**TITLE**

**Author list**

**ABSTRACT**

Epigenetic mechanisms regulate transcriptional output and play a vital role in cell fate decisions. Recent advances in multi-omics techniques have started to provide a view into how these processes are coordinated. In this work we continue these efforts and present single-cell ChIC&Meth (scCM-Seq), a novel method which simultaneously measures post-translational histone modifications and DNA methylation at the single-cell level. Combining bisulfite-free conversion and targeted MNase digestion, we are able to resolve the local correlations of different histone modifications and cytosine states at base-pair resolution. We apply scCM-Seq in a Fucci reporter line to track epigenetic changes and their interplay across the full cell cycle. Our combined measurements reveal that cells utilize different combinations of epigenetic control mechanisms to coordinate gene expression. In addition, our data provide the first direct evidence that kinetics of replication-coupled maintenance methylation are influenced by the local chromatin environment.

**Stuff to include**

* Supplementary table with mapping and deduplication rates
* MNase cutsite bias
* Spike-in conversion rate
* Correction of TAPS data
* Single-cell correlation for ChIC
* Single-cell correlation for methylation

**Stuff for text**

* DNMT1 impact of flanking sites
* DNMT1 de novo activity

**INTRODUCTION**

Eukaryotes package their DNA into chromatin to coordinate a number of crucial processes such as transcription, DNA repair and silencing of repetitive elements. Two of the most well-studied epigenetic marks involved in this regulation are post-translational histone modifications and the methylation of cytosine bases. Highlighting their importance, deregulation of epigenetic processes is commonly observed in a number of diseases, most notably cancer.

In order to guarantee stable phenotypes over time, intricate mechanisms are involved in the inheritance of histone marks and DNA methylation. During every replication of the genome, old histones are disassembled in front of the replication fork and re-integrated into the leading and lagging strands in roughly equal proportions. At the same time, DNMT1 travels with the replicative machinery and copies methylation patterns from the old onto the new strand. This maintenance methylation occurs in the context of symmetrical CpG dinucleotides. After DNA synthesis, methylated CpGs in the old strand are paired with unmodified CpGs in the nascent strand. DNMT1 recognized these hemimethylated sites, thereby providing a mechanistic basis for methylation inheritance.

There is sufficient evidence to suggest that the enzymatic properties of DNMT1 are reflected in genome-wide methylation patterns. For example, average DNA methylation is correlated with the flanking site preference of DNMT1 measured in vitro (Adam, 2021). However, there is little understanding of how its activity might be impacted by the local chromatin environment. DNMT1 itself is recruited by a number of different mechanisms, including the recognition of ubiquitin marks on histone H3, a mark placed by its partner UHRF1. Since methyltransferases cannot access nucleosome-bound DNA, it has been proposed that nucleosome insertion and DNMT1 accessibility might compete in newly synthesized regions of the genome (Petryk, 2020). In addition, modelling of kinetic rates of re-methylation after DNA replication has revealed large variability (Busto-Moner, 2020). This has prompted the suspicion that the local chromatin environment might impact the type and timing of maintenance methylation (Petryk, 2020). Further evidence in this direction comes from the discovery of partially methylated domains (PMDs). These large regions tend to become hypomethylated in cancer and aging and were found to co-localize with repressive, H3K9me3-marked chromatin (reference).

However, most evidence is correlative and there is no method to measure the epigenetic interplay in a time-resolved manner. Here we address this shortcoming and present [name], which allows read-out of histone modifications and DNA methylation from the same molecule at base-pair resolution in single cells. In addition to a thorough technical validation, we use our novel approach to measure the kinetics of DNA methylation in different chromatin contexts across the full cell cycle.

**RESULTS**

Our approach builds on single-cell chromatin cleavage (scChIC) and is outlined in Fig. 1A. Histone modification-specific antibodies are used to target MNase and the resulting fragments are ligated with barcoded adapters. After single-cell tagging, material is converted using Tet-assisted pyridine borane sequencing (TAPS) (Liu et al., 2018). This two-step process combines enzymatic oxidation and incubation with a chemical to convert 5mC to dihydroxyuracil (DHU). DHU is subsequently replaced by thymidine during amplification. Of note, due to the specific conversion of 5mC, TAPS is compatible with regular, i.e., unmethylated sequencing adapters. Illumina sequencing allows the extraction of multiple pieces of information for each read: (i) single-cell identity (barcode sequence), (ii) genomic location of histone modification (mapping position) and (iii) the methylation state of the original molecule (C to T transitions). Importantly, our data provide single-molecule in addition to single-cell resolution.

For technical validation, we produced scCM data for three different histone modifications in K562 cells (H3K9me3, H3K27me3 and H3K36me3). Mapping rates ranged from 90.5% to 98.8% (Supplementary Table x), about four times higher than single-cell bisulfite sequencing data (Clark et al., Nature Protocols 2017). We noted a strong bias for T or A as the first base (add data), consistent with previous reports on MNase cut site preference (**ref**). Cells were filtered based on the number of unique cut sites, TA fraction (MNase bias) and average methylation. Between 79% (H3K9me3) and 92% (H3K27me3) of cells passed our quality control criteria. TAPS conversion efficiency estimated based on fully methylated spike-ins ranged from 0.x and 0.y (add data).

We extracted histone and methylation profiles for the same single cells, Figure 1B shows the corresponding heatmaps for a 60 Mb region of chromosome 1. A more detailed zoom-in is provided in Figure S1. In addition to single-cell heatmaps, we aggregated signals into pseudo-bulk measurements which we compared to bulk data published by ENCODE. The number of MNase cuts in non-overlapping genomic bins of 100 kb were compared to normalized ChIP signal which revealed high concordance between the data sets. Genome-wide correlation (Pearsons *r*) ranged from 0.72 (H3K36me3) to 0.78 (H3K27me3), equivalent to stand-alone measurements obtained with single-cell CUT&RUN (Ku et al, 2019) and single-cell CUT&TAG (Kaya-Okur, 2019).

Methylation values obtained by TAPS were compared to whole-genome bisulfite sequencing WGBS data. For each histone mark, we assessed CpGs with at least 5x coverage in both data sets (Buys: filtering parameters?). Pseudobulk correlations were in the range of 0.98 to 0.99 (Figure S1), comparable to technical replicates of WGBS.

**Here we should add: UMI-based and spike-in based correction + a (supplementary) figure.** *Of note, the major artefact of TAPS is the underconversion of methylated bases. We used spike-ins to perform sequence context-aware correction. While genome-wide correlation remained similar, absolute differences were greatly diminished.*

To further validate our approach, we assessed nucleosome spacing patterns resulting from MNase digestion. Figure 1C shows the relationship between cut site spacing and genomic distance as a single-cell heatmap. Figure 1 D aggregates these data per histone mark, which reveals oscillatory patterns relating to nucleosome occupancy. While the phase (around 190 bp) is similar between marks and to previously published data in K562 cells (Pott, eLife, 2017), spacing and signal decay show subtle differences. Last, we investigated methylation values for the different data sets. Here, average methylation within H3K37me3 and H3K9me3 fragments (8 to 10%) is much lower than compared to H3K36me3 (50%). Again, these findings are in line with previously published reports on the high methylation of gene bodies (ref). Taken together, our data for K562 cells are well correlated with bulk reference data and thus accurately represent the underlying histone modification and DNA methylation landscapes. Of note, the quality of our measurements is comparable to single-cell single-omics techniques.

Next, we sought to apply scCM-Seq in a setting with dynamic epigenetic regulation. To this end, we chose to profile histone modifications and DNA methylation during cell cycle progression. In addition to the regulation of many genes, methylation patterns are maintained during S Phase. We therefore reasoned that our combined measurements might not only provide insights into epigenetic regulation of gene expression, but also the interplay of maintenance methylation and chromatin organization. To obtain high resolution data, we resorted to the RPE-FUCCI reporter system. This cell line expresses an orange fluorophore during G1 phase and a green fluorophore during G2 phase with a short window of concomitant expression of both markers in early S phase (Sakaue-Sawano, Cell, 2008). Of note, fluorophores are retained in the nucleus which allowed the recording of FANS parameters during sorting. Figure 2A shows cells which were profiled with scCM-Seq in FACS space.

**DISCUSSION**

**MATERIALS AND METHODS**

**Cell Culture**

K562 cells (ATCC® CCL-243TM) were cultured in RPMI 1640 GlutaMAXTM medium (Gibco, cat. no. 61870036), supplemented with 5% fetal bovine serum (Gibco, cat. no. A3382001), non-essential amino acids (Gibco, cat. no. 11140050) and Pen/Strep (Gibco, cat. no. 15140122). hTERT-RPE1-FUCCI cells were grown in adherent culture with DMEM/F12 GlutaMAXTM medium (Gibco, cat. no. 10565018) supplemented with Pen/Strep and 10% fetal bovine serum. TrypLETM Express Enzyme (Gibco, cat. no. 12605010) and PBS were used for passaging of RPE1-Fucci cells.

**Single-cell histone profiling**

*Buffer composition*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Component** | **Manufacturer** | **Cat. No.** | **WB 1** | **WB 2** | **WB 3** |
| **H2O** | Invitrogen | AM9932 |  |  |  |
| **HEPES** | Gibco | 15630080 | 20 mM | 20 mM | 20 mM |
| **NaCl** | Invitrogen | AM9760G | 150 mM | 150 mM | 150 mM |
| **Spermidine** | Sigma | S2626-5G | 0.5 mM | 0.5 mM | 0.5 mM |
| **Tween-20** | Sigma | P1379-100ML | 0.05% | 0.05% | 0.05% |
| **Protease inhibitor** | Roche | 5056489001 | 1 tablet  (per 50 ml) | 1 tablet  (per 50 ml) |  |
| **EDTA** | Invitrogen | 15575020 | 2 mM |  |  |

\* all values correspond to final concentrations

*Fixation, cell permeabilization and long-term storage*

All steps were performed in Protein LoBind tubes (Eppendorf, cat. no. 0030108094 and 0030122216). Cells were harvested in 15 ml tubes and washed twice with PBS. Fixation was performed by resuspending the pellet in 300 µl PBS per 106 cells. Then, 700 µl ice-cold absolute ethanol (Manufacturer, cat. no.) per 106 cells was added while vortexing gently. Cells were fixed at -20°C for two hours, washed twice with WB 1 and transferred to 0.5 ml tubes. Cells were stored at -80°C in WB 1 supplemented with 10% DMSO.

*Antibody binding, Pa-MNase incubation and FANS*

Protein A-MNase fusion protein (Pa-MNase) was expressed in bacterial culture and purified as outlined in Zeller et al [REF]. Cells were thawed, washed twice with WB 1 and resuspended in WB 1. Histone modification-specific antibodies were added to the reaction (see below for details). Incubation was performed overnight at 4°C with gentle agitation, effectively stripping the cell membrane and releasing nuclei through the presence of Tween-20. Nuclei were washed once and resuspended in 500 µl of WB 2. Pa-MNase (3 ng/ml final) and Hoechst 34580 (5 µg/ml final), were added to each sample, followed by incubation for 60 min at 4°C with gentle agitation. Cells were washed twice with WB 2, resuspended in 500 µl of WB 3 and filtered through a 70 µm strainer (Corning, cat. no. 431751) and transferred to FACS tubes (Manufacturer, cat. no.).

|  |  |  |  |
| --- | --- | --- | --- |
| **Antibody** | **Manufacturer** | **Catalogue no.** | **Concentration** |
| **anti-H3K4Me1** | Abcam | ab8895 | 1:400 |
| **anti-H3K9Me3** | Abcam | ab8898 | LOOK UP |
| **anti-H3K27Me3** | NEB | 9733S | 1:200 |
| **anti-H3K36Me3** | LOOK UP | LOOK UP | 1:2000 |

*Fluorescence-assisted nuclei sorting (FANS)*

Ahead of sorting, 384-well hard-shell plates (Manufacturer, cat. no.) were prepared for sorting by adding 10 µl of sterile filtered mineral oil (Sigma Aldrich, cat. no. 69794-500ML) per well using a Tecan Freedom EVO® liquid handler. Nuclei in WB 3 were sorted into 384-well plates on a BD InfluxTM cell sorter. Hoechst signal was used to select for K562 cell in G1 phase. Four gates were used for RPE1-Fucci cells to sample evenly from early G1, late G1, S and G2 phase, respectively. Four to eight wells were left empty as controls in all plates. After sorting, cells were spun down for one minute at 2,000 g.

*Processing of single-cell plates*

All pipetting steps outlined below were performed using an Innovadyne Nanodrop II robotic liquid handler. After each dispension step, plates were sealed with aluminium sealers (Manufacturer, cat. no.) and spun down for one minute at 2,000 g to fuse droplets.

*Pa-MNase activation and Proteinase K digest*

MNase digestion was initiated by adding 100 nl of WB 3 supplemented with 2 nM CaCl2 to each well. Plates were incubated for 30 min at 4°C. Digestion was stopped by dispensing 100 nl of the following solution (final concentrations): nuclease-free water; 40 mM EGTA (Thermo Fisher, cat. no. 15425795); 1.5% NP-40 (Manufacturer, cat. no.) and 2 mg/ml Proteinase K (Invitrogen, AM2548). Plates were incubated in PCR machines: 20 min at 4°C; 6 hours at 65°C; 2 min at 80°C; hold at 4°C. Plates were kept at -80°C until further processing.

*Blunting*

100 nl of the following mix was added to each well (volumes per well): 2 nl Klenow, large fragment (NEB, cat. no. M0210L); 2 nl T4 PNK (NEB, cat. no. M0201L); 5 nl dNTP solution (Promega, cat. no. U1515); 30 nl ATP 10 mM (NEB, cat. no. P0756S); 30 nl PNK Buffer 10x (NEB, cat. no. M0201L); 10 nl MgCl2 25 mM (Thermo Fisher, cat. no. 4398828); 5 nl PEG8000 50% (Promega, cat. no. V3011); 1.5 nl BSA 20 mg/ml (NEB, cat. no. B9000S); 14.5 nl nuclease-free H2O. Incubation: 30 min at 37°C; 20 min at 75°C; hold at 4°C.

*A-tailing*

200 nl of the following mix was added to each well (volumes per well): 1 nl AmpliTaq 360 DNA Polymerase (Applied Biosystems, cat. no. 4398818); 2 nl T4 PNK (NEB, cat. no. M0201L); 1 nl dATP (Promega, U1205); 10 nl DTT 0.1 M (part of Invitrogen cat. no. 18064022); 14 nl Tris 1 M pH 8.0 (Invitrogen, cat. no. 15568025); 20 nl ATP 10 mM (NEB, cat. no. P0756S); 25 nl KCl 2M (Invitrogen, cat. no. AM9640G); 1 nl MgCl2 1M (Invitrogen, cat. no. AM9530G); 10 nl PEG8000 50% (Promega, cat. no. V3011); 1 nl BSA 20 mg/ml (NEB, cat. no. B9000S); 115 nl nuclease-free H2O. Incubation: 15 min at 37°C; 10 min at 72°C; hold at 4°C.

*Dispension of barcoded adapters*

Per well, 50 nl of 5 µM barcoded adapter was added using a Mosquito HTS Nanolitre Liquid handler (ttplabtech). Adapters were manufactured by IDT, see below for an example sequence. A list of all adapters is provided in Supplementary Table Sx. Adapters contain the following features: forked sequence to prevent adapter-adapter ligations (underlined, dotted), T7 promoter (underlined, solid), RA5 Illumina primer binding site (italic), 3 random nucleotides as UMI, an 8 bp cell-specific barcode (bold) and a single-base T overhang.

Top Strand:

5’-GGTGATGCCGGTAATACGACTCACTATAG*GGAGTTCTACAGTCCGACGAT*CNNN**ACACACTA**T

Bottom Strand:

5’-p**TAGTGTGT**NNN*GATCGTCGGACTGTAGAACTCCC*TATAGTGAGTCGTATTACCGGC*GAGCTT*

*Adapter Ligation*

150 nl of the following mix was added to each well (volumes per well): 25 nl T4 Ligase 400,000 U/ml (NEB, cat. no. M0202L); 3 nl MgCl2 1M (Invitrogen, cat. no. AM9530G); 45 nl DTT 0.1 M (part of Invitrogen cat. no. 18064022); 20 nl ATP 10 mM (NEB, cat. no. P0756S); 5 nl PEG8000 50% (Promega, cat. no. V3011); 1 nl BSA 20 mg/ml (NEB, cat. no. B9000S); 51 nl nuclease-free H2O. Incubation: 20 min at 4°C; 16 hours at 16°C; 10 at 65°C; hold at 4°C.

*Pooling of plates*

Plates were inverted and placed in pooling plates (Clickbio VBLOK200) pre-coated with 3 ml of sterile filtered mineral oil. Plates were spun for two minutes at 500 g and the liquid phase transferred to fresh 1.5 ml Eppendorf tubes. Carry-over mineral oil was removed with the following washing procedure: 500 µl of n-Butanol (Manufacturer, cat. no.) were added, tubes inverted multiple times and spun down for one minute at 5,000 g. The butanol phase containing mineral oil was taken off with a P1000 pipette. This procedure was repeated for a total of three times. Then, 500 µl of ether (Manufacturer, cat. no.) were added. Tubes were vortexed and spun down using a table-top centrifuge. After removal of ether with a P1000 pipette, tubes were left open briefly at room temperature to allow evaporation of left-over ether. Next, DNA was purified by incubating for 10 minutes with 0.8x volumes of Ampure XP beads (Beckman Coulter, cat. no. A63881) pre-diluted 1:4 in bead binding buffer (1 M NaCl, 20% PEG8000, 20 mM Tris pH 8.0, 1 mM EDTA). Beads were pelleted and washed twice with 80% ethanol (Manufacturer, cat. no.). Beads were air-dried and resuspended in 19 µl of nuclease-free H2O. The supernatant was transferred to a fresh 0.5 ml Eppendorf tube. Material was stored at -20°C until further processing.

**Methylation profiling**

*Preparation of spike-ins*

To produce fully methylated lambda phage DNA, the following reaction was assembled in 0.5 ml DNA lo-bind Eppendorf tubes: 1 µg of unmethylated lambda phage DNA (Promega, cat. no. D1521); 5 µl NEB Buffer 2 10x (NEB, cat. no. M0226S); 1 µl SAM 32 mM (NEB, cat. no. M0226S); 2 µl M.SssI 4,000 U/ml (NEB, cat. no. M0226S); topped up to 50 µl with nuclease-free H2O. Incubation: 2 hours at 37°C. After 2 hours an additional 1 µl of SAM and 0.5 µl of M.SssI were added followed by further incubation for 2 hours at 37°C. DNA was cleaned with 1x volume of Ampure XP beads. The above reaction, including the top-up of enzyme and SAM, was repeated with the purified material as input, followed by a final 1x volume Ampure XP bead cleanup and elution in 20 µl of nuclease-free H2O. Next, methylated DNA was subjected to NlaIII restriction with the following reaction: 1 µl NlaIII 10,000 U/ml (NEB, cat. no. R0125S); 5 µl CutSmart Buffer 10x (NEB, cat. no. R0125S); 24 µl of nuclease-free H2O. Incubation: 2 hours at 37°C; 20 min at 65°C; hold at 4°C. Material was cleaned up with 1x volumes of Ampure XP beads and the concentration was measured with a Qubit 3 Fluorometer (Invitrogen). Pre-annealed adapter (see below for sequence) was added to the sample in a ratio of 10:1 (based on the measured concentration and assuming full digestion to 180 bp fragments). Next, ligation was performed by addition of the following: 2.5 µl T4 DNA ligase 400,000 U/ml (NEB, cat. no. M0202L); 5 µl T4 DNA ligase buffer 10x (NEB, cat. no. M0202L); volume topped up to 50 µl with nuclease-free H2O. Ligation was performed for 20 minutes at room temperature followed by heat inactivation for 10 min at 65°C. Material was cleaned up twice with 0.8x volumes of Ampure XP beads. Fully methylated and adapter-ligated spike-ins were diluted to a concentration of 7 pg/µl.

NlaIII adapter top Strand:

5’-GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNACACACTACATG

NlaIII adapter bottom Strand:

5’-pTAGTGTGTNNNGATCGTCGGACTGTAGAACTCCCTATAGTGAGTCGTATTACCGGC

*TET1 enzyme production*

Catalytic domain of mouse Ten-eleven translocation methylcytosine dioxygenase 1 (mTET1CD) was expressed as outlined by Liu et al. (REF). Briefly, FLAG-tagged protein was expressed in Expi293F cells (Gibco, cat. no. 13479756). After lysis, protein is bound with Anti-Flag M2 Affinity Gel (Sigma, cat. no. A2220) and purified on gravity chromatography columns (Bio-Rad, cat. no. 7321010) according to the manufacturer’s specifications. Protein is concentrated on Amicon® Ultra-4 Centrifugal Filter units (Merck, cat. no. UFC803024) followed by buffer exchange with Bio-Spin® P-30 Gel Columns (Bio-Rad, cat. no. 7326231). Protein was stored at -80°C in 20 mM HEPES pH 8.0, 150 mM NaCl, 1 mM DTT and 30% Glycerol (Manufacturer, cat. no.)

*TAPS conversion and clean-up*

Reaction buffer for TAPS consists of (final concentrations): 167 mM HEPES (Gibco, cat. no. 15630080); 333 mM NaCl (Invitrogen, cat. no. AM9760G); 3.3 mM alpha-Ketoglutarate (Sigma-Aldrich, cat. no. K3752-5G); 6.67 mM L-ascorbic acid (Sigma-Aldrich, cat. no. 95210-50G); 4 mM ATP (part of Thermo Fisher Scientific, R0441); 8.33 mM DTT (part of Invitrogen cat. no. 18064022). The following reaction was assembled on ice: 19 µl of pooled material, 1 µl of methylated lambda spike-in, 15 µl of TAPS reaction buffer, 3.33 µl of 1.5 mM Fe2+ solution, 12 µl of mTET1CD. Samples were incubated for 80 min at 37°C. Then, 1 µl of Proteinase K 20 mg/ml was added per reaction, followed incubation for 15 min at 55°C. Next, samples were cleaned up with 2x volumes of Ampure XP DNA beads and eluted in 19.67 µl of nuclease-free H2O. The above reaction and Proteinase K digest were repeated once followed by a clean-up with 2x volumes of Ampure XP DNA beads and elution in 33.75 µl. Sample was transferred to fresh 1.5 ml Eppendorf tubes. Then, 10 µl of NaAc 3M pH 4.3 (produced in-house) and 6.25 µl of pyridine borane solution 10 M (Sigma Aldrich, cat. no. 179752-5G) were added to the reaction mix. Samples were incubated for 16 hours at 37°C in a thermal shaker set to 850 rpm.

After pyridine borane incubation, reactions were cleaned up with oligo clean & concentrator columns (Zymo, cat. no. D4060) according to the manufacturer’s protocol with the following adaptations: samples were topped up to 200 µl with nuclease-free H2O and 400 µl of oligo-binding buffer and 800 µl of ethanol were used per column. Samples were eluted twice with pre-warmed (60°C), nuclease-free H2O. Then, volumes were reduced to 9.6 µl in a SpeedVac chamber. Cleaned-up samples were kept at -20°C until library preparation.

**Sequencing library preparation**

*In-vitro transcription (IVT)*

TAPS-converted and cleaned up samples were subjected to in-vitro transcription (IVT) by adding 14.4 µl of IVT reaction mix (2.4 µl UTP, 2.4 µl TTP, 2.4 µl GTP, 2.4 µl ATP, 2.4 µl Buffer 10x, 2.4 µl Enzyme; all part of MEGAscriptTM T7 Transcription Kit, Invitrogen, cat. no. AMB13345) followed by incubation for 14 hours at 37°C (with lid temperature set to 70°C). Next, 6 µl of H2O and 3 µl of Turbo DNAse (part of MEGAscriptTM T7 Transcription Kit) were added and samples incubated for 15 min at 37°C to digest template DNA. Amplified RNA (aRNA) was fragmented by adding 7.88 µl of fragmentation buffer (200 mM Tris-Acetate, pH 8.1; 500 mM KaOAc; 150 mM MgOAc) followed by incubation for 90 s at 94°C. Samples were immediately chilled on ice and 4.13 µl of 0.5 M EDTA pH 8.0 (Invitrogen, cat. no. 15575020) was added to capture Mg2+. Then, aRNA was cleaned with 0.8x volumes of RNAClean XP beads (Beckman Coulter, cat. no. A63987) and eluted in 6 µl of nuclease-free H2O. In order to assess RNA yield and quality, 1 µl of aRNA was run on a Bioanalyzer (Agilent RNA 6000 Pico Kit, cat. no. 5067-1513).

Reverse transcription and library amplification

After quality control, 5 µl of aRNA were combined with 0.5 µl of 10 mM dNTP solution (Promega, cat. no. U1515) and 1 µl of random hexamer RT primer 20 µM (sequence: GCCTTGGCACCCGAGAATTCCANNNNNN, IDT). Samples were heated to 65°C for 5 minutes and then immediately chilled on ice. 6.5 µl of primed sample were combined with 2 µl First Strand Buffer 5x, 1 µl DTT 0.1 M, 0.5 µl of SuperScriptII 200 U/µl (all part of Invitrogen cat. no. 18064022) and 0.5 µl of RNAseOUT (Invitrogen, cat. no. 10777019). Incubation: 10 min at 25°C; 60 min at 42°C; hold at 4°C. Then, 2 µl of barcoded RPIx primer (see below for example, Supplementary Table Sx for all sequences) was added to each sample. Library PCR is performed by adding 11 µl nuclease-free H2O, 25 µl of NEBNext Ultra II Q5 Master Mix 2x (NEB, cat. no. M0492L) and 2 µl of 10 µM RP1 primer (see below for sequence). Samples are amplified with 10 to 13 cycles of PCR, dependent on histone modification and aRNA yield. PCR settings: 30 s at 98°C; 10 to 13 x [10 s at 98°C, 30 s at 60°C, 30 s at 72°C]; 10 min at 72°C; hold at 4C. Amplified DNA was cleaned with two subsequent 0.8x AMPure XP bead cleanups and eluted in 15 µl of nuclease-free H2O. Concentration and size distribution were measured on a Qubit 3 Fluorometer and Bioanalyzer (Agilent High Sensitivity DNA kit, cat. no. 5067-4626), respectively. Samples were pooled and sequenced on the Illumina NextSeq2000 platform.

Barcoded RPIx primer (IDT):

5’-CAAGCAGAAGACGGCATACGAGAT-[6bp]-GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA

RP1 library PCR primer (IDT):

5’- AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA

**Data processing**

**Acknowledgements**

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* **USEQ**

**Text Main Figures**

**Fig.1 ChIC-TAPS enables multiplexed profiling of histone modifications and DNA methylation in single cells**

**A**, Schematic of single-cell ChiC-TAPS. MNase is targeted to histone modifications via specific antibodies. Single nuclei are sorted into 384-well plates and subsequent steps performed using a robotic liquid handler. First, MNase digestion is initiated through addition of Ca2+. Then, Proteinase K digestion, blunting, A-tailing and adapter ligation create barcoded fragments. Material from one plate is pooled, followed by conversion of methylated cytosines to dihydroxyuracil (DHU). After sequencing library amplification, DHU is replaced by thymidine (T). **B**, Heatmaps showing data for histone modifications (left) and DNA methylation (right) obtained from the same single cells across a 60 Mb region on chromosome 1. Colored traces above heatmaps correspond to the averaged signal across cells and are accompanied by reference profiles (ENCODE ChIP-Seq and WGBS, respectively). Tick marks under the heatmaps indicate locations of genes. **C**, Heatmap visualizing nucleosome spacing in single cells. Pairwise distances between cut sites (mapping position of Read 1) are calculated per single cell and z-score normalized. The striped vertical pattern corresponds to cuts located roughly one nucleosome distance away. **D**, Similar to C, this figure shows the probability of detecting another MNase cut site given a specific genomic distance aggregated across cells within the same histone modification. Oscillations show periodicities of approximately 180 to 190 bp with slight differences in peak location and signal decay between histone modifications. **E**, Similar to D but taking advantage of the multiplexed nature of [insert name], methylation levels (beta, fraction methylated) are plotted with respect to the position cut site.

**Text Supplemental Figures**

**References**