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🌐 snmCAT_V2 V.2

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protocol .

Human Cell Atlas Method Development Community

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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.

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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.

- **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
DTT:

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_5:
/5Biosg/AAGCAGUGGUAUCAACGCAGAGUACUTTTTTUTTTTTUTTTTTUTTTTTUTTTTTVN
N6_3: /5Biosg/AAGCAGUGGUAUCAACGCAGAGUACNNNNNN
TSO_4: /5Biosg/AAGCAGUGGUAUCAACGCAGAGUGAAUrGrGrG
ISPCR23_3: /5SpC3/AAGCAGUGGUAUCAACGCAGAGU

Background

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This protocol is based on the original 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. 10X 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   1. Transfer tissue sample or pre-ground tissue powder into the Dounce containing 3 ml of ^{10m}
      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 ice-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. Before do NOMe treatment, it's better to count the nuclei number either using
       hemocytometer or automated cell counter.
    2. Transfer less than 1 million nuclei per reaction to a new tube, centrifuge at 1000 x g for 10
       min at 4°C to spin down the nuclei.
    3. Remove supernatant and resuspend in 50 µL DPBS.
    4. Then make the 1st cycle NOMe reaction:

A	B
GpC Methyltransferase mix (per Rxn)	**ul vol.**
Nuclei mix	50
GpC Methyltransferase Buffer (10X)	15
S-adenosylmethionine (SAM 32mM)	0.15
GpC MTase	15
H2O	70

Incubate at 37°C for 8 mins

4. 2nd cycle NOMe reaction:

A	B
GpC Methyltransferase mix (per Rxn)	**ul vol.**
1st GpC treated nuclei mixture	150
GpC Methyltransferase Buffer (10X)	15
S-adenosylmethionine (SAM 32mM)	0.15
GpC MTase	15
H2O	120

Incubate at 37°C for 8 mins

5. Stop the reaction in 1ml ice-cold DPBS and centrifuge at 1000 x g for 10min at 4°C

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

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     Prepare mCT master mix:

A	B	C	D
Reagent	1 Rxn (µl)	384 Rxns (with 100% extra, µl)	8 x 384 Rxns (with 63% extra, µl)
Number of 384w plates		1	8
Rxn		800	5000
5X First-Strand Buffer	0.2	160	1000
0.1M DTT	0.05	40	250
1% Triton X-100	0.1	80	500
25mM MgCl2	0.1	80	500
500mM NaCl2	0.06	48	300
5-methyl-dNTP (10mM)	0.05	40	250
dT30VN_5 (100 µM)	0.012	9.6	60
N6_3 (100 µM)	0.02	16	100
TSO_4 (48 µM)	0.05	40	250
RNaseOUT40U/µl	0.025	20	125
SUPERaseIn 20U/µl	0.025	20	125
Superscript II RT*	0.05	40	200
H2O	0.258	206.4	1340
Total	1	800	5000

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** .

> For high RNA aboundance tissue or cell types, RNaseOUT, SUPERaseIn and Superscript II RT can be cut to 0.01 ul per reaction.

FACS   2h

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

Reverse Transcription   2h

Incubate with a thermocycler

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A	B	C
Temperature	Time	Cycles
25°C	5 mins	1x
42°C	90 mins	1x
50°C	2 mins	10x
42°C	2 mins	
85°C	5 mins	1x
4°C	∞	

PCR Amplification   1h

12   Prepare cDNA amplification mix:

A	B	C	D
Reagent	1 Rxn (µl)	384 Rxns (with 60% extra, µl)	8 x 384 Rxns (with 30% extra, µl)
Number of 384w plates		1	8
Rxns	1	600	4000
KAPA2G Buffer A (5X)	0.8	480	3200
ISPCR23_3 (100 µM)	0.024	14.4	96
KAPA2G Robust HotStart DNA Polymerase (5 U/µL)	0.016	9.6	64
H2O	2.16	1296	8640
Total	3	1800	12000

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	

*Different cell types have a various RNA quantity per cell or nucleus. The optimal cycle number for cDNA amplification needs to be optimized for specific cell types or experiments.

From our experiences:

Mouse and human neuronal nuclei 11- 13 cycles
Mouse and human non-neuronal nuclei 13 - 15 cycles
P120 Mouse non-neuronal nuclei 15 cycles
P120 Mouse neuronal nuclei 11 cycles
Human culture fibroblast and induced neurons 11-13 cycles
Human H1 and HEK293 whole cell 11 cycles
Human H1 and HEK293 nuclei 11-13 cycles

UDG Diegestion   30m

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

16 The NGS mapping pipeline and analysis tools can be found in the packages coded by Hanqing:
Yap: https://hq-1.gitbook.io/mc/
Allcools: https://lhqing.github.io/ALLCools/intro.html