Version 1

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# snmCAT_V1 V.1

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ABSTRACT

To comprehensively assess the molecular phenotypes of single cells in tissues, we devised single-nucleus methylCytosine, Chromatin accessibility and Transcriptome sequencing (snmCAT-seq) and applied it to various sample sources, like culture cells, fresh/frozen mice tissues (brain, liver, pancreases etc) and postmortem human frontal cortex tissue.

PROTOCOL CITATION

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GUIDELINES

For details, please refer to the publications below:

Luo, C., Rivkin, A., Zhou, J., Sandoval, J.P., Kurihara, L., Lucero, J., Castanon, R., Nery, J.R., Pinto-Duarte, A., Bui, B., et al. (2018). Robust single-cell DNA methylome profiling with snmC-seq2. Nat. Commun. 9, 3824.

Luo, C., Liu, H., Xie, F., Armand, E.J., Siletti, K., Bakken, T., Fang, R., Doyle, W.I., Hodge, R.D., Hu, L., et al. (2019). Single nucleus multi-omics links human cortical cell regulatory genome diversity to disease risk variants. bioRxiv.

• For troubleshooting: feel free to leave comments or message directly.

MATERIALS TEXT

- **Reagents**

RNaseOUT™ Recombinant Ribonuclease Inhibitor (ThermoFisher Scientific 10777019)
SUPERase• In™ RNase Inhibitor (ThermoFisher Scientific AM2694)
Protease Inhibitor Cocktail (Sigma-Aldrich P8340)
Hoechst 33342 Solution (20 mM) (ThermoFisher 62249)
OptiPrep™ Density Gradient Medium (Sigma-Aldrich D1556)
Dounce tissue grinder set (2 mL) (Sigma-Aldrich D8938)
Dounce tissue grinder set (7 mL) (Sigma-Aldrich D9063)
UltraPure™ BSA (50 mg/mL) (ThermoFisher AM2618)
DPBS (1X) (ThermoFisher 14190144)
GpC Methyltransferase M.CviPI (NEB M0227L) (optional)
1% Triton X-100

Superscript II Reverse Transcriptase (ThermoFisher Scientific 18064071)
5-methyl-dCTP (NEB N0356S)
Deoxynucleotide (dNTP) Solution Set (NEB N0446S)
KAPA2G Robust HotStart PCR Kit (Roche KK5517)
10X Uracil DNA Glycosylase (UDG) (Enzymatics G5010L)

anti-NeuN-488 clone A60 (Millipore MAB377)

- **DNA oligos (HPLC purified, synthesized by IDT).**

In the original snmCAT-seq protocol (ChongyuanLuo, et al. *BioRxiv* 2019, https://www.biorxiv.org/content/10.1101/2019.12.11.873398v1),
RT primers and TSO oligos were synthesized with a 5'-C3 Spacer. However, in recent experiments, we found a 5'-biotin spacer is necessary to prevent the concatenation of oligo molecules. We speculate the reduced efficiency for 5'-C3 Spacer in preventing oligo concatenation is due to certain composition changes in commercial enzymes used in the protocol.

dT30VN_4_5bio/5Biosg/AAGCAGUGGUAUCAACGCAGAGUACUTTTTTUTTTTTUTTTTTUTTTTTUTTTTTVN
N6_2_5bio/5Biosg/AAGCAGUGGUAUCAACGCAGAGUACNNNNNN
TSO_3_5bio/5Biosg/AAGCAGUGGUAUCAACGCAGAGUGAAUrGrG+G
TSO_4_5bio/5Biosg/AAGCAGUGGUAUCAACGCAGAGUGAAUrGrGrG
ISPCR23_2/5SpC3/AAGCAGUGGUAUCAACGCAGAGU

## Background

1 This protocol is based on the originial protocol named as snmC2T-seq from the BioRxiv paper (ChongyuanLuo, et al. *BioRxiv* 2019) https://www.biorxiv.org/content/10.1101/2019.12.11.873398v1 and SMART-seq3 (Hagemann-Jensen, *et al.Nat Biotechnol* 2020, https://www.protocols.io/view/smart-seq3-protocol-bcq4ivyw.

Reagents and oligo sequence can be found in Materials part.

## Nuclei preparation 50m

2 Sample preparation:

- We typically grind tissue samples with liquid nitrogen ahead of time and stored at -80°C.
- For smaller mouse tissues, we usually snap freeze the fresh dissected samples and store at -80°C.
- For culture cells, we typically pellet either suspension cells or dissociated adherent cells, aspirate supernatant then store at -80°C.

In recent experiments, we found the RNA integrity from frozen human tissues may various. DO RIN analysis in bulk tissue before starting the experiments will be helpful to know the sample quality.

2.1 Prepare the stock solutions for nuclei isolation, stored at **4 °C** :

1. Diluent buffer : Tris-Cl pH 8.0 (120 mM), KCl (150 mM), MgCl2(30 mM)
2. NIB: Tris-Cl pH 8.0 (10 mM), KCl (25 mM), MgCl2 (5 mM), Sucrose (250 mM)

2.2 Prepare the following solutions freshly before each experiment:

1. NIB_plus **On ice** : NIB + DTT (1 mM) + Proteinase inhibitor (0.5X) + SUPERase• In ( 1:1000 dilution) + RNaseOUT ( 1:1000 dilution)
2. NIBT **On ice** : NIB_plus + 0.1% Triton X-100
3. 50% Iodixanol **Room temperature** : 5 vol. Optiprep (60% Iodixanol) + 1 vol. Dilutent
4. 25% Iodixanol **Room temperature** : 1 vol. 50% Iodixanol + 1 vol. NIB
5. DPBS + RNase inhibitor **On ice** : DPBS + SUPERase• In ( 1:1000 dilution) + RNaseOUT ( 1:1000 dilution)

2.3 Pre-chilling steps:

- Plunge the Dounce and Pestles on ice (in a 50ml tube to avoid contamination from ice). Transfer 3ml of NIBT buffer to the Dounce in ice and let them chill for 10 min.
- Pre-chill 2 ml and 5 ml low retention microcentrifuge tube **On ice**
- Cooling down the swing bucket rotor for centrifuging **4 °C** .

3 10m

1. Transfer tissue sample or pre-ground tissue powder into the Dounce containing 3 ml of NIBT.
2. Gently do douncing with a loose pestle (A) 40 times and then with a tight pestle (B) 40 times without introducing bubbles.
3. Mix the suspension with 2 ml of 50% Iodixanol by pipetting in 5ml cold microcentrifuge tube.
4. Slowly pipette 1ml of cell mixture onto 500 µl 25% Iodixanol cushion, 5 tubes in total.
5. Centrifuge at 10,000 g for 20 min at 4°C using a swing rotor.

Before adding cell mixture, we usually aliquot the 500 µl 25% Iodixanol cushion into 2 ml low retention microcentrifuge tubes and centrifuge at 10,000 g for 5 min to sharp the liquid interface.

4 Depending on specific experiment, proceed either Section A or B or C or A+B or C+A or C+A+B

Section_A_Nuclei staining_ONLY 10m

5 1. Remove supernatant. Re-suspend the pellet in 1 ml of ice-cold DPBS + RNase Inhibitors.
2. Add Hoechest 33342, then incubate on ice for 5 min.

Section_B_Ab staining 30m

6 1. Remove supernatant. Resuspend the pellet in 900 µl of DPBS + RNase inhibitors and 100 µl UltraPure BSA (50 mg/ml).

2. Add specific amount of nucleus antibodies and incubate on ice for 20 min.
(For mouse/human neurons, 1 µl AlexaFluor 488 conjugated anti-NeuN clone A60 is used)

Section_C_NOMe treatment 20m

7
1. Remove supernatant. Resuspend each pellet in 200 µl of ice-cold NIB.
2. Count the number of nuclei. Divide the nuclei suspension to vials each containing 1M nuclei.
3. Centrifuge at 2000 x g for 10 min at 4°C.
4. Remove supernatant. Resuspend the pellet in 200 µl of GpC methyltransferase + RNase Inhibitors reaction.
5. Incubate at 37°C for 10 min.
6. Add 800 µl of ice-cold DPBS + RNase Inhibitors to the GpC methyltransferase reaction.

| A | B |
|---|---|
| **GpC Methyltransferase mix (per Rxn)** | **ul vol.** |
| 1M Sucrose | 60 |
| GpC Methyltransferase Buffer (10X) | 20 |
| S-adenosylmethionine (SAM 32mM) | 2 |
| GpC MTase | 20 |
| H2O | 98 |

Pre_sorting 15m

8
1. Centrifuge at 1000 x g for 10 min at 4°C.
2. Remove supernatant. Resuspend the pellet in 1ml DPBS + RNase inhibitors.
3. Filter with 40 um Cell strainer

Ready to run sorting

Prepare collection plates 30m

9
Prepare mCT master mix:

| A | B | C | D |
|---|---|---|---|
| Reagent | 1 Rxn (µl) | 384 Rxns (with 100% extra, µl) | 8 x 384 Rxns (with 100% extra, µl) |
| Number of 384w plates | | 1 | 8 |
| | | | |
| 5X First-Strand Buffer | 0.2 | 153.6 | 1228.8 |
| 0.1M DTT | 0.05 | 38.4 | 307.2 |
| 1% Triton X-100 | 0.1 | 76.8 | 614.4 |
| 50mM MgCl2 | 0.05 | 38.4 | 307.2 |
| 5-methyl-dNTP (10mM) | 0.05 | 38.4 | 307.2 |
| dT30VN_4 (100 µM) | 0.01 | 7.68 | 61.44 |
| N6_2 (100 µM) | 0.02 | 15.36 | 122.88 |
| TSO_3 (100 µM) | 0.02 | 15.36 | 122.88 |
| RNaseOUT 40U/µl | 0.01 | 7.68 | 61.44 |
| SUPERaseIn 20U/µl | 0.01 | 7.68 | 61.44 |
| Superscript II RT | 0.01 | 7.68 | 61.44 |
| H2O | 0.47 | 360.96 | 2887.68 |
| Total | 1 | 768 | 6144 |

Use Beckman i7 robot to distribute mct reaction buffer to 384-plates:

Add **1 µl** RT mix into each well of a 384 well plate.

Quick centrifugation the plates and keep **On ice** .

FACS 2h

10 Sort single nuclei using BD Influx or other sorters into 384 well plates on one-drop single mode.

Reverse Transcription 2h

11 Incubate with a thermocycler

| A | B |
|---|---|
| 25°C | 5 mins |
| 42°C | 90 mins |
| 70°C | 15 mins |
| 4°C | ∞ |

PCR Amplification 1h

12 Prepare cDNA amplification mix:

| A | B | C | D |
|---|---|---|---|
| Reagent | 1 Rxn (µl) | 384 Rxns (with 30% extra, µl) | 8 x 384 Rxns (with 30% extra, µl) |
| Number of 384w plates | | 1 | 8 |
| | | | |
| mCT-seq RT reaction | 1 | 500 | 4000 |
| KAPA2G Buffer A (5X) | 0.8 | 400 | 3200 |
| ISPCR23_2 (100 µM) | 0.024 | 12 | 96 |
| KAPA2G Robust HotStart DNA Polymerase (5 U/µL) | 0.016 | 8 | 64 |
| H2O | 2.16 | 1080 | 8640 |
| Total | 4 | 2000 | 16000 |

Add **3 µl** RT mix into each well of a 384 well plate.

Incubate with a thermocycler

| A | B | C | D |
|---|---|---|---|
| Step | Temperature | Time | Cycles |
| Initial denaturation | 95 °C | 3 min | 1x |
| Denaturation | 95 °C | 15 sec | 11-15x |
| Annealing | 60°C | 30 sec | |
| Elongation | 72 °C | 2 min | |
| Final Elongation | 72 °C | 5 min | 1x |
| Hold | 4 °C | Hold | |

*Each cell type contains a different RNA quantity per cell or nucleus. The optimal cycle number for cDNA amplification needs to be optimized for each cell type.

Mouse and human neuronal nuclei - 14 cycles
Mouse and human non-neuronal nuclei - 16 cycles
Human H1 and HEK293 whole cell - 12 cycles
Human H1 and HEK293 nuclei - 15 cycles

## UDG Diegestion

13 Prepare uracil digestion mix:

| A | B | C | D |
|---|---|---|---|
| Reagent | 1 Rxn (µl) | 384 Rxns (with 50% extra, µl) | 8 x 384 Rxns (with 50% extra, µl) |
| UDG (G5010) | 0.5 | 287.5 | 2300 |
| EB buffer | 0.5 | 287.5 | 2300 |
| Total | 1 | 575 | 4600 |

Add **1 µl** RT mix into each well of a 384 well plate and incubate at 37°C for 30 mins.

## Bisulfite conversion

14 Add **25 µl** Zymo direct bisulfite conversion reagent into each well of a 384 well plate.
Incubate with a thermocycler

| A | B |
|---|---|
| Temperature | Time |
| 98 °C | 8 min |
| 64 °C | 3.5 hrs |
| 4 °C | hold |

## snmC-Seq2 library preparation

15 Proceed to the snmC-seq2 library preparation protocol.
https://www.protocols.io/view/methyl-c-sequencing-of-single-cell-nuclei-snmc-seq-pjvdkn6