

Jul 22, 2024

Human Neuronal Nucleus Isolation for Single-Nucleus Transcriptomic Profiling (10x Genomics)

DOI

dx.doi.org/10.17504/protocols.io.4r3l2q3p4l1y/v1

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DOI: dx.doi.org/10.17504/protocols.io.4r3l2q3p4l1y/v1

Protocol Citation: Satoshi Ishishita, Allan-Hermann Pool 2024. Human Neuronal Nucleus Isolation for Single-Nucleus Transcriptomic Profiling (10x Genomics). **protocols.io** <https://dx.doi.org/10.17504/protocols.io.4r3l2q3p4l1y/v1>

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Protocol status: Working

We use this protocol and it's working

Created: July 16, 2024

Last Modified: July 22, 2024

Protocol Integer ID: 103467

Keywords: single-nucleus RNA-seq, neuronal nucleus suspension, human tissue, spinal cord

Abstract

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

Equipment and Reagents

1 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
- 70-micron Corning Cell Strainer (#431751)
- 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)

2 Reagents

- Roche Protector RNase Inhibitor (Millipore Sigma RNAINH-RO)
- 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

3 NMDG-Hepes-ACSF

- NMDG (93 mM)
- KCl (2.5 mM)
- NaH₂PO₄ (1.2 mM)
- NaHCO₃ (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)
- MgSO₄ (10 mM, prepare 2M stock that is good for 6-months at 4°C)
- CaCl₂ (1 mM, prepare 2M stock that is good for 6-months at 4°C)
- Kynurenic acid Na-salt (1 mM)

4 Nuclear Buffer



- Sucrose (320 mM)
- Tris-HCl (pH=7.4) (10 mM)
- MgCl₂ (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:

- DTT (dithiothreitol, 1 mM)
- Roche Protector RNase Inhibitor (0.1 U/uL)

5 Lysis Buffer

- Nuclear buffer
- Triton-X100 (0.1%)

6 1M Sucrose Cushion

Sucrose (1 M)
Tris-HCl (pH=7.4) (10 mM)
MgCl₂ (3 mM)
NaCl (10 mM)
BSA (nuclease free) (0.50%)
Kollidon VA64 (1%)

Water (molecular biology grade) Fill to 50 mL
Do NOT Filter sterilize!

7 Resuspension Buffer

- Sucrose (320 mM)
- Tris-HCl (pH=7.4) (10 mM)
- MgCl₂ (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)

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 and RNA-se 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 and RNA-se 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.

9 **2. Dissect out tissue**

Place snap frozen brain tissue into 100 mm tissue culture dissection dish on a layer of dry ice in a larger 150 mm dish. Microdissect out desired tissue parts and cut into small 1.5 mm^3 cubicles. Drop the latter into ice-cold NMDG-Hepes-ACSF in 1.5 mL collection tubes on ice.

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.

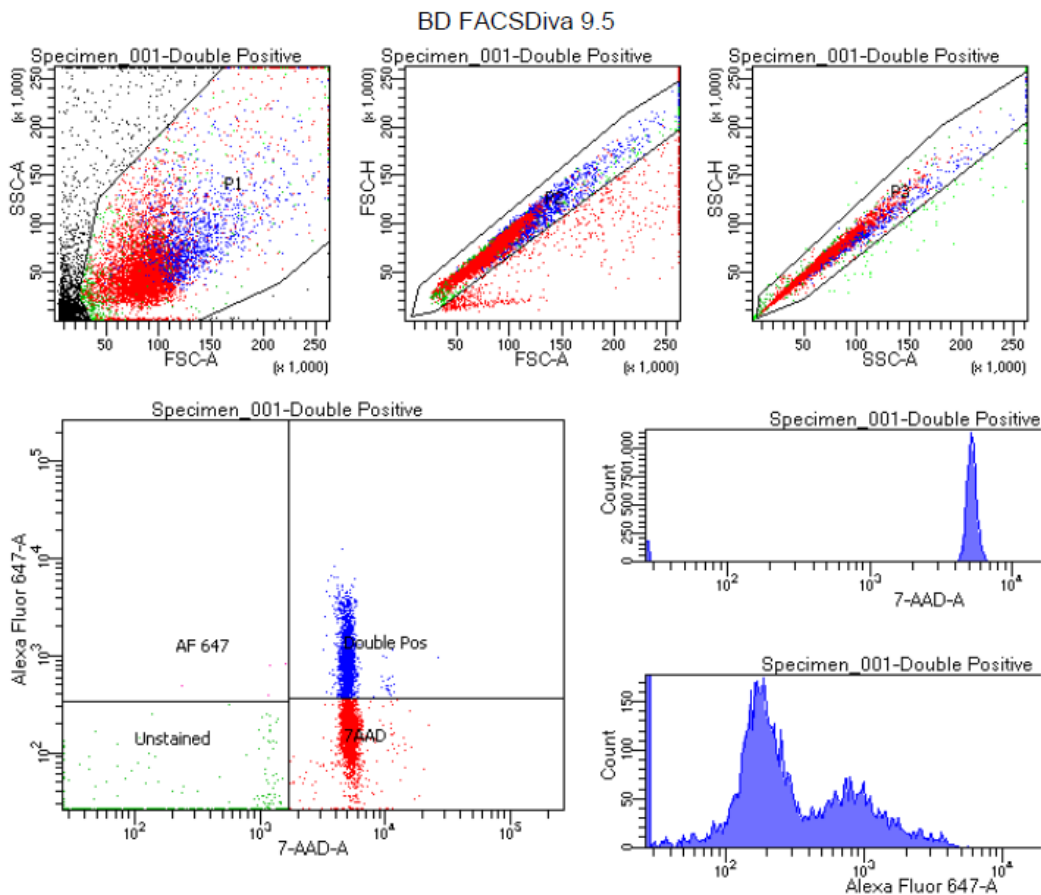
13 **6. Stain nuclei for FACS sorting**

- 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).



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

15 8. Concentrate nuclei



- Spin FACS sorted nuclei down at 500g for 5 min at 4°C in a spin-out rotor and gently resuspend in desired buffer for 10x profiling (e.g. Resuspension Buffer above). Note 1 - post-FACS centrifugation is ok with human neuronal nuclei but not recommended with post-FACS mouse nuclei.
- During optimization stage, visualize nuclei with a 40x objective in brightfield and make sure resulting nuclei have predominantly (~85%+) round intact nuclear membranes.
- Optional: gently pipette the suspension with 150 micron fire polished Pasteur pipette to get rid of any remaining nuclear clumps.
- Proceed to 10x Genomics or other profiling.