Feb 06, 2026

Nuclear Isolation for Single-Nucleus Multiomic Profiling of Mouse Brain and Spinal Cord

DOI
https://dx.doi.org/10.17504/protocols.io.e6nvwnmo9vmk/v1

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DOI: https://dx.doi.org/10.17504/protocols.io.e6nvwnmo9vmk/v1

Protocol Citation: Liliana Cano Gomez, Satoshi Ishishita, Allan-Hermann Pool 2026. Nuclear Isolation for Single-Nucleus Multiomic Profiling of Mouse Brain and Spinal Cord. **protocols.io** https://dx.doi.org/10.17504/protocols.io.e6nvwnmo9vmk/v1

Protocol status: Working
We use this protocol and it's working

Created: February 06, 2026

Last Modified: February 06, 2026

Protocol Integer ID: 242786

Keywords: single-nucleus RNA-seq, neuronal nucleus suspension, spinal cord, neuronal nuclei, nuclei, single-cell multiome, tissue preparation, snATAC-seq, 10x Genomics, nucleus multiomic profiling of mouse brain, nucleus multiomic profiling, neuronal nuclei, spinal cord protocol generating suspensions of mouse, mouse brain, central nervous system tissue, spinal cord protocol generating suspension

Funders Acknowledgements:
National Institute of Mental Health
Grant ID: R01MH134799
Rita Allen Foundation Scholars Award in Pain
Grant ID: N/A
McKnight NBD Award
Grant ID: N/A
UTSW Endowed Scholars Program
Grant ID: N/A

Abstract

Protocol generating suspensions of mouse neuronal nuclei (NeuN+) from central nervous system tissue for single-nucleus multiomic profiling.

Materials

Equipment

- Kimble Dounce Kontes tissue-grinder set (DWK 885300-0000)
- 50 ml Oakridge tubes (#0556214D)
- 15 mL Falcon tubes (Fisher #352097)
- 50 mL Falcon tubes (Fisher #352070)
- 1.5mL LoBind Eppendorf Tubes
- 40-micron Corning Cell Strainer (#431750)
- Fire polished glass Pasteur pipettes (VWR #14672-380, polished in an open gas flame down to ~600 micron, 300 micron and 150 micron tip opening sizes)
- Magnetic stand: Biorad Surebeads Magnetic Stand or equivalent

'Reagents

- Roche Protector RNase Inhibitor (Millipore Sigma RNAINH-RO)
- EDTA free Protease Inhibitor: Roche cOmplete Mini-Tablet # 11873580001 (for preserving nucleosomal structure)
- BioLegend anti-NeuN antibody (#608452, for labeling neuronal nuclei)
- Alexa Fluor 647 Microscale Protein Labeling Kit (ThermoFisher #A30009)
- 1 mg/ml 7-AAD (nuclear labeling)
- 1M DTT (dithiothreitol, prepare fresh every couple of months and store at -20°C)
- Ultrapure RNA-se free/ DNA-se free water

Solutions

1 **NMDG-Hepes-ACSF**

- NMDG (93 mM)
- KCl (2.5 mM)
- NaH_2PO_4 (1.2 mM)
- $NaHCO_3$ (30 mM)
- HEPES (20 mM)
- Glucose (25 mM)

Bring pH to between 7.3 - 7.4 with 10N HCl and filter sterilize (good for 2 weeks at 4°C).

On the morning of tissue preparation add the following components (final concentration):

- Na-Ascorbate (5 mM)
- Thiourea (2 mM)
- Na-pyruvate (3 mM)
- $MgSO_4$ (10 mM, prepare 2M stock that is good for 6-months at 4°C)
- $CaCl_2$ (1 mM, prepare 2M stock that is good for 6-months at 4°C)
- Kynurenic acid Na-salt (1 mM)

2 **50x Protease Inhibitor Stock**

Dissolve 1 cOmplete minitablet in 1mL of ddH2O)

3 **Nuclear Buffer**

- Sucrose (320 mM)
- Tris-HCl (pH=7.4) (10 mM)
- $MgCl_2$ (3 mM)
- NaCl (10 mM)
- BSA (RNAse free) (0.50%)
- Kollidon VA64 (1 %)
- Ultrapure water, fill to 50 mL and 0.22 micron filter sterilize.

Morning of run:

- add DTT (dithiothreitol, 1 mM final concentration)
- add Roche Protector RNAse Inhibitor (0.1 U/uL)
- add 50x Protease Inhibitor (1x final concentration)

4 **Lysis Buffer (0.75 mL per sample)**

- Nuclear buffer
- Triton-X100 (0.1%)

5 **1M Sucrose Cushion (12 mL per sample)**

- Sucrose (1 M)
- Tris-HCl (pH=7.4) (10 mM)
- $MgCl_2$ (3 mM)
- NaCl (10 mM)
- BSA (nuclease free) (0.50%)
- Water (Ultrapure) Fill to 50 mL

Do NOT Filter sterilize!

Add:
- DTT (dithiothreitol, 1 mM final concentration)
- Roche Protector RNAse Inhibitor (0.1 U/uL, final concentration)
- add 50x Protease Inhibitor (1x final concentration)

6 **Resuspension Buffer (only for snRNA-seq, do not use for 10x Genomics Multiome)**
- Sucrose (320 mM)
- Tris-HCl (pH=7.4) (10 mM)
- $MgCl_2$ (1 mM)
- NaCl (10 mM)
- BSA (RNAse free) (0.50%)
- Ultrapure water, fill to 50 mL and 0.22 micron filter sterilize.

Morning of run:
- DTT (dithiothreitol, 1 mM)
- Roche Protector RNAse Inhibitor (0.1 U/uL)

7 **Prepare ConA beads for nuclear concentration**
- Gently resuspend the ConA beads and transfer 11 µL per sample to a 1.5 ml tube . Immediately add 100 µL/sample ice cold Bead Activation Buffer, and pipette to mix. Place on ice and wait for about 30-60min. Place the tube on magnet until slurry clears and pipette to remove supernatant.
- Repeat wash one more time.
- Resuspend beads in 11 µL (per sample) ice cold Bead Activation Buffer. Keep activated ConA beads on ice until needed.

Protocol

8 **1. Prepare solutions and equipment**
- Prepare 50 mL of NMDG-HEPES-ACSF from pre-prepared stock by adding (Na-Ascorbate, Thiourea, Na-pyruvate, $MgSO_4$, $CaCl_2$ and Kynurenic acid Na-salt) and place on ice.

- Prepare Nuclear Buffer (add DTT, RNA-se inhibitor and protease inhibitor to preprepared solution) and place on ice.
- Prepare Lysis Buffer from Nuclear Buffer (add Triton-X100 to 0.1% of final volume) and pipette 0.75 mL into a Kontes tissue grinder.
- Prepare 1M sucrose cushion (add DTT, RNA-se inhibitor and protease inhibitor to preprepared solution) and place on ice.
- Pre-cool centrifuge to 4°C.
- Place 100 mm dissection dish into a 150 mm dish with dry ice.
- Activate the ConA beads and place on ice.

9 **2. Dissect out tissue**

Anesthetize mouse with isoflurane and decapitate. Rapidly extract nervous system region of interest and transfer to ice-cold carbogenated (95% O_2 and 5% CO_2) NMDG-HEPES-ACSF in 1.5 mL collection tubes on ice. Microdissect out the specific region of interest in ice-cold NMDG_ACSF and cut the tissue into small 1.5 mm^3 cubicles.

10 **3. Generate nuclear suspension**

- Transfer tissue pieces into the Lysis Buffer in the Kontes tissue grinder.
- Apply 5 strokes with the loose pestle followed by 15 strokes with the tight pestle.
- Place a 70-micron cell strainer on a 50 mL Falcon tube and pre-wet with 500 µL of Nuclear Buffer.
- Add 250 µL of Nuclear Buffer to the tissue grinder.
- Mix nuclear suspension in tissue grinder twice with a 600-micron fire polished glass capillary and transfer through the cell strainer.
- Wash tissue grinder with 750 µL Nuclear Buffer and transfer again through the cell strainer.
- Wash cell strainer with final 750 µL Nuclear Buffer.

11 **4. Spin nuclei down and resuspend in fresh Nuclear Buffer**

- Spin nuclei down for 5 min at 500g at 4°C in a spin-out rotor.
- Remove supernatant and resuspend in fresh 3 mL Nuclear Buffer.

12 **5. Purify nuclei with a sucrose cushion centrifugation**

- Transfer 12 mL of sucrose cushion into a 50 mL Oakridge tube.
- Gently layer the nuclear suspension from the previous step on the sucrose cushion (avoid mixing of the layers).
- Centrifuge the tubes at 3200g at 4°C for 20 minutes in a spin-out rotor.
- After centrifugation, pour out the supernatant by decanting in one smooth motion and drying out the neck of the Oakridge tube with a Kimwipe.
- Resuspend the nuclear pellet in 300 µL of ice-cold Nuclear Buffer and mix gently with a 300 micron fire polished Pasteur pipette.
- Transfer purified nuclear suspension to a new 15 mL tube on ice.

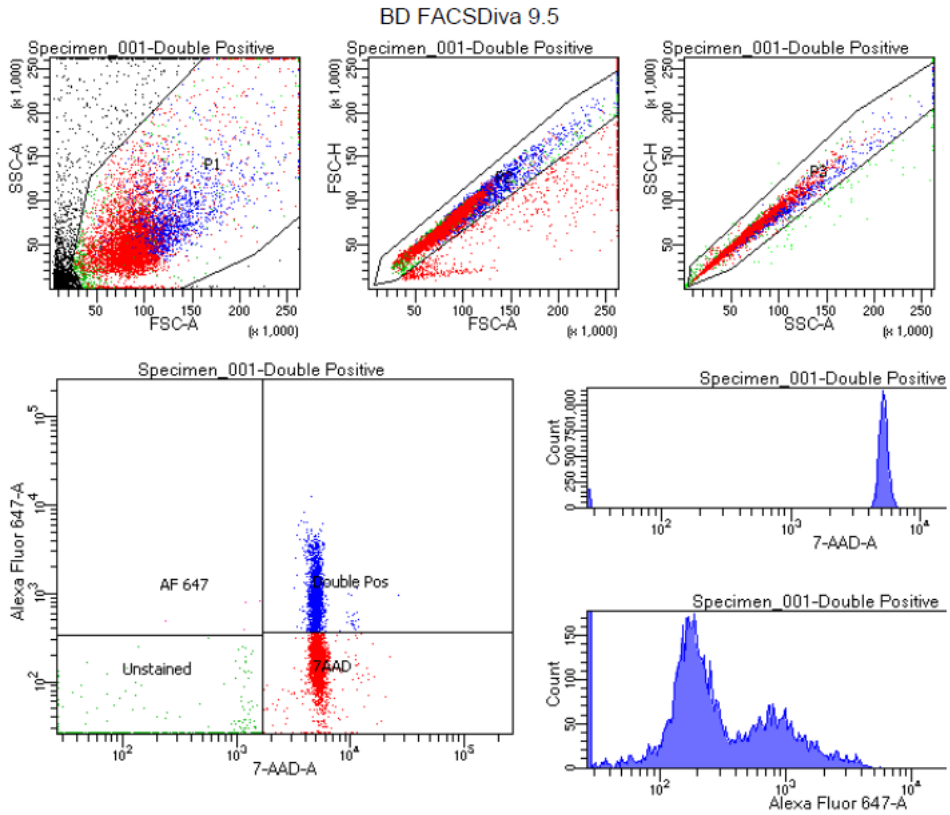
13 **6. Stain nuclei for FACS sorting**

- Add 3 µL of anti-NeuN-Alexa-647 Ab and 3 µL of 7-AAD to the nuclear suspension.

- Incubate the nuclear suspension at 4°C (not on ice!; keep dark as 7-AAD is light sensitive).
- Bring nuclear suspension volume to 3 mL with Nuclear Buffer.
- Spin nuclei down at 500g for 5 min at 4°C in a spin-out rotor.
- Remove most of supernatant and resuspend nuclei in 3 mL of Nuclear Buffer.
- Place suspension at 4°C for 5 min.
- Spin nuclei down at 500g for 5 min at 4°C.
- Remove supernatant and resuspend nuclei in 0.5 mL of Nuclear Buffer.
- Gently triturate with 150 micron glass Pasteur pipette to declump any nuclei.
- Add 1 mL of Nuclear Buffer and take to FACS sorting.

14 **7. FACS sorting**

- Sort at low pressures (4 or 5 PSI on BD sorters).
- Select for 7-AAD and Alexa 647 double-positive nuclei (note - perform compensation with single-stains of dyes before the actual profiling run to get clear separation between double-positive neuronal nuclei and single-positive non-neuronal nuclei).
- Collect 120 000+ nuclei



Sorting gates for isolating neuronal nuclei (double positive for 7-AAD and Alexa-647)

15 **8. Concentrate nuclei**

(note: mouse nuclei are fragile after sorting)

- Add 12 microL of prepared ConA beads to FACS sorted nuclei and gently mix with pipette.
- Incubate on ice for 10 minutes
- Put tube on magnet for 1-2 minutes to concentrate the nuclei.
- Aspirate the supernatant and rapidly suspend nuclei with 15 microL of 1x Nucleus buffer (10x Genomics Next Gem Multiome Kit, aim for 4000 nuclei/microL).
- Triturate gently wtih 150 micron glass capillary for 10 times
- Proceed to 10x single-cell multiomic profiling.

Acknowledgements

The authors are grateful to the UTSW Moody Flow Cytometry Facility for technical advice and help.