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Single-Nuclei Isolation from Adult Mouse Ventral Midbrain

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We use this protocol and it's working

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Abstract

We use this protocol to isolate single nuclei from frozen tissue. It is compatible with Chromium Fixed RNA Kit (10x Genomic).

Materials

Required buffers and reagents:

1. Homogenize buffer I
   - HBSS (Thermo Fisher 14175095)
   - 0.2 U/ul RNase Inhibitor (40U/ul, Millipore Sigma, 3335402001)
2. Nuclei Extraction Buffer (Miltenyi Biotec, 130-128-024)
3. Homogenize buffer II (**HMII**)
   - 0.25 M sucrose
   - 25 mM KCL
   - 5 mM MgCl2
   - 20 mM Tris-HCL
   - pH7.8
4. 50% Iodixanol working solution (**WS**)
   - 5 vol of OptiPrep
   - 1 vol of the diluent (150mM KCL, 30mM MgCl2, 120 mM Tris-HCL, pH7.8)
5. 40% Iodixanol-Sucrose-KCL-MgCL2 solutions
   - 4 vol of WS (800ul/sample)
   - 1 vol of HMII (200ul/sample)
6. Nuclei Wash and Resuspension Buffer
   - 1X PBS (Life Technologies: 10010-023)
   - 1% BSA (ThermoFisher: AM2616)
   - 0.2 U/ul RNase Inhibitor (Millipore Sigma, 3335402001)
   - Ultrapure Water (Life Technologies: 10977-015)
7. Fixation Buffer
   - 4% Formaldehyde
   - Conc. Fix & perm buffer (10x genomics PN 2000517)
   - Ultrapure Water
8. Quenching Buffer
   - Conc. Quench Buffer (10x Genomics PN 2000516)
   - Ultrapure Water
9. Enhancer
10. 50% glycerol

Others:

1. Centrifuge with fixed angle rotor (3000rcf, 4 °C)
2. Centrifuge with swing bucket rotor (1.5ml/15ml adaptor, 500rcf, 4 °C)
3. Dry ice for tissue short storage
4. Wet ice for whole process
5. 15ml tubes/ 2-ml sharp bottomed tubes/ 1.5-ml low binding tubes
6. Hand-held TissueRuptor (TEM: 22573)
7. Disposable tips (STERILE OMNI TIP)

8. Wide-bore glass pipette (pre-autoclaved)
9. 40 µm-strainer (with adapter)
10. Trypan blue
11. Cell counter
12. 200ul/1000ul pipette & tips with filter.
13. Heat bath (65°C)
14. 70% ethanol
15. RNase ZIP
16. PPE (gloves/masks)
17. Trash beaker

Before start

(Always keep in mind of RNase free)

Dissociation & Release Nuclei

1 Add 3ml Homogenize buffer I to 15ml tube, transfer tissue into the tube, homogenize on ice with hand-held TissueRuptor (TEM: 22573) with disposable tips (STERILE OMNI TIP), at lowest setting, 5 sec on, 5 sec off, repeat 4 times, store on ice.

2 Centrifuge the homogenates at 500rcf for 5 minutes at 4°C and remove supernatant.

3 Add 3 mL chilled Nuclei Extraction Buffer (Miltenyi Biotec, 130-128-024) to the tissue. Resuspend the pellet using a wide-bore glass pipette, mix gently and incubate on ice for 15min. Triturate 10x by pipetting up and down with wide-bore glass pipette during the incubation.

4 Filter homogenate using a 40 µm-strainer (with adapter) to a new 15ml tube. (Optional: check residue on the strainer, consider recover it). Centrifuge the nucleus at 500rcf for 5 minutes at 4°C and remove supernatant.

5 Add 900 uL homogenate buffer II, resuspend the pellet and mix 10x using a regular-bore pipette tip.

6 Proceed to Nucleus Purification.

Nucleus Purification (Optiprep, 60% Iodixanol)

7 Add 900uL 40% Iodixanol to 900uL homogenates, mix well and transfer the sample into 2-ml sharp bottomed tube.

8 Centrifuge the mixture containing the nucleus at 3000 rcf for 30 min at 4°C.

9 Carefully (!!!) remove supernatant (watch out for cell debris on the tube wall), using a regular-bore pipette tip, add 200uL Nuclei Wash and Resuspension Buffer, resuspend the nuclei pellets, transfer to a new 1.5-ml low binding tube.

10 Add 800uL Nuclei Wash and Resuspension Buffer. Gently suspend the pellet.

11 Check the quality and purity of the nucleus (Target nuclei concentration is 700-1,200 nuclei/µL). QC can be done with microscope/ trypan blue cell counting/ fluorescence cell counting (Luna cell counter).

12 Centrifuge nuclei at 500 rcf for 5min at 4°C (Swinging bucket !!!).

13 (optional) repeat washing step 4) for 1-2 times in total.

14 Proceed to fixation.

Nuclei Fixation/ Storage

15 Add 1 ml Fixation Buffer to the sample pellet and pipette mix 5x.

16 Incubate for 16-24 h at 4°C.
*DO NOT agitate or mix the sample during incubation.
*Fixation time and temperature should be consistent across all samples in an experiment.

17 Centrifuge at 500 rcf for 5 min at room temperature (Swinging bucket !!!). Remove the supernatant without disturbing the pellet.

18 Add 1 ml chilled Quenching Buffer to the sample pellet and pipette mix 5x and keep on ice.

19 Thaw Enhancer (included in the 10x Genomics Kit_PN-1000414) for 10 min at 65°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use. (Once thawed, Enhancer can be kept at 42°C for up to 10 min.)

20 Add 0.1 volume pre-warmed Enhancer to fixed nuclei in Quenching Buffer (included in the 10x Genomics Kit PN-1000414).

21 Add 50% glycerol for a final concentration of 10%. Pipette mix. Store at -80°C for up to 6 months.