

Mapping *In vivo* Nascent Chromatin with EdU and Sequencing

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Summary

The epigenome has been mapped in different cell types to understand the relationship between the chromatin landscape and the control of gene expression. However, most mapping studies profile a large population of cells in various stages of the cell cycle, which results in an average snapshot of the chromatin landscape. Chromatin itself is highly dynamic, undergoing rapid changes during active processes like replication, transcription, repair, and remodeling. Hence, we need methods to map chromatin as a function of time. To address this problem in the context of replication, we developed the method MINCE-seq (Mapping In vivo Nascent Chromatin using EdU and sequencing). MINCE-seq is a genome-wide method that uses the passage of replication fork as a starting point to map the chromatin landscape as a function of time. MINCE-seq can measure chromatin dynamics in the time scale of minutes and at the resolution of individual nucleosome positions and transcription factor binding sites genome-wide.

Key Words

Replication, chromatin dynamics, transcription factor, genomics

Introduction

Chromatin states are defined by the position and identity of proteins on the genome, including transcription factors (TFs) and nucleosomes. Chromatin states reflect genome function as evidenced by recent epigenome mapping efforts in many cell types using short-read sequencing(1). Epigenome mapping is usually performed on a population of cells that are at various states of activity. Hence, the chromatin states mapped are ensemble averages. However, chromatin is disrupted and reassembled constantly and on time-scales of seconds to minutes due to active processes such as remodeling, replication, repair, and transcription. Every protein-DNA contact in the genome is broken at least once during the cell cycle when the replicative helicase unwinds double-stranded DNA to supply single-stranded DNA to the DNA polymerase for replication. Thus, replication represents a starting point for establishment of TF and nucleosome landscapes genome-wide on newly replicated naked DNA. Furthermore, because the pre-existing chromatin landscape is disrupted during replication, replication represents a challenge to the cell to maintain specific chromatin landscapes through successive cell divisions. To understand how distinctive chromatin landscapes are maintained through replication, we need to be able to map the nascent chromatin landscape established right after replication and then track its maturation over time.

Epigenome mapping has been performed using a variety of DNA enzymes including methyl transferases(2), transposases(3, 4), and nucleases(5, 6). One enzyme that has been used for decades for mapping nucleosomes, and more recently for TFs, is Micrococcal Nuclease (MNase), an endo-exo nuclease from *Staphylococcus aureus*. MNase, when added to intact nuclei in the presence of Ca^{2+} , generates minimal

protections of protein-DNA complexes. Subjecting DNA fragments obtained after MNase treatment of nuclei to paired-end short-read sequencing enables determination of both the length of the protected fragment and its position in the genome. Because TFs usually protect short DNA fragments (<50 bp) and nucleosomes protect around 150 bp, computationally filtering the sequencing data based on fragment lengths yields genome-wide maps of both TFs and nucleosomes from a single experiment. These maps show distinct patterns of nucleosomes and TFs at promoters, enhancers, and replication origins (Figure 1).

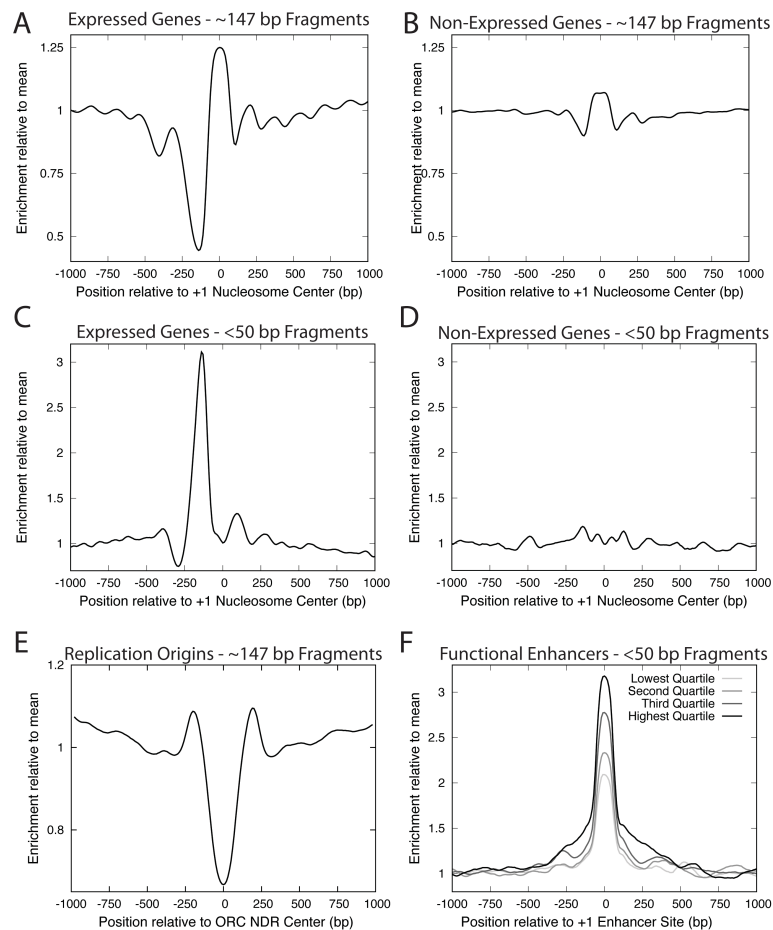


Figure 1. Chromatin landscapes reflect genome function. **A)** Average profile of 147 ± 5 bp fragments from MNase-seq data from *Drosophila* S2 cells (combined data from GEO accessions GSM1974516 and GSM1974518) over promoters of active genes show an array of nucleosomes upstream and downstream of the promoters, and

nucleosome depletion at the promoters. **B)** Average profile of 147 ± 5 bp fragments from MNase-seq data over promoters of inactive genes shows no nucleosome depletion at promoters and no nucleosome arrays upstream or downstream of the promoters. **C)** Average profile of 25-50 bp fragments from MNase-seq data over promoters of active genes show a peak with high enrichment coinciding with the nucleosome depleted region. **D)** Average profile of 25-50 bp fragments from MNase-seq data over promoters of inactive genes shows no peak at the promoter. **E)** Average profile of 147 ± 5 bp fragments from MNase-seq data over origin recognition complex (ORC) binding sites shows nucleosome depletion. ORC binding sites correspond to origins of replication. **F)** Average profile of 25-50 bp fragments from MNase-seq data over functional enhancers (as defined by STARR-seq) shows a peak whose enrichment correlates with quartiles of enhancer strength; the highest quartile of enhancer strength has the peak with highest enrichment at the enhancer site followed by second, third, and then the lowest quartiles.

To map the newly replicated chromatin landscape, we developed the method “Mapping *In vivo* Nascent Chromatin with EdU and sequencing” (MINCE-seq)(7). In MINCE-seq, we combine high-resolution mapping of the chromatin landscape using MNase with metabolic labeling with Ethynyl-deoxyUridine (EdU)(8). EdU has an alkyne group that can be covalently attached to an azide group *ex vivo* using the bio-orthogonal click chemistry method. EdU is incorporated by active replication forks in place of thymidine, enabling high-stringency purification of newly-replicated DNA genome-wide from asynchronous cultures. EdU and MNase can be used in most metazoan cell types, making MINCE-seq highly transferrable to different experimental systems. EdU labeling can be followed by a thymidine chase for various time intervals, thus enabling us to track chromatin landscape as a function of time after replication genome-wide.

Applying MINCE-seq to *Drosophila* S2 and BG3 cell lines, we observed nucleosomes to replace TFs at active promoters and enhancers genome-wide due to the action of replication-coupled chromatin assembly machinery. This finding implies that the steady state chromatin landscape at active regions is established by a

competition between transcription factors and nucleosomes. We present here the detailed experimental methodology of MINCE-seq (schematic in Figure 2) that should be applicable to any desired cell type.

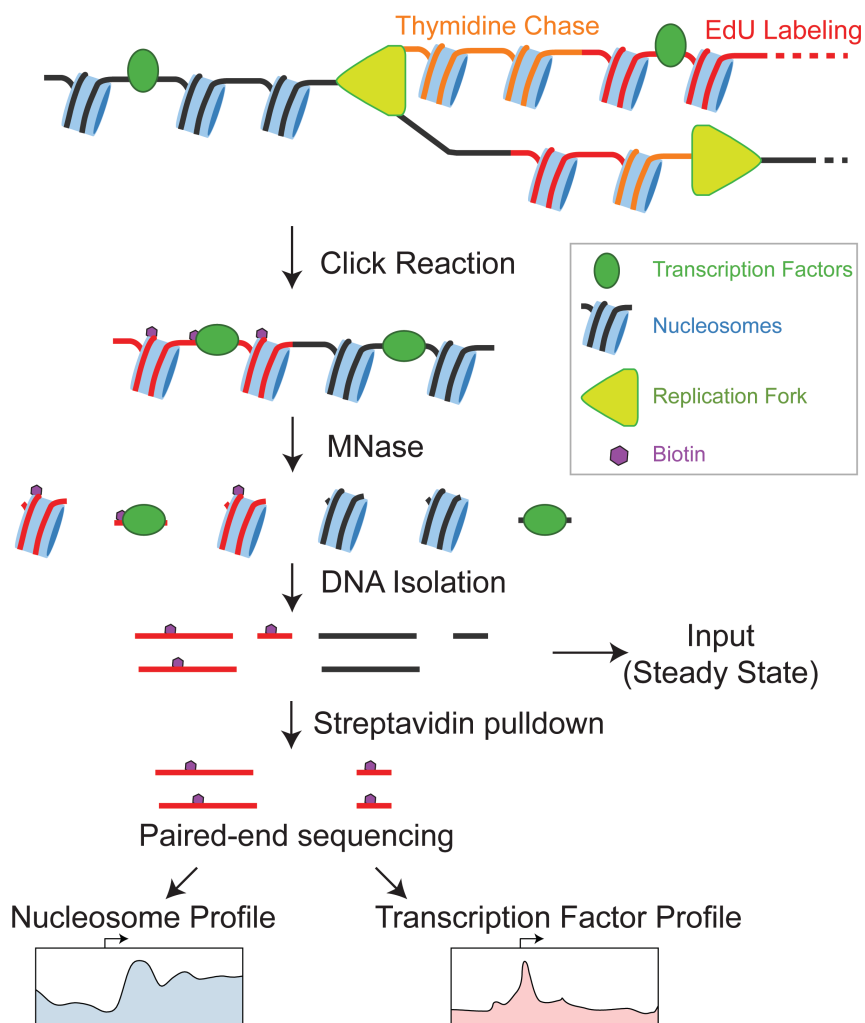


Figure 2. Schematic of the MINCE-seq protocol

Materials

EdU Labeling and click reaction

1. Mid-log-phase cultured cells
2. 10 mM 5-ethynyl-2'-deoxyuridine solution in dimethyl sulfoxide (DMSO). Can be stored at -20°C

3. 20 mM thymidine solution in DMSO
4. Phosphate-buffered saline (PBS): 11.9 mM phosphates, 137 mM NaCl, 2.7 mM KCl. Keep ~500 mL of PBS in ice before starting the experiment for cold washes. For formaldehyde fixation and permeabilization with Triton X-100, keep some PBS at room temperature.
5. Formaldehyde – 37% or 16% (Pierce, methanol-free) solutions – dilute to 1% in PBS just before cross-linking.
6. 1.25 M Glycine
7. Triton X-100
8. 30% BSA in saline (w/v, Sigma)
9. 100 μ M Biotin TEG azide (Berry & Associates) solution in DMSO
10. Sodium ascorbate – freshly prepared solution of 10 mM in water
11. Copper sulfate – freshly prepared solution of 2 mM in water

Chromatin preparation and DNA isolation

1. Lysis buffer, final concentrations in water:
 - a. 1% sodium dodecyl sulfate (SDS) w/v
 - b. 0.5 M sodium chloride
 - c. 100 mM Tris-HCl pH 8
 - d. 2 mM ethylenediaminetetraacetic acid (EDTA)
2. Dilution buffer, final concentrations in water:
 - a. 1% Triton X-100 v/v
 - b. 3 mM calcium chloride
 - c. 2 mM EDTA

3. Micrococcal nuclease (Sigma), 0.2 Units/ μ L stock solution in water
4. 0.2 M ethylene glycol-bis(β -aminoethyl ether)-tetraacetic acid (EGTA)
5. 10% SDS w/v
6. Proteinase K (Thermo Fisher Scientific, cat. no. EO0492)
7. Phenol-chloroform-isoamyl alcohol 25:24:1 v/v
8. RNase A (DNase- and Protease-free) 10 mg/mL solution
9. 300 mM sodium acetate
10. 20 mg/mL glycogen from mussels (Roche)
11. Isopropanol
12. TE buffer
 - a. 10 mM Tris pH 8
 - b. 1 mM EDTA
13. 200 proof ethanol

Isolation of biotinylated DNA

1. 2X Wash and Bind (2X WB) buffer in water (dilute this buffer 1:1 with water to obtain 1X WB buffer):
 - a. 10 mM Tris-HCl pH 7.5
 - b. 1 mM EDTA
 - c. 2 M NaCl
 - d. 0.2% Tween 20
2. Streptavidin M-280 dynabeads (Invitrogen)

General equipment

1. Refrigerated centrifuges that can accommodate 15 mL tubes at speeds up to 1,100 rcf and tabletop refrigerated centrifuges that can accommodate 1.5 mL tubes at speeds up to 16,000 rcf
2. Nutating and end-over-end mixers
3. Probe sonicator
4. Heating blocks/ water baths at 37°C and 65°C

Methods

1. Culture cells to mid-log-phase at the start of the experiment (For S2 cells, $4-6 \times 10^6$ cells/mL of media). The scale of the experiment with S2 cells is 2 x 150 cm² flasks with 30 mL of media in each, to a total of $240-360 \times 10^6$ cells.
2. Start the experiment by adding EdU to the cells in the culture flask, to a final concentration of 10 μ M (1:1000 dilution from the stock). Replace the cells in the incubator for the duration of EdU labeling. EdU labeling for *Drosophila melanogaster* S2 and BG3 cells is performed for 10 minutes.
3. After EdU labeling, if performing thymidine chase, quickly change media. For S2 and BG3 cells, which are semi-adherent, the media collected from the flask is spun-down in an aseptic polypropylene tube. The supernatant is discarded and the pelleted cells are resuspended in fresh media and added back to the flask. Thymidine is then added to a final concentration of 20 μ M (1:1000 dilution from the stock).

4. After the desired time of chase (or if not performing chase, immediately after EdU labeling), harvest cells and pellet them at 600 rcf.
5. Wash cells once with cold PBS.
6. Resuspend the cells with 10 mL of 1% formaldehyde in PBS in a 15 mL polypropylene conical tube. Cross-link cells for 15 minutes in a nutating mixer.
7. Quench with glycine: add glycine to a final concentration of 250 mM, 1:5 dilution from stock and mix in a nutating mixer for five minutes.
8. Pellet the cross-linked cells at 1100 rcf and wash thrice with PBS.
9. Permeabilize cells by resuspending the cell pellet in 4 mL of PBS + 0.25% Triton X-100 and placing in a nutating mixer for 30 minutes at room temperature.
10. Pellet cells at 1100 rcf for 4 minutes and wash once with 4 mL of cold 0.5% BSA in PBS.
11. Assemble the click reaction by resuspending cells in PBS and adding the click reagents in the following order (final volume of reaction is 450 μ L): Biotin TEG azide, sodium ascorbate and finally copper sulfate. Make the sodium ascorbate and copper sulfate stock solutions fresh just before setting up the click reaction. The stock and final concentrations of click reagents are presented in Table 1. Place on a nutating mixer for 1 hour.

Table 1. Click reaction recipe

Reagent	Stock Concentration	Required Concentration	Volume of stock to be added
PBS	N/A	N/A	414 μ L
Biotin Azide	2 mM	100 μ M	22.5 μ L
Sodium ascorbate	1 M	10 mM	4.5 μ L
Copper sulfate	100 mM	2 mM	9 μ L
Total volume			450 μL

12. Pellet the cells at 1100 rcf for 4 minutes and discard the supernatant.
13. Wash the cells three times with 5 mL of cold PBS to thoroughly remove copper.
14. Resuspend the cells gently in 200 μ L of cold lysis buffer and incubate for 10 minutes on ice.
15. Add 1.8 mL of cold dilution buffer to the cells and mix well (total 2 mL).
16. Sonicate the lysed cells to solubilize the chromatin in ice using a probe sonicator (Branson digital sonifier) at 30% intensity, 2.5s ON and 5s OFF for 40 seconds.
17. Split into four 0.5 mL samples in 1.5 mL tubes and incubate at 37°C for 5 minutes.
18. To each of the four samples add 1, 2, 4, and 8 μ L of Micrococcal Nuclease (concentration of 0.2 U/ μ L) respectively and incubate for 10 minutes.
19. Quench the nuclease reaction by adding EGTA to a final concentration of 2 mM (1:100 dilution of 0.2 M stock).
20. Add Proteinase K (20 μ g), SDS to a final concentration of 1% (1:10 dilution of stock) and mix well.
21. Incubate overnight at 65°C to reverse the crosslinks and degrade the protein.
22. The next day, add an equal volume of Phenol:Chloroform:Isoamyl alcohol mix (25:24:1 ratio) and spin at 16,000 rcf in a tabletop centrifuge for 10 minutes.
23. Separate the aqueous phase into a new tube and add 2 μ L of RNase A.
24. Incubate at 37°C for 15 minutes to degrade the RNA molecules in the sample.
25. Add an equal volume of phenol:chloroform:isoamyl alcohol mix (25:24:1 ratio). Mix well by pipetting and then spin at max speed (16,000 rcf) in a tabletop centrifuge for 10 minutes.

26. Separate the aqueous phase and add sodium acetate to a final concentration of 30 mM (dilute from 300 mM stock solution), 2 μ L of glycogen, and isopropanol to a final concentration of 70%. Mix well by inverting the tube several times.
27. Precipitate the DNA by spinning at 16,000 rcf at 4°C for 30 minutes in a tabletop centrifuge.
28. Discard the supernatant and wash the pellet with 75% ethanol (v/v) in TE buffer: to the DNA pellet, first add 800 μ L of 200-proof ethanol then add 400 μ L TE. Mix well by inverting the tube several times and precipitate by spinning at 16,000 rcf at 4°C for 15 minutes.
29. Discard the supernatant and air dry the DNA pellet. Resuspend the dry pellet in 400 μ L TE; take a 25 μ L aliquot to serve as input, which can be diluted to a final volume of 100 μ L.
30. Analyze the size of the input DNA by 2% TBE agarose gel electrophoresis to determine the MNase concentrations at which di- and tri-nucleosomes are preserved. These samples can be used for streptavidin pull-down.
31. To the remaining DNA (in 375 μ L) add an equal volume of 2X WB buffer.
32. Use 25 μ L of streptavidin magnetic beads for each sample. Wash the beads in 1 mL of 1X WB buffer and resuspend to original volume in 1X WB buffer.
33. Add 25 μ L of washed streptavidin magnetic beads to each sample of DNA.
34. Rotate at room temperature for 30 minutes and place the tubes on the magnet.
35. Save the unbound fraction and wash the beads 3 times. Resuspend the beads with 1 mL of 1X WB buffer and rotate at room temperature for 5 minutes for each wash.

36. Resuspend the beads in 20 μ L of TE. Add SDS to a final concentration of 1% and 20 mg of Proteinase K per mL of the reaction and incubate at 65°C for 15 min.
37. Add an equal volume of phenol:chloroform:isoamyl alcohol mix (25:24:1 ratio) and spin at 16,000 rcf in a tabletop centrifuge for 10 minutes.
38. Separate the aqueous phase and add sodium acetate to a final concentration of 30 mM (dilute from 300 mM stock solution), 2 μ L of glycogen and isopropanol to a final concentration of 70%. Mix well by inverting the tube several times.
39. Precipitate the DNA by spinning at 16,000 rcf at 4 °C for 30 minutes in a tabletop centrifuge.
40. Discard the supernatant and air-dry the DNA pellet. Resuspend the dried pellet in 20 μ L of nuclease-free water and proceed for library preparation using a published protocol (5).

Notes

1. Optimize the EdU labeling time for the chosen cell type: Sufficient EdU labeling for robust isolation of newly replicated DNA depends on the fraction of cells in S-phase, the number of active replication forks and the length of EdU labeling. Early S-phase has a higher number of active replication forks but would lead to poor sampling of inactive regions of the genome as they replicate in late S-phase. Longer EdU labeling would result in a higher amount of DNA labeled, but would miss the early events post-replication. The replication fork moves at ~2 kb/min(9). So for 10 minutes of labeling, we capture events that occur within ~20 kb from the replication fork.

2. Before proceeding to the biotin pull-down, perform a dot blot analysis using 1-3 μL of input DNA to confirm efficient biotin labeling. Biotin labeling could be inefficient either due to an insufficient fraction of cells in S-phase or due to an inefficient click reaction. Use biotinylated DNA as positive control.
3. Perform agarose gel electrophoresis to elucidate the degree of MNase digestion to choose a sample with optimal digestion. Nascent chromatin is more sensitive to nuclease digestion(10), so a lower level of digestion of bulk chromatin (as indicated by agarose gel electrophoresis of input DNA) would be preferable for MINCE-seq.
4. Strategies to increase the proportion of cells in S-phase include treatment with hydroxy urea (HU) to block cells in S-phase then release into media without HU, a single thymidine block, and for S2 cells, letting them reach stationary phase before splitting into fresh media(11).

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