DEMONSTRATED PROTOCOL

Cell Isolation from Fresh Tissue for Single Cell Sequencing Applications

Overview

This protocol outlines the process for isolating, cleaning, counting, and preparing single cells from fresh tissue for the single-cell sequencing assays listed below. The protocol is species-agnostic, with recommendations tailored to different tissue types. Some optimization of Singulator protocol parameters and post-isolation cleanup steps may be needed based on the species, tissue type, and condition of the fresh tissue. Refer to Appendix 1 for tissue-specific recommendations.

Compatible Downstream Applications

The assays listed below have been validated to work with cells isolated with the Singulator™ Platform. Cells isolated with the Singulator™ Platform are expected to be compatible with most downstream single-cell applications. For more information, including customer published protocols and publications, visit our website at s2genomics.com/resources.

Platform	Assay	Part Number
10x Genomics	Chromium Next GEM Single Cell 3’	1000269 (4 rxns) 1000268 (16 rxns)
10x Genomics	Chromium GEM-X Single Cell Gene Expression (3’)	1000686 (4 rxns) 1000691 (16 rxns)
Parse	Evercode Whole Transcriptome V3	ECWT3100 (Mini) ECWT3300 ECWT3500 (Mega)

Reagents and Consumables

Vendor	Item	Part Number
S2 Genomics	Cell Isolation Bundle (24 samples)	100-289-261
	Brain Reagent, 20 mL	100-064-304
	Lung Reagent, 20 mL	100-064-413
	Liver Reagent, 20 mL	100-064-522
	Kidney Reagent, 20 mL	100-064-631
	Spleen Reagent, 20 mL	100-064-740
	Intestine Reagent, 20 mL	100-064-849
	Skin Reagent, 20 mL	100-254-082
	Tumor Reagent, 20 mL	100-253-955
Millipore Sigma	40 µm Flowmi Cell Strainer	BAH136800040
Eppendorf	DNA LoBind Tubes	0030122275
VWR	15 mL High Performance Centrifuge Tubes	21008-089
	Pipette Tips RT LTS 1000 µL – Low Retention	30389219
	Pipette Tips RT LTS 250 µL – Low Retention	30389250
	Pipette Tips RT LTS 20 µL – Low Retention	30389226

	Bovine Serum Albumin – Lyophilized Powder (Molecular Biology Grade, Nuclease and Protease Free)	97061-420
	Percoll Density Gradient Media	89428-01
Revvity	Cellometer K2 Fluorescent Cell Counter	-
	SD025 Counting Chambers	CHT4-SD025
	ViaStain AO/PI Staining buffer	CS2-0106
pluriSelect	pluriStrainer 30 mm	43-50030-03
ThermoFisher	Gibco (Ammonium-Chloride-Potassium) ACK Lysing Buffer	A1049201
	Fetal Bovine Serum (FBS), qualified, heat inactivated	16140071
	DMEM (Dulbecco's Modified Eagle Medium), high Glucose	11965092
Corning	HBSS (Hank's Balanced Salt Solution) 1X without Calcium, Magnesium, and Phenol Red	21-0022-CM
	RPMI 1640 Medium	10-040-CV
	Phosphate Buffered Saline (PBS) without Calcium and Magnesium	21-040-CV
	Phosphate Buffered Saline (PBS) 10x without Calcium and Magnesium	46-013-CM

Getting Started

Prepare Buffers

Cell Reagent Buffer* Fully dissolve desired tissue-specific Cell Reagent in 20 mL of appropriate buffer (See Table 1) at room temperature by inverting vial for 20 minutes. Do not pre-heat Cell Reagent Buffer. Prepare 3 mL aliquots in 15mL conical tubes and store at 4°C for up to one week or at -20°C for up to 6 months.	**Per 6 Samples**
Tissue-Specific Cell Reagent (20 mL vial)	-
Appropriate Buffer (See Table 1)	20 mL
Total	20 mL

** Prepare fresh.*

Optional Buffers

Optional buffers listed below for Debris Removal steps in Section C. See Table 1 below for tissue-specific recommendations.

Stock Percoll Solution	**Per 1 Sample**
PBS 10x	100 µL
Percoll	900 µL
Total	2 mL

Percoll Debris Removal Solution (place on Ice)	**Per 1 Sample**
PBS 1X	2.1 mL
Stock Percoll Solution	900 µL
Total	3.0 mL

Tips and Best Practices

1. Use Table 1 to determine cartridge, Singulator protocols, appropriate buffers, RBC lysis incubation time, and post-cleanup procedures on specific tissues.

Table 1

Tissue	Recommended Tissue Mass	Cell Reagent	Singulator Protocol	Appropriate Buffer	RBC Lysis Incubation Time (on ice)	Debris Removal Step
Brain	150 mg	Brain Reagent, 20mL	Mouse Brain Cells V2	DMEM	1 minute	Required
Liver	200-400 mg	Liver Reagent, 20mL	Mouse Liver Cells V2	HBSS	2 minutes	Required
Kidney	150-300 mg (whole kidney)	Kidney Reagent, 20mL	Mouse Kidney Cells V2	HBSS	5 minutes	Optional
Tumor	50-200 mg	Tumor Reagent, 20mL	Tumor Cells V2	DMEM	2 minutes	Optional
Skin	150-200 mg	Skin Reagent, 20mL	Mouse Skin Cells V2	DMEM	2 minutes	Optional
Spleen	50-100 mg (whole spleen)	Spleen Reagent, 20mL	Mouse Spleen Cell V2	HBSS	2 minutes	Not Required
Lung	50-200 mg	Lung Reagent, 20mL	Mouse Lung Cells V2	DMEM	2 minutes	Not Required

2. Process fresh tissue immediately upon tissue collection.
3. For optimal yields, refer to recommended tissue masses in Table 1. Samples smaller than recommendations may result in decreased viability and yield. Samples larger than recommendations may cause clogging of the filter unit; proceed with caution.
4. Place all tubes on ice containing cells and downstream reagents.
5. Minimize time as much as possible between steps to maintain cell viability.
6. Use a swinging bucket centrifuge to pellet cells and prevent cells from smearing against sides of centrifuge tubes. This will maintain cell integrity and viability.
7. Resuspend pellets by gently pipetting to avoid shearing the cells.
8. Cell wash buffer has 1% BSA solution to maintain cell viability and prevent clumping during processing.

Cell Isolation from Fresh Tissue

A. Singulator Setup

1. Obtain a Cell Isolation Cartridge and equilibrate to room temperature.
2. Prepare buffers as described in **Getting Started** section.
3. Turn on the Singulator by pressing the power button located at the top of the tablet interface.
4. Pre-heat the Singulator by sliding the toggle in the upper right of the **User Home Screen** to **Heat** and tap the icon to initiate pre-heating. The bar will turn orange indicating pre-heating is in progress and will turn green and display **On √** upon completion.
5. Once the Singulator is pre-heated, select the desired protocol.
    a. Select **Run a Protocol** from the **User Home Screen**.
    b. Select the **Cells** button to toggle to cell protocols.
    c. Select **appropriate Cell Isolation Protocol** (See Table 1).
    d. Select **Next**.

e. On the **Run Notes Screen**, add notes if desired to be saved in the internal log files, then select **Next**.

6. Using the red knob, pull and slide down the tube holder of the Single Shot Mechanism next to the designated processing bay. Remove the cap and place a 15 mL conical tube containing 3 mL of room temperature Cell Reagent Buffer into the right side of the Single Shot Mechanism, in the slot labeled "Enzyme".
7. Add 6 mL of appropriate buffer (See Table 1) to a 15 mL conical and place into the left side of the Single Shot Mechanism, in the slot labeled “Buffer”.

B. Cell Isolation

1. Obtain fresh tissue and mince into 1-2mm^2 pieces using a scalpel, razor blade, or scissors.
2. Remove the Cell Isolation Cartridge from its sealed packaging, remove the cap from the cartridge, place the minced tissue to the bottom the Dissociation Chamber, and replace the cap.

   Tip: If tissue is sticky and difficult to transfer, add 100 µL of appropriate buffer to the sample when transferring to help keep tissue from sticking to the bottom of the cartridge.
3. Lift the door open of the Singulator and slide out the Cartridge Tray while lifting the red knob.
4. Place the cartridge on the Cartridge Tray and turn the white cartridge lock counterclockwise to lock cartridge into place.
5. Slide in the Cartridge Tray by pushing the back of the tray until the red knob fully drops into place. DO NOT USE THE RED KNOB TO PUSH THE CARTRIDGE TRAY.
6. Close the door of the Singulator.
7. Select Run – the cell isolation takes approximately 30-65 minutes depending on protocol selected.
8. After completion of the run, the instrument will display a Run Complete Screen. Raise the door and then, lift the red knob up, slide the Cartridge Tray and cartridge out of the instrument. Close the door. Rotate the cartridge lock clockwise and remove the cartridge from the Cartridge Tray.
9. Pierce foil seal of the Output Chamber with a 1 mL pipette and retrieve the sample and place into a cold 15 mL conical tube. Make sure to tap the cartridge on the benchtop to get any remaining sample out of the filter unit. There will be approximately 5.5 mL of sample.
10. Centrifuge sample at 300 g for 5 minutes at 4°C in a swinging bucket rotor.
11. Remove used 15 mL conical tubes, now filled with rinse water, from Single Shot Mechanism and discard.

C. RBC Lysis

This protocol uses an ACK Lysis Solution for lysing red blood cells. For samples with a pellet that is not visible after centrifugation, or a pellet with no RBCs present (indicated by a lack of red color), an RBC lysis step is not recommended. Proceed to Section D.

1. Remove the supernatant after centrifugation and gently resuspend pellet in 1 mL cold ACK Lysing Solution.
2. Incubate for recommended time (See Table 1) on ice.
3. Add 6 mL of appropriate buffer (See Table 1).
4. Centrifuge sample at 300 g for 5 minutes.

D. Debris Removal (optional) and Resuspension in Cell Resuspension Buffer

This protocol includes an optional gentle density gradient centrifugation step to remove debris from cell samples. See Table 1 for tissue specific recommendations. For tissue samples where debris removal is not recommended, samples under 20 mg, or with a pellet that is not visible after centrifugation, proceed directly to Step D.4 for resuspension in Cell Resuspension Buffer.

1. Remove supernatant and gently resuspend the pellet in 3 mL of Percoll Debris Removal Solution.
2. Centrifuge sample at 300 g for 10 minutes (with brake setting set to 0 or 1). Once complete, remove sample from centrifuge, taking care not to disturb the possible floating 'debris cake' at the top of the supernatant.
3. Using wide bore tips, carefully remove the floating 'debris cake' and supernatant.

   Tip: Using a razor blade or scissors, cut the wide bore pipette tip to increase the orifice, and remove the supernatant in 300-500 µl increments. Make sure to completely remove supernatant without letting debris fall onto the cell pellet. Near the end, use a standard (non-wide) bore pipette tip to remove the supernatant close to the pellet.
4. Gently resuspend the pellet in 1 mL of Cell Resuspension Buffer and place on ice.
5. Filter with a 40 µm Flowmi strainer to remove aggregates and remaining debris, and place in a cold 1.5 mL Eppendorf tube.

E. Counting and Dilution

Follow manufacturer's instructions to obtain cell count using fluorescence method, briefly described below. Adjust cell concentration based on manufacture's recommendations for desired downstream application.

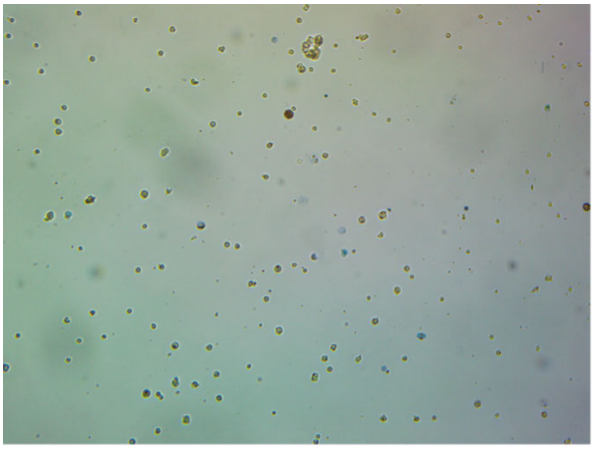
1. Remove coverslip from Nexcelom counting slide.
2. Mix 30 µL of sample with 30 µL of ViaStain AO/PI dye, and place 20 µL of the mixture in each side of the counting slide.
3. Insert the counting slide into the Cellometer K2 cell counter.
4. Open the matrix software on K2 laptop. Select **K2_AOPI_Primary Cells** assay and enter dilution factor of 2.
5. Select **Preview**.
6. Using knob on right side of the instrument adjust focus until cells appear in "**Good Focus**" according to the **Cellometer Focus Guide**.
7. Select **Count**.
8. Adjust concentration of cell suspension with Cell Resuspension Buffer to desired concentration for downstream assay. Strain through 30 µm pluriSelect strainer if needed to remove clumps prior to loading single-cell assays.

Results

Representative Images:

Human Lung Tumor Cells

(10x Magnification)



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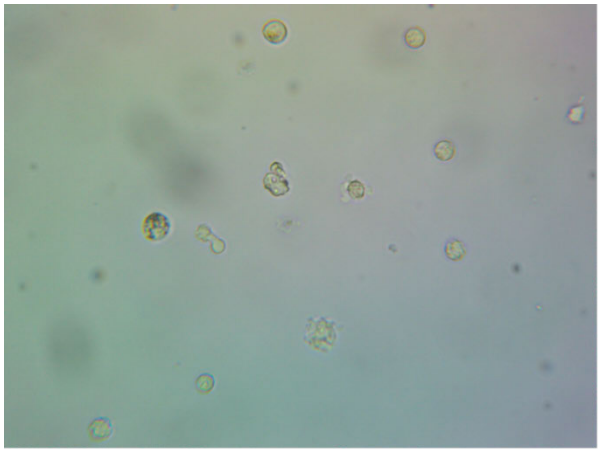
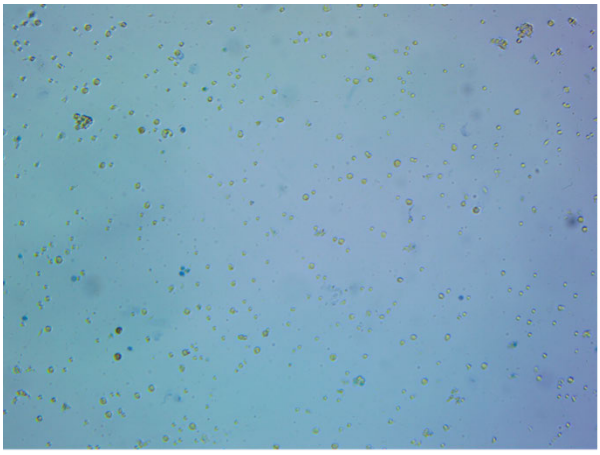


Mouse Lung Cells

(10x Magnification)



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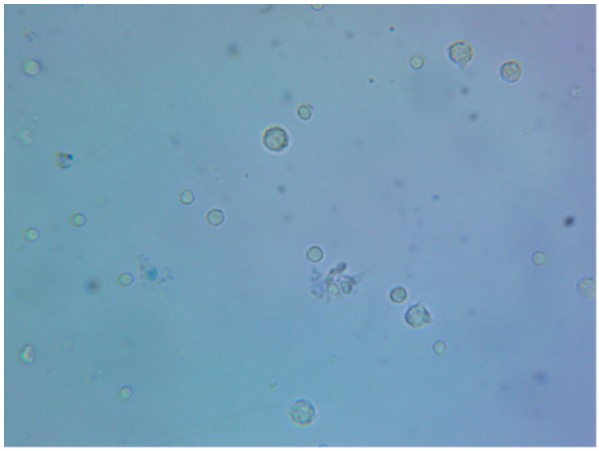

Mouse Skin Cells

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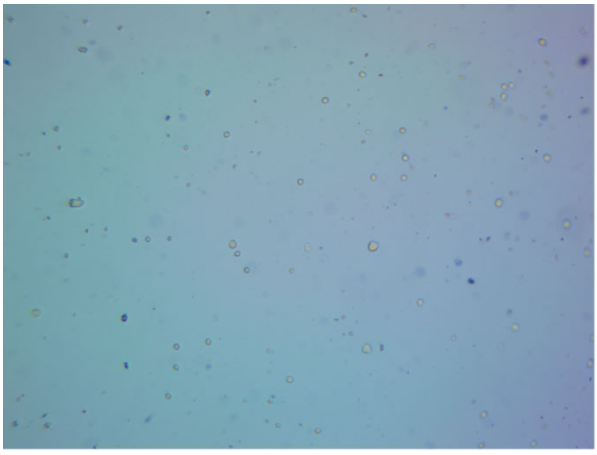

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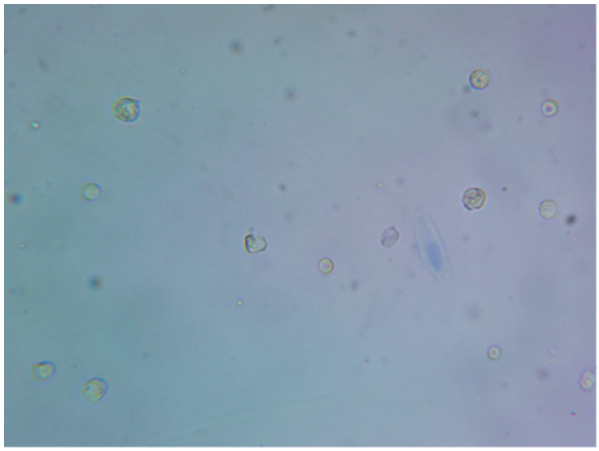

Mouse Kidney Cells

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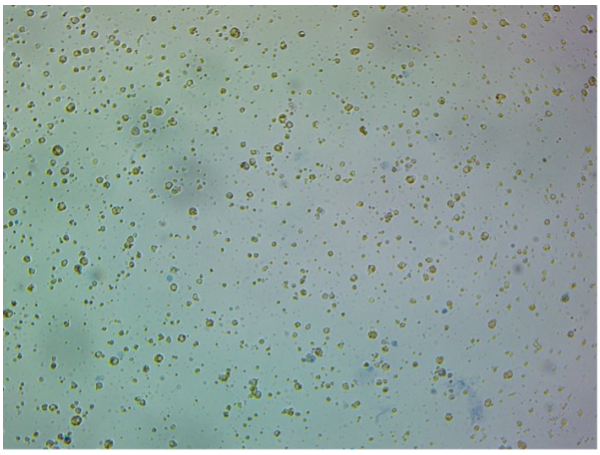

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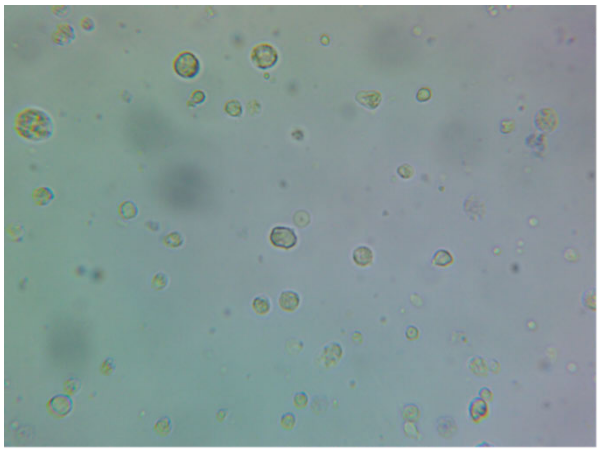
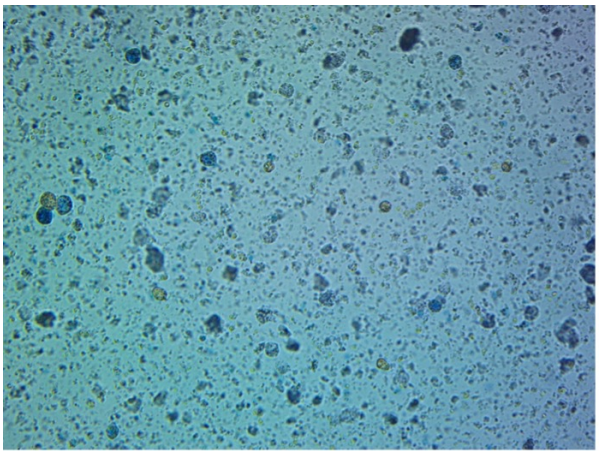


Mouse Liver Cells

Before Debris Removal
(10x Magnification)



After Debris Removal
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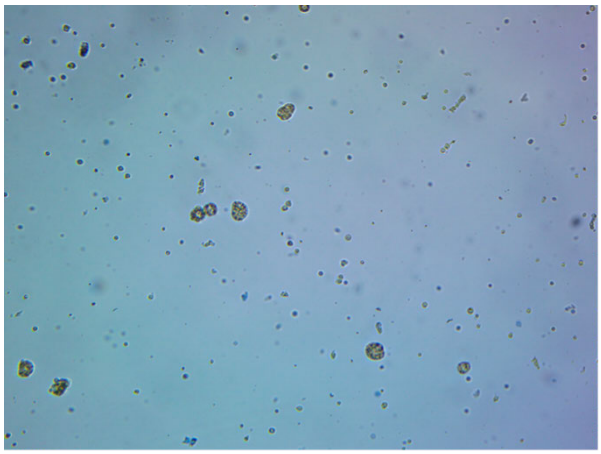

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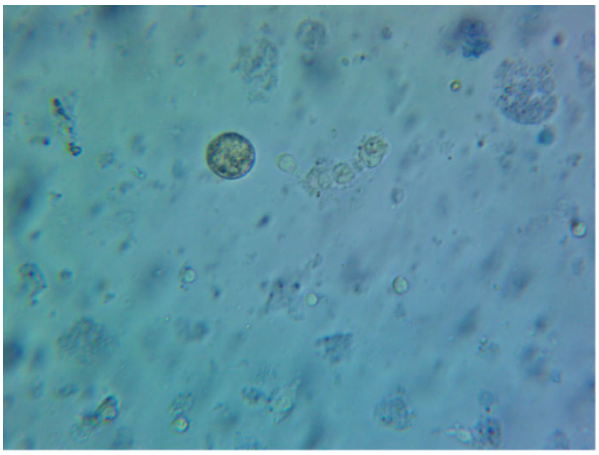

After Debris Removal
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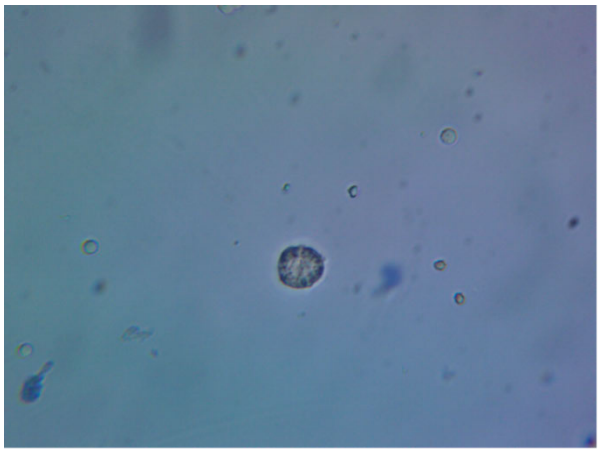
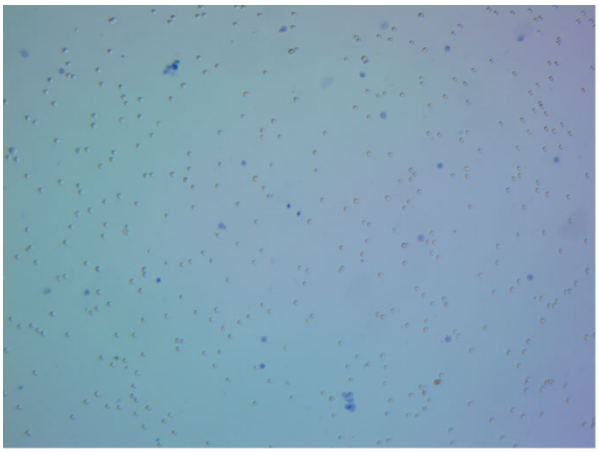


Mouse Spleen Cells

(10x Magnification)



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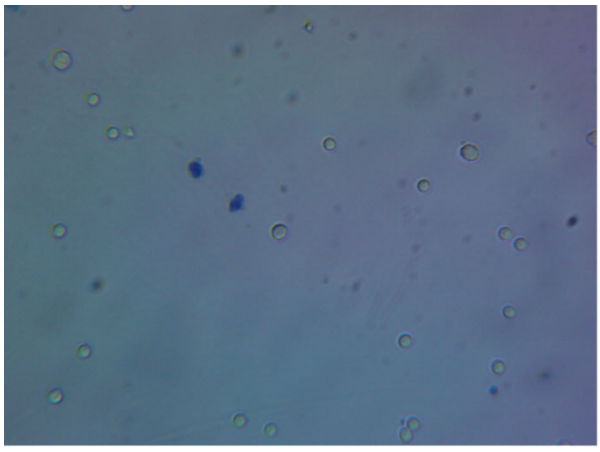
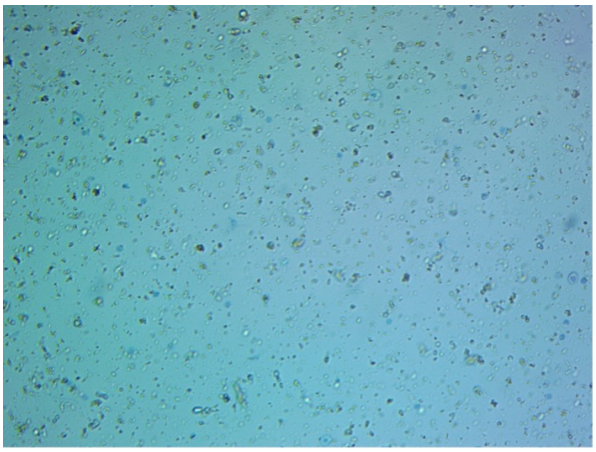


Mouse Brain Cells

Before Debris Removal
(10x Magnification)



After Debris Removal
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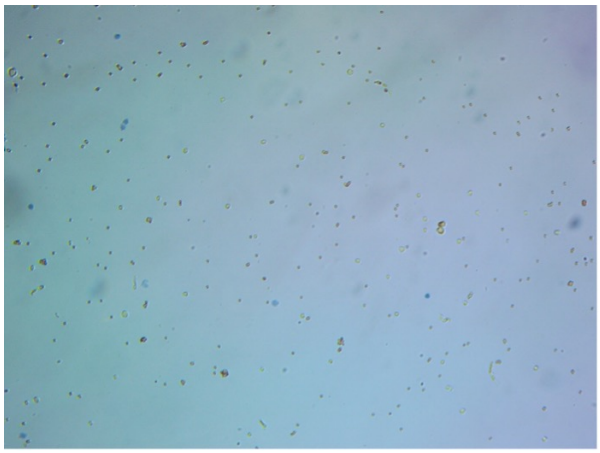

Before Debris Removal
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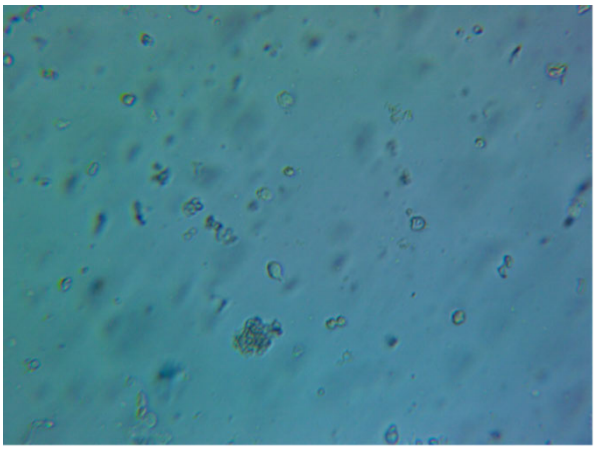

After Debris Removal
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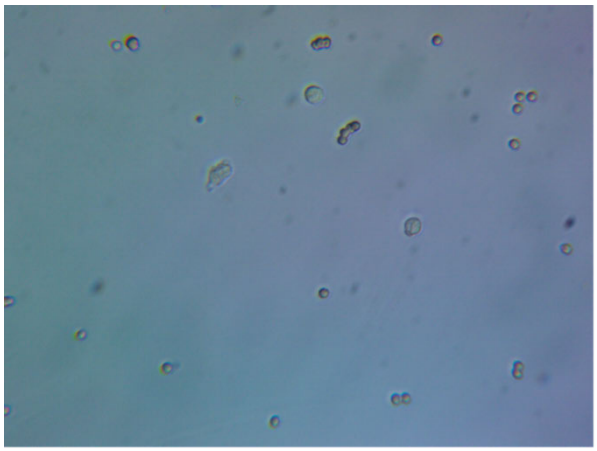


Appendix 1 – Tissue Specific Recommendations and Notes

Tissues listed below have been tested with the Singulator Platform. For more information, including customer published protocol and publications, visit our website s2genomics.com/resources.

Tissue	Recommendations	Notes
Brain	• See Table 1 for protocol recommendations	• Non-neuronal type cells are preferentially isolated due to fragility of neuron cell type. If gene expression from neuronal cell type is desired we recommend nuclei isolation. Follow "Demonstrated Protocol Nuclei Isolation from Frozen Mouse Brain Tissue for Single Nuclei Sequencing Applications"
Heart	• See "Demonstrated Protocol: Cell Isolation from Mouse Heart"	• Requires Large Cell Isolation Cartridge.
Intestine	• See "Demonstrated Protocol: Cell Isolation from Mouse Intestine"	• Requires pretreatment of tissue.
Kidney	• Use 2 mL of ACK Lysis buffer in step C.1 and incubate on ice for 3-4 minutes in Step C.3. • Additional wash in 3 mL of Cell Resuspension Buffer can be done in place of Steps D.1 through D.3. to remove debris.	• Increased RBC lysis time may be required.
Liver	• Debris removal is required, see Section D.	• Non-parenchymal cells are preferentially isolated due to fragility of parenchymal cell types. If gene expression from parenchymal cell types is desired we recommend nuclei isolation. Follow "Demonstrated Protocol Nuclei Isolation from Frozen Tissues for Single Nuclei Sequencing Applications".
Organoids	• Pellet and resuspend in 100 µL of appropriate buffer and add to cartridge in Step B.2. • After testing, determine if reduced incubation time is required. See Appendix 2 – Issues and Recommendations for further optimizations.	• Reduced incubation time likely required. See Appendix 2 – Issues and Recommendations if viability is low.
Spleen	• See Table 1 for protocol recommendations.	
Skin	• See Table 1 for protocol recommendations.	• Ensure adequate mincing of tissue into 1mm^2 for efficient digestion.

Tumor	• See Table 1 for protocol recommendations.	• Ensure adequate mincing of tissue into 1mm^2 for efficient digestion. • Variability in yield and viability expected based on tumor quality.
Lymph	• See Table 1 and follow spleen recommendations.	

Tissues not listed above have not been validated for use with this demonstrated protocol. Please reach out to customer support at support@s2genomics.com to further discuss your desired tissue and application.

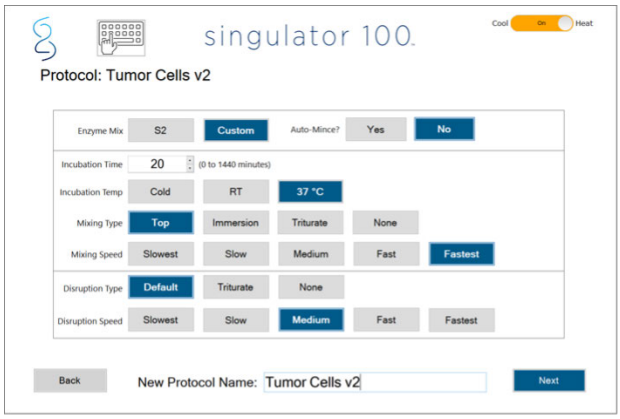
Appendix 2 – Issues and Recommendations

Issues	Recommendations
Lower yield than previously observed for an equivalent sample	• Calibrate instrument, see Singulator Instrument User Guide.
Insufficient yield for downstream application	• Increase tissue input mass • Ensure thorough mincing of tissue to 1-2mm^2.
Low Viability	• Ensure proper expiration of reagents. • Decrease incubation time and disruption speed (See Appendix 3 - Protocol Modification). • Consider alternative cleanup methods such as FACs, dead cell removal kits, or Levitation Technologies. • Skip RBC lysis steps C.1 through C.3.
High levels of debris after debris removal step	• Strain sample through 20 or 30 µm cell strainer before debris removal steps. • Perform additional wash of cells in 1 mL of Cell Resuspension Buffer 1 by centrifuging at 300 g for 5 minutes and removing supernatant after Step D.4. • Consider alternative cleanup methods such as FACs, positive or negative selection, or Levitation Technologies.
Red blood cells still present in cell suspension	• Repeat RBC lysis from Section C with a 1-minute lysis incubation time in Step C.1.

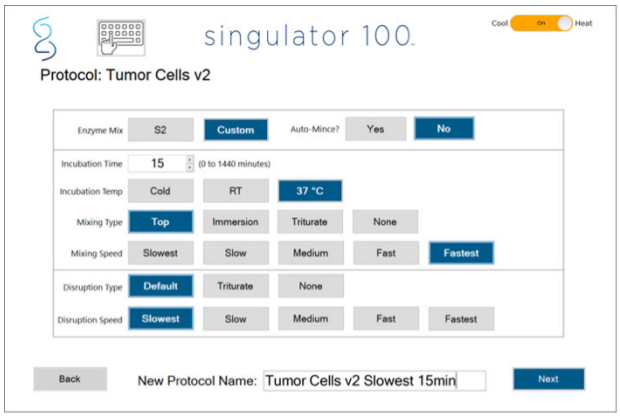
Appendix 3 – Protocol Modification

(Example - Decreasing incubation time and disruption speed modification)

Protocol modifications can be made by first selecting the desired protocol from the protocols screen by selecting Modify. Once desired modifications are selected, the protocol must be renamed and saved to proceed. Modified protocols will be saved in the protocols list for future use. See the Singulator User Guide for a full description of all modification parameters.



Original - “Tumor Cells V2”



Modified - “Tumor Cells V2 Slowest 15min”