Chapter 13

Genome-Wide Mapping of Protein–DNA Interactions on Nascent Chromatin

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Abstract

Chromatin immunoprecipitation (ChIP) is the most widely used method to analyze protein–DNA interactions in vivo. Coupled with next generation sequencing, ChIP-seq experiments map protein–DNA interactions in a genome-wide fashion. Here we describe a novel method called nasChIP-seq for mapping genome-wide occupancy of posttranslationally modified histones or transcription factors on newly replicated DNA.

Key words Chromatin immunoprecipitation, ChIP-seq, DNA replication, Nascent chromatin, Click chemistry

1 Introduction

ChIP assays utilize the specific interaction between an antibody and a protein of interest to pull down the fraction of genomic DNA occupied by this protein, achieving an enrichment of regions occupied by the protein out of all randomly sheared genomic regions [1]. The isolated DNA can be used to prepare a library and sequenced under high-throughput conditions to reveal the underlying sequence content. These sequences can then be mapped to a reference genome to locate the genomic regions harboring the sequence, which can be visualized in a genome browser to show the locations of the enriched occupied regions [2–4]. Tandem ChIP assays (re-ChIP or sequential ChIP) have been used to map the location of protein co-occupancy sites [5, 6], or the location of protein occupied sites on chromatin under a certain context, such as nascent (newly replicated) chromatin [7]. Petruk et al. used BrdU to label newly replicated DNA in fly embryos, followed by ChIP with antibodies targeting Trithorax group proteins and BrdU sequentially. Quantitative PCR (qPCR) allowed these authors to measure the occupancy of proteins on nascent chromatin [7]. Here we describe a method utilizing EdU to label newly replicated DNA

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followed by click chemistry to transform EdU into biotin on nascent DNA [8–11]. The replacement of BrdU with EdU enabled us to take advantage of the strong interaction between biotin and streptavidin for further isolation of labeled DNA. Furthermore, we carried out the click chemistry in a facilitative aqueous phase of purified ChIP DNA molecules, instead of the classical way to "click" in a suspension of formaldehyde-crosslinked nuclei [8–11], which severely limits the reaction components to access the EdU-labeled DNA. These key improvements allowed us to achieve very high labeling efficiency and pulldown of the nascent chromatin fraction. Here we describe two examples in which this technique is used for genome-wide mapping of histone modifications or protein occupancy on nascent chromatin. In one example we use 20 min of EdU labeling with 10 million synchronized *Drosophila* Kc167 cells to map the distribution of H3K4me3 on nascent chromatin (Fig. 1a). In the second example, we describe the mapping of CTCF occupancy on nascent chromatin with 20 min labeling of 10 million asynchronous H9 human embryonic stem cells (Fig. 1b).

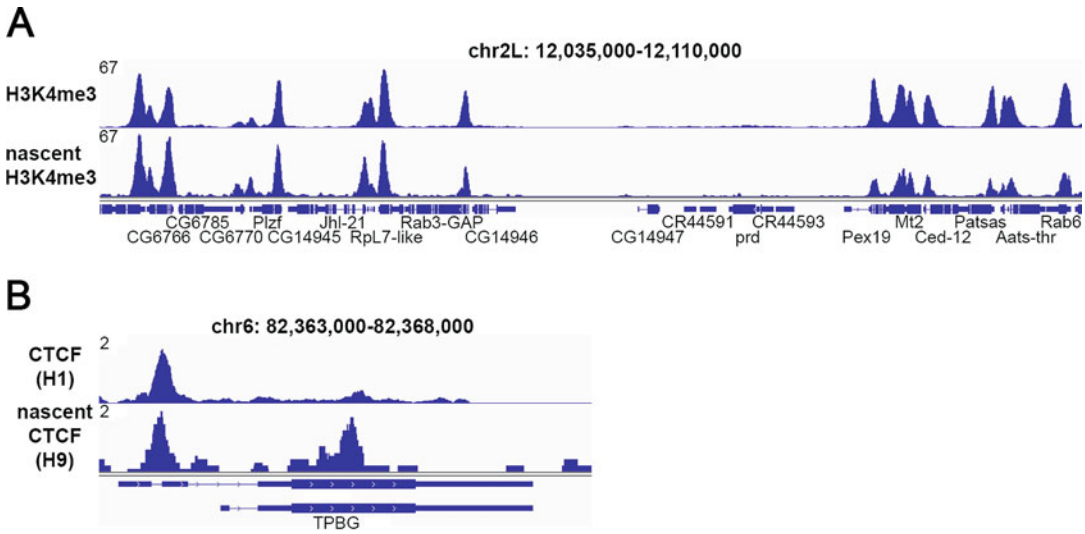

Figure 1, Xu and Corces

Fig. 1 Results from ChIP-seq on nascent chromatin. (**a**) A 75 kb genomic region of the *Drosophila* genome showing a published H3K4me3 ChIP-seq data in asynchronous Kc167 cells (upper track) [12], and our H3K4me3 nasChIP-seq data in synchronized Kc167 cells with 20 min of EdU labeling (lower track). The sequence read density was normalized to *per* one million reads (RPM). The left half of the genomic region shows full restoration of H3K4me3 on nascent chromatin within 20 min, while H3K4me3 is only partially restored in the right half of the region on nascent chromatin. (**b**) A 5 kb genomic region of the human genome showing a published CTCF ChIP-seq data in H1 hES cells (upper track) [13], and our CTCF nasChIP-seq data in H9 hES cells (lower track). The sequence read density was normalized to *per* one million reads (RPM). One conserved CTCF occupied site and one nascent chromatin-specific site are shown around the transcription start site of the TPBG gene

2 Materials

Prepare all solutions every 3 months unless otherwise noted. Molecular biology grade water is the solvent for all solutions unless otherwise noted.

2.1 Cell Synchronization, EdU Labeling, and Chromatin Immunoprecipitation

1. Kc167 cells (ATCC) or H9 cells (WiCell).
2. SFX-Insect cell culture media (HyClone) or StemPro hESC SFM (ThermoFisher).
3. 2 M hydroxyurea solution. Aliquot and store at −20 °C (*see* **Note 1**).
4. 40 mM EdU (5-ethynyl-2′-deoxyuridine) solution. Aliquot and store at −20 °C (*see* **Note 2**).
5. 1× DPBS (Dulbecco's Phosphate Buffered Saline).
6. Formaldehyde solution (37%). Store in the dark.
7. 1.25 M glycine solution. Store at 4 °C.
8. Anti-H3K4me3 antibody. Store at 4 °C.
9. Anti-CTCF antibody. Store at 4 °C.
10. Refer to [14] for other reagents for ChIP.

2.2 Click Chemistry

1. 1 mM Biotin azide in DMSO. Aliquot and store at −20 °C.
2. 100 mM $CuSO_4$ solution. Store at 4 °C.
3. 100 mM sodium ascorbate solution. Aliquot and store at −20 °C.
4. Microcentrifuge tubes.
5. Ethanol. Store at −20 °C.
6. 5 M NaCl solution.
7. 20 mg/ml glycogen solution. Aliquot and store at −20 °C.

2.3 Nascent DNA Pulldown

1. 70% ethanol solution. Store at 4 °C.
2. Dynabeads® MyOne™ Streptavidin C1. Store at 4 °C.
3. 2× binding and washing buffer: 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 2 M NaCl. Store at 4 °C.
4. 10 mM Tris–HCl (pH 8.0). Store at 4 °C.

2.4 Library Preparation

1. NEB End Repair Enzyme Mix. Store at −20 °C.
2. Klenow Fragment (3′ → 5′ exo$^-$). Store at −20 °C.
3. 1 mM dATP. Store at −20 °C.
4. T4 DNA Ligase. Store at −20 °C.
5. KAPA SYBR FAST qPCR Kit Master Mix (2×). Store at −20 °C.

6. Refer to [15] for sequence information on adaptors and PCR primers.
7. Agencourt AMPure XP beads.

3 Methods

All steps were performed at room temperature (25 °C) unless otherwise noted.

3.1 Cell Synchronization, EdU Labeling, and Chromatin Immunoprecipitation

1. **Kc167:** Cells were grown to 50% confluence in SFX medium at 25 °C in an incubator.

   H9: Cells were grown to 60–70% confluence in StemPro hESC medium at 37 °C in an incubator under 5% CO_2.
2. **Kc167**: Add hydroxyurea to cells at a final concentration of 2 mM, and culture cells in an incubator for 20 h (*see* **Note 3**).
3. **Kc167**: Premix EdU with fresh SFX medium to obtain a final concentration of 40 μM. Discard hydroxyurea-containing medium and wash the cells gently with DPBS once. Add EdU-containing medium to cells (*see* **Note 4**). Leave cells in the incubator for 20 min (*see* **Note 5**).

   H9: Premix EdU with fresh StemPro medium to obtain a final concentration of 50 μM. Prewarm the medium to 37 °C. Add EdU-containing medium to cells. Leave cells in the incubator for 20 min (*see* **Note 6**).
4. Premix formaldehyde with DPBS to obtain a final concentration of 1%. Discard EdU-containing medium, add 1% formaldehyde to cells.
5. After 10 min, add glycine solution to reach 0.125 M final concentration.
6. Proceed to ChIP with H3K4me3 or CTCF antibody respectively, and refer to [14] for details.

3.2 Click Chemistry

1. Centrifuge ethanol-precipitated ChIP DNA at 16,000 × *g* for 30 min at 4 °C.
2. Discard supernatant. Add 70% ice-cold ethanol. Invert the microcentrifuge tube several times to wash the pellet (*see* **Note 7**).
3. Centrifuge at 16,000 × *g* for 5 min.
4. Discard supernatant. Air-dry the pellet (*see* **Note 8**).
5. Add 174 μl 1×DPBS to the pellet. Incubate at 37 °C for 10 min.
6. Add these reagents to 174 μl DNA solution: 2 μl 1 mM biotin-azide (or DMSO, as control, *see* **Note 9**), 4 μl 100 mM $CuSO_4$ solution. Vortex briefly.

7. Add 20 μl 100 mM sodium ascorbate solution. Immediately vortex for 3 s (*see* **Note 10**).
8. Incubate at 37 °C for 1 h (*see* **Note 11**).
9. Add 9 μl 5 M NaCl solution and 2 μl glycogen. Vortex briefly. Add 500 μl ice-cold ethanol. Vortex vigorously. Incubate at −80 °C for 2 h.

3.3 Nascent DNA Pulldown

1. Centrifuge ethanol precipitated DNA at 16,000 × *g* for 30 min at 4 °C.
2. Discard supernatant. Add 70% ice-cold ethanol. Invert the microcentrifuge tube several times to wash the pellet (*see* **Note 12**).
3. Centrifuge at 16,000 × *g* for 5 min.
4. Discard supernatant. Air-dry the pellet (*see* **Note 8**).
5. Add 100 μl 10 mM Tris–HCl (pH 8.0) to the pellet. Incubate at 37 °C for 10 min.
6. Wash 5 μl Streptavidin C1 beads with 1 ml 1× binding and washing buffer. Remove supernatant on a magnetic stand. Resuspend the beads in 100 μl 2× binding and washing buffer. Mix 100 μl DNA solution with 100 μl bead suspension. Rotate on a rotor for 30 min.
7. Remove supernatant on a magnetic stand. Wash the beads with 1 ml 1× binding and washing buffer on a rotor for 10 min. Repeat this step once.
8. Remove supernatant on a magnetic stand. Wash the beads with 1 ml 10 mM Tris–HCl (pH 8.0) on a rotor for 5 min.
9. Gently suspend the beads in 24 μl dH_2O.

3.4 Library Preparation

1. Add 3 μl reaction buffer and 3 μl NEB End Repair Enzyme Mix to the bead suspension. Gently mix. Rotate on a rotor for 30 min (*see* **Note 13**).
2. Remove supernatant on a magnetic stand. Wash the beads with 1 ml 1× binding and washing buffer on a rotor for 10 min.
3. Remove supernatant on a magnetic stand. Wash the beads with 1 ml 10 mM Tris–HCl (pH 8.0) on a rotor for 5 min.
4. Gently resuspend the beads in 19 μl dH_2O. Add 3 μl reaction buffer, 6 μl 1 mM dATP, and 2 μl Klenow Fragment ($3' \rightarrow 5'$ exo^-). Gently mix. Rotate on a rotor at 37 °C for 30 min.
5. Repeat **steps 2** and **3**.
6. Gently resuspend the beads in 19 μl dH_2O. Add 3 μl reaction buffer, 5 μl 10 μM adaptors, and 3 μl T4 DNA ligase. Gently mix. Rotate on a rotor for 4 h.
7. Repeat **steps 2** and **3**.

8. Gently resuspend the beads in 21 μl dH_2O. Add 2 μl each of 10 mM primers, and 25 μl KAPA SYBR FAST qPCR Kit Master Mix (2×). Gently mix. Amplify on a qPCR instrument. Monitor the real-time amplification curve and stop the amplification appropriately (*see* **Note 14**).
9. Size-select and clean the DNA using Agencourt AMPure XP beads (Refer to product manuals.).
10. Send the library to sequencing service providers for quality assessment and high throughput sequencing.

4 Notes

1. Hydroxyurea will decompose in water. Make the stock solution monthly, aliquot into small volumes for single use, and store at −20 °C.
2. Short-term 37 °C incubation and vigorous vortexing are sometimes necessary to fully dissolve the 40 mM EdU stock solution stored at −20 °C.
3. Usually the cells will reach 70%–80% confluence after synchronization with hydroxyurea for 20 h. It should be noted that synchronization is only necessary for DNA replication timing-specific mapping. Our scheme of 20 min labeling after synchronization and release preferentially maps the genomic regions replicated during first 20 min after entry into S phase.
4. Kc167 cells attach loosely after synchronization with hydroxyurea. Be cautious not to perturb the cells when washing and changing the medium. Always pipette the medium through the side surface of the cultureware.
5. The labeling time is highly tunable. For high temporal resolution (the degree of nascentness), choose the shortest labeling time that generates a library with good signal-to-noise ratio after sequencing. For high yield of final DNA product, increase the labeling time appropriately. Also, cell harvesting can be done after a "chase" period following EdU labeling to map dynamic changes in protein occupancy on nascent chromatin.
6. Prewarm the EdU medium to 37 °C to ensure efficient labeling. When labeling cells in multiple pieces of cultureware at the same time, always place individual cultureware back in the incubator immediately after the EdU medium is added.
7. Washing with 70% ethanol helps to remove residual SDS (sodium dodecyl sulfate) carried over from elution buffer in ChIP. SDS is a strong inhibitor of click chemistry. Repeat this step if necessary.

8. 5–10 min are optimal for air-drying the DNA pellet. Overdrying will lead to low solubility of the pellet and overall reduction of final DNA yield.
9. Setting aside a DMSO reaction and making a mock library serves as a negative control for the nascent DNA pulldown step. In our hands, amplification of the DMSO library with the same number of PCR cycles yields very little DNA, ruling out the possibility that the nascent DNA after pulldown contains some carryover ChIP DNA, proving the nascent ChIP DNA faithfully reflects the enrichment of protein occupancy on nascent chromatin.
10. Sodium ascorbate is highly prone to oxidation. Always keep caps on when not using it. Protect from light. Make fresh stock solution monthly. Vortex immediately after adding it to the mixture, ensuring timely reaction with Cu^{2+} before any putative oxidation.
11. Oxygen interferes with click chemistry. Choose microcentrifuge tubes with good airtightness as reaction containers. Place tubes in an airtight container if necessary.
12. Washing with 70% ethanol helps to remove residual biotin azide carried over from click chemistry. Free biotin will reduce the ability of biotin-labeled DNA to bind to the streptavidin beads. Repeat this step if necessary.
13. We determined 30–40 μl as the optimal reaction volume in a 1.5 ml microcentrifuge tube. Volumes lower than this will lead to higher density of beads and higher chance of bead aggregation. Higher volumes will lead to spill of beads to the side surface of the microcentrifuge tube during rotation. Both conditions reduce the overall yield of final DNA.
14. PCR amplification with optimal cycle numbers is critical for the quality of the library. Insufficient amplification will lead to insufficient input material for sequencing and failure of the experiment. Overamplification will lead to higher PCR duplication level (piling up of clonally identical sequence) and reduce the overall cost-efficiency of sequencing.

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