DEMONSTRATED PROTOCOL

Nuclei Isolation from Frozen Mouse Brain Tissue for Single Nuclei Sequencing Applications

Overview

This protocol describes how to isolate, clean, count, and prepare single nuclei from fresh frozen mouse brain tissue for single-nuclei sequencing applications detailed below. Some optimizations of the Singulator™ protocol parameters may be needed based on the storage time and condition of the frozen tissue.

Compatible Downstream Applications

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	Nuclei Isolation Bundle with RNase Inhibitor V2 (8 samples)	100-291-422
	Nuclei Isolation Bundle with RNase Inhibitor V2 (24 samples)	100-288-798
	NIC+ Isolation Bundle with RNase Inhibitor V2 (8 samples)	100-291-531
	NIC+ Isolation Bundle with RNase Inhibitor V2 (24 samples)	100-288-807
	RNase Inhibitor V2	100-288-916
	Nuclei Debris Removal Stock Reagent	100-246-863
	Loading Buffer	100-257-006
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
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

Getting Started

Prepare Buffers

Diluted Nuclei Debris Removal Reagent	**Per Sample**
Nuclei Debris Removal Stock Reagent	200 µL
Nuclei Storage Reagent (NSR)	795 µL
RNase Inhibitor V2	5 µL
Total	1 mL

BSA Loading Buffer Prepare 10mL, sterile filter, and freeze (-20°C) in 1mL aliquots for up to 6 months	**Per 10 Samples**
Loading Buffer	10 mL
Bovine Serum Albumin (BSA)	100 mg
Total	10 mL

Supplemented Loading Buffer Thaw 1 mL aliquot of BSA Loading Buffer and add RNase inhibitor on the *day* of the nuclei isolation	**Per Sample**
BSA Loading Buffer – from frozen aliquot	975 µL
RNase inhibitor V2	25 µL
Total	1 mL

Tips and Best Practices

1. Use Table 1. To determine cartridge, protocol and volume of reagents based on input mass.

Table 1

Input Mass (mg)	Cartridge	Singulator Protocol	Required volume of NIR and NSR	Myelin Removal Step Pellet Resuspension Volume	RNase Inhibitor V2 Volume Added to Cartridge	Final Supplemented Loading Buffer Resuspension Volume
2-10	NIC+	Low Volume Nuclei Isolation V2	1.5mL (NIR) 2.5mL (NSR)	1mL of Diluted Debris Removal Reagent (if visible pellet is present)	75 µL	250uL
10-30	NIC+	Low Volume Nuclei Isolation V2	1.5mL (NIR) 2.5mL (NSR)	1mL of Diluted Debris Removal Reagent	75 µL	500uL
30-150	NIC+ or Nuclei Isolation Cartridge	Low Volume Nuclei Isolation V2	2.5mL (NIR) 2.5mL (NSR)	3mL of Diluted Debris Removal Reagent	75 µL or 100 µL (Additional RNase Inhibitor Required)	1mL
150-300	NIC+ or Nuclei Isolation Cartridge	Standard Nuclei Isolation V2	2.5mL (NIR) 2.5mL (NSR)	3mL of Diluted Debris Removal Reagent	100 µL (Additional RNase Inhibitor Required)	1mL

2. Store frozen tissue in liquid nitrogen for best results, or, if unavailable, at -80°C.
3. Place all tubes on ice for handling steps.
4. Minimize time as much as possible between steps to maintain nuclei integrity.

5. Use a swinging bucket centrifuge to pellet nuclei and prevent nuclei from smearing against sides of centrifuge tubes. This will maintain nuclei integrity.
6. Resuspend pellets by gently pipetting to avoid shearing the nuclei.
7. The Nuclei Isolation Bundle and NIC+ bundle were configured for use with the Low Volume Nuclei Isolation V2 protocol. When using the "Standard Nuclei Isolation Volume V2" additional RNase Inhibitor V2 (sold separately) will be required.
8. The Supplemented Loading Buffer is compatible for direct loading into the Chromium Next GEM Single Cell 3' v3.1 and Chromium GEM-X Single Cell Gene Expression (3') Assays.

Nuclei Isolation from Adult Mouse Brain Tissue

A. Singulator Setup

1. Place the appropriate cartridge(s) (see Table 1) in a -20°C freezer overnight or for at least 20 minutes before run(s).
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-cool the Singulator by sliding the toggle in the upper right of the **User Home Screen** to **Cool** and tap the icon to initiate pre-cooling. The bar will turn orange indicating pre-cooling is in progress and will turn green and display **On √** upon completion.
5. Obtain 2-300mg of frozen brain tissue from -80°C freezer and place on dry ice.
6. Ensure enough Nuclei Isolation Reagent (NIR) and Nuclei Storage Reagent (NSR) are present in Chiller unit, (see table 1).
7. Once the Singulator is pre-cooled, select the desired protocol.
    a. Select **Run a Protocol** from the User Home Screen.
    b. Select the **Nuclei** button to toggle to nuclei protocols.
    c. Select the appropriate protocol from 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**.

B. Nuclei Isolation

1. Remove the cartridge from the -20°C freezer, remove the cap from the cartridge, place the tissue and RNase Inhibitor V2 inside the Dissociation Chamber, and replace the cap. (See Table 1 for recommended volume)
2. Lift the door open of the Singulator and slide out the Cartridge Tray by 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 6.5 minutes.
7. Immediately 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 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 500g for 5 minutes at 4°C in a swinging bucket rotor.

C. Myelin Removal and Resuspension in Supplemented Loading Buffer

This protocol uses a gentle density gradient centrifugation to remove myelin and other debris from nuclei samples.

1. Remove the supernatant after centrifugation and gently resuspend pellet in cold Diluted Nuclei Debris Removal Reagent. (See Table 1 for recommended Volume)
    a. **Tip**: If there is no visible pellet after initial centrifugation skip myelin debris removal step and proceed to step C.4 below.
2. Centrifuge sample at 500g for 10 minutes (with brake setting 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 present in larger samples.
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 non-wide bore pipette tip to get closer to pellet.
4. Resuspend the pellet in Supplemented Loading Buffer (See Table 1 for recommended volume) 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.

D. Counting and Dilution

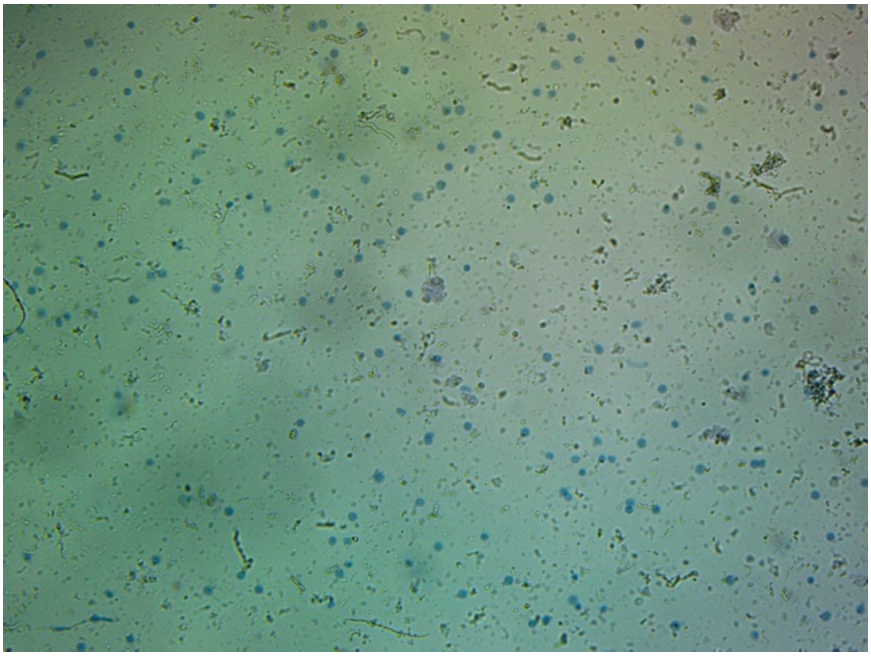
Follow manufacturers' instructions to obtain nuclei count using fluorescence method, briefly described below.

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.

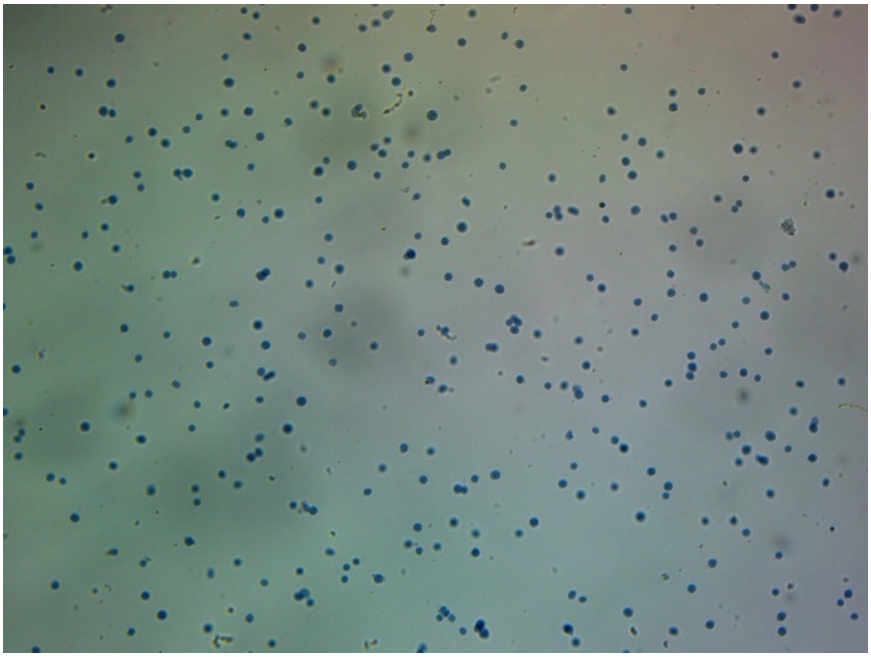
Results

Representative Counts and Images:

Before Myelin Removal
(10x Resolution)



After Myelin Removal
(10x Resolution)



K2 Nuclei Count (1:4 dilution) of 35 mg frozen mouse brain tissue

Count	Concentration (cells/mL) at Dilution Factor: 2	Mean Size (um)	
Live: 0	0.000 x10^6	.0	Viability: .0 %
Dead: 265	0.909 x10^6	8.9	
Total: 265	0.909 x10^6	8.9	

Sample Name: New Sample 2022/08/26-18:35:42
Time Stamp: 8/26/2022 6:35:49 PM
Assay Name: K2_AOPI_Primary Cells or Cell Lines
Assay Description: Total cell count and % viability using AO/PI staining

Total Nuclei Count = 3,630,000
Nuclei per mg = 103,714

K2 Fluorescent Image (PI)