**New Nuclei Isolation for Single Nuclei RNA Sequencing**

**(80μm sectioned frozen human brain tissue)**

**<https://www.nature.com/articles/nprot.2016.015>**

1. Preparation

Can be prepared ahead of time:

* Tissue sectioning: If enriched 200mg of tissue, if total population only, 50mg of tissue.
* 29% and 50% Iodixanol Preparation
* IDM and 1.5M Sucrose stock in cold room
* NIM1 to be made in advance
* Chill centrifuge (4°C)
* Clean RNA zap workspace (+UV dounce, pipettes and chip cover)
* Coat all tubes in 0.5% BSA in PBS and shake well (RT) prior to use
* SOX10 needs to be resuspended upon arrival
* 50x protease inhibitor needs to be resuspended upon arrival

To be made fresh (on ice) before starting:

* NIM2
* Homogenisation Buffer
* BSA Buffer

**Density Gradient and Sucrose**

|  |  |
| --- | --- |
| **1.5M Sucrose RNA-DNA free** | **Final Concentration** |
| **Sucrose (solid)**  | 25.67g |
| **DNAse-RNAase free H20** | Initially 25mL, dissolve, fill up to 50mL |
| **Total Volume** | 50mL |

**Iodixanol Medium (IDM)**

*Stable for 6 months at 4 degrees*

|  |  |  |
| --- | --- | --- |
| Component  | Volume (ul) | Final Concentration (mM) |
| 1.5M sucrose  | 5,000 | 250 |
| 2M KCl | 2,250 | 150 |
| 1M MgCl2 | 900 | 30 |
| 1M Tris buffer, pH 8.0 | 1800 | 60 |
| Nuclease- free water  | 20050 | - |
| Total Volume | 30,000 | - |

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| --- | --- | --- |
| **50% Iodixanol** | **Volume (mL)** | **Final Concentration** |
| **Iodixanol 60% (v/v)** | 12500 | 50% v/v |
| **IDM** | 2,500 | - |
| **Total volume** | 15,000 | - |

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| --- | --- | --- |
| **29% Iodixanol** | **Volume (mL)** | **Final Concentration** |
| **Iodixanol 29% (v/v)** | 7,250 | 29% v/v |
| **IDM** | 7,750 | - |
| **Total volume** | 15,000 | - |

**BSA for coating tubes (0.5% in PBS)**

|  |  |  |  |
| --- | --- | --- | --- |
| Component | Volume (mL) | Volume (mL) | Final Concentration |
| PBS | 11400 | 22800 | - |
| MACS BSA 10% | 600 | 1200 | 0.5% |
| Total Volume | 12000 | 24000 | - |

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| --- | --- | --- |
| Nuclei Isolation Medium #1 (NIM1) | Volume (μL)*Stable for 6 months 4 degrees*  | Final Concentration |
| 1.5M Sucrose  | 7500 | 250mM |
| 2M KCl | 562.5 | 25mM |
| MgCl2 1M | 225 | 5mM |
| 1M Tris Buffer, pH 8.0 | 450 | 10mM |
| Nuclease-free water  | 36262.5 | - |
| Total Volume | 45000 | - |

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| --- | --- | --- | --- | --- | --- |
| Nuclei Isolation Medium #2 (NIM1) | Volume (μL)1 sample | Volume (μL)2 samples | Volume (μL)3 samples | Volume (μL)4 samples | Final Concentration |
| NIM1 | 1958 | 3916 | 5874 | 7832 | 1x |
| 1mM DTT | 2 | 4 | 6 | 8 | 1 μM |
| 50x protease inhibitor cocktail | 40 | 80 | 120 | 160 |  |
| Total Volume | 2000 | 4000 | 6000 | 8000 | - |

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| --- | --- | --- | --- | --- | --- |
| Homogenisation Buffer  | Volume (μL)1 sample | Volume (μL)2 samples | Volume (μL)3 samples  | Volume (μL)4 samples  | Final Concentration |
| NIM2 | 1748 | 3496 | 5244 | 6992 | 1x |
| RNasin RNAse Inhibitor (40U/ μL) | 50 | 100 | 150 | 200 | 1U/ μL |
| Triton X-100 1% (v/v) | 200 | 400 | 600 | 800 | 0.1% |
| DAPI | 2 | 4 | 6 | 8 | 0.1% |
| Total Volume | 2000 | 4000 | 6000 | 8000 | - |

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| BSA BUFFER (PBS+ 1% BSA+ 1U/ μL RNase Inhibitor) | Volume (μL)1 sample | Volume (μL)1 sample(enriched)  | Volume (μL)2 samples(1+1) | Volume (μL)3 samples | Final Concentration |
| MACS BSA 10% | 130 | 510 | 640 | 390 | 1% |
| RNasin RNAse Inhibitor (40U/ μL) | 33 | 128 | 161 | 99 | 1U/ μL |
| Protease Inhibitor Cocktail (50x) | 26 | 102 | 128 | 78 | 1x |
| PBS (pH 7.4) | 1111 | 4360 | 5471 | 3333 | - |
| Total Volume | 1300 | 5100 | 6400 | 3900 | - |

1. **Nuclei Isolation**

In Hood

1. Place a pre-labelled Eppendorf on ice.
2. On ice, add 1000mL Homogenisation Buffer to tissue sample.
3. Pipette tissue, transfer to 2mL dounce.
4. Rinse tube with 200mL HB and add to the dounce.
5. 5 strokes pestle A
6. 10 strokes pestle B
7. Transfer homogenate to the labelled Eppendorf.

Out of Hood

1. Centrifuge at 1,000rcf for 8 mins at 4°C.

-During this time: Add 500ml 29% iodixanol to new Eppendorfs (3 Eppendorfs per sample). Add 750ml 50% iodixanol to a 50mL falcon. Place 70mm filter on top of 50mL falcon.

1. Remove most of the supernatant.
2. Add 750ul Homogenisation Buffer
3. Pipette mix to resuspend the pellet and filter through 70mm filter cap.
4. Pipette to mix 50% iodixanol and cell suspension (total volume 1500ul)
5. Layer 1/3 (500ul) of the nuclei/sucrose mix on top of each of the 3 tubes with 29% iodixanol.
6. Centrifuge 13,000g for 40min at 40c
7. After centrifugation, remove the supernatant, starting at the top.
8. Resuspend in 500ul BSA buffer, FACS filter (combining the 3 individual sample tubes).
9. Centrifuge 500g, 5mins at 40c
10. Resuspend in 1mL BSA buffer.
11. Pipette mix and FACS filter again.
12. Take 10mL nuclei from FACS tube, load onto cell imaging slide. View on microscope (EVOS 20X) to assess debris and clumping.
13. Take 800uL of sample and label as “enrichment "and the rest as “DAPI”. Fill both aliquots to 1mL with BSA buffer.
14. **Staining and FACS**
15. Add primary antibodies Neun (1in500-2ul) and Sox10 (1in250-4ul) and leave on for 1 hour in cold room.
16. FACS filter the DAPI sample and sort for DAPI positive on the Melody.
17. Centrifuge enrichment sample 5mins 500xg, remove supernatant and resuspend in 1mL BSA Buffer.
18. Add 488 anti-goat and 647 anti-mouse (1in1000) and incubate for 30mins in the dark.
19. Centrifuge enrichment sample 5mins 500xg, remove supernatant and resuspend in 1mL BSA Buffer.
20. Pass through filtered FACS tube.
21. Add 200ul of BSA buffer with inhibitor in the tubes the samples will be sorted into.
22. FACS Sort into negative, neun+, sox10+.
23. **Preparation for GEM generation**
24. Centrifuge all samples at 500 rcf for 5 min at 4°C. Neun+ and Sox10+ pellets with all supernatants removed to be stored at –80 freezer.
25. For DAPI only and negative samples, remove the supernatant without disrupting the pellet. Leave 30ul volume, pipette mix to resuspend.
26. Count cells on Luna Cell counter. In a tube strip prepare for each sample:

 -1ml Propidium Iodide stain

 -9ml nuclei suspension

 -Transfer 10ul stained nuclei to a Cell Counting Slide chamber.

 - Calculate: # cells/1000 = cells per μL.