Simultaneous single-cell chromatin architecture and gene expression sequencing by LiMCA

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Method Article

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Abstract

The concurrent measurement of chromatin organization and gene transcription within a single cell provides a unique opportunity to investigate their intricate interplay. We introduce Linking mRNA to Chromatin Architecture (LiMCA), a multi-omic assay that enables sensitive profiling of three-dimensional genome structure and gene expression at single-cell resolution with low-input samples. LiMCA employs a physical separation strategy to proceed with cytoplasm (mRNA) and nuclei, respectively, which ensures high sensitivity.

Introduction

Three-dimensional (3D) genome organization plays a crucial role in regulating gene expression\(^1\)\(^-\)\(^3\). Disruption of topologically associating domains (TADs) leads to aberrant gene activation, causing severe developmental defects and cancers\(^4\)-\(^6\). However, studies have shown that acute depletion of chromatin architectural proteins like CCCTC-binding factor (CTCF) or cohesin has only modest effects on gene expression\(^7\)-\(^9\). Additionally, investigations on drosophila embryos have demonstrated that chromatin conformation is independent of gene activation\(^10\),\(^11\). Consequently, it remains uncertain whether changes in chromatin folding directly cause alterations in gene expression. Chromatin organization is highly dynamic and stochastic as revealed by single-cell Hi-C\(^12\)-\(^16\) and microscopy studies\(^17\),\(^18\). Such heterogeneity may arise from variations in the transcriptional or epigenetic state between cells, thus multimodal measurements in single cells will enhance our understanding of the stochasticity and functional implications of genome folding.

Imaging-based techniques offer an inherent advantage by enabling concurrent examination of chromatin and RNA within the same cells. Several methods have been developed to investigate 3D chromatin organization and transcriptional activity at the single-cell level\(^19\)-\(^23\). While current methods are typically restricted to preselected genomic loci and genes, at most recover thousands of genomic loci and one thousand genes. Recently, sequencing-based methods have gained significant attention, and several approaches have been developed\(^24\)-\(^27\). Here we reposted a complementary method called LiMCA, which enables sensitive detection of both chromatin structure and gene expression with low-input samples.

Reagents

Tris-HCl (Invitrogen, AM9855G)
NaCl (Invitrogen, AM9760G)
Nuclease-free water (Invitrogen, AM9937)
MgCl\(_2\) (Invitrogen, AM9530G)
IGEPAL CA-630 (Sigma, I8896)
SUPERaseIn RNase inhibitor (Invitrogen, AM2694)
Dynabeads™ MyOne Carboxylic Acid (Invitrogen, 65011)
SuperScript RTase (Invitrogen, 18064014)
dNTP mix (NEB, N0447S)
rAlbumin (NEB, B9200S)
GTP (Thermo Scientific, R0461)
Betaine (Sigma, B0300-1VL)
KAPA HiFi HotStart ReadyMix (Roche, KK2602)
Paraformaldehyde (EMS, 15714-S)
Glycine (Sigma, G7126)
cOmplete, EDTA-free Protease Inhibitor Cocktail (Sigma, 4693132001)
Sodium dodecyl sulfate (Sigma, 71736)
Triton X-100 (Sigma, 93443)
NlalII (NEB, R0125L)
T4 DNA ligase (NEB, M0202L)
dithiothreitol (Sigma, 646563)
Protease (QIAGEN, 19157)
Tn5 transposase (Vazyme, S111-01)
Nextera Transposome (Vazyme, TD502)
Multiplex Oligos Set 4/5 for Illumina (Vazyme, N321/N322)
Q5 High-Fidelity 2X Master Mix (NEB, M0492L)
DNA Clean and concentrator (ZYMO, D4014)
AMPure XP beads (Beckman, A63882)
40% (w/w) Polyethylene glycol 8000 (Sigma P1458)
Reagents could be stored at 4°C for several months

10% IGEPAL CA-630
1 M NaCl

Reagents could be stored at -20°C for several months

2 M Glycine
Oligos for Reverse transcription and Amplification

Reagents could be stored at -80°C for several months

Template switching oligos
Assembled META20 transposome

Equipment

T100 Thermal Cycler (Bio-Rad)
Benchtop Centrifuge (Eppendorf)
Swing bucket centrifuge (Eppendorf)
Qubit Fluorometric Quantification (ThermoFisher)
Magnetic stands (MosBio)
Fragment analyzer (Agilent)
Micropipetts (0.1-2 μl, 0.5-10 μl, 2-20 μl, 10-100 μl, 20-200 μl, 100-1000 μl)
DNA LoBind tubes (1.5 mL, Eppendorf #L200934G)
MAXYMum Recovery PCR Tubes (Axygen, PCR-02-L-C 0.2mL)

Procedure
A. Day1: preparation of single-cell chromosome conformation capture and mRNA

1. Prepare soft cell lysis buffer (Could be stored at -20°C for several months)

30 μL NaCl (1 M)

25 μL Tris-HCl (1 M, pH 8.3)

45 μL IGEPAL CA-630 (10%)

900 μL Nuclease-free H2O

2. Prepare soft cell lysis buffer supplemented with RNase inhibitor (7 μL per cell, following reagents for 48 cells)

392 μL soft cell lysis buffer

9.8 SUPERaseIn (20 U/μL)

3. Isolate single cells by mouth pipetting

1) Aliqout 7 μL soft cell lysis buffer containing RNase inhibitor to UV-treated MAXYMum Recovery PCR tubes (label as g, such as GM_001g). Keep on ice.

2) Pick single cell into the lysis buffer, and incubate on ice for 30 min.

3) At the end of incubation, vortex for 1 min.

4. Single-cell cytoplasm-nucleus separation

1) Centrifuge at 500 x g for 5 min at 4°C with a swing bucket centrifuge.

2) Carefully transfer 5 μL supernatant into a new UV-treated MAXYMum Recovery PCR tube, (label as c, such as GM_001c). Store at -80°C until cDNA processing.

5. Crosslinking with PFA

1) Prepare 2.5% paraformaldehyde (PFA) by adding 50 μL 32% PFA to 590 μL 1 x PBS.

2) Aliquot 8 μL 2.5% PFA to the remaining 2 μL lysis buffer containing the nucleus.

3) Gently vortex and incubate at room temperature for 10 min.
4) At the end of incubation, add 10 μL 0.25 M glycine (diluted in 1 x PBS) supplemented with 0.2 μL Dynabeads MyOne Carboxylic Acid.

5) Gently vortex and incubate at room temperature for 5 min.

6) Centrifuge at 500 x g for 5 min at 4°C using a swing bucket centrifuge, carefully discard 17 μL supernatant.

6. Restriction enzyme digestion

1) Prepare Hi-C lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.2% IGEPAL CA-630).

2) Wash the crosslinked with 17 μL Hi-C lysis buffer supplemented with 1 x Protease inhibitor.

3) Centrifuge at 500 x g for 5 min at 4°C, carefully discard 17 μL supernatant.

4) Add 2 μL 0.75% SDS, gently vortex and incubate at 62°C for 10 min.

5) At the end of incubation, add 5 μL 4% Triton X-100 and incubate at 37°C for 15 min.

6) At the end of incubation, add 10 μL digestion mix (2 x rCutSmart buffer, 4 U/μL NlaIII).

7) Gently vortex, and incubate at 37 °C for 2 hours.

7. Proximity ligation

1) Centrifuge at 500 x g for 5 min at 4°C, carefully discard 17 μL supernatant.

2) Wash once with 20 μL 1 x T4 DNA ligase buffer.

3) Add 17 μL DNA ligation mix (1x T4 DNA ligase buffer, 10 U/μL T4 DNA ligase, 1x rAlbumin).

4) Gently vortex, and incubate at room temperature for 2.5 hours.

8. Cell lysis

1) At the end of incubation, centrifuge at 500 x g for 5 min at 4°C, carefully remove 18 μL supernatant without disturbing the magnetic beads.

2) Add 2 μL Triton lysis buffer (20 mM Tris-HCl pH 8.0, 40 mM NaCl, 30 mM DTT, 2 mM EDTA, 0.2% Triton X-100, 3 mg/mL QP).

3) Gently cortex, incubate as 50°C for 1 hour, 65°C for 1 hour and 70°C for 15 min.

4) Store at -80°C before amplification.
B. Day2: mRNA and gDNA amplification and library preparation

Part1: cDNA Smart-seq2 amplification and library preparation

1. Reverse transcription

1) Add 1.25 μL oligo-dT and dNTP mix (1 μM oligo-dT30VN (Supplementary Table 1), 2 mM dNTP mix) to 5 μL supernatant containing cytoplasmic mRNA, incubate at 70°C for 5 min and hold at 4°C.

2) Add 7 μL reverse transcription mix (1 x SSII first-strand buffer, 1 U/μL RNase inhibitor, 10 U/μL SSII RTase, 1 mM GTP, 5 mM DTT, 1 M Betaine, 6 mM MgCl2, 1 μM Template switch oligo (Supplementary Table 1)), incubate as 42°C for 90 min and 10 cycles of [50°C, 2 min; 42°C, 2 min], followed by 72°C for 5 min.

2. cDNA amplification

1) After reverse transcription, add add 14.75 μL amplification mix (14 μL KAPA HiFi Hotstart mix, 0.28 μL 10 μM ISPCR primer (Supplementary Table 1), 0.47 μL nuclease-free water).

2) Vortex thoroughly, and incubate as [98°C, 3 min 21 cycles of [98°C, 20 s, 65°C, 30 s, 72°C, 4 min], 72°C, 5 min]

3) After amplification, purify with 0.7x AMPure XP beads, and elute with 20 μL elution buffer. Quantify with Qubit fluorometer.

4) Typical cDNA Amplicon see Fig. 1a.

3. Library preparation with Vazyme TD502

1) Aliquot 5 ng amplified cDNA as input for Tn5 transposition.

2) Prepare transposition mix (2 μL 5 x TTBL, 2.5 μL TTE Nextera transposome, 5.5 μL input cDNA).

3) Mix thoroughly, and incubate at 55°C for 10 min.

4) Add 2.5 μL TSstop buffer.

5) Add 17.5 μL amplification mix (1x Q5 master mix, 0.5 μM UDPxxx i5 index (Supplementary Table 1), 0.5 μM UDPxxx i7 index (Supplementary Table 1)).

6) Amplify as 72°C, 5 min; 98°C, 30s; 9 Cycles[98°C, 10s; 62°C, 30s; 72°C, 1 min], 72°C, 5 min, 4°C hold.

7) Pool multiple cells, and purify with ZYMO DCC5, elute with 50 μL elution buffer.
8) Size selection: first purify with 0.55 x AMPure XP beads, then followed by 0.25 x AMPRe XP beads. Quantify with Qubit fluorometer.

9) Typical cDNA library see Fig. 1b.

4. Sequencing.

cDNA library was sequenced on Illumina NovaSeq 6000 platform with Pair End 150 bp, each cell sequenced with 2 million reads.

**Part2: gDNA whole genome amplification and library preparation**

1. Assemble META 20 transposome.

META transposome was assembled as previously described\(^\text{17, 28}\).

1) Dilute Vazyme Tn5 transposase by adding 60 \(\mu\)L Tn5 storage buffer (50 mM HEPES pH 7.2, 0.1 M NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 50% glycerol) to 10 \(\mu\)L Tn5 transposase (Vazyme, S111-01).

2) Anneal META 20 transposon to nal 5 \(\mu\)M.

Dissolve META 20 Adaptors (Supplementary Table 1) into 50 \(\mu\)M and mix with equal mole. Prepare Annealing mix (50 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 5 \(\mu\)M META 20 Adaptors, 5 \(\mu\)M 19 bp ME). Mix thoroughly, then run the annealing program (95°C, 1 min, gradual cooling, -0.1°C /3s, 700 cycles to 25°C, hold at 4°C).

3. Mix annealed META 20 transposon and diluted Tn5 transposase with equal mole. And incubate at room temperature for 30 min, protected from light.

4. Dilute assembled META 20 transposome tenfold with transposome dilution buffer (50 mM NaCl, 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 20% glycerol).

2. Transposition.

1) Prepare 2 x Trans8k buffer (20 mM Tris-HCl pH 8.0, 10 mM MgCl\(_2\), 16% (w/w) Polyethylene glycol 8000).

2) Add 6 \(\mu\)L transposition mix (5 \(\mu\)L 2 x Trans8k and 1 \(\mu\)L diluted META 20 transposome (final 0.3 nM dimer)) to 4 \(\mu\)L lysis buffer containing gDNA. Mix thoroughly and incubate as 55°C for 10 min, hold at 4°C.
3) Tn5 were removed by adding 2 μL Tn5 removal mix (250 mM NaCl, 37.5 mM EDTA, 2 mg/mL QIAGEN protease), incubate as 50°C, 30 min; 70°C, 15 min.

3. Preamplification

1) Add 14 μL amplification mix (1 x Q5 high-fidelity master mix, 0.1 μM each META 20 primer (Supplementary Table 1), 3.5 mM MgCl$_2$) to each tube.

2) Mix thoroughly and incubate as 72°C, 5 min; 98°C, 30 s; 13 cycles [98°C, 10 s; 65°C, 1 min, 72°C, 2 min], 72°C, 5 min.

4. Adaptor incorporation

1) Add 1 μL EXO1 exonuclease to each tube to remove excess primer, incubate as 37°C, 30 min; 80°C, 20 min.

2) Prepare adaptor incorporation mix (3 μL 2 x Q5 high-fidelity master mix, 2 μL 50 μM META 40 primer mix (Supplementary Table 1)), add 5 μL to each tube, and incubate as 98°C, 30 s; 2 cycles [98°C, 10 s; 65°C, 1 min; 72°C, 2 min], 72°C, 5 min; hold at 4°C

5. Library preparation

1) Add 1 μL EXO1 exonuclease to each tube to remove excess primer, incubate as 37°C, 30 min; 80°C, 20 min.

2) Prepare adaptor library preparation mix (5 μL 2 x Q5 high-fidelity master mix, 2 μL DM5XX, 2 μL DM7XX), add 9 μL library preparation mix to each tube, mix thoroughly and incubate as 98°C, 30 s; 2 cycles [98°C, 10 s; 65°C, 1 min, 72°C, 2 min], 72°C, 5 min, hold at 4°C.

6. Purification

1) Purify with ZYMO DCC5, elute with 20 μL elution buffer, quantify with Qubit fluorometer.

2) Pool multiple cells, further purify with 0.8 x AMPure XP beads.

7. Sequencing

gDNA library was sequenced on Illumina NovaSeq 6000 platform with Pair End 150 bp, each cell sequenced with 10-20 million reads.

**Troubleshooting**

**Time Taken**
Anticipated Results

References


**Figures**
Figure 1

Fig. 1 Amplicon and library fragment size distribution.

a, Typical cDNA amplicon. b, Typical cDNA library. c, Typical gDNA library. Fragments were analyzed with Fragment Analyzer DNF-474.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- LiMCAOligos.xlsx