ItChIP-simultaneous indexing and tagmentation-based ChIP-seq

Shanshan Ai

Institute of Molecular Medicine, Beijing Key Laboratory of Cardiometabolic Molecular Medicine, Peking University, Beijing 100871, China

Haiqing Xiong

Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China

Chen C. Li

Institute of Molecular Medicine, Beijing Key Laboratory of Cardiometabolic Molecular Medicine, Peking University, Beijing 100871, China

Yingjie Luo

Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China

Yaxi Liu

Institute of Molecular Medicine, Beijing Key Laboratory of Cardiometabolic Molecular Medicine, Peking University, Beijing 100871, China

Xianhong Yu

Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China

Aibin He (✉ ahe@pku.edu.cn)

Institute of Molecular Medicine, Beijing Key Laboratory of Cardiometabolic Molecular Medicine, Peking University, Beijing 100871, China. Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China <https://orcid.org/0000-0002-3489-2305>

Method Article

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Abstract

Single-cell measurement of chromatin states, including histone modifications and non-histone protein binding, remains challenging. We present a low-cost, efficient ChIP-seq (simultaneous indexing and tagmentation-based ChIP-seq, itChIP), compatible to both low-input and single cells for profiling chromatin states. This single-cell itChIP approach combines chromatin opening, simultaneous cellular indexing and chromatin tagmentation in a single tube, processing samples with tens of single cells in rarity or with thousands of single cells per assay, and the entire procedure can be finished in two days. The sc-itChIP data acquire ~9,000 unique reads per cell, sufficiently capturing the earliest epigenetic priming along cell fate transition and the basis for cell-type specific enhancer usage. Our results demonstrate that itChIP is a generalizable technology for single-cell chromatin profiling of epigenetically heterogeneous cell populations in many biological processes. This step-by-step protocol is related to the publication “Profiling chromatin state by single-cell itChIP-seq” in Nature Cell Biology.

Introduction

Profiling epigenetic states largely relies on measurement of chromatin occupancy of covalently modified histones, histone regulators, and transcription factors (TFs)¹⁻⁵. Among them, chromatin immunoprecipitation followed by massively parallel sequencing (ChIP-seq) is the method of choice, allowing the rapid identification of transcriptional regulatory elements genome-wide⁶⁻⁹. Standard ChIP-seq requires a million or more cells. Reducing the required input to one to hundreds of cells would open up an array of new applications. For instance, understanding the transcriptional regulatory networks that control lineage specification in developing embryos would be enabled by using ChIP-seq to probe lineage-specific enhancers from low amounts of cells. To define differentiation trajectories and potentials of rare spatiotemporal progenitor cells, single-cell RNA-seq (scRNA-seq) techniques have become widely used to separate subpopulations from transcriptomically heterogeneous cell populations¹⁰⁻¹³. However, a robust single-cell ChIP-seq tool remains lacking to understand epigenetic heterogeneity in complex tissue or cell populations at single cell level.

In the past decade, epigenomic studies have seen great strides towards reduced input sample size, including nano-ChIP¹⁴, UNI-NChIP¹⁵, STAR ChIP¹⁶, MOWChIP¹⁷, CUT&RUN¹⁸ and ChIL-seq¹⁹, but development of a generalizable, low-cost, and robust method effective on single cells or small cell populations lags behind. Microfluidic systems have been used to profile histone marks by ChIP-seq using as few as 100 cells¹⁷. A study by Rotem et al. demonstrated the proof-of-concept use of microfluidic systems for single-cell ChIP (Drop-ChIP). However, at ~800 reads per cell, the sparse data yield from Drop-ChIP limits its potential applications²⁰.

Through overcoming these limitations, we present simultaneous indexing and tagmentation of opened/relaxed chromatin-based ChIP-seq (itChIP-seq)(Figure 1), which enables genome-wide profiling of histone modifications and non-histone proteins from ultra-low input (as few as 100 cells) and various histone modifications of single-cell samples (Figure 2), enabling processing rare cell populations of less

than 100 single cells and abundant samples of thousands of single cells per assay. Furthermore, itChIP eliminates the requirement for specialized microfluidic devices and is generalizable and affordable for most biomedical research laboratories.

Reagents

BI21 (DE3)

Formaldehyde Solution (SIGMA, F8775-500ML)

Glycine (AMRESCO, 0167-1KG)

Phosphate Buffered Daline (PBS) (Hyclone, SH30256.01)

Sodium butyrate (SIGMA, 303410-100G)

Collagenase Type 2 (Worthington, LS004177)

H3K4me3 antibody (Millipore, Cat.No: 04-745)

Trypsin (Gibco, 27250-018)

Ampicillin Na (AMRESCO, VT0236)

IPTG (INALCO, 1758-1400)

HEPES free acid (AMRESCO, 0511-1KG)

Glycerol (SIGMA, 49767-100ML)

Triton X-100 (SIGMA, T8787-50ML)

EDTA-free Protease Inhibitor Cocktail (Roche, 04693132001)

Phenyl Methane Sulfonyl Fluoride (PMSF) (AMRESCO, 0754-5G)

Dithiothreitol (DTT) (INALCO, 1758-9030)

TRIS (AMRESCO, 0497-5KG)

TAPS (SIGMA, T-5130)

Magnesium chloride solution (MgCl_2) (SIGMA, 68475-100ML)

Potassium chloride (KCl) (XiLong SCIENTIFIC, 7447-40-7)

Calcium chloride dihydrate (CaCl_2)

(XiLong SCIENTIFIC, 10035-04-8)

Sodium chloride (NaCl) (SIGMA, 303410-100G)

tRNA (Invitrogen, cat# AM7119)

N,N-Dimethylformamide (DMF) (SIGMA, D4551-250ML)

Sodium deoxycholate (SIGMA, D6750)

SODIUM DODECYL SULFATE (SDS) (AMRESCO, 0227-1KG)

Proteinase K (AMRESCO, 0706-100MG)

KAPA HiFi HotStart ReadyMix (BIOSYSTEMS, KM2602)

AMPure XP (BECKMAN COULTER, A63881)

Streptavidin M280 beads (Invitrogen, 60210)

Dynabeads protein A (Invitrogen, 10001D)

H3K27me3 antibody (Millipore, Cat.No: 07-449)

H3K27ac antibody (Abcam, Cat.No: ab4729)

EZH2 antibody (CST, Cat.No: S246S)

RNA polymerase II CTD antibody (Abcam, Cat.No: ab5408)

Equipment

1.5 ml tube (Axygen, MCT-150-C)

0.2 ml 8-strip PCR tube (Axygen, PCR-0208-C)

DynaMag™-PCR Magnet

Qubit 2.0 Fluorometer (Thermo Fisher Scientific)

Centrifuge (Eppendorf)

Thermal cycler (Eppendorf)

ThermoMixer

Qsonica, Q800R

Procedure

Reagents Setup

• Hypotonic Buffer

20 mM HEPES(PH7.9)

10 mM KCl

10% Glycerol

0.2%NP-40

0.3% SDS

• 5XTMgCl-DMF

50 mM TAPS-NaOH(PH8.3)

25 mM MgCl₂

50% DMF

• Releasing Buffer

0.8% SDS

1% TX-100

2 mM EDTA

20 mM Tris-HCl(PH8.0)

200 mM NaCl

• Low Salt Buffer

1% TX-100

2 mM EDTA

50 mM HEPES(PH7.9)

150 mM NaCl

0.1% Sodium Deoxycholate

• High Salt Buffer

1% TX-100

2 mM EDTA

50 mM HEPES(PH7.9)

500 mM NaCl

0.1% Sodium Deoxycholate

• ChIP Elution Buffer

50 mM Tris-HCl(PH8.0)

10 mM EDTA

1% SDS

Detailed Experiment Procedure for Low Input ItChIP

The important steps of the procedure for low-input itChIP were optimized as shown in Figure 3.

• Cell preparation and chromatin opening.

1. Sort fixed 100-1,000,000 cells into 5 µl Hypotonic Buffer containing 0.3% SDS in 200 µl tubes.
2. Open chromatin: incubation the cell at 62°C for 10 min in an Eppendorf ThermoMixer with agitation at 600 rpm.
3. Quench SDS: add 1.2 µl of 10% Triton X-100 to the samples and incubated at 37°C for 60 min in a PCR thermal cycler.

• Chromatin tagmentation.

4. Tn5 tagmentation: add 2 µl 5XTMgCl-DMF, 3 µl 12.5 µM Tn5 Transposase and 0.8 µl ddH₂O to the 6.2 µl cell mixture (using 10-fold dilution of Tn5 for the cell number less than 1,000, Table 1). The reaction was performed at 37°C for 1 h, followed by addition of 2 µl 250 mM EDTA and incubation at RT for 20 min to stop Tn5 reaction.

• Chromatin release.

5. Collect the sample by centrifugation at 12,000 g for 3 min at 4°C, get rid of the supernatant and resuspend the nuclear pellet with 20 µl Releasing Buffer (0.8% SDS, 2 mM EDTA, 20 mM Tris-HCl pH8.0,

200 mM NaCl) by pipetting ~30 times to disperse the nuclei.

6. Incubate the nuclear lysis at RT for 30 min, followed by brief sonication at 20% power for 4 cycles (15 s ON and 30 s OFF per cycle; Q800R2, Qsonica) to aid in solubilize chromatin.

7. Dilute the SDS concentration by adding 100 µl ChIP Dilution Buffer to the chromatin mixture before centrifugation at 20,000 for 15 min at 4°C to collect soluble chromatin for immunoprecipitation.

• Antibody-based chromatin immunoprecipitation.

8. Add 0.1~1.0 µg antibody to the tagmented soluble chromatin mixture and incubated at 4°C overnight.

9. Meanwhile, prepare 4 µl Dynabeads protein A (Invitrogen, 10001D, 30 mg/ml) beads and block the beads with 150 µl 1% BSA/PBS overnight at 4°C.

10. Next day, transfer the 4 µl protein A beads to the chromatin samples and incubate at 4°C for 2 h with gentle rotation.

11. Wash beads: the beads were washed thrice with 150 µl cold Low Salt Buffer and once with 150 µl cold High Salt Buffer.

12. Transfer the chromatin-antibody-beads complex to new tubes in 150 µl cold Low Salt Buffer, and the beads was collected by magnet stand to facilitate removing supernatant.

13. Suspend the complex with 100 µl ChIP Elution Buffer, and reverse-crosslinked at 70°C for 3 h on a thermal mixer at 1500 rpm.

14. Add 0.1 mg/ml proteinase K to the DNA mixture and incubate at 55°C for 3 h.

15. Purify the DNA with phenol-chloroform. The purified DNA can be directly used for library preparation.

• Bio-itChIP.

Continue from step 7.

7-8. Add NaCl to the tagmented soluble chromatin to final 350 mM.

7-9. Add 10 µl pre-blocked (1% BSA/PBS block the beads at 4°C for 2 h) streptavidin M280 beads to the tagmented soluble chromatin at 4°C for 2 h.

7-10. Wash beads: wash the beads once with 150 µl 2% SDS/PBS, and thrice with 150 µl cold High Salt Buffer.

7-11. Transfer the chromatin-antibody-beads complex to new tubes in 150 µl cold High Salt Buffer, and the beads was collected by magnet stand to remove supernatant.

7-12. Suspend the complex with 100 µl ChIP Elution Buffer, and the ChIPed DNA was purified as above.

• Nextera library preparation and sequencing of low-input itChIP/bio-itChIP.

16. Amplify the purified ChIPed DNA by addition of 1 µl 25 µM i5 index primer (5'-AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTC-3'), 1 µl 25 µM i7 index primer (5'-CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG-3') (Table 2) and 25 µl 2X KAPA master mix to the 23 µl purified ChIPed DNA and incubation at 72°C for 5 min, 98°C for 45 s, 14-19 cycles of (98°C for 15 s, 63°C for 30 s, 72°C for 1 min), and final 72°C extension for 5 min.

17. After PCR, purify the library with 1 X AMPure XP beads once. Size selection was carried out by first 0.5X AMPure XP beads to remove >1kb fragments, and second 0.5X AMPure XP beads to the supernatant to obtain 200-1000 bp fragments for sequencing.

18. Sequence the libraries with paired-end 150-bp reads on Hiseq X-ten or Novaseq 6000 platform (Illumina).

Single-cell itChIP

• Single cell chromatin opening.

Follow the procedures described above: Step 1-3.

• Single cell chromatin tagmentation and indexing.

19. Tn5 tagmentation: add 2 µl 5XTMgCl-DMF, 2 µl 1.25 µM Tn5-T5 (5'-TCGTCGGCAGCGTCTCCACGC[T5]GCGATCGAGGACGGCAGATGTGTATAAGAGACAG-3'), 2 µl 1.25 µM Tn5-T7 (5'-GTCTCGTGGGCTCGGCTGTCCCTGTCC[T7]CACCGTCTCCGCCTCAGATGTGTATAAGAGACAG-3') (Table 3), and ddH₂O with 10 ng yeast tRNA (Invitrogen, cat# AM7119) to 10 µl for each well for tagmentation at 55°C for 30 min.

20. Stop the reaction by adding 2 µl 250 mM EDTA to each well, mix well and incubate at RT for 20 min.

• Single cell chromatin release and pooling of indexed single cells.

21. Combine each well of single cell into 1.5 ml Eppendorf tube and the nuclei were collected by centrifugation at 12,000 g for 3 min at 4°C.

22. Discard the supernatant and resuspend the nuclear pellet with 20 µl Releasing Buffer by pipetting ~30 times to disperse the pellets.

23. Incubate the nuclear lysis at RT for 30 min, followed by brief sonication at 20% power for 4 cycles (15 s ON and 30 s OFF per cycle) to solubilize chromatin.

24. Dilute the SDS concentration by adding 100 µl ChIP Dilution Buffer to the chromatin mixture before centrifugation at 20,000 for 15 min at 4°C to collect soluble chromatin for immunoprecipitation.

• Pooled single cell chromatin immunoprecipitation.

25. Add 0.1~1.0 µg antibody to the indexed soluble chromatin mixture from pooled single cells and incubate at 4°C overnight, then immunoprecipitation was performed following the low-input itChIP procedure.

• Single-cell Nextera library preparation for sequencing using a custom recipe.

26. Amplify the pooled single cell ChIPed DNA by addition of 1 µl 25 µM i5 index primer (5'-AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCTGCGCAGCGTC-3'), 1 µl 25 µM i7 index primer (5'-CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG-3') and 25 µl 2X KAPA master mix to the 23 µl purified ChIPed DNA and incubation at 72°C for 5 min, 98 °C for 45 s, 18-20 cycles of (98°C for 15 s, 63 °C for 30 s, 72°C for 1 min), and final 72°C for 5 min.

27. After PCR, purify the library with 1 X AMPure XP beads once. Size selection was carried out by first 0.5X AMPure XP beads to remove >1kb fragments, and second 0.5X AMPure XP beads to the supernatant to obtain 200-1000 bp fragments for sequencing.

• Custom sequencing of single-cell Nextera libraries.

28. Quantify the libraries by qPCR.

29. Load 10 pM libraries per lane of HiSeq 2500 (Illumina) using custom sequencing primers (Table 3) following read lengths: 69 bp (Read1) + 43 bp (Index1) + 37 bp (Index2) + 69 bp (Read2) (Supplementary Fig. 6). The first 8 bp of Index1 correspond to the T7 barcode and the last 8 bp to the i7 barcode. The first 8 bp of Index2 correspond to the i5 barcode and the last 8 bp of Index2 correspond to the T5 barcode. About 10% PhiX and 15% spike-in library in which we replaced universal connector A/B sequences with 21/27 bp N were pooled with single-cell itChIP libraries for sequencing to balance the constant bases within each detection cycle.

• Single-cell Truseq library preparation for sequencing using the Illumina standard workflow.

30. Prepare the Truseq libraries by 2 rounds of PCR amplification (Fig. 4).

31. Add 22.3 µl PCR Mix 1 (10 µl Q5 reaction buffer (NEB), 10 µl Q5 high GC enhancer (NEB), 1 µl 10 mM dNTP mix, 0.3 µl Q5 polymerase, 0.5 µl 25 µM connector forward primer (5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGTCTGCGCAGCGTCTCCACGC-3'), 0.5 µl 25 µM connector reverse primer (5'-GACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTCTCGTGGGCTCGGCTGTCCCTGT-3')) to 27.7 µl ChIPed DNA and the reaction was set up at 72°C for 5 min, 95°C for 30 s, 18 cycles of (98°C for 10 s, 63°C for 30 s, 72°C for 1 min), and final 72°C extension for 5 min.

32. After the first round of PCR, remove the excessive connector primers by addition of 0.5 µl 20 U/µl Exol (NEB) and incubation at 37°C for 30 min and 72°C for 20 min to inactivate Exol.

33. Perform second round of PCR by addition of 9.5 µl PCR Mix 2 (2 µl Q5 reaction buffer (NEB), 2 µl Q5 high GC enhancer (NEB), 0.5 µl 10 mM dNTP mix, 0.1 µl Q5 polymerase, 1 µl 10 µM NEB Index primer (5'-CAAGCAGAAGACGGCATACGAGAT[index]GACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'), 1 µl 10 µM NEB universal Primer (5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3', 2.9 µl ddH₂O) to 50.5 µl of PCR products and incubation at 95°C for 30 s, 5 cycles of (98°C for 10 s, 63°C for 30 s, 72°C for 1 min, and 72°C for 5 min.

34. Purify the library with 1X AMPure XP beads once and size selection was carried out by first 0.5X AMPure XP beads to remove >1 kb fragments, and second 0.5X AMPure XP beads to the supernatant to obtain 200-1000 bp fragments for sequencing.

35. Sequence the resulting libraries using standard Truseq recipe for paired-end 150-bp reads on Hiseq X-ten or Novaseq 6000 platform (Illumina).

Troubleshooting

1.

Step: 4.

Problem: Small amount of soluble chromatin with larger fragment size.

Possible reason: Tn5 complex lose its potency.

Solution: Increase the amount of Tn5 complex for tagmentation or prepare new Tn5 transpose complex.

2.

Step: 8.

Problem: ChIP-qPCR exhibits lower signal or higher background.

Possible reason: The antibody amount is not optimal.

Solution: Optimize the antibody concentration for immunoprecipitation.

Time Taken

Day 1

• Cell preparation and chromatin opening.

- Chromatin tagmentation.
- Chromatin release.
- Antibody-based chromatin immunoprecipitation.

Day 2

- Wash beads and Purify ChIPed DNA.
- Library preparation for sequencing.

Anticipated Results

1. The library exhibits good ChIP-qPCR enrichment before sequence.
2. Sequence results shows good enrichment of positive signals

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Figures

Figure 1

Work flow of itChIP-seq experimental design.

Figure 2

Work flow of sc-itChIP-seq design.

Figure 3

Optimization of key steps in the itChIP protocol. a, b. Optimization of the SDS concentration for chromatin opening. 10,000 ESCs after fixation for 7 min were treated with SDS as indicated concentrations at the step of chromatin opening at 62°C for 10 min, quenched with Triton X-100, tagmented with Tn5 complex, and briefly sonicated to release chromatin followed by antibody based immunoprecipitation and DNA extraction by phenol-chloroform for both soluble and insoluble chromatin after reverse-crosslinking for size examination on agarose gel. We picked the condition yielding desired DNA fragments of < 1 kb (a), as those > 1 kb were undesired for sequencing, and higher ChIP-qPCR enrichment (b) for itChIP. 0.3% SDS was selected according to this criterion. P values were calculated by one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. c, d. Optimization of the required amount of Tn5 complex for efficient tagmentation. The same as in (a), except for the indicated Tn5 amount. We picked the condition yielding DNA fragments of < 1 kb (c) and higher ChIP-qPCR

enrichment (d) for itChIP. 3 μ l of 12.5 μ M Tn5 transposase was selected for 10,000 cells according to this criterion. P values were calculated by one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. e, f. Optimization of the temperature for chromatin opening. The same as in (a), except for the indicated chromatin opening conditions, 37°C for 1 h and 62°C for 10 min. We picked the condition yielding DNA fragments of < 1 kb (e) and higher ChIP-qPCR enrichment (f) for itChIP. In this regard, 37°C for 1 h and 62°C for 10 min exhibited no difference. For simplicity, we used 62°C for 10 min to open chromatin in this study for all the itChIP experiments. P values were calculated by two-tailed paired t-test. g, h. Optimization of tagmentation conditions. The same as in (a), except for the indicated temperature and time for tagmentation. We picked the condition yielding DNA fragments of < 1 kb (g) and higher ChIP-qPCR enrichment (h) for itChIP. In this regard, both 37°C for 1 h and 55°C for 30 min can be used for chromatin tagmentation. For simplicity, we used 37°C for 1 h for chromatin tagmentation in this study for all the itChIP experiments. P values were calculated by two-tailed paired t-test. Optimal conditions were boxed. Dish lines in a, c, e, g indicate the position of 1 kb DNA marker. In b, d, f and h, data represent means $\pm$ s.d. from n=3 biological replicates. Arrowhead indicates free adaptors.

Figure 4

Overview of the design of mosaic Truseq library preparation for sequencing using Illumina's standard recipe.

Supplementary Files

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