User Demonstrated Plant Nuclei Isolation for Single Cell RNA Sequencing

Legal Notices

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Scale Biosciences
5601 Oberlin Dr., Suite 110
San Diego, CA 92121, United States
www.scale.bio
support@scale.bio

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Introduction

This User Demonstrated Protocol (UDP) uses the standard low-volume fixation protocol and has been developed to isolate and process high-quality nuclei from plant samples tested on *Arabidopsis thaliana*, *Zea mays*, *Sorghum bicolor*, *Medicago truncatula*, and *Brachypodium distachyon*, for downstream single-cell RNA-sequencing with the ScaleBio Single Cell RNA Sequencing Kit. The steps and buffer compositions in this protocol have been optimized to obtain robust, high-yield nuclei from normally difficult to work with samples. We recommend carrying out pilot experiments with specific samples using this protocol to evaluate yield and quality of nuclei before proceeding with the ScaleBio Sample Fixation Kit protocol.

Disclaimer

The protocol steps and deviations recorded in this User Demonstrated Protocol have not been tested at Scale Bio. This protocol was written and developed in conjunction with the Birbaum Lab, NYU, based on Guillotin B, et al 2023. See References on page 14. Please refer to this paper for additional information about this method. Scale Bio cannot guarantee optimal performance using this method and may not be able to provide support for sub-optimal runs or workflow questions.

Best Practices

- Perform all steps on ice and all centrifugations at 4°C.
- Start with fresh material for optimal nuclei quality.
- Liquid nitrogen flash-frozen samples are acceptable.
- For samples stored in RNAlater, wash twice with water in a petri dish. While RNAlater preserves nuclei, it decreases final quality.
- Limit starting material to reduce debris. 15 mg of fresh roots yield 40,000–50,000 nuclei; plan accordingly. Plant leaves tend to yield more, and hypocotyls yield much less due to their large, elongated cells.
- Examine samples under a microscope to assess the debris. In cases of excessive debris, follow the optional steps outlined in Filtration and Wash on page 10 of the procedure.
- DO NOT grind the samples with a traditional ceramic mortar and pestle; this increases debris and hampers nuclei quality.
- Use a swing bucket centrifuge to ensure efficient nuclei recovery.
- Aim to complete the protocol within 40 minutes for optimal results. Processing times exceeding 90 minutes result in lower data quality.
- Handle Fixation Reagent, DEPC, and methanol in a chemical fume hood.

Required Materials

Consumables and Reagents Manufactured by ScaleBio

ScaleBio Single Cell RNA Sequencing Kit v1.1 (PN 950884) Consumables and Reagents:

Kit Module	Consumable	Part Number	Storage Temp
ScaleBio Sample Fixation Kit- Module A (PN 2020002)	Wash Buffer	202100001	–20°C
ScaleBio Sample Fixation Kit- Module A (PN 2020003)	Fixation Reagent	202110001	4°C

Consumables and Reagents Manufactured by Other Vendors

Consumable or Reagent	Supplier	Part Number
1x PBS without calcium or magnesium	Various	Various
DEPC (purity ≥96%)	VWR	E174
DMSO (anhydrous)	Various	Various
Methanol (purity ≥99%)	Various	Various
Cell counting dye	Various	Various
Pipette tips (nuclease-free, filtered, low retention for P1000, P200, P20)[a]	Various	Various
0.2-mL PCR tube strips (nuclease-free)	Various	Various
1.5-mL DNA LoBind tubes[a]	Eppendorf	0030108418
5-mL DNA LoBind tubes[a]	Eppendorf	0030108310
Single edge razor blades	Gravey	40475
60-mm Petri dish	Various	Various
2-mL Dounce homogenizer/tissue grinder with Pestle A and B	Kimble	885300-0002
Blue plastic pestles	Various	Various
20-µm filter	CellTrics	04-0042-2315
10-µm filter	PluriSelect	43-10010-50
Sucrose	Fisher	S5-500
Ficoll	Sigma	F4375
Dextran	Sigma	31389
Tris HCl	Sigma	T2694
MES	Sigma	M8250
$MgCl_2$	Sigma	63069
KCl	Sigma	P5405

Consumable or Reagent	Supplier	Part Number
NaCl	Sigma	S3014
Spermine	Sigma	S3256
Spermidine	Sigma	S0266
Triton X-100	Sigma	X100
Phenylmethylsulfonyl fluoride (PMSF)	Sigma	93482
Plant protease inhibitors	Sigma	P9599
BSA	Sigma	A3912
EDTA	Sigma	324504
DTT	Biochemica	A1101
RNase inhibitor	Promega	N2611
RNase-free water	Various	Various

a. Required for best assay performance.

Recommended Equipment

Item	Supplier	Part Number
Pipettes (P1000, P200, P20, P10, P2)	Various	Various
Vortex mixer	Various	Various
Cell counter	Various	Various
Centrifuge with temperature control and swing-bucket rotor (1.5-mL tubes, 96-well deep-well plates)	Various	Various
Fluorescent Microscope with DAPI visualization capability and magnification up to 63x	Various	Various

Buffer Composition

Lysis Buffer

Lysis Buffer (600 µL/sample)	Stock Solution	Per mL	10 mL
0.3M Sucrose	Solid	102 mg	1.02 gm
1.25% Ficoll	Solid	12.5 mg	0.125 gm
2.5% Dextran	Solid	25 mg	0.25 gm
15 mM Tris HCl pH8	1M	15 µL	150 µL
20 mM MES	Make 1M in RNase-free water	20 µL	200 µL
10 mM MgCl2	Make 1M in RNase-free water	10 µL	100 µL
60 mM KCl	Make 1M in RNase-free water	60 µL	600 µL

Lysis Buffer (600 µL/sample)	Stock Solution	Per mL	10 mL
15 mM NaCl	Make 5M in RNase-free water	3 µL	30 µL
0.5 mM Spermine	Make 100 mM in RNase-free water	5 µL	50 µL
0.5 mM Spermidine	Make 100 mM in RNase-free water	5 µL	50 µL
0.1% Triton X-100	Make 10% in RNase-free water	10 µL	100 µL
RNase-free water		to 1 mL	to 10 mL
Add right before use			
1% DEPC	pure	10 µL	100 µL
5 mM DTT	1 M	5 µL	50 µL
1 mM Phenylmethylsulfonyl fluoride (PMSF) (only stable for 30 min)	0.1 M	1 µL	10 µL
1% Plant protease inhibitors	100%	1 µL	10 µL
0.4% BSA (increase to 1% if nuclei are clumpy)	10%	40 µL	400 µL
RNase inhibitor (0.4 units/µL)	40 units/µL	10 µL	100 µL

Wash Buffer

Wash Buffer (500 µL/sample)	Stock Solution	Per mL
0.3 M Sucrose	2.5 M	120 µL
15 mM Tris HCl pH8	1 M	15 µL
60 mM KCl	Make 1 M in RNase-free water	60 µL
15 mM NaCl	Make 5M in RNase-free water	3 µL
0.5 mM Spermine	Make 100 mM in RNase-free water	5 µL
0.5 mM Spermidine	Make 100 mM in RNase-free water	5 µL
15 mM MES	Make 1 M in RNase-free water	15 µL
0.1% Triton X-100	10%	10 µL
RNase-free water		to 1 mL
Add right before use		
5 mM DTT	1 M	5 µL
1 mM Phenylmethylsulfonyl fluoride (PMSF) [only stable for 30 min]	0.1 M	1 µL
1% Plant protease inhibitors	100%	1 µL

Wash Buffer (500 µL/sample)	Stock Solution	Per mL
0.4% BSA	10%	40 µL
RNase inhibitor (0.2 units/µL)	40 units/µL	5 µL

Final Resuspension Buffer

Final Resuspension Buffer (100 µL/sample)	Stock Solution	Per mL
0.3 M Sucrose	2.5 M	120 µL
15 mM Tris HCl pH8	1 M	15 µL
60 mM KCl	Make 1 M in RNase-free water	60 µL
15 mM NaCl	Make 5M in RNase-free water	3 µL
2 mM EDTA	0.5 M	4 µL
0.5 mM Spermine	Make 100 mM in RNase-free water	5 µL
0.5 mM Spermidine	Make 100 mM in RNase-free water	5 µL
15 mM MES	Make 1 M in RNase-free water	15 µL
RNase-free water		to 1 mL
Add right before use		
5 mM DTT	1 M	5 µL
1% Plant protease inhibitors	100%	1 µL
1% BSA	10%	100 µL
RNase inhibitor (0.2 units/µL)	40 units/µL	5 µL

Protocol

Root Preparation

1. Place samples directly into a pre-chilled 60-mm plastic petri dish **on ice** containing 300 µL of lysis buffer.
2. Mince samples with two razor blades for at least 8–10 minutes **on ice** until no visible pieces remain.

Tissue Homogenization

Two homogenization options are provided. Test Option A with your samples. If it results in nuclei that are crushed or broken, test Option B, which is gentler but generates less nuclei.

Option A (Eppendorf Tube Homogenization)

1. Transfer the minced tissue to a pre-chilled 1.5-mL Eppendorf tube containing a pre-chilled blue plastic pestle using a P1000 pipette. If starting material is limited, wash the petri dish with **100 µL** of lysis buffer and transfer the wash to the Eppendorf tube.
2. Perform 20 up-and-down strokes with a twisting motion using the blue pestle to further homogenize the tissue.
3. Incubate the homogenate **on ice** for **2–3 minutes** to complete cell lysis.
4. Perform 10 additional up/down strokes with the pestle to release remaining nuclei.

Option B (Dounce Homogenizer)

1. Transfer the minced tissue to a pre-chilled 2-mL Dounce tissue grinder (Kimble) using a P1000 pipette. If starting material is limited, wash the petri dish with 100 µL of lysis buffer and transfer the wash to the Dounce grinder.
2. Perform 10 up-and-down strokes with pestle A to further homogenize the tissue.
3. Incubate the homogenate in the Dounce grinder **on ice** for **3–5 minutes** to complete cell lysis.
4. Perform 5 additional strokes with pestle A to release any remaining nuclei.
5. For *Arabidopsis* only: Use pestle B and perform 10 up-and-down strokes. Do not use pestle B for maize or sorghum due to the risk of nuclei degradation.

Filtration and Wash

1. Filter the homogenate through a 20-µm CellTrics filter into a pre-chilled 1.5-mL Eppendorf tube.
2. Wash the filter with **200 µL** of lysis buffer, ensuring complete transfer of the liquid to the tube.
3. Centrifuge the sample at **500 x g** for **10 minutes**. For samples with high debris, perform **Additional Purification** (below) before performing this step.

   NOTE: Adapt the centrifugation speed depending on the genome size of the species used, larger genomes such as maize (2.4 GB) have denser nuclei that pellet at 500 x g than the less dense *Arabidopsis* (135 MB) that pellet at 1000 x g. For *Medicago* (430 MB) centrifuge at 800 x g.

   For Additional Purification: For high debris, centrifuge the sample for 3 minutes at 50 x g to pellet debris. Carefully transfer the supernatant to a new tube, leaving approximately 50 µL behind to avoid transferring debris. Proceed with step 3 as indicated.

4. Carefully remove the supernatant without disturbing the pellet, leaving only a few microliters of liquid.
5. Add **300 µL** of Wash Buffer to the pellet and gently resuspend the nuclei using a P1000 pipette until no clumps are visible.
6. Centrifuge the sample at 500 x g for 5 minutes. For samples with high debris, optioinally perform **Additional Purification** (below) before performing this step.

   NOTE: Adapt the speed to your species of interest.

   For Additional Purification: For high debris, centrifuge the sample for **3 minutes** at **50 x g** to pellet debris. Carefully transfer the supernatant to a new tube, leaving approximately 50 µL behind to avoid transferring debris. Proceed with step 6 as indicated.
7. Carefully remove the supernatant without disturbing the pellet, leaving only a few microliters of liquid.

   For Additional Purification: After performing step 7, optionally follow these steps:

   a. Add **200 µL** of Wash Buffer to the pellet and resuspend by gentle pipetting.

   b. Carefully layer the resuspended nuclei on top of **500 µL** of cushion buffer (same composition as Wash Buffer but with 1.7 M sucrose instead of 0.3 M) in a fresh 1.5-mL Eppendorf tube. Ensure slow and gentle layering to maintain a distinct interface.

   c. Centrifuge at **1000 x g** at **4°C** for **5 minutes**.

   d. Carefully remove the supernatant, as the nuclei will have pelleted at the bottom of the tube, while chloroplasts and other debris will remain in the supernatant. Remove as much supernatant as possible without disturbing the pellet.

   e. Continue with step 8.
8. Add **50 µL** of final buffer to the pellet and gently resuspend the nuclei by flicking the tube or gently pipetting.
9. Filter the sample through a 10-µm PluriSelect strainer into a fresh pre-chilled 1.5-mL Eppendorf tube. Apply gentle pressure with your thumb on top of the filter to aid filtration. If starting material is limited, wash the tube and filter with an additional 10 µL of final buffer.

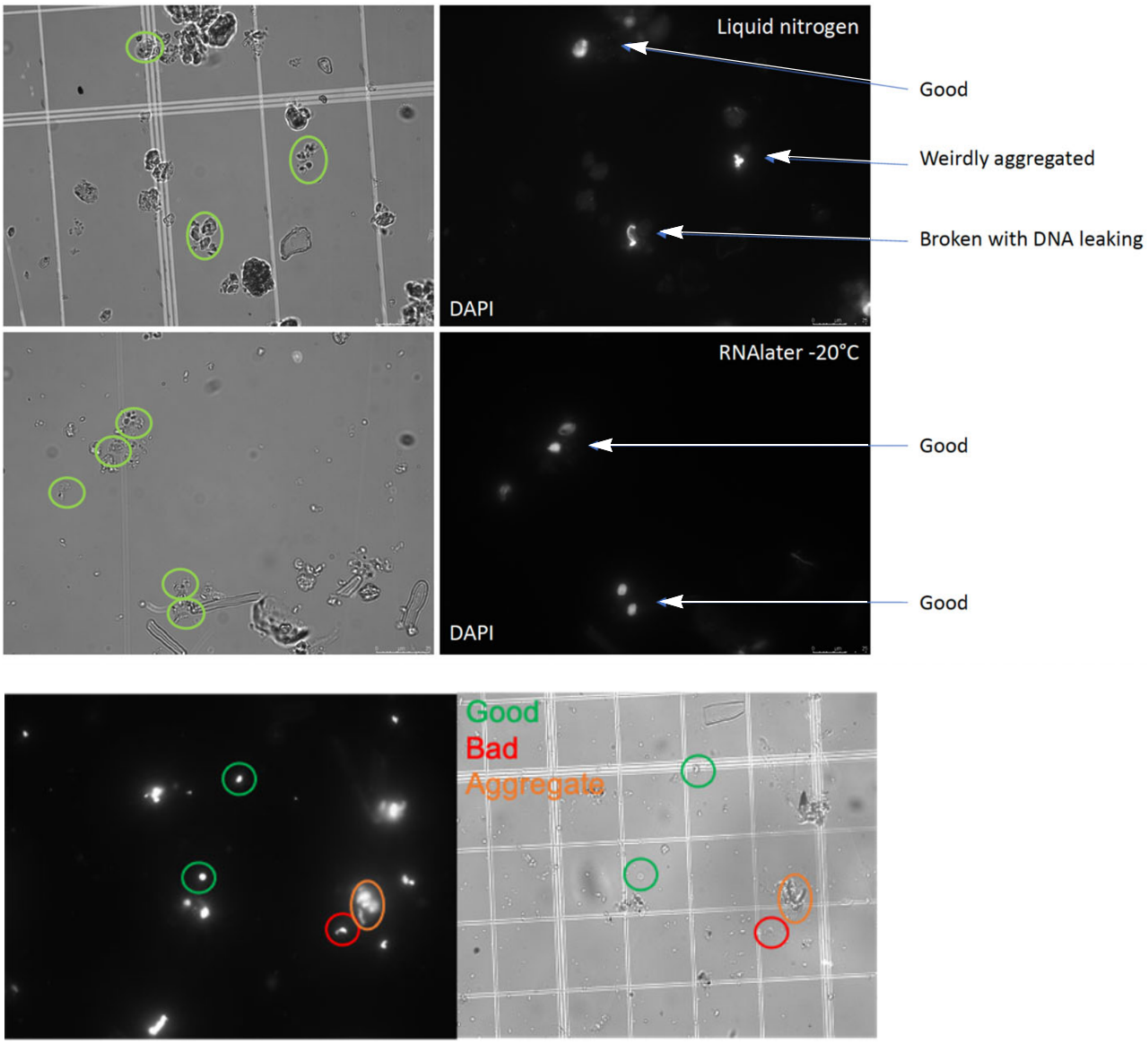
Nuclei Counting

Manual counting using DAPI dye and a fluorescent microscope is recommended for best results, as using an automated counter such as Countess tends to overestimate nuclei counts and confound nuclei with debris.

1. Mix **1 µL** of DAPI stock solution with **9 µL** of purified nuclei to achieve a final DAPI concentration of 300 nM.
2. Apply **9 µL** of the DAPI working solution to a clean hemocytometer.
3. Count nuclei at 40x or 63x magnification. Use a standard coverslip for 63x magnification, as the hemocytometer coverslip is too thick.
4. Under brightfield illumination, visually identify and memorize the boundaries of a red square in the field of view. This serves as a reference for subsequent fluorescence imaging.
5. Switch to the DAPI filter. Count all well-defined, round nuclei within the pre-identified square's boundaries. Adjust the z-plane (focus) during counting to ensure comprehensive enumeration, as nuclei may adhere to the coverslip or hemocytometer bottom.
6. Repeat counts in 6–8 squares and calculate the average.

7. Expect approximately 50,000 nuclei from 15 mg of fresh root tissue. Refer to the Best Practices on page 5. For nuclei counts between 100,000 and 400,000, proceed with the ScaleBio Low Volume Fixation for Single Cell RNA Sequencing Protocol (Doc. 1020807).

Examples of good vs bad nuclei



ScaleBio Low Volume Fixation

Preparation of Complete Cell Fixation Solution

1. In a chemical fume hood, reconstitute the Fixation Reagent by adding 50 µL of DMSO to one tube of Fixation Reagent. The lyophilized reagent appears as a white pellet at the bottom of the tube.
2. Vigorously vortex the tube at high speed until the reagent is fully dissolved. This may take several minutes. Ensure complete dissolution before proceeding.
3. For each sample, prepare the fixation mix as follows:

Reagent	Volume (µL)
Methanol	222
Reconstituted Fixation Reagent	5.5
DEPC	2.2

4. Vortex the mix thoroughly and keep it **on ice**. Use immediately.
5. Add **200 µL** of the Complete Cell Fixation Solution to the 1.5-mL tube containing the resuspended nuclei. Pipette up and down three times to make sure nuclei don't stick together.
6. Incubate the tube **on ice** for **15 minutes**, gently inverting the tube every **5 minutes** to mix. A white precipitate may appear, which is normal.
7. Add **500 µL** of Wash Buffer to the tube and resuspend the cells by flicking the tube. The white precipitate should disappear.
8. Centrifuge the tube at **500 x g** for **5 minutes** at **4°C** (1000 x g for Arabidopsis). For nuclei or small cells, consider centrifuging for 8–10 minutes or increasing the speed to maximize recovery.
9. Carefully remove the supernatant without disturbing the cell pellet, leaving approximately 30 µL of residual volume.
10. Add **250 µL** of Wash Buffer and resuspend the cells by flicking the tube.
11. Centrifuge the tube at **500 x g** for **5 minutes** and **4°C**.
12. Carefully remove the supernatant without disturbing the pellet, leaving approximately 20–30 µL of residual volume. Resuspend the cells by flicking the tube.
13. Transfer 2–5 µL of the cell suspension to a 0.2-mL PCR tube for cell counting.
14. Freeze the remaining sample at **–80°C** for future use with ScaleBio Single Cell RNA Sequencing Kit.

Additional Information

Notes

We recommend using the following read structure for sequencing to improve genome alignment:

Read Structure	Cycles
Read 1	34
Index 1	10
Index 2	10
Read 2	150

Acknowledgment

This protocol was written in conjunction with the Birbaum Lab, NYU.

References

Guillotin B, Rahni R, Passalacqua M, et al. A pan-grass transcriptome reveals patterns of cellular divergence in crops. *Nature*. 2023;617(7962):785–791.

Document Revision History

Document Revision	Revisions Date	Description of Change
1325131 Rev A	May 2025	Initial release