DEMONSTRATED PROTOCOL

Nuclei Isolation from Adult Mouse Brain Tissue for Single Cell RNA Sequencing

Overview

This protocol outlines how to isolate, wash, and count single nuclei from adult neural tissue for use with 10x Genomics Single Cell RNA protocol. The protocols described here are expected to be compatible with many tissue types. Additional optimization may be required when working with new sample types.

Additional Guidance

Consult Demonstrated Protocol – Cell Preparation Guide (Document CG000053) for Tips & Best Practices and Technical Note – Guidelines on Accurate Target Cell Counts (Document CG0000091) for more information on determining accurate cell counts.

Cells carry potentially hazardous pathogens. Follow material supplier recommendations and local laboratory procedures and regulations for the safe handling, storage and disposal of biological materials.

Preparation - Buffers

Lysis Buffer Prepare fresh, maintain at 4°C	Stock	Final	10 ml
Tris-HCl (pH 7.4)	1 M	10 mM	100 µl
NaCl	5 M	10 mM	20 µl
$MgCl_2$	1 M	3 mM	30 µl
Nonidet P40 (for Sigma 74385, prepare a 10% stock)	10%	0.1%	100 µl
Nuclease-free Water	-	-	9.75 ml
Nuclei Wash & Resuspension Buffer Prepare fresh, maintain at 4°C	**Stock**	**Final**	**50 ml**
BSA Solution	5%	1%	10 ml
RNase Inhibitor*	40 U/µl	0.2 U/µl	0.25 ml
1X PBS	-	-	39.75 ml

*Verify RNase Inhibitor stock concentration

Additional Buffers maintain at 4°C
LS Column Calibration Buffer (PBS + 0.5% BSA)
Sucrose Cushion Buffer I (2.7 ml Nuclei PURE 2M Sucrose Cushion Solution with 300 µl Nuclei PURE Sucrose Cushion Buffer)

Specific Reagents & Consumables

Vendor	Item	Part Number
Thermo Fisher Scientific	UltraPure BSA (50 mg/ml)	AM2616
	Trypan Blue Stain, 0.4%	T10282
	Countess II FL Automated Cell Counter	AMAQAF1000
	Countess II FL Automated Cell Counting Camber Slides	C10228
Millipore-Sigma	Trizma Hydrochloride Solution, pH 7.4	T2194
	Sodium Chloride Solution, 5M	59222C
	Magnesium Chloride Solution, 1M	M1028
	Nonidet P40 Substitute	74385
	Protector RNase Inhibitor	3335399001
	Live/Dead Cell Double Staining Kit	04511-1KT-F
	Nuclei PURE 2M Sucrose Cushion Solution	NUC201-1KT
	Nuclei PURE Sucrose Cushion Buffer	NUC201-1KT
Bel-Art	Flowmi Cell Strainer, 40 µm	H13680-0040
Corning-Cellgro	Phosphate-Buffered Saline 1X without Calcium & Magnesium	21-040-CV
Miltenyi Biotech	MACS SmartStrainers, 30 µm	130-098-458
	Myelin Removal Beads II, Human, Mouse, Rat	130-096-733
	LS Columns	130-042-401
	MACS MultiStand	130-042-303
	MidiMACS Separator	130-042-302

This list may not include some standard laboratory equipment.

Tips & Best Practices

Factors Influencing Nuclei Recovery
To recover the expected number of nuclei, maximize input cell viability, minimize cell and nuclei handling time, accurately count nuclei, and pipette the correct volume into the Single Cell Master Mix when executing 10x Genomics Single Cell protocols. Consult Technical Note Guidelines for Accurate Target Cell Counts Using 10x Genomics Single Cell Solutions (Document CG000091) for more details.

Lysis Condition

- Centrifugation speed/time, the number of wash steps, and methods of debris removal (e.g. filtration, myelin removal, or flow cytometry) may require optimization for a specific sample type.
- Assess lysis efficacy via microscopy after incubation.
- Optimize lysis time when working with new cell/tissue types.
- Avoid over lysis as it could lead to nuclei aggregation and clumping.
- Cell lysis should be carried out on the ice and using chilled reagents.

Washing & Resuspension

- Use sufficient volumes to maintain concentrations <5,000 nuclei/µl in Nuclei Wash and Resuspension Buffer when washing and resuspending nuclei.
  Maintaining nuclei at higher concentrations may cause aggregation and clumping.
- The recommended Nuclei Wash and Resuspension Buffer contains BSA, to minimize nuclei losses and aggregation, and RNase Inhibitor to inhibit RNases during nuclei isolation and purification.
- The presence of BSA and RNase Inhibitor in the Nuclei Wash and Resuspension Buffer results in a translucent/opaque rather than clear aqueous phase after breaking the GEMs with Recovery Agent. This is normal and will not impact downstream operations.

Pipette Tips

- Use wide-bore pipette tips to avoid cellular shearing and premature lysis. However, generating single nuclei suspensions from pellets or clumps is best achieved using a regular-bore pipette tip to break up aggregates.
- Pipette gently and slowly during resuspension steps to minimize physical damage to nuclei from shearing forces.

Aggregate & Debris Removal

- Filter lysed tissues and nuclei suspensions with an appropriate cell strainer to remove cellular debris and nuclei aggregates.
- The MACS SmartStrainer is recommended as it generally causes minimal changes to the nuclei concentration. However, a volume loss of 100 µl or more can occur.
- For low nuclei suspension volumes, the Flowmi Tip Strainer is recommended to minimize volume losses.
- Nuclei concentrations may decrease by up to 40%, depending on suspension volume and strainer type. Measure the nuclei concentration before and after straining.
- When lysing neuronal tissue, large quantities of myelin debris are produced. Depending on the age of the tissue, reduction of myelin debris may improve the cleanliness of final nuclei preparation.
- Myelin removal can be done by using Myelin Removal Beads II (Human, Mouse, Rat) in combination with chilled reagents.

Nuclei Visualization & Counting

- Visualize nuclei suspensions to determine nuclei concentration and viability, suspension quality, and nuclei sizes prior to use in 10x Genomics Single Cell protocols.
- Use the Countess II FL Automated Cell Counter for determining nuclei concentrations for most applications. Sample types with very small nuclei or high levels of aggregation may require alternative counting methods.
- Perform two different counting and viability assays when characterizing a sample type for the first time.
- **Trypan Blue Staining:** Stain nuclei with trypan blue and count the nuclei concentration and viability using the Countess II FL Automated Cell Counter.
- **Fluorescent Dye Staining:** Stain nuclei with fluorescent dyes and measure cell viability using a tissue culture microscope and automated cell counting software.
- Manual counting using a hemocytometer may be used as an additional method.

Nuclei Concentration for Optimal Performance

- The total number of suspended nuclei used as input to 10x Genomics Single Cell protocols is determined by the nuclei recovery target. Consult the applicable 10x Genomics Single Cell protocol to determine these relationships.
- The optimal input nuclei concentration is 700-1,200 nuclei/µl.
- If possible, bring the input nuclei suspension to a concentration that is optimal for the dynamic range of counting technique used (manual or automated), allows for 3-4 reproducible counts (where the standard deviation is <25%), and requires pipetting 2.5-15 µl nuclei suspension into the Single Cell Master Mix.
  Pipetting nuclei suspension volumes <2.5 µl increases variance due to pipetting inaccuracy, while volumes >15 µl increases the risk of introducing unwanted debris or inhibitors.
- Use final single nuclei suspension to estimate the number of input nuclei because nuclei are inevitably lost during washing and resuspension steps.

Flow Cytometry

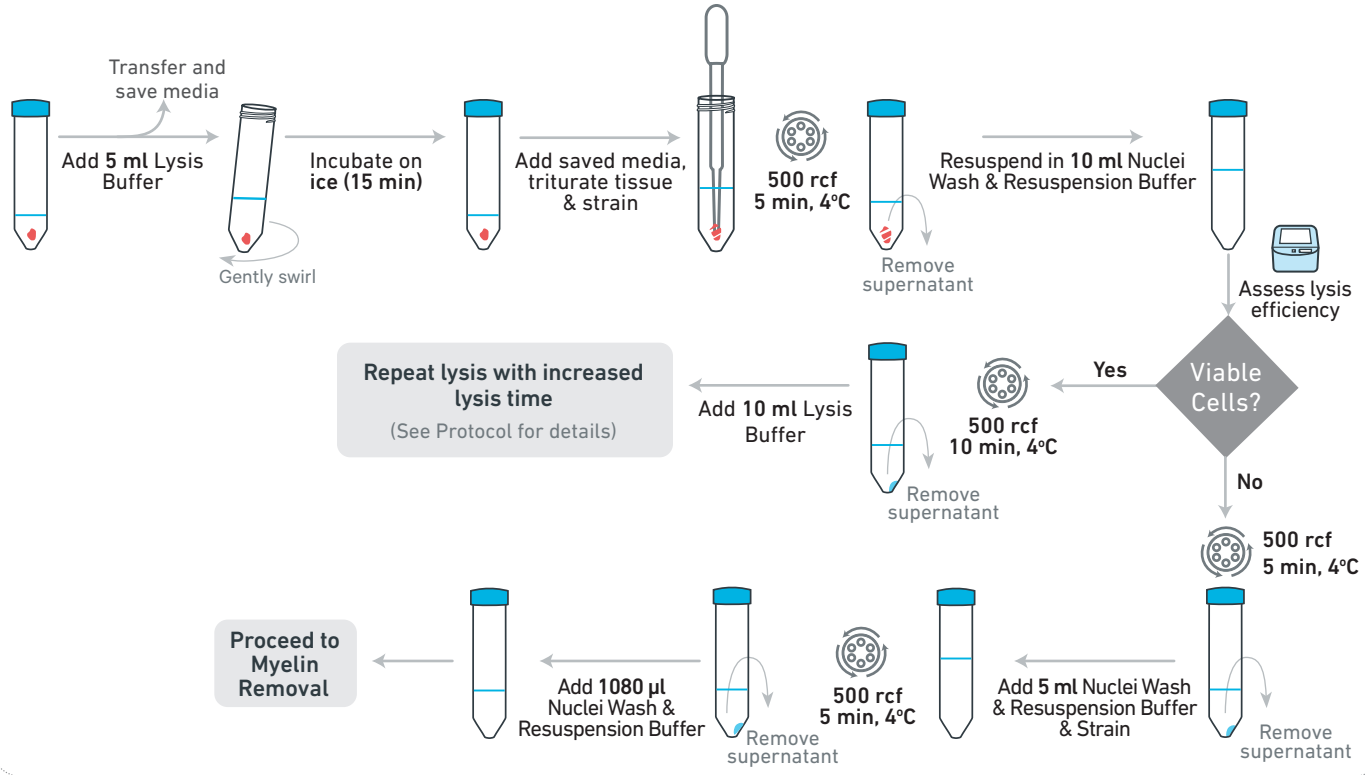
- If a sample concentration and volume allows, flow cytometry may further improve the purity of nuclei suspensions used as input for the 10x Genomics Single Cell protocols.
- Visually inspect the sorted nuclei under a microscope and re-count the nuclei suspension using a cell counter or hemocytometer prior to pipetting into the Single Cell Master Mix. Nuclei counts that are based on the flow cytometer are inaccurate.

Nuclei Control Sample

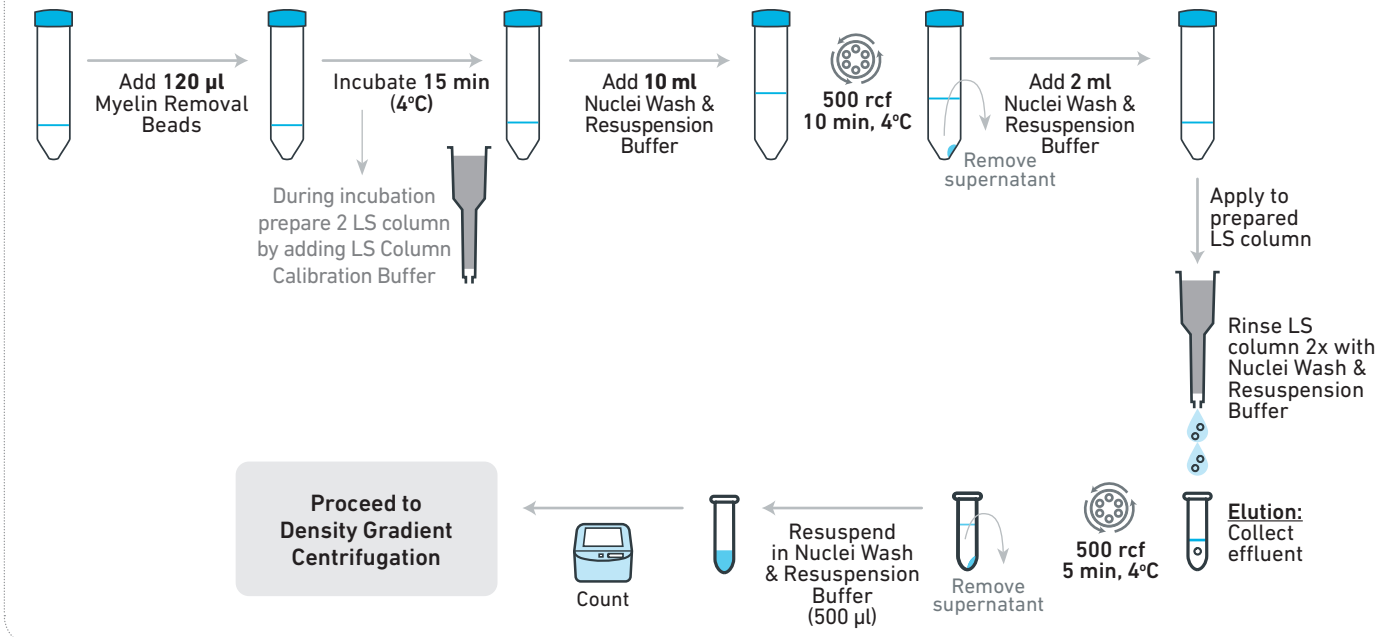
- To measure success and assess the reproducibility of these protocols, it is recommended to run a quality control sample in parallel with each experimental sample.
- Recommended quality control samples include cultured cell lines (e.g. human HEK293T cells) that are of high quality (>90% viable) and yield a sufficient number of nuclei (i.e. >1 million nuclei).

Protocol Overview : Nuclei Isolation from Adult Mouse Brain Tissue

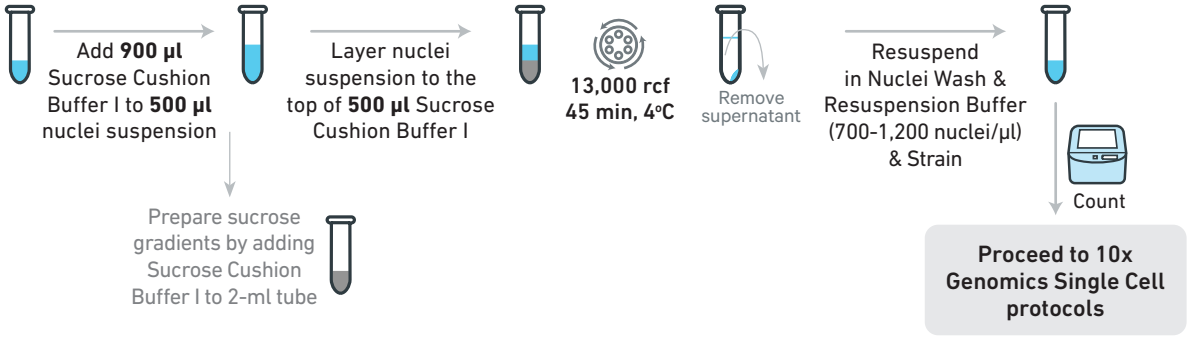
1.1 Tissue Lysis & Nuclei Washing



1.2 Myelin Removal



1.3 Density Gradient Centrifugation



Nuclei Isolation from Adult Mouse Brain Tissue

Cell Preparation & Sourcing: This protocol was demonstrated using fresh neuronal tissue (Combine Cortex, Hippocampus and Ventricular Zone) from adult mouse. Fresh adult mouse brain tissue was shipped on cold packs and used immediately upon receipt.

1.1 Tissue Lysis & Washing of Nuclei

a. Using a 10-ml serological pipette, gently transfer the tissue along with the Hibernate E/B27/GlutaMAX (HEB) medium to a new 50-ml centrifuge tube.

b. Transfer the HEB medium from the tissue to a new 50-ml tube, leaving only enough medium to cover the tissue. Keep the HEB medium on ice for step d.

c. Add **5 ml** chilled Lysis Buffer to the tissue and lyse the tissue on ice for **15 min.** Gently swirl to mix, repeat 2x during incubation.

d. Add **5 ml** HEB medium saved from step b back to the lysed tissue.

e. Triturate the tissue: Aspirate the tissue with the medium into a fire polished silanized Pasteur pipette and **immediately** dispense the contents back into the tube. Repeat 10-15x.

f. Use a 30 µm MACS SmartStrainer to remove cell debris and large clumps.

g. Centrifuge the nuclei at **500 rcf** for **5 min** at **4°C**.

h. Remove the supernatant without disrupting the nuclei pellet.

i. Using a 10-ml serological pipette, add **10 ml** Nuclei Wash and Resuspension Buffer and gently pipette mix 10x.

j. Assess lysis efficiency and viability by staining the cells with trypan blue and by using the Countess II FL Automated Cell Counter/microscopy.

If a high fraction of viable cells is still present:

- Centrifuge at **500 rcf** for **5 min** at **4°C**.
- Add **10 ml** chilled Lysis Buffer and incrementally increase the lysis time, monitoring efficacy via microscopy.
- After optimal lysis, repeat steps f - h.

k. Centrifuge at **500 rcf** for **5 min** at **4°C**.

l. Remove the supernatant without disrupting the pellet.

m. Using a 10-ml serological pipette, add **5 ml** Nuclei Wash and Resuspension Buffer and gently pipette mix 10x.

n. Use a 30 µm MACS SmartStrainer to remove cell debris and large clumps.

o. Centrifuge at **500 rcf** for **5 min** at **4°C**.

p. Remove the supernatant without disrupting the pellet.

q. Using a regular-bore pipette tip, add **1080 µl** Nuclei Wash and Resuspension Buffer and gently pipette mix 10x.

r. Proceed directly to Myelin Removal.

1.2 Myelin Removal

This protocol was demonstrated using sample sizes compatible with Myelin Removal Beads II and a single LS Column. The volumes of buffer, Myelin Removal Beads II, and the number of LS columns depend on the age and mass of the tissue and should be adjusted according to the manufacturer's instructions.

a. Add 1**20 µl** Myelin Removal Beads II to the resuspended nuclei from step 1.1q. Mix thoroughly with a **wide-bore** pipette tip. DO NOT vortex.

b. Incubate for **15 min** at **4°C**.

c. During incubation, prepare two LS columns, each with **3 ml** LS Column Calibration Buffer.

d. After incubation is complete, dilute the nuclei suspension (containing Myelin Removal Beads II) with **10 ml** Nuclei Wash and Resuspension Buffer (using a 10-ml serological pipette) and gently pipette mix 5x.

e. Centrifuge the nuclei at **500 rcf** for **10 min** at **4°C.**

f. Remove the supernatant without disrupting the nuclei pellet.

g. Resuspend the pelleted nuclei in **2 ml** Nuclei Wash and Resuspension Buffer.

h. Apply **1 ml** nuclei suspension to each LS column.

i. Wash each column twice with **1 ml** Nuclei Wash and Resuspension Buffer.

j. Collect the effluent into two 5-ml tubes.

k. Centrifuge the nuclei at **500 rcf** for **5 min** at **4°C**.

l. Remove the supernatant without disrupting the nuclei pellet.

m. Using a **regular-bore** pipette tip, add **500 µl** Nuclei Wash and Resuspension Buffer to each 5-ml tube. Gently pipette mix 10x or until nuclei are completely suspended.

n. Determine the nuclei concentration using a Countess II FL Automated Cell Counter or hemocytometer.

If the concentration in each tube is <1 x 10^7 total nuclei:

- Centrifuge at **500 rcf** for **5 min** at **4°C**.
- Remove the supernatant.
- Add **500 µl** Nuclei Wash & Resuspension Buffer.

o. Proceed directly to Density Gradient Centrifugation.

1.3 Density Gradient Centrifugation

a. Add **900 µl** Sucrose Cushion Buffer I to each tube containing **500 µl** of resuspended nuclei from step 1.2n. Pipette mix 10x using a regular-bore pipette tip.

b. Prepare two sucrose gradients by adding **500 µl** Sucrose Cushion Buffer I to two 2-ml tubes.

c. Carefully layer each of the **1,400 µl** nuclei suspensions from step a to the top of each tube containing Sucrose Cushion Buffer I. DO NOT mix.

d. Centrifuge the sucrose gradient containing the nuclei at **13,000 rcf** for **45 min** at **4°C**.

e. Carefully remove supernatant leaving **100 µl** in each tube. Using a regular-bore pipette tip, resuspend the nuclei pellets.

f. Using a regular-bore pipette tip, add **1 ml** Nuclei Wash and Resuspension Buffer or an appropriate volume to achieve the target nuclei concentration of 700-1,200 nuclei/µl.

Gently pipette mix 10x or until nuclei are completely suspended.

g. Use a 40 µm Flowmi Cell Strainer to remove cell debris.

h. Determine the nuclei concentration using a Countess II FL Automated Cell Counter or hemocytometer.

i. If nuclei concentration is <500 nuclei/µl (5×10^5 nuclei/ml), adjust the volume accordingly.

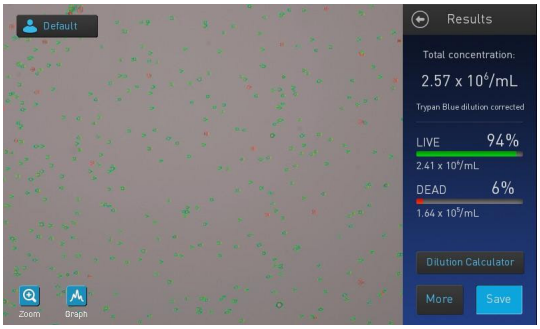
j. Once the target nuclei concentration of 700-1,200 nuclei/µl is obtained, place the nuclei on ice.

k. Proceed **immediately** with the 10x Genomics Single Cell protocol and minimize the time between nuclei preparation and chip loading.

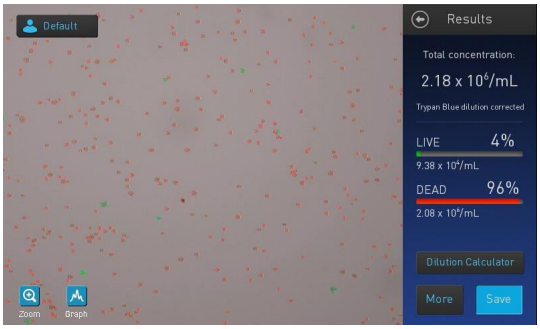
Results

Representative Staining Images

Pre Lysis



Post Lysis



Troubleshooting

Problem	Possible Solution
High fraction of viable cells post cell lysis	Incrementally increase the lysis time and monitor lysis efficacy microscopically
Low nuclei recovery	Use a swing bucket rotor for centrifugation steps.
Nuclei aggregation & clumping	Optimize the lysis time and avoid over-lysis Maintain concentrations <5,000 nuclei/µl when washing and resuspending nuclei Filter nuclei suspensions with an appropriate cell strainer

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