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

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

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

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Abstract

Protocol for generating suspensions of fixed 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) / can replace with 50 mL Falcon tube
- 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) // can replace with regular micropipetting.

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

3 **Protocol outline**

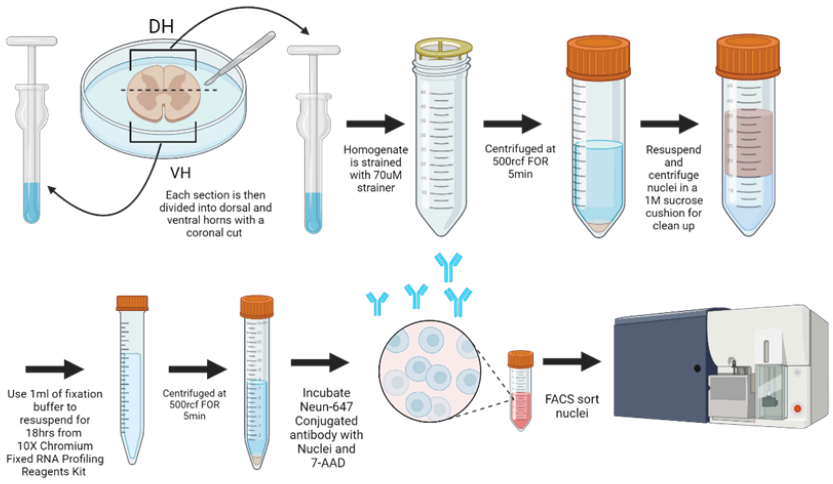

Figure 1: Protocol outline with spinal cord as sample central nervous system tissue.

Solutions

4 **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)

5 **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:

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

6 **Lysis Buffer**

- Nuclear buffer
- Triton-X100 (0.1%)

7 **1M Sucrose Cushion**

Sucrose (1 M)
Tris-HCl (pH=7.4) (10 mM)
$MgCl_2$ (3 mM)
NaCl (10 mM)
BSA (nuclease free) (0.50%)
Kollidon VA64 (1%)

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

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.

13 **6. Fix nuclei**

- 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 nuclei with 1mL of Fixation buffer from the **10X fixation of cells & nuclei for chromium fixed RNA profiling (CG000478)** and transfer resuspension to a 15mL Falcon tube and incubate for 18hr at 4°C.

14 **7. Stain nuclei for FACS sorting**

- Centrifuge tube at 500g for 5min at 4°C to wash off the fix.
- Resuspend in 300 µL of Nuclear Buffer.
- Add 3 µL of anti-NeuN-Alexa-647 Ab (conjugate Alexa-647 to Biolegend anti-NeuN antiobody prior to use) 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.

15 **8. 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).

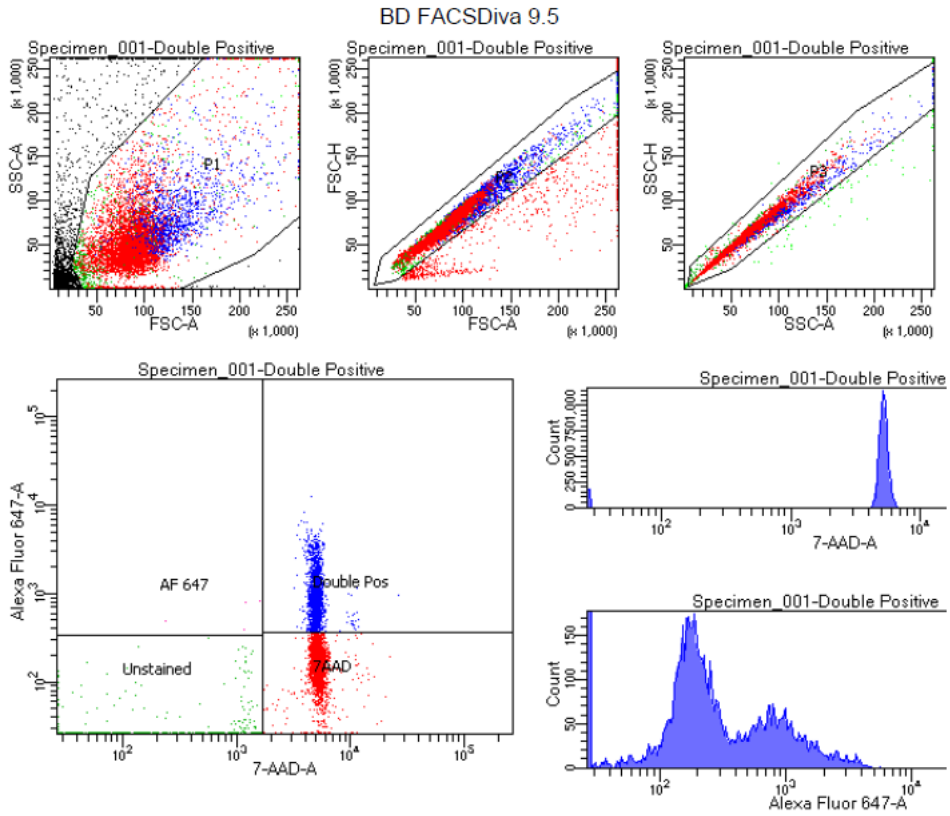

Figure 2: Sorting gates for isolating neuronal nuclei (double positive for 7-AAD and Alexa-647)

16 **9. Store or prepare nuclei for profiling**

- Post FACS sorting, spin collected nuclei at 850g for 5 min at 4°C and resuspend the nuclei with 1 mL of quenching buffer and follow the instructions of the 10X fixed nucleus profiling kit to freeze the samples long term or short-term depending on the timeline for sequencing steps.
- Proceed to 10x Genomics or other profiling.