

Single-cell multi-omic profiling of chromatin conformation and DNA methylation

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Abstract

The ability to profile epigenomic features in single cells is facilitating the study of the variation in transcription regulation at the single cell level. Single cell methods have also facilitated the generation of cell-type resolved transcriptomic and epigenetic profiles of lineages derived from complex heterogeneous samples. However, integrating different epigenetic features remain challenging, as many current methods profile a single data type at a time. Furthermore, some epigenetic features, such as 3D genome organization, are intrinsically variable between single cells of the same lineage, so it remains unclear how well these methods may resolve cell-types from complex mixtures. Here we describe a method for profiling 3D genome organization and DNA methylation in single cells. This protocol accompanies Lee et al. (Nature Methods 2019) after peer review to aid potential users in applying the method to their own samples.

Introduction

Three-dimensional genome architecture is a critical feature of gene regulation in metazoan organisms¹⁻³. Despite the increasing utility of these datasets, most existing chromatin conformation maps are generated from cell lines *in vitro* or from bulk tissues *in vivo*⁴⁻⁸. While cell line data has enabled a greater understanding of the general principles of chromatin organization, it cannot fully represent the diversity of cell types that arise *in vivo*. Recent efforts using single-cell “omics” technologies aim to resolve this challenge by generating single-cell data from complex tissues that are then partitioned into the relevant distinct cell types *in silico* using data dimensionality reduction and clustering algorithms⁹⁻¹². However, it remains unclear whether single-cell Hi-C profiles will be suitable for partitioning into constituent cell types *in vivo*.

In contrast to single cell Hi-C data, single-cell DNA methylome datasets enable high-resolution cell-type classification, allowing the reconstruction of epigenomic maps from cell types in primary human tissues^{13,14}. 3C or HiC methods capture chromatin configuration by performing proximity ligation with restriction digested genomic DNA in crosslinked nuclei¹⁵. DNA methylation (mC) is fully preserved in the chimeric DNA molecules produced by 3C or HiC. Therefore, it is feasible to jointly

detect both long-range ligation junction and mC by analyzing DNA molecules generated by 3C or HiC using bisulfite sequencing. Joint analysis of chromatin conformation and mC can also facilitate the study of cross-talk between the two epigenomic features.

Here we describe a method, single-nucleus methyl-3C sequencing (sn-m3C-seq), to jointly profile chromatin conformation and DNA methylation from the same cell. As described in the associated publication, this method allows generates single cell chromatin conformation and DNA methylation profiles that are of equivalent quality as existing unimodal technologies. We have applied these to complex tissue samples such as human prefrontal cortex to generate cell type resolve DNA methylation and 3D genome profiles from the human brain.

Reagents

Reagents

General Reagents

- HyClone™ HyPure™ Molecular Biology Grade (MB) Water (GE Life Sci. cat. no. SH30538.03)
- 200-Proof (100%) Ethanol (Koptec cat. no. V1001)

Tissue in situ Hi-C

- Triton-X 100 (Sigma 93443)
- Hoechst (Life Technologies H3570)
- 10X NEB Buffer 4 (NEB B7004S).
- NlaIII restriction enzyme (NEB R0125)
- 10X NEB T4 DNA ligase buffer (NEB B0202)
- 400U/μl T4 DNA Ligase (NEB M0202)
- 2X Protease Inhibitor (Roche 11 873 580 001)

Collection of single nuclei by Fluorescence-activated Cell Sorting (FACS)

- UltraPure™ BSA (50 mg/mL) (ThermoFisher AM2618)

- anti-NeuN AlexaFluor488 (Millipore MAB377X)
- M-Digestion Buffer (Zymo cat. no. D5021-9)
- Proteinase K (Zymo cat. no. D3001-2-D)
- Proteinase K Storage Buffer (Zymo cat. no. D3001-2-B)
- Unmethylated Lambda DNA (Promega cat. no. D1521, 100pg/μL)

Bisulfite conversion

- CT Conversion Reagent (Zymo cat. no. D5003-1)
- M-Solubilization Buffer (Zymo cat. no. D5021-7)
- M-Dilution Buffer-Gold (Zymo cat. no. D5006-2)
- M-Reaction Buffer (Zymo cat. no. D5021-8)
- M-Binding Buffer (Zymo cat. no. D5021-7)
- M-Wash Buffer (Zymo cat. no. D5040-4)
- M-Desulphonation Buffer (Zymo cat. no. D5040-5)
- M-Elution Buffer (Zymo cat. no. D5007-6)

Random primers

- HPLC purified Random primers were ordered from Integrated DNA Technologies (IDT)

P5L_AD002_H /5SpC3/TTCCCTACACGACGCTCTCCGATCTCGATGT(H1:33340033)(H1)(H1)(H1)
(H1)(H1)(H1)(H1)(H1)

P5L_AD006_H /5SpC3/TTCCCTACACGACGCTCTCCGATCTGCCAAT(H1:33340033)(H1)(H1)(H1)
(H1)(H1)(H1)(H1)(H1)

P5L_AD008_H /5SpC3/TTCCCTACACGACGCTCTCCGATCTACTTGA(H1:33340033)(H1)(H1)(H1)
(H1)(H1)(H1)(H1)(H1)

P5L_AD010_H /5SpC3/TTCCCTACACGACGCTCTCCGATCTTAGCTT(H1:33340033)(H1)(H1)(H1)
(H1)(H1)(H1)(H1)(H1)

P5L_AD001_H /5SpC3/TTCCCTACACGACGCTCTCCGATCTATCACG(H1:33340033)(H1)(H1)(H1)

(H1)(H1)(H1)(H1)(H1)

P5L_AD004_H /5SpC3/TTCCCTACACGACGCTCTCCGATCTTGACCA(H1:33340033)(H1)(H1)(H1)

(H1)(H1)(H1)(H1)(H1)

P5L_AD007_H /5SpC3/TTCCCTACACGACGCTCTCCGATCTCAGATC(H1:33340033)(H1)(H1)(H1)

(H1)(H1)(H1)(H1)(H1)

P5L_AD012_H /5SpC3/TTCCCTACACGACGCTCTCCGATCTTTGTA(H1:33340033)(H1)(H1)(H1)

(H1)(H1)(H1)(H1)(H1)

Sera-Mag Solid Phase Reversible Immobilization (SPRI) beads

- Sera-Mag SpeedBeads Magnetic Carboxylate Modified (GE Healthcare cat. no. 45152105050250)
- Poly(ethylene glycol) PEG 8000 (Sigma cat no. 89510-250G-F)
- TE buffer pH=8.0 (Ambion cat. no. AM9858)
- 5M NaCl
- 1M Tris-HCl pH=8.0
- 0.5M EDTA pH=8.0
- AMPure XP beads (Beckman Coulter cat no. A63881)
- 100 bp DNA ladder (New England Biolabs cat no. N3231L)

snmC-seq2 library preparation

- Blue Buffer (10x) (Enzymatics cat. no. P7010-HC-L)
- Klenow Exo- (50U/μL) (Enzymatics cat. no. P7010-HC-L)
- Deoxynucleotide Solution Mix (10mM each dNTP) (NEB cat. no. N0447L)
- Exonuclease I (20U/μL) (Enzymatics cat. no. X8010L)
- Shrimp Alkaline Phosphatase (rSAP) (1U/μL) (NEB cat. no. M0371L)
- Sera-Mag SPRI beads
- M-Elution Buffer (Zymo cat. no. 5007-6)

- EB buffer (Qiagen cat. no. 19086)
- Accel-NGS® Adaptase™ Module (Swift Bio cat. no. 330384)
- P5 Indexing Primer (IDT custom DNA oligo, standard desalted)
- P7 Indexing Primer (IDT custom DNA oligo, standard desalted)
- KAPA HiFi HS RM (Kapa cat. no. KK2602)
- Qubit dsDNA BR Assay Kit (Thermo Fisher cat. no. Q32850)

Reagent Setup

-

Ethanol, 80%

To 40mL of 200-proof Ethanol, add 10μL MB water in a 50mL tube. Keep solution sealed when not in use. Prepare ~6 tubes fresh before each library preparation.

Digestion Buffer Plates (for FACS sorting)

In a 50mL tube, combine 15mL M-Digestion Buffer (incubate for 10 minutes at 37°C to dissolve precipitate) with 14mL MB water. Resuspend one tube Proteinase K with 1mL Proteinase K Resuspension Buffer and add 1mL to the digestion buffer mix. Add 10μL λ DNA to aid determination of bisulfite conversion efficiency. Vortex to mix. Aliquot 2μL per well to 20 384-well plates to use for FACS sorting of stained nuclei (TECAN script). Plates can be stored at 4C.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script

-2uL_Digestion_Buffer.

Sera-Mag Solid Phase Reversible Immobilization (SPRI) Beads

Mix Sera-Mag SpeedBeads and transfer 1mL to a 1.5ml tube.

Place SpeedBeads on a magnetic stand until clears and carefully remove the supernatant. Wash the beads twice with 1ml of TE. For each wash, remove the tube from the magnet and mix by inversions.

Resuspend the washed beads in 1ml of TE.

Add 9g of PEG 8000 to a new 50ml sterile conical tube.

Add 10ml of 5M NaCl to the 50ml tube.

Add 500 μ l of 1M Tris-HCl pH=8.0 and 100 μ l of 0.5M EDTA pH=8.0 to the 50ml tube.

Mix until all dissolves into solution.

Add 1ml of resuspended SpeedBeads to the 50ml tube and fill the volume with MB water.

Test against AMPure XP beads using 100bp DNA ladder.

CT Conversion Reagent

Add 7.9 mL M-Solubilization Buffer and 3 mL M-Dilution Buffer to a bottle of CT Conversion Reagent.

Shake vigorously at room temperature to fully dissolve before adding 1.6 mL M-Reaction Buffer.

M-Wash Buffer

Add 288mL 200-proof Ethanol to four bottles of M-Wash buffer. Invert to mix. Make fresh each time.

Extra buffer can be stored at room temperature.

Random Primer solution

35 μ L random primer stock (100 μ M) in 7mL M-Elution buffer (500nM final primer concentration).

Aliquot to eight 96-well plates.

Random Priming Master Mix

To have sufficient reagents for robotic preparation of 3,072 reactions, prepare 3600 reactions of Random Priming Master Mix in a 15mL tube and vortex to mix. Aliquot 180 μ L of master mix into each well of one 96-well plate. Keep sealed on ice until use. Do not store.

Reagent	Vol. per reaction	Vol. for 3,600 reactions
Blue Buffer (10x)	1 μ L	3600 μ L

Klenow exo- (50U/μL)	0.25 μL	900 μL
dNTP (10mM each)	0.5 μL	1800 μL
MB water	3.25 μL	11700 μL

Exo/rSAP Master Mix

To have sufficient reagents for robotic preparation of 3,072 reactions, prepare 3600 reactions of Exo/SAP Master Mix in a 15mL tube and vortex to mix. Aliquot 53μL master mix to each well of one 96-well plate. Keep sealed on ice until use. Do not store.

Reagent	Vol. per reaction	Vol. for 3,600 reactions
Exonuclease 1 (20U/uL)	1 uL	3600 uL
rSAP (1U/uL)	0.5 uL	1800 uL

Adaptase Master Mix (using Accel-NGS® Adaptase™ Module)

To have sufficient reagents for robotic preparation of 3,072 reactions, prepare 3600 reactions of Adaptase Master Mix in a 15mL tube and vortex to mix. Aliquot 48 uL master mix to each well of on 96-well plate. Keep sealed on ice until use. Do not store.

Reagent	Vol. per reaction	Vol. for 3,600 reactions
EB	4.25 uL	1912.5 uL
Buffer G1	2 uL	900 uL
Reagent G2	2 uL	900 uL
Reagent G3	1.25 uL	562.5 uL
Enzyme G4	0.5 uL	225 uL
Enzyme G5	0.5 uL	225 uL

PCR Primer Mix

Sequences of indexing primers with unique dual barcodes are provided in Supplementary Table 2. Each PCR Primer Mix contains a P5L indexing primer (600 nM) and a P7L indexing primer (1 μ M).

Equipment

Equipment

- GenteMACS M-Tube (Miltenyi Biotec 130-096-335)
- 40 μ m nylon cell strainer (Corning 431750)
- Mortar and pestle (Fisher S337621)
- Formaldehyde (Fisher F79500)
-
- 384-Well Hardshell PCR Plate Clear, 20 Pcs/pk (Thermo Fisher cat. no. 4483285)
- 96-Well Hardshell PCR Plate GPLE, 20 Pcs/pk (Thermo Fisher cat. no. 4483348)
- Zymo-Spin 384 Well Plate, 2 pack (Zymo cat. no. C2012)
- 2.0mL 96-well Deep Well Polypropylene Plate, Sterilized (USA-SCI. cat. no. 1896-2110)
- Reservoir Single Well 96 Bottom High Profile, Clear (Axygen cat. no. RES-SW96-HP)
- Reservoir Single Well 96 Bottom Low Profile, Clear (Axygen cat. no. RES-SW96-LP)
- 15mL Centrifuge Tubes (Olympus cat. no. 28-103)
- 50mL Centrifuge Tubes (Olympus cat. no. 28-106)
- 1.5 μ L Eppendorf Tubes (Thermo Fisher cat. no. 02-681-320)
- 300 μ L 8-Strip Tubes
- Microamp Clear Adhesive Film, 100pc (Thermo Fisher cat. no. 4306311)
- Microporous Film, -20C to 80C, 50p, sterile (USA-SCI. cat. no. 2920-1010)
- Speedball Deluxe Soft Rubber Brayer, 4 inches (Statesville N.C.)
- 37°C Incubator
- 384-well and 96-well Compatible Thermocycler
- DynaMag™-96 Side Magnet (Thermo Fisher cat. no. 12331D)

- DynaMag™ -2 Magnet (Thermo Fisher cat. no. 12321D)
- Allegra 25R Centrifuge with S5700 2x96 Swinging Bucket Rotor (Beckman Coulter cat. no. 368954)
- All centrifuge steps at 5000xg for 5 minutes at room temperature unless otherwise specified
- (Optional) Axygen DITI 50µL STE.FIL. Robotics Tips (Thermo Fisher cat. no. EVF-50-RS)
- (Optional) Axygen DITI 180µL STE.FIL. Robotics Tips (Thermo Fisher cat. no. EVF-180-RS)
- (Optional) TECAN Freedom Evo 100 Base Unit with MultiChannel™ Arm MCA 96
- (Optional/Required for TECAN use) Collection Spacer/Adapter, Type 2, 26.5mm, Te-VacS (Tecan US Inc. cat. no. 10760662)

Procedure

Tissue Fixation

1. Place mortar and pestle, 15 mL conical tube, and a metal spatula in dry ice for 15 minutes. (Do this in a styrofoam box with lid on to prevent condensation).
2. Pulverize the brain sample using the mortar and pestle.
3. Scrape the powdered tissue using a metal spatula into a 15 mL conical tube and resuspend the tissue in 5 mL of PBS.
4. Add 276 µL of 37% formaldehyde and incubate at room temperature for 10 minutes with slow rotation.
5. Add 447 µL of 2.5 M glycine solution to quench the reaction. Incubate at room temperature for 5 minutes with slow rotation.
6. Centrifuge for 10 minutes at 1000 rpm at 4°C. Discard supernatant into an appropriate collection container.
7. Resuspend cells in 1mL of PBS and transfer the sample to 1.75 mL eppendorf tube. Spin for 5 minutes at 1000 rpm at 4°C. Discard supernatant and flash-freeze cell pellets in liquid nitrogen or dry ice.
8. Either proceed to the rest of the protocol or store cell pellets at -80°C.

Lysis and Restriction Digest

Lysis Buffer:

50 μ L 1M CaCl₂

30 μ l 1M MgAc

40 μ l 0.5M EDTA

10 μ l 0.5M EGTA

100 μ l 1M Tris-HCl pH 8

400 μ l 50X stock protease inhibitors (Roche)

10 μ l 1M DTT

10 μ l 0.1M/ml PMSF

9.35 mL Water

*Dissolve one tablet - Complete protease inhibitor in 1mL water to get 50X stock.

* Add 400 μ l of 10% Triton-X 100 to 8 mL of Lysis buffer to make Lysis buffer with 0.4% Triton-X.

9. Prior to starting the protocol, place a M-tube (Miltenyi Biotec 130-096-335) and a 50 mL conical tube in ice for 15 minutes. Prepare lysis buffer without and with 0.4% Triton-X 100 (Sigma 93443) and place it in ice. (For processing one crosslinked pellet, prepare 5 mL of each of Lysis buffer without and with 0.4% Triton-X 100).

10. Add 1 mL of ice-cold lysis buffer to one crosslinked pellet of cells and resuspend the cells. Transfer the sample to a M-Tube.

11. Add another 2 mL of the lysis buffer to the M-tube. Insert the M-tube to gentleMACS Dissociator and run the gentleMACS factory-set program: "Protein-M-tube-1.0".

12. Transfer the M-tube to ice. Pour the sample through a 40 μ m nylon cell strainer (Corning 431750) over the 50 mL conical tube kept in ice. Add 3 mL of Lysis buffer with 0.4% Triton-X to the M-Tube.

Close the M-tube with its cap and invert gently a couple of times to wash the tube and pour it over the

cell strainer.

13. Pool both the lysis buffer together (one without Triton-X and the other with 0.4% Triton-X) to make a 0.2% Triton-X solution.

14. Wash the cell strainer with 2 mL of Lysis buffer with 0.2% Triton-X. Discard the cell strainer.

15. Centrifuge the 50 mL conical tube at 1000 rpm for 10 minutes at 4 C. Discard the supernatant carefully. (Remove the supernatant carefully using vacuum. Do not invert the tube to discard the supernatant).

16. Resuspend the pellet in 1 mL of Lysis buffer with 0.2% Triton-X and transfer it to a 1.75 mL Eppendorf tube.

17. Centrifuge at 2500 G for 10 minutes at 4 C. Discard the supernatant.

18. Gently resuspend pellet in 50 μ l of 0.5% sodium dodecyl sulfate (SDS) and incubate at 62°C for 10 minutes, shaking.

19. After heating is over, add 145 μ l of H₂O and 25 μ l of 10% Triton X-100 to quench the SDS. Mix well, avoiding excessive foaming. Incubate at 37°C for 15 minutes, shaking.

20. Add 25 μ l of 10X NEB Buffer 4 (NEB B7004S). Then add 20 μ l (200U) of NlaIII restriction enzyme (NEB R0125) and digest chromatin ON at 37°C, shaking.

Proximity Ligation and Staining

21. Incubate at 62°C (shaking) for 20 minutes to inactivate NlaIII, then cool to room temperature.

22. Add 900 μ l of ligation master mix to sample

Ligation Buffer:

* 663 μ l H₂O

* 120 μ l 10X NEB T4 DNA ligase buffer (NEB B0202)

* 100 μ l 10% Triton X-100

* 12 μ l 10mg/mL Bovine Serum Albumin

* 5 μ l 400U/ μ l T4 DNA Ligase (NEB M0202)

23. Mix by inverting and incubate at room temperature for 4 hours with slow rotation.
24. Add 15 μL of 0.1 $\mu\text{g}/\mu\text{L}$ of Hoechst (Life Technologies H3570) for the last 30 minutes of the ligation step.
25. Spin the sample at 2500 G for 5 minutes and discard the supernatant. Resuspend the pellet in 600 μL of PBS. Add 6 μL of 0.1 $\mu\text{g}/\mu\text{L}$ of Hoechst to the sample.
26. Pass the sample through a 40 μm nylon cell strainer (Corning 431750) into another 1.75 mL eppendorf tube.
27. Centrifuge sample for 5 minutes at 2500g, aspirate supernatant carefully
28. (Optional) If using cellular markers for FACS: Resuspend pellet in 900 μL of DPBS + RNase inhibitors
29. (Optional) If using cellular markers for FACS: Add 100 μL of UltraPure BSA (50mg/mL)
30. (Optional) If using cellular markers for FACS: Incubate on ice for 20 minutes
31. (Optional) If using cellular markers for FACS: add appropriate antibodies. For example, to enrich for neural populations from human brain samples, we add 1 μL of AlexaFluor 488 conjugated anti-NeuN antibody, incubate on ice for 20 minutes.
32. (Optional) If using cellular markers for FACS: Centrifuge sample for 5 minutes at 2500g, aspirate supernatant carefully
33. Resuspend pellet in 1mL of DPBS + RNase inhibitors

Cell Sorting

34. Prepare 384 well plates for cell sorting as described in "Reagents Setup" of "Reagents" section.
35. Sort cells using FACS into 384 well plates. Nuclei are first gated based on Forward Scatter (FSC) and Side Scatter (SSC) pulse height, then multiplet exclusion gating was applied (forward scatter and side scatter pulse width). Finally, nuclei of specific DNA content were selected (e.g. 2N) by virtue of Hoechst fluorescence intensity.

36. After sorting, plates are incubated at 50°C for 20 minutes in a 384 well PCR block with heated lid for proteinase K digestion.

37. At this point, plates can be sorted at -20°C until ready for further processing.

Bisulfite Conversion

Timing ~ 5h

38. Add 15µL CT conversion reagent to each well of 384-well plate. Pipette up and down for 8 times to mix the sample.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script -

15uL_Conversion_Buffer

39. Seal the plate with adhesive film and quick spin for 10s at 2,000xg at room temperature. Place the plate in a thermocycler and run the following program:

98°C 8 min

64°C 3.5hrs

4°C Hold

40. Load 80µL M-Binding buffer to each well of 384-Well DNA Binding Plate.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script - 80uL_Binding Buffer

41. Transfer bisulfite conversion reactions to Zymo-Spin 384 Well DNA Binding Plate. Pipette up and down for 8 times to mix the sample. Place the 384-Well DNA Binding Plate on a 2.0mL 96-well Deep Well Plate and centrifuge for 5 min at 5,000g. Discard the flow through by decanting.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script -
17uL_Conversion_Rxn_To_Binding_Buffer.

42. Add 100µL M-Wash Buffer to each well of 384-Well DNA Binding Plate. Centrifuge for 5 min at 5,000g and discard the flow through by decanting.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script -
100uL_M_Wash_Buffer.

43. Add 50µL M-Desulphonation Buffer to each well of 384-Well DNA Binding Plate. Incubate at room temperature for 15 min. Centrifuge for 5 min at 5,000g and discard the flow through by decanting.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script -
50uL_Desulphonation_Buffer.

44. Add 100µL M-Wash Buffer to each well of 384-Well DNA Binding Plate. Centrifuge for 5 min at 5,000g and discard the flow through by decanting.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script -
100uL_M_Wash_Buffer.

45. Repeat step 7.

46. Place 384-Well DNA Binding Plate on new 384-well PCR plate. Add 7µL Random Primer Solution. Each quadrant of 384-well plate is barcoded with a distinct indexed random primer (Fig. 2). Every two 384-well plates receive a complete set of all eight indexed random primers. Incubate for 5 min at room temperature. Centrifuge for 5 min at 5,000g and discard the 384-Well DNA Binding Plate.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script - 7uL_Primer_Elution.

47. Seal the plate with adhesive film and store at -20°C for up to 1 week.

Random-primed DNA synthesis

Timing ~2h

48. Denature the samples by placing 384-well PCR plate on a thermocycler and run the following program.

95°C 3 min

Immediately place the plate on ice for 2 minutes.

49. Add 5µL Random Priming Master Mix to each well of the 384-well PCR plate. Vortex and quick spin for 10s at 2,000xg.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script - 5uL

Random_Priming_Mix.

50. Place the plate in a thermocycler and run the following program:

4°C 5 min

25°C 5 min

37°C 60 min

4°C Hold

Inactivation of free primers & dNTP

51. Add 1.5µL Exo/rSAP Master Mix to each well of the 384-well PCR plate. Vortex to mix the samples

and quick spin for 10s at 2,000xg.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script - 1uL_Exo_rSAP_MM.

52. Place the plate in a thermocycler and run the following program

37°C 30 min

4°C Hold

Sample clean-up

53. Add 73.6µL (0.8x) SPRI Beads to each well of four clean 96-well PCR plates.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script -

BP_Add_To_Clean_96well.

54. Transfer samples from two 384-well plates to each 96-well plate. The eight quadrants (from two 384-well plates) barcoded with distinct indexed random primers are combined. Mix the samples by vortexing and incubate for 5 minutes at room temperature, then quick spin for 10s at 2,000xg.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script -

BP_Transfer_Lib_384well_To_96well.

55. Place 96-well plates on DynaMag™-96 Side Magnet, let stand until solution in wells is clear of beads (~5 minutes). Remove supernatant and wash beads 3x with 150µL fresh 80% EtOH. Remove all EtOH, remove plates from magnet, and let beads dry at room temperature. DO NOT overdry beads.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script -

BP_2Plate_150uL_EtOH_Wash.

56. Add 10µL EB buffer and resuspend beads by pipet. Vortex and incubate for 5 minutes at room temperature, then quick spin for 10s at 2,000xg. Place back on magnet and let stand until solution is

clear of beads (~5 minutes).

57. Remove 10uL supernatant to a clean 96-well PCR plate.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script - 10uL_96Well_Elution.

Adaptase reaction

58. Denature the samples by placing 96-well plates on a thermocycler and run the following program.

95°C 3 min

Immediately place the plate on ice for 2 minutes.

59. Add 10.5µL Adaptase Master Mix to each well of the 96-well PCR plate. Vortex and quick spin for 10s at 2,000xg.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script - 10uL_25uL_Adaptase_KAPA. Enter 10.5uL for the volume transferred.

60. Place the plate in a thermocycler and run the following program:

37°C 30 min

95°C 2 min

4°C Hold

Library amplification

61. Add 5uL PCR Primer Mix.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script - 5uL_PCR_Primer.

62. Add 25µL 2x KAPA HiFi Mix. Vortex and quick spin for 10s at 2,000xg.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script -

10uL_25uL_Adaptase_KAPA. Enter 25uL for the volume transferred.

63. Place the plate in a thermocycler and run the following program:

a. 95°C 2 min

b. 98°C 30s

c. 98°C 15s

d. 64°C 30s

e. 72°C 2 min

Go to step c 14 times

f. 72°C 5 min

g. 4°C Hold

Library clean-up

64. Sample cleanup. Add 40µL (0.8x) SPRI Beads to each well of four 96-well PCR plates. Transfer contents of one 96-well plate to another to combine four 96-well plates to two 96-well plates. Vortex and incubate for 5 minutes at room temperature, then quick spin for 10s at 2,000xg.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script -

BP_Add_Beads_And_Combine_96well.

65. Place 96-well plates on DynaMag™-96 Side Magnet, let stand until solution in wells is clear of beads (~5 minutes). Remove supernatant and wash beads 2x with 150µL fresh 80% EtOH. Remove all EtOH, remove plate from magnet and let beads dry at room temperature. DO NOT overdry beads.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script -

BP_2Plate_150uL_EtOH_Wash (stop script after two wash steps).

66. Add 25µL EB and resuspend beads by pipet. Vortex and incubate for 5 minutes at room temperature, then quick spin for 10s at 2,000xg. Place back on magnet and let stand until solution is

clear of beads (~5 minutes). Remove 25uL supernatant to a clean 96-well PCR plate.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script - 20uL_96Well_Elution.

67. Add 20µL (0.8x) SPRI Beads to each well of two 96-well PCR plates. Transfer contents of one 96-well plate to another to combine two 96-well plates to one 96-well plate. Vortex and incubate for 5 minutes at room temperature, then quick spin for 10s at 2,000xg.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script - BP_Add_Beads_And_Combine_96well.

68. Place 96-well plate on DynaMag™-96 Side Magnet, let stand until solution in wells is clear of beads (~5 minutes). Remove supernatant and wash beads 2x with 150µL fresh 80% EtOH. Remove all EtOH, remove plate from magnet, and let beads dry at room temperature. DO NOT overdry beads.

(Optional) This step can be automated using TECAN Freedom Evo 100 with script - BP_1Plate_150uL_EtOH_Wash.

69. Add 20µL EB buffer and resuspend beads by pipet. Vortex and incubate for 5 minutes at room temperature, then quick spin for 10s at 2,000xg. Place back on magnet and let stand until solution is clear of beads (~5 minutes). Combine 20µL eluent from all wells in each column of the 96-well plate (8 wells per column, 12 columns) into 12 1.5µL Eppendorf tubes.

70. Add 128µL (0.8x) SPRI Beads to each 1.5µL Eppendorf tube. Pipette to mix and incubate for 5 minutes at room temperature.

71. Place 1.5µL tubes on DynaMag™-2 Magnet, let stand until solution in tubes is clear of beads (~5 minutes). Remove supernatant and wash beads 2x with 200uL fresh 80% EtOH. Remove all EtOH, remove tubes from magnet, and let beads dry at room temperature. DO NOT overdry beads.

72. Add 40µL EB and resuspend beads by pipet. Incubate for 5 minutes at room temperature. Place tubes back on magnet and let stand until solution is clear of beads (~5 minutes). Remove 40uL supernatant to 12 clean 1.5µL Eppendorf tubes.

73. Measure concentration of each 1.5µL Eppendorf tube with Qubit dsDNA BR Assay Kit. Normalize library concentrations and pool for sequencing.

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Figures

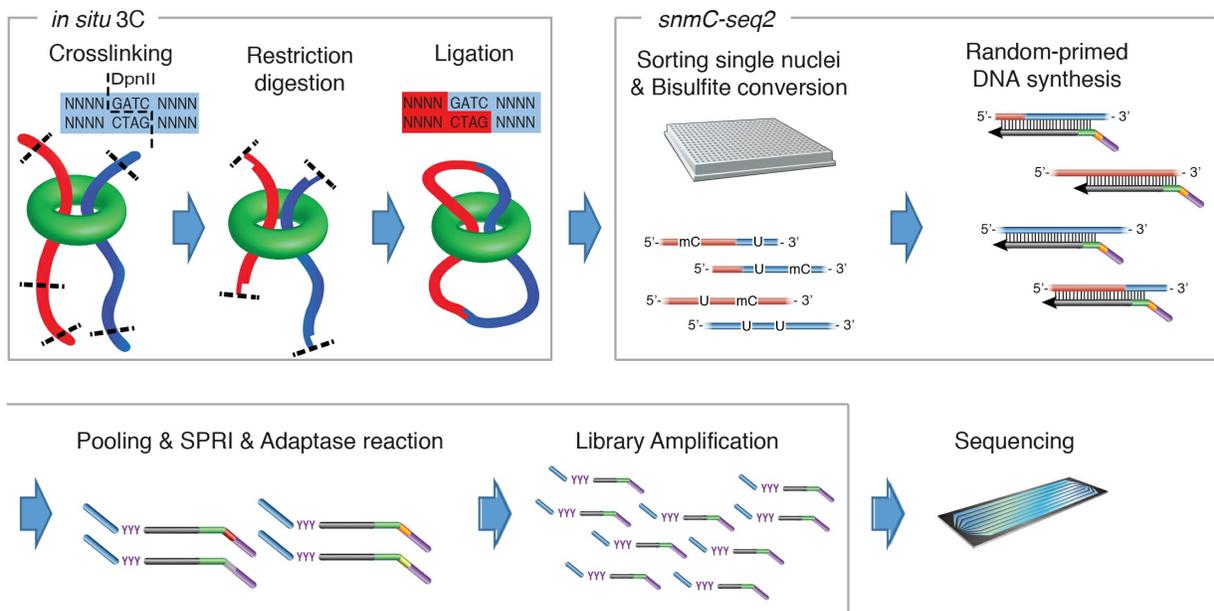
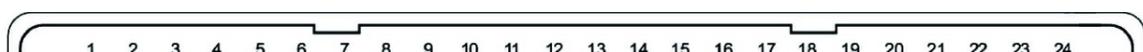


Figure 1

Overview of scm3C-seq method. Tissue samples (or cell lines) are first processed into a single cell suspension and fixed with formaldehyde. *in situ* Hi-C is then carried out, including digestion and ligation of chromatin fragments. Nuclei are sorted into 384 well plates. Nuclei are processed by proteinase K and unmethylated cytosines are converted to uracil using bisulfite. Single nuclei are barcoded using random primed DNA synthesis which incorporates a 5' sequencing adaptor. 384 well plates are pooled into 96 well plates where 3' adaptors are added using adaptase, and finally PCR amplified using barcoded primers. Finally Illumina sequencing is carried out after pooling, cleaning, and quantifying the library.



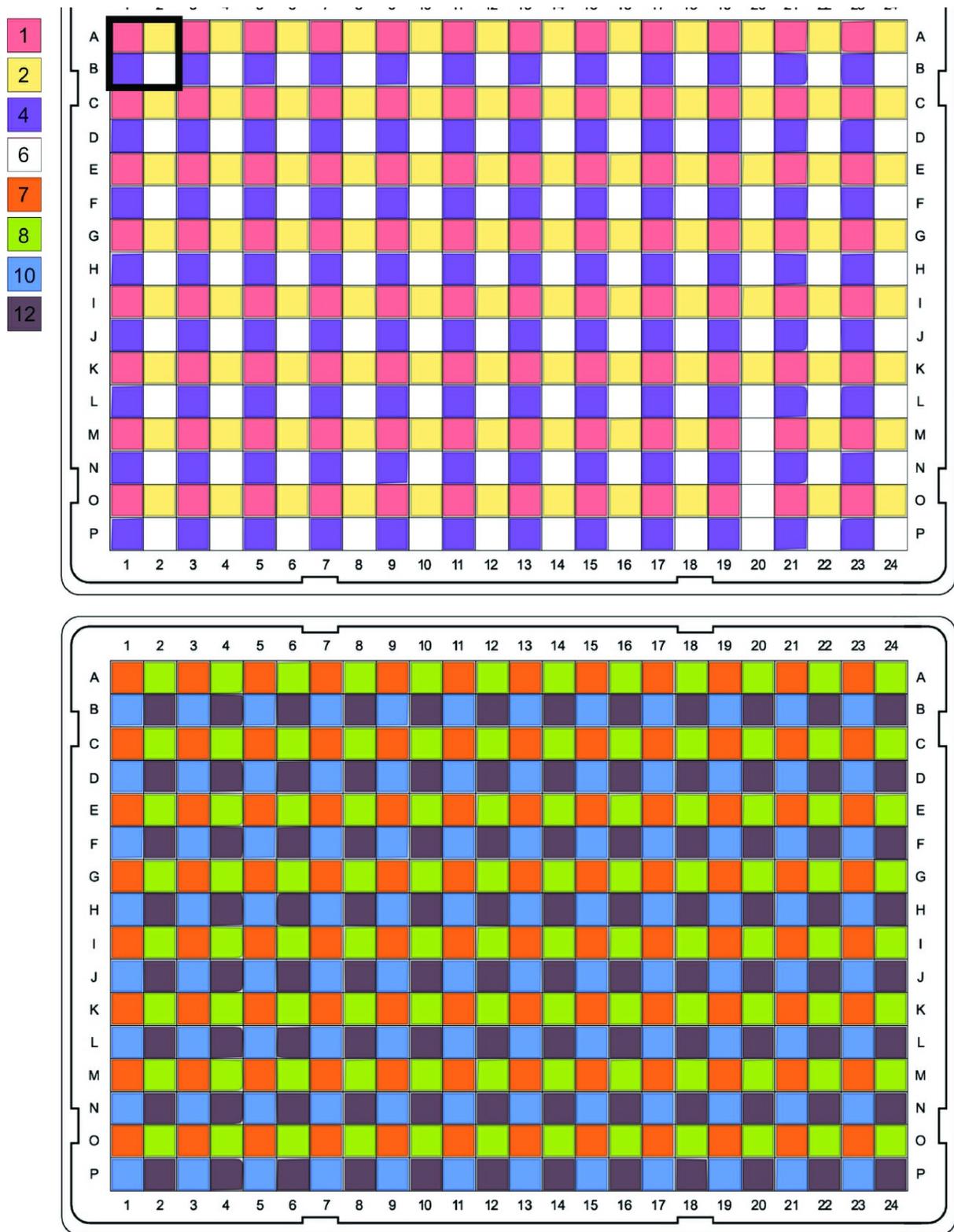


Figure 2

Schematics of single-cell barcoding with indexed random primers. Using a TECAN Freedom Evo 100 with MultiChannel Arm MCA 96, each indexed random primer is added into a quadrant of 384-well DNA Binding Plate. The color of each quadrant indicates the random

primer barcode.

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