Nuclei Isolation Protocol for snRNASeq

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1. Keep tissue frozen in -80 degree freezer and maintain adequate conditions
2. Take out 3M dithiothreitol (DTT) aliquot from -20 degree freezer to thaw at room temperature
3. Retrieve ultracentrifuge (UC) rotor/buckets from 4 degree fridge or cold room, switch on UC and activate vacuum with rotor inside to maintain 4 degree temperature
4. Prepare Lysis & Sucrose Buffers in 50ml Falcon tubes:
    - Lysis: add 5µl 3M DTT to 30ml lysis buffer (for 3 samples)
    - Sucrose: add 5µl 3M DTT to 30ml sucrose buffer (for 3 samples)
5. Add 10µl of RNase inhibitor (RRI) to 990µl of lysis buffer (W/ DTT) in a 2ml eppendorf tube, do this for each sample
6. Take brain sample out of -80 freezer and keep on dry ice
7. Pipette 1ml of lysis+RNase inhibitor buffer (from step 5) into one of the douncers
8. Cut one brain sample as per project requirements (only about 50mg of sample is needed for single-nuclei sequencing), add to douncer, dounce 40-50x or until tissue is fully broken down. If tissue is very large, dip tissue into DPBS to help with dissection
    - Note: If pooling 3-4 brain samples together, ~20mg of tissue from each unique brain sample is sufficient. These brain samples go into one douncer.
    - All douncers, forceps, scalpels, and tips used with human tissue are soaked in a 75% bleach solution for >8hrs before cleaning and/or discarding.
9. Pipette 4ml of lysis buffer into the douncer, dounce 20x or until sample solution is homogeneous
10. Transfer sample from the douncer to an UC tube using a serological pipet
11. Repeat steps 7-10 for each sample
12. Underlay each sample in an UC tube with 9ml sucrose buffer
    - To underlay sucrose in UC tube: Draw up 9ml of sucrose buffer into a 10ml serological pipet. Insert the pipet tip into the UC through the sample all the way until the tip of the pipet is pressed against the bottom of the tube. Holding the tip against the bottom of the tube, slowly "prime" the serological pipet to release some of the sucrose (press the "down" button on the pipet gun in short gentle bursts to release only a small amount of sucrose buffer, just enough that you start to see it on the

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bottom of the tube). You will see the sucrose form a clear, separated layer below the sample. Slowly release more sucrose buffer, raising the pipet tip as you go so the tube does not overflow, but always keeping the tip below the sucrose line to maintain the separation of the sucrose and sample layers. When you have dispensed almost all of the 9ml of sucrose buffer, the sample will be very close to the top of the tube. Be very careful here not to cause the tube to overflow. When you dispense the last drops of sucrose, a bubble may come out of the pipet tip and float up to the top of the sample, this is fine and if you are gentle the bubble should not disrupt the layers of separation. If you cannot dispense the last of the sucrose without overflowing the tube, you do not have to dispense every last drop. The tube should now have 9ml of clear sucrose buffer on the bottom and 5ml of sample solution on the top, with a distinct line of separation between the two layers.

13. Balance tubes with additional lysis buffer. Add necessary extra buffer gently to the top of the sample layer so as not to disrupt the gradient (samples placed opposite each other in the rotor must have ≤0.03g weight difference between tubes)
14. Place tubes into UC rotor buckets and start UC spin for 1hr, 24,000rpm, 4 degrees
15. Clean up work space, return unused tissue to -80 freezer
16. Label eppendorf 2ml tubes with sample IDs for each sample
17. Combine 200µl 5% BSA in DPBS + 25µl RRI + 775µl DPBS into each of the two eppendorf tubes from step 16
18. Retrieve samples from UC, place samples on ice. Check for white halo at bottom of tubes
19. Vacuum supernatant: start with debris layer at interface between lysis and sucrose layers, then remove lysis buffer. Circle the vacuum tip around the side of the tube at the interface, vacuum away the sucrose. As you approach the bottom, tilt the tube so the sucrose drips away from the halo at the bottom of the tube
20. Immediately add 1ml resuspension solution from step 17 directly to halo and leave on ice for 5-10 minutes
21. Resuspend the pellet 50 times: take from the bottom of the tube and resuspend on the wall. Go slowly and gently
22. Transfer each sample to a 1.5ml eppendorf tube
23. Add 1µl DAPI to each sample, wrap in tin foil and keep on ice
24. Prepare collection tubes for fluorescence-activated nuclei sorting (FANS): Coat 1.5ml eppendorf tubes with 5% BSA in DPBS. Remove any residual BSA at bottom of tubes
25. FANS purification: collect DAPI-positive nuclei using Aria or Tyto cell sorter, then return to lab

- Collect 300,000 DAPI+ nuclei into collection tubes prepared in step 24
- Note % dead count of nuclei, and concentration of the dead population (nuclei/ml)

26. Spin down nuclei for 5min, 2500 x g, 4 degrees
    - If using Tyto cell sorter, skip this step and go to step 28
27. Remove supernatant with pipet, leaving over 50-100µl of supernatant in the tube, and resuspend pellet gently using the leftover supernatant
28. Count nuclei using automated cell counter. Mix 10ul Trypan Blue and 10ul of collected nuclei and pipette 10ul of solution into cell counting slide
    - Adjust focus and brightfield setting to optimally visualize and count sample (e.g. brightfield 42, focus ~60)
29. Continue with 10x Chromium Next GEM Single Cell 3' v3.1 (Dual Index) Protocol (CG000315 Rev A)
30. Use the nuclei concentration obtained from the cell counter in step 28 and the Cell Suspension Volume Calculator Table on page 27 of the protocol to determine the volume of the cell suspension to input into the protocol, according to desired Target Cell Recovery

Reagents and Equipment Details

Lysis Buffer (400ml solution)

43.76g Sucrose
2ml 1M $CaCl_2$
1.2ml 1M $Mg(Ace)_2$
80µl 0.5M EDTA
4ml 1M Tris pH 8
4ml 10% Triton
Adjust to 400ml w/ autoclaved milliQ H_2O

Sucrose Buffer (400ml solution)

246.24g Sucrose
1200µl 1M $Mg(Ace)_2$
4ml 1M Tris HCL pH 8
Adjust to 400ml w/ autoclaved milliQ H_2O

3M dithiothreitol (DTT)

231.38mg DTT
500µl H_2O

Takara Bio Recombinant RNase Inhibitor (RRI) (Cat. 2313)

ThermoFisher Scientific Sorvall WX80+ Ultracentrifuge (Cat. 75000080) with SureSpin 630 (17mL) Rotor (Note: This rotor is discontinued but SureSpin 632 Rotor seems comparable Cat. 75003031)

Beckman Coulter Polypropylene Centrifuge Tubes ⅝ x 3 ¾ in. (Ref. 361707)

Invitrogen DAPI (4′,6-diamidino-2-phenylindole) (Ref. D1306)

Biosciences BD FACSAria Cell Sorter or **Miltenyi Biotec MACSQuant Tyto Cell Sorter**

ThermoFisher Scientific Countess™ 3 FL Automated Cell Counter (Cat. AMQAF2000)