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Multiplexed snRNA-seq from frozen human brain samples V.2

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We use this protocol and it's working

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Abstract

This is a protocol to save your experiment and successfully demultiplex nuclei using CellPlex, the cholesterol-modified-oligo (CMO) barcodes from 10x Genomics on your nuclei from snap-frozen tissue.

Generally speaking, nuclei from snap-frozen tissue perform really poorly with CMOs. This crucial information was not in their initial documentation when the product was launched, leading to costly losses.

However, if you are multiplexing samples from different human donors, there is a way of saving your data and still demultiplexing your samples quickly and reliably. By taking advantage of known SNPs in the human genome, it is possible to demultiplex samples originating from different donors using freemuxlet.

Materials

Sectioned or finely chopped frozen human tissue (10-30 mg).

Solutions (Detailed recipes here)

- Lysis Buffer (*LB*, 3ml/sample)
- Wash and Resuspension Buffer (*WRB*, 7ml/sample)
- Sucrose Buffer (10ml/sample)

2000U of RNAse inhibitor/sample

Before start

Important: If you are designing an experiment and planning to multiplex your nuclei from frozen tissue, you should avoid using Cellplex altogether. It's better to design your experiment using freemuxlet in combination with DNA sequencing or bulk RNAseq from each donor. If you already used CellPlex and your barcoding is terrible, this protocol is for you.

Preparation

10m

1 Check if the Rotor SW32Ti rotor is at 4°C

2 Prepare your working area: Clean the workbench and pipettes with RNAse Zap. 1m

3 Add RNAse inhibitor (Sigma cat.# 3335402001**)** to LB and WRB (0.2U/ul).

4 Put lysis buffer and sucrose solutions on ice.

Nuclei Isolation

4h

5 Use glass dounce homogenizer (Thomas Scientific; Catalog # 3431D76; size A). Put douncer on ice, pipette 1mL of lysis in the douncer. Transfer tissue either using spatula or P1000 pipette with cut tip and additional lysis buffer. Bring the total volume of lysis buffer in the douncer to 3mL. On ice

6 Dounce tissue on ice with 10 strokes or until no chunks of tissue are visible. On ice

7 Transfer homogenized tissue in lysis buffer into a labeled thick wall ultracentrifuge tube on ice (Beckman Coulter; 355631). On ice

8 Carefully pipette 9 mL of Sucrose solution to the bottom of the tube containing Lysis buffer. Be careful not to introduce bubbles. You should see two clearly separated phases: sucrose on the bottom and cloudy homogenate on top. On ice

9 When you are done with all samples weigh them and bring to the same weight by adding Lysis buffer.

10 Load the samples to SW32Ti rotor (needs to be swing bucket). If using less than 6 samples still balance with empty buckets. 2h 30m

107163 rcf, 4°C, 02:30:00 , 29500 RPM on SW32Ti Rotor

11 After the spin, transfer samples on ice and carefully remove the supernatant using a P200 tip cut at an angle and vacuum. Make sure not to touch the bottom (stick to the wall and tilt the tube), but remove all the liquid. Carefully pipette 200uL of WRB on the bottom. Wait 20 min on ice. 20m

12 Meanwhile, transfer materials to the tissue culture room. Prepare eppendorf tubes with 10ul of DAPI for each sample.

13 Add 800ul of WRB (for a total of 1ml of WRB)and resuspend cells. On ice

14 Filter twice using Miltenyi Pre-separation filters (30um). (130-041-407)

15 Add 10ul of each sample to 10ul of DAPI. Count nuclei in each sample using a hemocytometer.
You should have at least 10^5 nuclei/sample

Expected result

1mg of human cortex typically yields ~10^4 cells

CellPlex Barcoding 1h 30m

16 Thaw *Cell Multiplexing Oligo* at room temperature. Vortex for 5 sec and centrifuge for 5 sec.

17 Centrifuge nuclei using a swing bucket rotor 500 rcf, 4°C, 00:10:00 10m

18 Remove supernatant 5m
Add 100ul of Cell Multiplexing Oligo (room temperature) to the nuclei. Gently pipette mix 10-15x to resuspend. Incubate for 5 mins.
Store the remaining Cell Multiplexing Oligo at -20C

19 Add 1.9 mL of cold WRB supplemented with RNAse inhibitor. Gently pipette mix

20 Centrifuge cells using a swing bucket rotor 500 rcf, 4°C, 00:10:00 10m

21 Remove supernatant and add 2ml of cold WRB.

22 Centrifuge cells using a swing bucket rotor 500 rcf, 4°C, 00:10:00 10m

23 for a total of 2 washes

24 Based on starting concentration and assuming a ~50% cell loss, add an appropriate volume of cold WRB to obtain a final concentration of ~1500 cells/ul.

25 Pool samples together and count the total number of cells using a hemocytometer. Load 49,500 cells/10x well to aim for 30k cells

Demultiplexing with popscle/freemuxlet

26 After sequencing your libraries, align your reads with CellRanger and make sure to enable the option to generate .BAM files

27 Familiarize yourself with popscle/freemuxlet inputs, outputs and vignetes on its Github page:

https://github.com/statgen/popscle

28 Use the BAM files as inputs to freemuxlet. A .VCF file (a reference file with the SNPs) is also needed as an input for freemuxlet and can be obtained from 1000 genomes, filtering for high variant confidence, Minor Allele Frequency (MAF 0.01) and exonic variants.

29 After running, identify the droplet barcodes that were not assigned as singlets and remove these cells from your analysis

30 In order to identify which donor identified in freemuxlet corresponds to each sample, you can simply check for the amount of each CMO in all cells of each donor. Despite CMO labeling being too weak to demultiplex samples by itself, you can use it to match donors identified on freemuxlet with each sample.