Demonstrated Protocol: Nuclei Isolation and Cleanup from Frozen Mouse Brain Tissue for Single Nuclei Sequencing Applications

Overview

This protocol describes how to isolate, clean, count, and prepare single nuclei from mouse brain tissue for single nuclei sequencing applications. Some optimizations of Singulator™ 100 parameters may be needed based on the storage time and condition of the frozen tissue.

Reagents and Consumables

Vendor	Item	Part Number
S2 Genomics	NIC+ Nuclei Isolation Cartridge	100-215-389
	Nuclei Isolation Reagent, 25	100-063-396
	Nuclei Storage Reagent, 25	100-063-405
	RNase Inhibitor	100-247-435
	Nuclei Debris Removal Stock Solution	100-246-863
	Loading Buffer	100-257-006
Millipore Sigma	Flowmi Cell Strainer	BAH136800040
Eppendorf	DNA LoBind Tubes	0030122275
VWR	15 mL High Performance Centrifuge Tubes	21008-089
	Pipette Tips RT LTS 1000 □L – Low Retention	30389219
	Pipette Tips RT LTS 250 □L – Low Retention	30389250
	Pipette Tips RT LTS 20 □L – 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

Required Buffers

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

BSA Loading Buffer Prepare 10 mL and freeze (-20°C) in 1 mL aliquots for up to 6 months	Per 10 Samples
Loading Buffer	10 mL
BSA	100 mg
Total	10 mL

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

Tips and Best Practices

1. This protocol was validated using fresh frozen mouse brain tissue of varying sizes from 10-150 mg.
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. Use wide bore pipette tips and resuspend pellets gently to avoid shearing the nuclei.
7. Standard volume protocols may be used but will require 4 mL equivalent of RNase inhibitor. The low volume protocols use 1 mL of NIR (isolation) and 2 mL of NSR (wash).
8. The supplemented loading buffer has the appropriate magnesium concentration for loading into the 10x 3’ GEX assay. Load nuclei directly into G chip in the supplemented loading buffer.

Nuclei Isolation from Adult Mouse Brain Tissue

A. Singulator Setup

1. Place the Nuclei Isolation Cartridge(s) in a -20°C freezer for at least 20 minutes before run(s).
2. Prepare buffers as described in the Required Buffers 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 10-150 mg 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, at least 3 mL for each run.
    a. Check the reagents in the Reagent Chiller to determine enough NIR and NSR is available in the bottles for the run(s).
    b. For Single-Shot nuclei protocols, make sure a 15 mL conical tube with 3 mL of NIR is in the Enzyme slot and a 15 mL conical tube with 3 mL of NSR is in the Buffer slot.
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 **Low Volume Nuclei Isolation** or **Single-Shot Low Volume Nuclei Isolation** 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**.

B. Nuclei Isolation

8. Remove the chilled NIC+ cartridge from the -20°C freezer, remove the cap from the cartridge, place the tissue and 75 □L RNase Inhibitor inside the Dissociation Chamber, and replace the cap.
9. Lift the door open of the Singulator and slide out the Cartridge Tray by lifting the red knob.
10. Place the cartridge on the Cartridge Tray and turn the white cartridge lock counterclockwise to lock cartridge into place.
11. 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**.
12. Close the door of the Singulator.
13. Select **Run** – the nuclei isolation takes approximately 6.5 minutes.
14. 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.
15. Pierce foil seal of the Output Chamber with a 1 mL pipette and retrieve the sample (~2.5 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.
16. Centrifuge sample at 500g for 5 minutes at 4°C in a swinging bucket rotor.

C. Myelin Removal

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

17. Remove the supernatant after centrifugation and gently resuspend pellet in 3 mL of cold diluted Nuclei Debris Removal Solution.
18. Centrifuge sample at 500 g for 10 minutes with no brake! Once complete, remove sample from centrifuge taking care not to disturb the floating 'debris cake' at the top of the supernatant.
19. Using wide bore tips, carefully remove the floating debris cake and supernatant.

    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.
20. Resuspend the pellet in 1 mL of Supplemented Loading Buffer, and place on ice.
21. 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

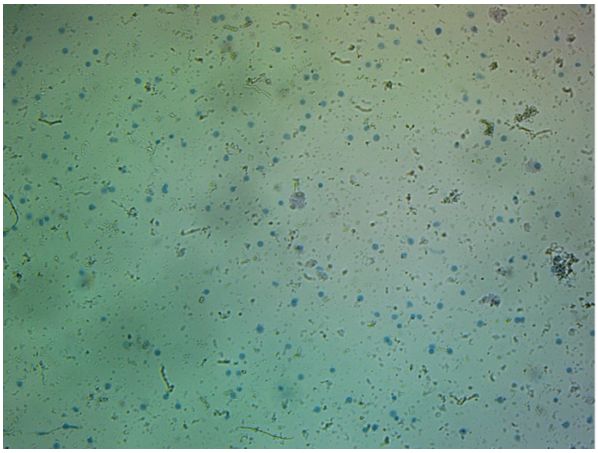
Follow manufacturers' instructions to obtain nuclei count using a fluorescence method, briefly described below.

1. Remove coverslip from a Nexcelom counting slide.
2. Mix 30 µL of the sample with 30 µL of ViaStain AO/PI dye, and place 20 µL in each side of counting slide.
3. Insert counting slide into the Cellometer K2 cell counter.
4. Open the matrix software on the 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 to desired concentration for downstream snSeq applications. Strain through 30 µm pluriSelect strainer if needed to remove clumps prior to single cell chip loading.

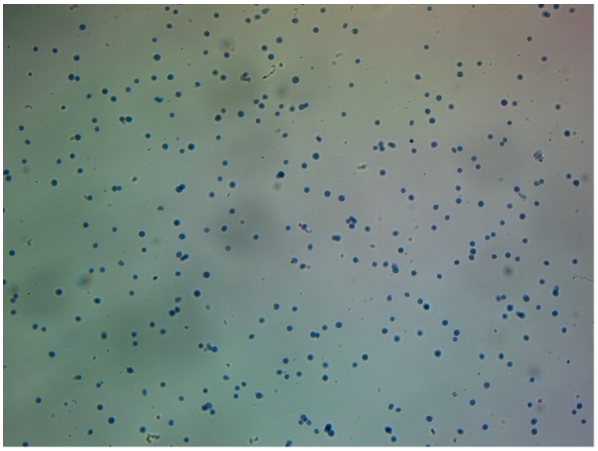
Results

Representative Counts and Images

Before Myelin Removal (10x Resolution)



After Myelin Removal (10x Resolution)



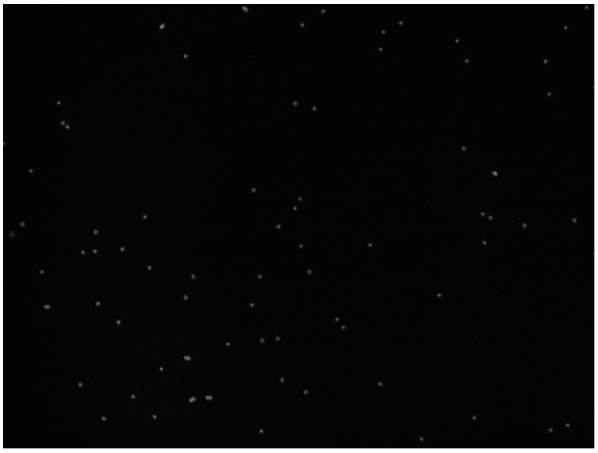
K2 Nuclei Count (1:4 dilution) of 35 mg frozen mouse brain tissue

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)

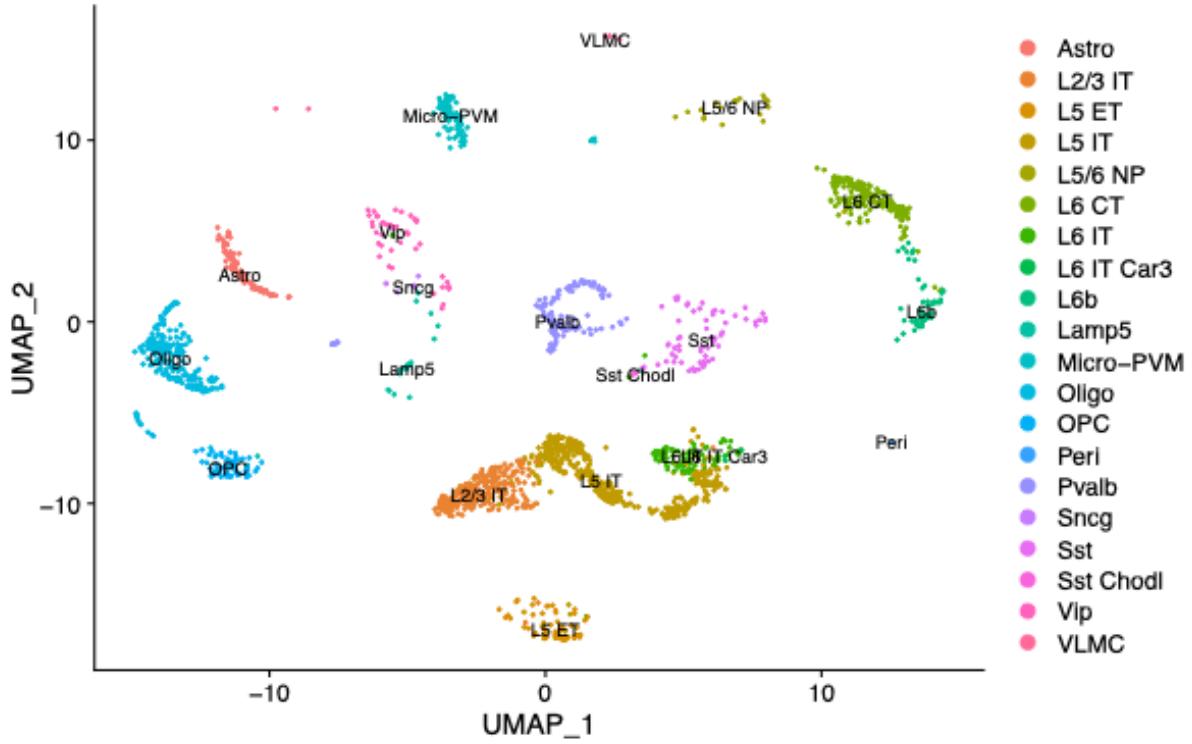


Sequencing

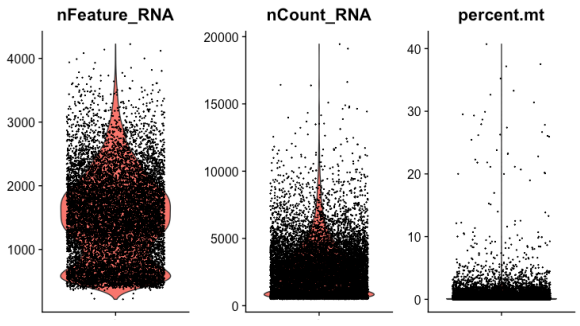
Samples were loaded into the 10x Genomics 3' Gene Expression assay targeting 10,000 nuclei and manufacturer's instructions for library preparation followed. Single cell library QC was done using the Agilent TapeStation 4150 with the D1000 assay.

For the data in this demonstrated protocol, the final library was sequenced on a NovaSeq V1.5 SP to read depth of 22,054 reads per cell. Data was analyzed using Seurat 4.0. Ambient RNA was removed using SoupX to provide a corrected count matrix. The median genes per cell was 1,581 with 68% of reads in cells with low mitochondrial contamination.

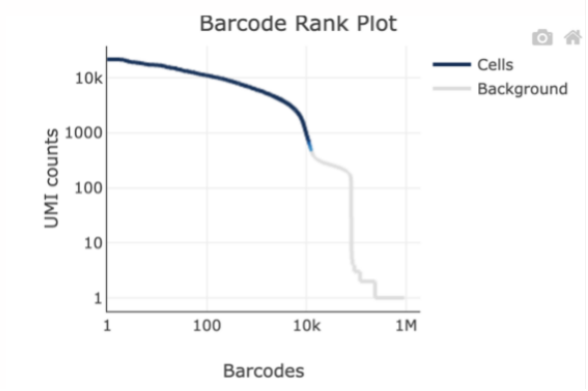
Clustering and SubClass Cell Type Labeling



Sequencing QC Metrics



Barcode Rank Plot (Cell Ranger Web Summary)



The corrected count matrix was then integrated with Azimuth mouse cortex reference (Yao, Liu, Xie, Fischer, et al, bioRxiv 2020) for a reference-based mapping pipeline that performs normalization, visualization, cell annotation, and differential expression (Hao et al, Cell 2021). As the figure above shows, 20 well defined annotated clusters were generated.

Summary

Nuclei can readily be isolated from frozen mouse brain using the S2 Genomics Singulator Systems, and with the accompanying reagents, Nuclei Debris Removal Stock Solution (100-246-863) and Loading Buffer (100-257-006), can provide clean, high-quality samples that generate robust single nuclei RNA-Seq data. The NIC+ cartridge and protocol facilitate high yield with over 100,000 nuclei isolated per mg of tissue, and the debris removal and loading buffer solutions offer a quick and reproducible method for cleaning samples for single nuclei processing solutions.