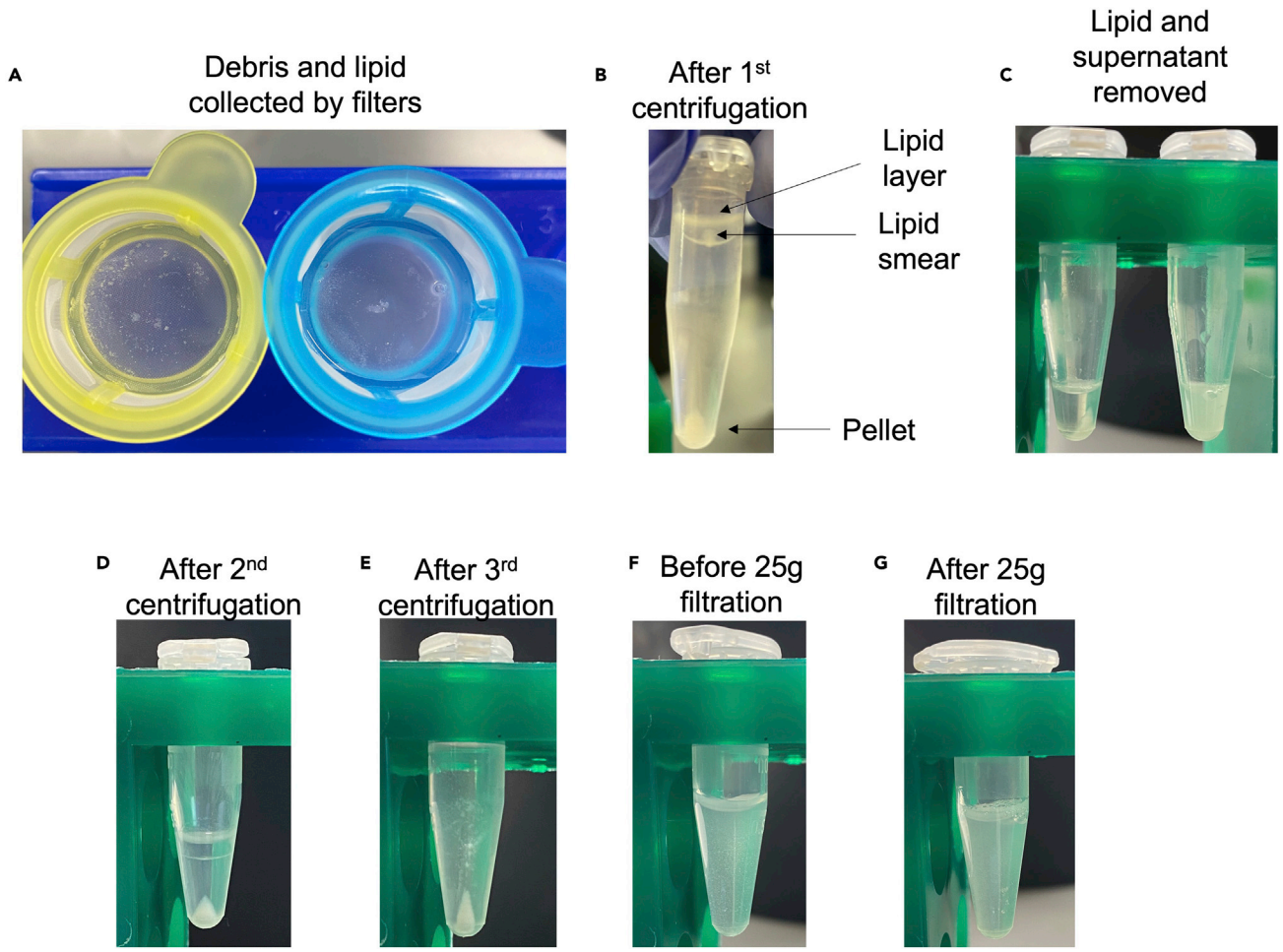

Figure 2. Nuclei Isolation

(A) 100μm (left) and 40μm (right) filters after homogenate solution has been filtered through (step 8).

(B) WAT homogenate after initial centrifugation (step 9). Lipid layer and lipid smear can form at the top. Nuclei pellet can be seen at the bottom of the Eppendorf. N.B. Pellet can also be smaller than one in the picture.

(C) WAT homogenate after removal of lipid layer and supernatant. Tube on the right is after re-suspension in ~50μL (step 11–13).

(D) Nuclei pellet after 2nd centrifugation (step 14).

(E) Nuclei pellet after the 3rd centrifugation step (step 19).

(F) Nuclei after being resuspended in nuclei resuspension buffer before 25g filtering (step 22).

(G) Nuclei after 25g filtering (step 23).

16. Remove supernatant and leave ~50μL.
17. Resuspend 50μL pellet thoroughly by pipetting up and down (approximately 30 times).
18. Add 950μL of Nuclei Resuspension buffer and mix with pipetting.
19. Centrifuge the nuclei at 2700g for 10 min at 4°C (Figure 2E).

Note: This centrifugation step can be reduced to 1000g.

20. Remove supernatant and leave ~50μL.
21. Resuspend 50μL pellet thoroughly by pipetting up and down (approximately 30 times).
22. Add 450μL of Nuclei Resuspension buffer and mix with pipetting (Figure 2F).
23. Filter nuclei solution through 25g syringe 10 times (Figure 2G).

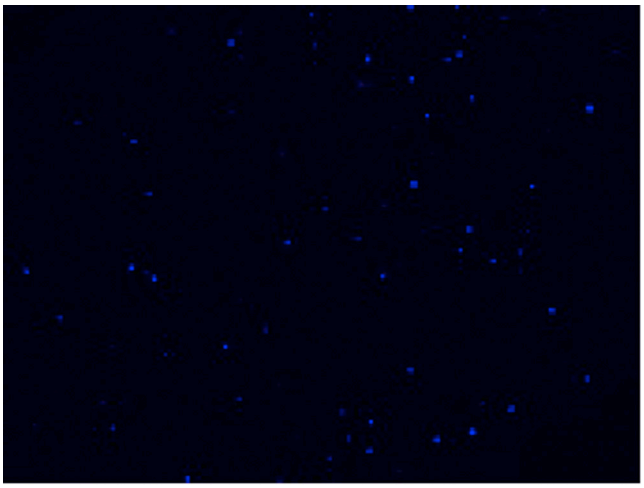

Figure 3. Isolated Nuclei from human WAT

Nuclei isolated from human subcutaneous WAT and stained with NucBlue and counted with Countess 3 Automated Cell Counter. Image is jpg readout from the Countess 3.

⚠ CRITICAL: Be careful not to let the 25g syringe touch the plastic of the 1.7mL LoBind Tube. This step helps to prevent nuclei aggregation.

Staining and counting nuclei

Timing: 15 min

The following steps detail how to stain and count the number of nuclei extracted.

24. Add 1 drop of ReadyProbes NucBlue (Hoechst 33342) to the 500 µL nuclei solution and leave on ice protected from light for 15 min.

Note: It is possible to count the nuclei extracted using DAPI under a microscope.

25. Mix nuclei solution with pipetting before immediately adding 10µL of nuclei solution to countess slide.
26. Count nuclei with Countess 3 Automated Cell Counter (Figure 3).

Note: Typical yield is 180K nuclei at a concentration of 360K/mL.

27. To ensure accurate nuclei loading on the ICELL8 CX, we recommend diluting the sample with DPBS (−/−) to 200K/mL and re-counting for validation.

ICELL8 CX single nucleus RNA sequencing

Timing: 1–2 days

The following steps describe how to load the nuclei isolation onto ICELL8 CX platform and the parameters used for detecting nanowells that contain a single nucleus.

28. Nuclei preparation is mixed with reagents from TakaraBio USA SMART-Seq® ICELL8® Application Kit – 5 Chip (including second diluent (100x), BSA (1%), RNase Inhibitor (40 U/µL), SMART-Seq ICELL8 CDS) and DPBS (−/−) to achieve a final concentration of 40K nuclei/mL.

Table 1. CellSelect software settings to detect a single nucleus	
Image processing	
Extra Segmentations	6
Open Iterations After Threshold	2
Scale Steps	6
Second Derivative Scale	2
Thresholds	200
Use Dual Scale Segmentation	True
Misc	
Algorithm	V2
Elongation or Suspicious Circularity	0.65
Is Standard Layout	True
Preprinted Barcodes	True
Post processing	
Detect Reflections Aggressively	False
Expected Cell Size	50
Expected Cell Size Range	100
Ignore when Size is Less	10
Min Distance From Well Center	0
Minimum Confidence	0.80
Suspicious Circularity	0.65
Well Detection Radius	155

29. Dispense nuclei solution onto ICELL8 350v chip as per manufacturer's instructions.
30. Confirmation of single nucleus candidacy is checked using ICELL8 CellSelect software. The following settings used to detect which nanowells contain a single nucleus can be found in Table 1 (Figure 4).

    Note: The algorithm can include some false negatives that are usually identified as no cells even when there is a cell. By ordering the cells by type and confidence, it is advised to check for non-cell nanowells that do contain a nuclei and manually push these through using cellselect software.

31. Single nuclei RNA-Seq libraries are prepared according to the recommendation of the manufacturer. (https://www.takarabio.com/documents/User%20Manual/SMART/SMART-Seq%20ICELL8%20cx%20Application%20Kit%20User%20Manual.pdf).

    Note: TakaraBio have recently updated their technology to SMART-Seq Pro. (https://www.takarabio.com/documents/User%20Manual/SMART/SMART-Seq%20Pro%20Application%20Kit%20User%20Manual.pdf). This nuclei isolation technique has also been validated by SMART-Seq Pro.

32. The single nuclei RNA-Seq libraries are quantified for cDNA content using Qubit™ 1X dsDNA Assay Kits, high sensitivity (HS) and broad range (BR) and following manufacturers instructions. Typical yield is approximately 20 ng/μL.

    Note: Total DNA yield is lower with SMART-Seq Pro and is likely due to different PCR steps and number of cycles used.

33. cDNA library is diluted to 2 ng/μL and checked for quality using High sensitivity DNA kit on Agilent Bioanalyzer following manufacturer's instructions.
    a. For a good quality library, we expect to have >80% of total bp to between 200–1000bp and overall concentration to be between 5–10mM.

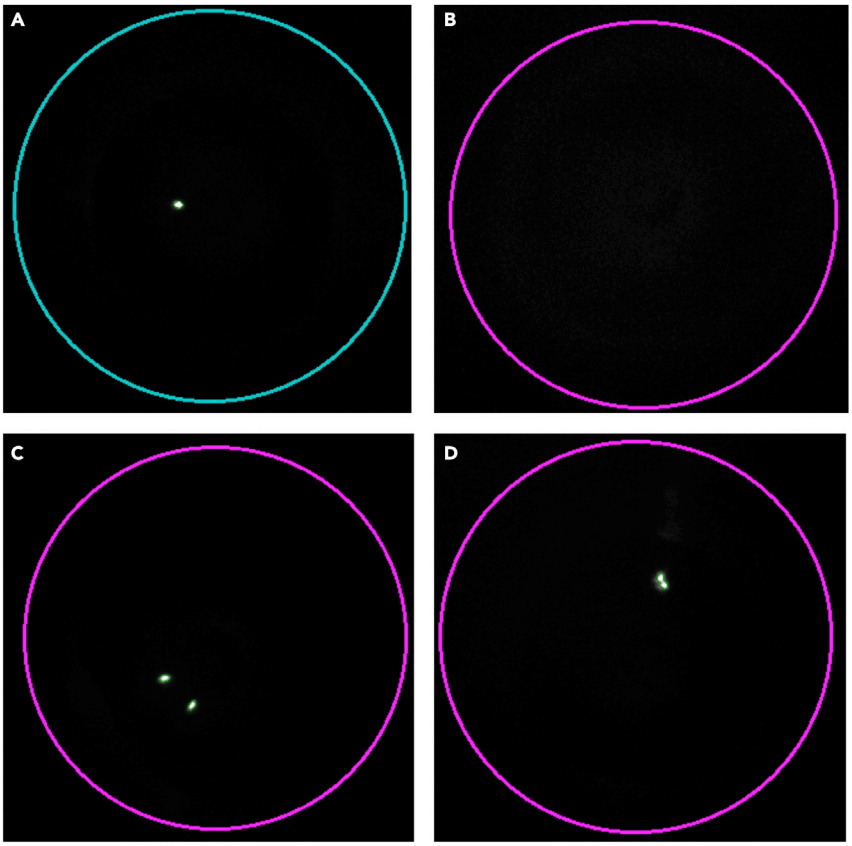

Figure 4. Confirmation of single cell candidature using TakaraBio USA Cellselect software

(A) A nanowell showing a single nucleus. These nanowells are selected for downstream processing.

(B) A nanowell void of nuclei.

(C) A nanowell with more than one nucleus.

(D) A nanowell with more than one nucleus clustered together.

34. Two snRNA-Seq libraries can be sequenced simultaneously with Illumina HiSeq 4000 aiming for 250 million paired end reads.

Note: The library is compatible with any Illumina sequencing platform using dual 8nt barcode. We typically sequence two snRNA-libraries (index A kit and index B kit) on a single HiSeq lane. If ICELL8 full length libraries would be pooled with other types of library, please check the barcoding scheme to avoid demultiplexing issue (page 39–44 https://www.takarabio.com/documents/User%20Manual/SMART/SMART-Seq%20ICELL8%20cx%20Application%20Kit%20User%20Manual.pdf).

EXPECTED OUTCOMES

From 100mg of frozen abdominal subcutaneous WAT, we expect to get between 60,000–250,000 nuclei. This number can vary on the phenotype of the donor, i.e., BMI, age and sex and the quality of the biopsy. For full-length snRNA-Seq profiling of WAT using the ICELL8cx, it is necessary to isolate at minimum 40,000 nuclei per sample. After nuclei dispense onto a 350v chip using the ICELL8cx, we typically achieve between 1200–1600 single nuclei nanowells that can be submitted for downstream cDNA library generation. This number can vary depending on the cleanliness of the preparation and the accuracy of nuclei quantification. Following cDNA library generation, it is expected to have a cDNA library concentration between 9–25 ng/μL cDNA libraries can be

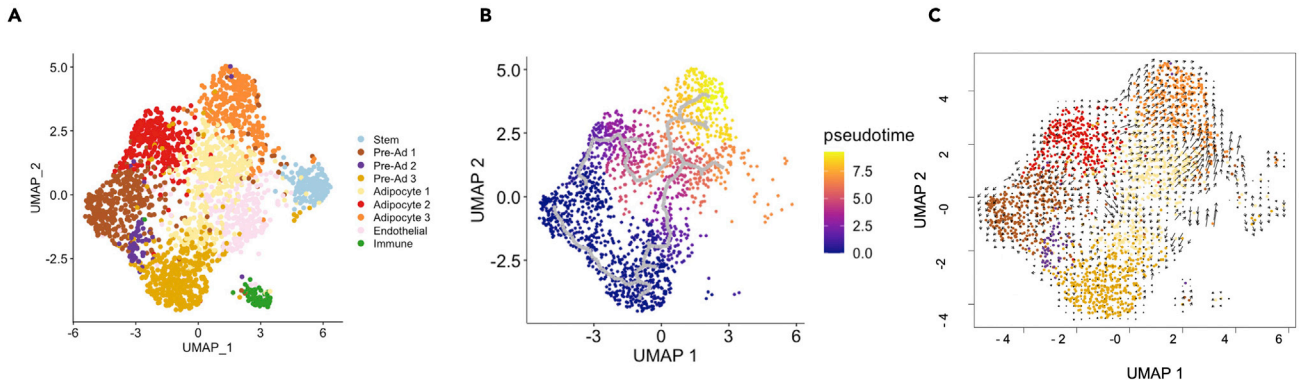

Figure 5. snRNA-Seq from human WAT

(A) UMAP plot showing different cell types detected from human WAT using snRNA-Seq. Figure reprinted with permission from Whytock et al., 2022.[1]

(B) Pseudotime analysis showing adipocyte differentiation *in vivo* using snRNA-Seq.

(C) RNA velocity analysis of pre-adipocyte and adipocyte subpopulations using snRNA-Seq.

sequenced with concentrations as low as 2 ng/μL. For a good quality cDNA library, we expect to have >80% of total bp to between 200–1000bp and overall concentration to be between 5–10nM. Sequencing snRNA-Seq libraries on an Illumina HiSeq 4000 achieves approximately 120K reads per cell and 3000–6000 genes detected per nuclei.

By performing full-length snRNA-Seq on frozen human subcutaneous WAT, we were able to profile all major cell types (Figure 5A), detect adipocyte heterogeneity, track *in vivo* adipocyte differentiation using pseudotime and RNA velocity analysis (Figures 5B and 5C). snRNA-Seq data was analyzed using publicly available R packages. For more details, refer to Whytock et al., (2022).[1] Quality control was performed with R package scran.[3] Intergration and clustering were performed with R package Seurat.[4] CogentAP™ analysis pipeline (Takara Bio, USA) was used for de-multiplexing and generation of alignment bam files for each single cell. Loom files for each sample were produced with velocyto[5] from bam files. Trajectory was accomplished with scvelo[6] and monocle3.[7–11]

LIMITATIONS

One of the main limitations of using ICELL8cx for snRNA-Seq analyses is the reduced number of nuclei profiled (1200–1600 nuclei) in comparison to other platforms such as 10X Genomics Chromium (≤∼10,000 nuclei). This limits the possibility of profiling rarer or lower proportion cell types. However, this approach results in a greater number of genes detected per nuclei and more detailed analysis of intron and exon regions. This information permits better trajectory profiling and the potential for alternate splicing and isoform calling analysis.

By using single nuclei rather than single cell RNA-Seq, we lose the capability of capturing mRNA in the cell cytoplasm and other organelles. Nonetheless, recent literature has suggested that scRNA-Seq and snRNA-Seq are comparable in detecting cell types.[12,13] By using snRNA-Seq, we were able to transcriptionally profile adipocytes that are incompatible with the dispense step on the ICELLcx as a whole cell due to their large lipid content.

TROUBLESHOOTING

Problem 1

Nuclei aggregation (Figure 6).

Potential solution

This problem may occur if a large number of nuclei are isolated from the tissue or filtration steps are not sufficient to dissociate the nuclei or the sample is left sitting for prolonged periods.

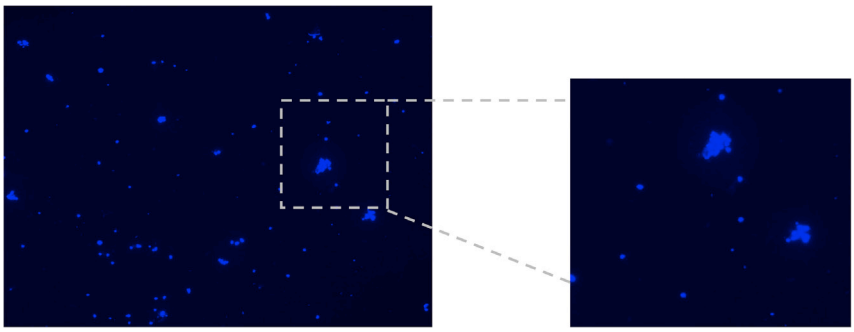

Figure 6. Aggregated Nuclei
Nuclei isolated from human subcutaneous WAT and stained with NucBlue and counted with Countess 3 Automated Cell Counter. Image is jpg readout from the Countess 3. Aggregated nuclei appear in clusters.

If the concentration is high (above 200K/mL), dilute some of the nuclei solution down (do not go below 100k/mL) and repeat 25g syringe filtration step (step 23). Repeat dilution and syringe filtration step as necessary until nuclei are less aggregated.

The ICELL8Cx system will automatically detect clustering of aggregated nuclei, and these will not be processed downstream. If there are only a few aggregated nuclei present in the preparation, these will be filtered out and it is okay to proceed.

Ensure that each pellet is completely resuspended in the solution. The pellet should be re-suspended in a small volume ~50μL before additional homogenization or nuclei resuspension buffer is added.

Work quickly and efficiently to prevent nuclei aggregating together. ICELL8cx dispense should occur within 20 min after isolation.

Problem 2
Presence of cell debris (Figure 7).

Potential solution
The 100μm and 40μm filters and 25g syringe filter are there to remove the debris. If debris is still present, an extra 70μm filter can be introduced during step 8.

If cell debris still appears in the nuclei isolation, it is advised to reduce the speed of the centrifuge. This may reduce the amount of cell debris accumulation. However, this may result in a lower nuclei yield.

Problem 3
Too much lipid.

Potential solution
Taking the time to pipette off any accumulated lipid following the first centrifugation step is one of the most important steps for having a clean preparation downstream (step 10).

If lipid still appears after the second centrifugation, transfer the homogenate solution to new LoBind tubes after each wash to help minimize lipid contamination.

Problem 4
Low nuclei yield.

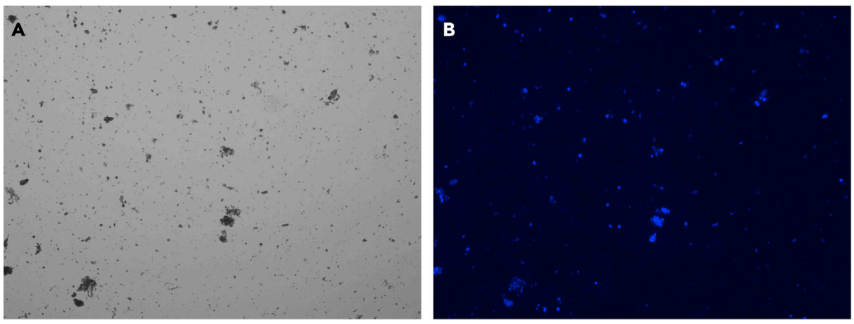

Figure 7. Presence of cell debris in nuclei preparation

(A) The brightfield image on the Countess 3 Automated Cell counter shows the presence of cell debris.

(B) The complementary NucBlue staining of the same image is unable to identify all cell debris present.

Potential solution

Due to the fibrotic nature of human WAT, we believe the pulverization step aids in releasing progenitor cells from the extra-cellular matrix and results in higher nuclei yield following homogenization. Ensure that the time is taken to completely pulverize the tissue in LN_2 to help increase nuclei yield (step 2).

Every step of this protocol has been optimized for 100mg of human WAT. If more tissue is available, it is advised to run 2 × 100mg samples in parallel and combine nuclei at the end rather than increasing the starting material for one protocol execution. Having too much tissue may lead to more nuclei aggregation and lipid accumulation.

If the pellet is left too long before removing the supernatant and re-suspending, nuclei can be lost in the supernatant. It is advised to work quickly during these steps and to remove the supernatant on both LoBind tubes before resuspension (steps 10–12).

Removing the 40µm filter step (step 8C) can help to increase nuclei yield but may also result in an increase in cell debris.

Problem 5

Low starting material.

Potential solution

Obtaining high-quality (i.e., clean and void of excess blood clots and collagen) WAT biopsies from some humans can be difficult, particularly if they are lean males. If the total amount of biopsy collected is low, the starting material for the protocol could be reduced to 50mg, although obtaining a sufficient number of nuclei for cDNA library preparation is not guaranteed.

We advise at least 40K total nuclei are isolated from WAT for successful dispense on the ICELL8cx. We typically achieve 180K from a 100mg WAT; therefore, it may be possible to start with 50mg. If using 50mg we would advise homogenizing in 1mL of homogenization buffer rather than 2mL (step 1) and resuspend into a low final volume of 300µL (step 22).

Problem 6

Low number of candidates on the nanowell chip.

Potential solution

If an optimal number of nuclei was obtained from the isolation technique but low number of candidate nanowells were selected on the ICELL8cx, this is likely due to a counting error or heterogenous nuclei solution.

It is recommended to dilute the sample in DPBS (−/−) to 200K/mL and recount the nuclei on the Countess prior to preparing the sample for ICELL8cx dispense (step 27). Samples that are too highly concentrated can lead to inaccuracies in the count.

Remix the sample prior to ICELL8cx dispense to ensure a homogenous solution (step 28).

The CellSelect software can sometimes incorrectly identify a nanowell as a false negative, i.e., it contains a single nuclei but identifies it as empty or having more than one nuclei. Order the nanowells by type and confidence to manually check for nanowells that do contain a single nucleus. These false negatives often occur in nanowells identified as 'No cells' or 'Too many cells'.

We suggest using an empty nanowell chip for cell dispensing for a mock run if this the first time performing a WAT nuclei dispense on the ICELL8cx.

Problem 7

Low cDNA concentration using updated TakaraBio SMART-Seq Pro technology.

Potential solution

This protocol has been validated using TakaraBio SMART-Seq technology. If you are using TakaraBio's updated SMART-Seq Pro technology for ICELL8cx it is possible their modified reagents may negatively interact with the EDTA and $MgCl_2$ in the nuclei resuspension buffer.

To avoid this issue we recommend a wash step after the nuclei have been incubated with the NucBlue dye (step 24) by centrifuging at 1000g for 5 min followed by resuspension in DPBS(−/−).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Lauren Sparks (Lauren.Sparks@adventhealth.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique dataset or code.

ADDITIONAL RESOURCES

Samples used during this protocol optimization were obtained from the following clinical trial (NCT04034706).

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AUTHOR CONTRIBUTIONS

Methodology, K.L.W., A.D., M.H., M.F.P., Y.S.; investigation, K.L.W., A.D., Y.S., G.Y.; validation, K.L.W., A.D., M.H., R.X.Y., Y.S.; writing – original draft, K.L.W.; writing – review and editing, A.D., Y.S., R.X.Y., M.H., L.M.S.; visualization, R.Y., K.L.W.; supervision, M.J.W., S.R.S., L.M.S.; funding acquisition, S.R.S., M.J.W., L.M.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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