DEMONSTRATED PROTOCOL

Cell Isolation from Intestine Tissue for Single Cell Sequencing Applications

Overview

This protocol outlines the process for isolating, cleaning, counting, and preparing single cells from fresh intestine tissue for single-cell sequencing assays listed below. Some optimizations 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 1.0 for optimization recommendations. This protocol incorporates the Large Cell Isolation Cartridge, which features larger filtration units specifically designed to minimize the loss of cardiomyocytes during the filtration process.

Compatible Downstream Applications

The assays listed below have been tested 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' v3.1	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)	100-289-370
	Intestine Reagent (20mL)	100-064-304
Millipore Sigma	Flowmi Cell Strainer	BAH136800040
	EDTA, Disodium Sale, Dihydrate, Molecular Biology Grade	0030122275
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	97061-420
Revvity	Cellometer K2 Fluorescent Cell Counter	-
	SD025 Counting Chambers	CHT4-SD025
	ViaStain AO/PI Staining buffer	CS2-0106
pluriSelect	pluriStrainer 30 µm	43-50030-03
ThermoFisher	Gibco (Ammonium-Chloride-Potassium) ACK Lysing Buffer	A1049201
	Fetal Bovine Serum (FBS), qualified, heat inactiviated	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
	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

Intestine Cell Reagent Buffer* – Fully dissolve Intestine Reagent in 20 mL of DMEM at room temperature by inverting vial for 20 minutes. Do not heat Intestine 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**
Intestine Reagent (20 mL vial)	-
DMEM	20 mL
Total	20mL

** Each vial provides enough Intestine Reagent Buffer for 6 cell isolation runs. If using a frozen Intestine Reagent Buffer aliquot, thaw and maintain at room temp.*

Tissue Pre-Treatment Buffer – Prepare 15 mL and vortex until completely dissolved. (Keep at Room Temp)	**Per 1 Sample**
PBS (No Calcium or Magnesium)	15mL
EDTA, Disodium Salt, Dihydrate	11.6mg
Total	15 mL

PBS – Prepare 100 mL and place on Ice.	**Per 1 Sample**
PBS (No Calcium or Magnesium)	100mL
Total	100 mL

Cell Resuspension Buffer* – Prepare 1 mL and sterile filter. (Place on Ice)	**Per 1 Sample**
Appropriate Buffer (See Table 1)	900 µL
Fetal Bovine Serum (FBS)	100 µL
Total	1 mL

**Prepare fresh.*

Optional Buffers

Optional buffers listed below for Debris Removal steps in Section C.

Stock Percoll Solution	**Per 1 Samples**
PBS 10x	100 µL
Percoll	900 µL
Total	**1.0 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. Store frozen tissue in liquid nitrogen for best results, or, if unavailable, at -80°C.
2. Ensure to completely wash and remove contaminants from tissue in Section B – Tissue Preparation and Pre-Treatment, inadequate cleansing with reduce yield and viability.
3. Place all tubes on ice for handling steps.
4. Minimize time as much as possible between steps to maintain cell viability.
5. 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.
6. Resuspend pellets gently to avoid shearing the cells.

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 on 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 the **Intestine Cell Isolation Protocol V2** protocol.
    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 Intestine Cell Reagent Buffer into the right side of the Single Shot Mechanism, in the slot labeled "**Enzyme**".
7. Add 6 mL of DMEM to a 15 mL conical and place into the left side of the Single Shot Mechanism, in the slot labeled "**Buffer**".

B. Tissue Preparation and Pre-Treatment

1. Obtain 10–12 cm of fresh intestine tissue and place it in a sterile petri dish with 10 mL of cold PBS.
2. Gently flush the intestine with 10 mL of cold PBS by inserting a pipette tip into the intestinal tube. Repeat three times or until all fecal matter is removed.
3. Using dissection scissors, cut the intestine lengthwise to open it. Transfer the tissue to a new petri dish with 15 mL of cold PBS. Swirl and agitate the tissue with forceps to remove mucus and contaminants. Repeat 2–3 times with fresh PBS and petri dishes until the buffer remains clear.
4. Transfer the tissue to a new petri dish and cut it into 2–3 mm pieces using a scalpel or razor blade.
5. Add 15 mL of Tissue Pre-Treatment Buffer to the tissue pieces and incubate for 5 minutes at room temperature.
6. Rinse the tissue pieces with PBS to remove the Pre-Treatment Buffer. Pour the tissue and buffer through a 100 µm cell strainer placed over a 50 mL conical tube. Rinse the tissue remaining in the strainer with 20 mL of fresh PBS.

    Tip: Thorough rinsing of the tissue pieces is essential to remove any remaining Pre-Treatment Buffer, as residual buffer can inhibit enzymatic digestion.

C. Cell Isolation

1. 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 DMEM to the sample when transferring to help keep tissue from sticking to the bottom of the cartridge.
2. Lift the door open of the Singulator and slide out the Cartridge Tray while lifting the red knob.
3. Place the cartridge on the Cartridge Tray and turn the white cartridge lock counterclockwise to lock cartridge into place.
4. 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.**
5. Close the door of the Singulator.
6. Select **Run** – the nuclei isolation takes approximately 45 minutes.
7. 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.
8. Pierce foil seal of the Output Chamber with a 1 mL pipette and retrieve the sample (~5.5 mL) 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.
9. Centrifuge sample at 300g for 5 minutes at 4°C in a swinging bucket rotor.
10. Remove used 15 mL conical tubes, now filled with rinse water, from Single Shot Mechanism and discard.

D. RBC Lysis

This protocol uses an ACK Lysis Solution for lysing red blood cells. For samples under 20 mg, those 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 E.

1. Remove the supernatant after centrifugation and gently resuspend pellet in 1 mL of cold ACK Lysing Solution
2. Incubate for 3-4 minutes on ice.
3. Add 6mL of DMEM to suspension.
4. Centrifuge sample at 300g for 5 minutes at 4°C in a swinging bucket rotor.

E. Debris Removal (Optional) and Resuspension in Supplemented Loading Buffer

This protocol includes an optional gentle density gradient centrifugation step to remove debris from intestine cell samples. For samples under 20mg, or with a pellet that is not visible after centrifugation, proceed directly to Step E.4 for resuspension in Cell Resuspension Buffer.

1. Remove the supernatant after centrifugation and gently resuspend pellet in 3 mL of Percoll Debris Removal Solution.

2. Centrifuge sample at 300g for 10 minutes (with brake setting to 0 or 1) Once complete, remove sample from centrifuge taking care not to disturb the floating 'debris cake' at the top of the supernatant.
3. Using wide bore tips, carefully remove the floating debris cake and supernatant.
    a. **Tip**: Using razor blade or scissors, cut the wide bore pipette tips to increase the orifice, and remove the supernatant in 300-500 µl increments to remove debris more easily. Make sure to completely remove supernatant without letting debris fall onto the nuclei pellet. Near the end, use a standard (non-wide) bore pipette tip to remove the supernatant close to pellet.
4. Gently resuspend the pellet in 1 mL of Cell Resuspension Buffer, and place on ice.

F. Counting and Dilution

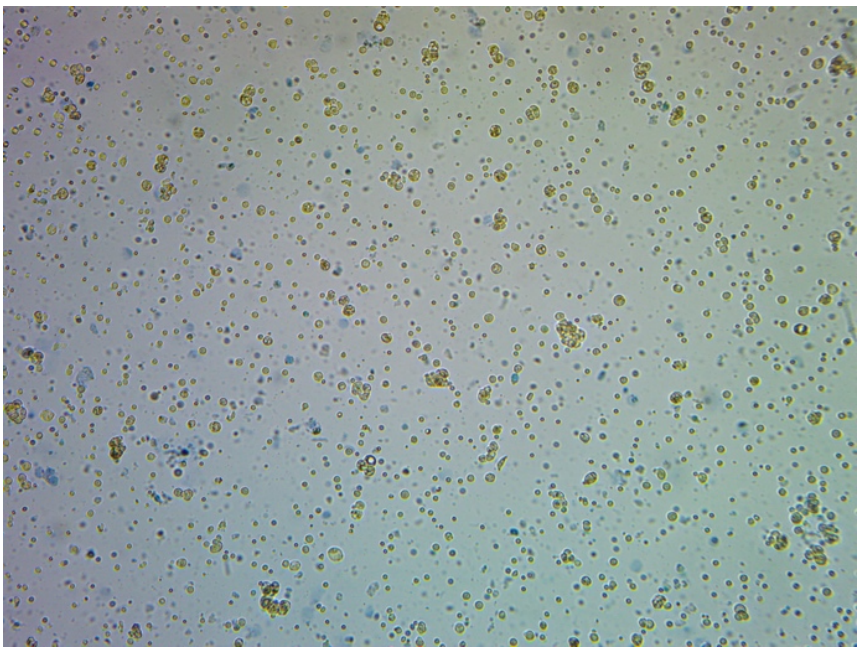
Follow manufacturers' instructions to obtain nuclei count using fluorescence method, briefly described below.

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 in each side of 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 nuclei appear in **"Good Focus"** according to the **Cellometer Focus Guide**.
7. Select **Count**.
8. Adjust concentration of nuclei suspension with loading buffer to desired concentration for downstream applications. Strain through 30 µm pluriSelect strainer if needed to remove clumps prior to loading single nuclei assay.

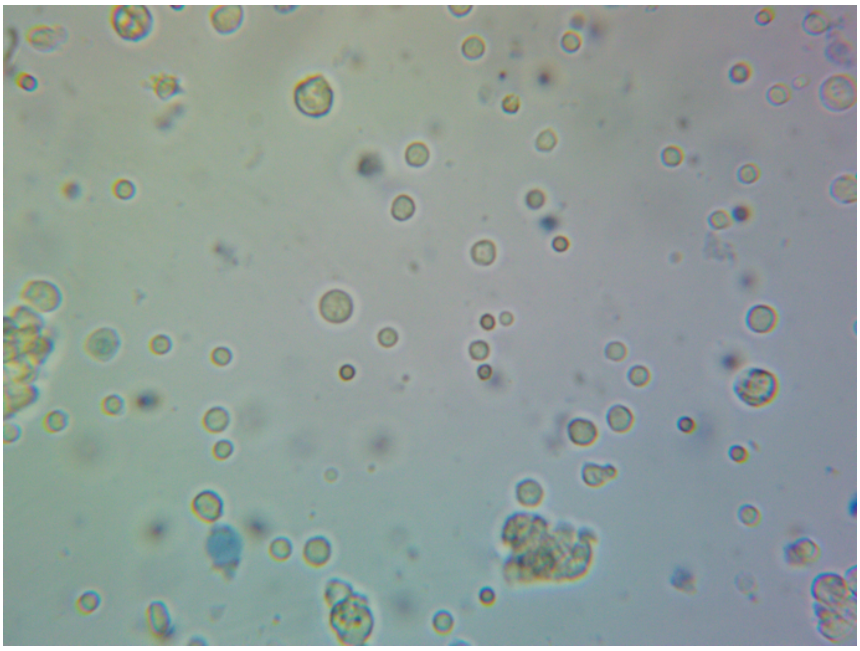
Results

Representative Images:

Mouse Intestine Cells
(10x Resolution)



Mouse Intestine Cells
(10x Resolution)



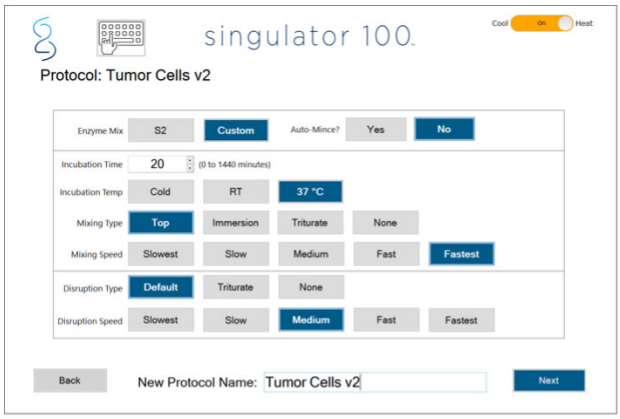
Appendix 1 – Issues and Recommendations

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 2 - Protocol Modification). • Consider alternative cleanup methods such as FACs, dead cell removal kits, or Levitation Technologies. • Skip RBC lysis steps D.1 through D.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 E.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 D.2.

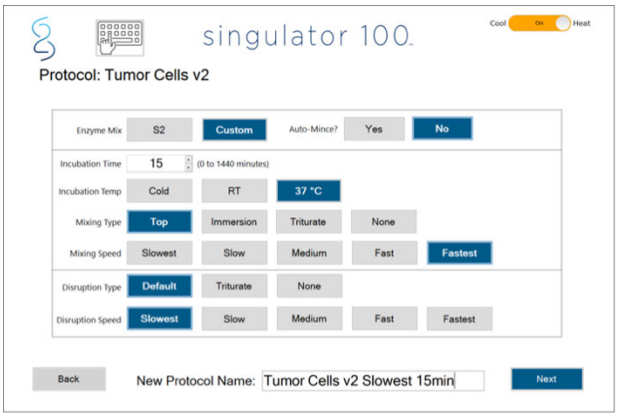
Appendix 2 – Protocol Modification

(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”