CoTECH for single-cell joint detection of transcriptome and chromatin occupancy

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Method Article

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Abstract

Here we present CoTECH, a high-throughput co-assay that measures chromatin occupancy and transcriptome in single cells. The CoTECH method adopts a combinatorial indexing strategy to enrich chromatin fragments of interest as reported in CoBATCH in combination with a modified Smart-seq2 procedure to simultaneously capture the 3’ mRNA profiles in the same single cells. The whole experimental procedure can be handled within three days. The CoTECH acquires data quality of 1000-9000 unique mapped reads (DNA partition) and 1500-4000 expressed genes (RNA partition) per cell. Experimentally linking chromatin occupancy to transcriptional outputs and inferred molecular association between multimodal omics datasets made possible by CoTECH enables reconstructions of higher dimensional epigenomic landscape, providing new insights into epigenome-centric gene regulation and cellular heterogeneity in many biological processes. This step-by-step protocol is related to the publication “Single-cell joint detection of chromatin occupancy and transcriptome enables higher-dimensional epigenomic reconstructions” in Nature Methods.

Introduction

Single-cell transcriptome sequencing (scRNA-seq) has revolutionized our understanding of cellular heterogeneity in physiological and pathological biological processes 1-7. The transcriptome and cell identities of different cell types/states with the same DNA are determined by multilayers of epigenetic information. Recently, technologies for single-cell profiling of multi-dimensional chromatin states have been developed, such as various scChIP-seq techniques for DNA-binding proteins and histone modifications 8-12, scATAC-seq for chromatin accessibility 13, MNase-seq for nucleosome positioning 14, scBS-seq for DNA methylome 15 and scHiC for higher-order chromatin structure 16. Although these methods measure multiple modalities of single cells, each provides only specific layers of cellular heterogeneities. To build connections across these layers in single cells, innovative computational platforms emerge to integrate unpaired single-cell omics datasets and project different molecular information into a common latent space 17,18. However, existing strategies require priori knowledge-based correspondence across multimodal omics datasets from different experiments, limiting the ability to reconstruct an accurate view of functional relationship between different modalities of epigenomic features and gene expression as well as the crosstalk between different epigenetic layers.

More recently, unlike those profiling molecular layers one at a time, new approaches have been developed to experimentally link the transcriptome and epigenome by simultaneously measuring multi-omics in the same single cells, making it possible to precisely analyze single-cell-resolved epigenomic regulation of gene expression and cell fate decisions. For example, several various single-cell co-assays (combined scATAC-and scRNA-seq) for joint analysis of accessible chromatin and gene expression have been developed, permitting inference of the correlation between cis-regulatory elements and putative target genes 19-22.
Apart from chromatin accessibility, other important molecular layers of the epigenome are covalent modifications to histones and chromatin occupancy of DNA binding proteins, providing a critical guidance for determining transcriptional outcomes\textsuperscript{23}. Two elegant studies demonstrated the proof-of-concept application of simultaneously quantifying of protein–DNA interactions and transcriptome in single cells by scDam&T-seq and scCC\textsuperscript{24,25}. Both methods rely on a transgene expressing transcription factors or chromatin binding proteins tethered to Escherichia coli DNA adenine methyltransferase (Dam) or piggyBac transposase for scDam&T-seq and scCC, respectively, limiting the likelihood of wide adoption and the potential implementations, in particular for studying clinical human samples. Moreover, the same strategy cannot measure various histone modifications in single cells. Here, we develop a high-throughput method for single-cell joint detection of chromatin occupancy and transcriptome. This approach, named CoTECH (combined assay of transcriptome and enriched chromatin binding), adopts a combinatorial indexing strategy\textsuperscript{9,26} to enrich chromatin fragments of interest as reported in CoBATCH in combination with a modified Smart-seq2 procedure\textsuperscript{27} to simultaneously capture the 3' mRNA profiles in the same single cells. To demonstrate the utility of CoTECH, we used it to study the relationship between multiple histone modifications and gene expression in mouse embryonic stem cells (mESCs) and the regulatory basis of endothelial-to-hematopoietic transition (EHT) in two waves of hematopoietic cells. Experimentally linking chromatin occupancy to transcriptional outputs and inferred molecular association between multimodal omics datasets made possible by CoTECH enables reconstructions of higher dimensional epigenomic landscape, providing new insights into epigenome-centric gene regulation and cellular heterogeneity in many biological processes.

**Reagents**

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

Sodium butyrate (SIGMA, 303410-100G)

Trypsin (Gibco, 27250-018)

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 (MgCl₂) (SIGMA, 68475-100ML)
Potassium chloride (KCl) (XiLong SCIENTIFIC, 7447-40-7)
Potassium hydroxide (KOH) (XiLong SCIENTIFIC, 1310-58-3)
Calcium chloride dihydrate (CaCl₂) (XiLong SCIENTIFIC, 10035-04-8)
Sodium chloride (NaCl) (SIGMA, 303410-100G)
Spermidine (Sigma, S2501-1G)
Concanavalin A coated beads (Bangs Laboratories, BP531)
Manganese (II) chloride tetrahydrate (MnCl₂) (XiLong SCIENTIFIC, 13446-34-9)
Digitonin (Sigma, Cat# D141-500MG)
Bovine Serum Albumin (Sigma, A1933-100G)
dNTP mix (Fermentas, R0192)
SuperScript IV reverse transcriptase (Invitrogen, 18090200)
Exonuclease I (NEB, M0293L)
Recombinant RNase inhibitor (Takara, 2313B)
Betaine (Sigma, B3501)
DAPI (Invitrogen, S36942)
PEG 8000 (Sigma, 89510-250g-F)
N,N-Dimethylformamide (DMF) (SIGMA, D4551-250ML)
SODIUM DODECYL SULFATE (SDS) (AMRESCO, 0227-1KG)
Proteinase K (AMRESCO, 0706-100MG)
KAPA HiFi DNA polymerase (BIOSYSTEMS, KK2102)
AMPure XP (BECKMAN COULTER, A63881)
H3K27me3 antibody (Millipore, Cat.No: 07-449)
H3K27ac antibody (Abcam, Cat.No: ab4729)
H3K4me3 antibody (Millipore, Cat.No: 04-745)
PE/Cy7 anti-mouse CD31 antibody (BioLegend, Cat.No: 102524)
Rabbit Control IgG antibody (Abclonal, Cat.No: AC005)
Donkey anti-Rabbit-555 antibody (Invitrogen, Cat.No: A31572)
TIANquick Mini Purification Kit (TIANGEN, DP203-02)
QIAquick Gel Extraction kit (Qiagen, 28706)

**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
CFX96 Real-Time System

**Procedure**

**Sample preparation**

1. Harvest cells (from tissues or cultured cell line), digest cells into single-cell suspension, wash and resuspend cells by PBS or 1%BSA/PBS. Fix cells by adding -20°C methanol (Sigma) drop by drop to a final concentration of 90%. The prepared sample can be stored at -80°C for later use.

*Attention: Buffers and reagents used below are RNase free.*
**Binding cells to Concanavalin A beads**

2. ConA beads activation: Wash 5-20 µl Concanavalin A (ConA) beads with 1 ml Binding Buffer (20 mM HEPES pH 7.9, 4 mM KCl, 0.4 mM CaCl$_2$ and 0.4 mM MnCl$_2$) twice. Resuspend beads with 5-20 µl Binding Buffer before apply them to the cell mixture.

   *Attention: Wash tubes with 1% BSA/PBS prior to use and use low-retention tubes if possible. This operation can prevent cell/beads loss.*

3. Centrifugate the cells at 1000 G, 4°C for 5 min. Resuspend the cells with 1 ml Wash Buffer (20 mM HEPES pH 7.5, 300 mM NaCl, 0.5 µM spermidine and 10 mM sodium butyrate) and add 5~20 µl activated ConA beads to the cell suspension.

4. Incubate the cells-beads mixture at RT for 10-20 min.

**Binding antibody**

5. Collect cells by magnetic stand. Wash cells by 1 ml Wash Buffer once, put the tube on the magnetic stand and pull off all liquid.

6. Resuspend cells with 100 µl Antibody buffer (supplied 2 mM EDTA, 0.01% Digitonin, 0.05% TX-100, 2% proteinase inhibitor cocktail (Roche), 1% recombinant RNase inhibitor (TAKARA) in Wash Buffer). Add 0.5 µl antibody against protein of interest, and incubate the cell suspension at 4°C for ~3h.

   *Attention: (Optional) Binding a 2$^{nd}$ antibody is recommended if 1$^{st}$ antibody has low affinity against protein A.*

**Binding PAT**

7. Wash cells twice with 180 µl Dig-Wash Buffer without TX-100 (Wash Buffer supplemented with 0.01% Digitonin) and resuspend cells with 500 µl 1% BSA / PBS.
8. Prepare PAT incubation plate: Add 70 µl Dig-Wash buffer to each well which contains individual combinatorial barcoded 6 µg/ml PAT-T5 and 6 µg/ml PAT-T7.

Attention: Dilute 37.5 µM stocked barcoded PAT by Dig Wash Buffer (Wash Buffer supplemented with 0.01% Digitonin, 0.05% TX-100, 2% proteinase inhibitor cocktail (Roche), 1% recombinant RNase inhibitor (TAKARA) and 1 mM DTT) at 1:445 to a final concentration of 6 µg/ml. Add 35 µl diluted PAT-T5 and 35 µl diluted PAT-T7 to each well.

9. FACS or pipette 500-2,000 cells into each well.

Attention: Cell number in each well should be equivalent, which can be scaled from 500/well to 2,000/well

10. Place the plate on a rotator and incubate at 4°C for 1 h.

11. Put the plate on a 96-well magnetic stand and pull off all liquid. Wash cells twice by 180 µl Dig-Wash buffer (Wash Buffer supplemented with 0.01% Digitonin and 0.05% TX-100).

Attention: Change tips every pipetting to prevent barcode contamination.

**Targeted tagmentation**

12. Resuspend cells with 10 µl cold Reaction Buffer (10 mM TAPS-NaOH pH 8.3, 5 mM MgCl₂, 2% proteinase inhibitor cocktail, 1% Recombinant RNase inhibitor, 1 mM DTT and 10 mM sodium butyrate) by pipetting.

13. Tagmentation: Incubate the plate at 25°C for 1 h in a thermal cycler. The reaction system is gently mixed once after 30-min incubation.

14. Add 10 µl 40 mM EDTA to each well and mix well. Incubate the plate at 4°C for 15 min.
15. Add 1 µl 250 mM MgCl₂ to each well and incubate the plate at 4°C for 5 min.

16. Collect cells by magnetic stand. Briefly wash cells by 100 µl 1% BSA/PBS and discard all liquid.

Reverse transcription

17. Add 2 µl barcoded RT-lysis Buffer (1.2 µl PBS, 0.5 µl 10 mM dNTP mix, 0.05 µl RNase inhibitor (Takara), 0.25 µl 5 µM barcoded oligo dT, and 0.01% Digitonin) to each well, and gently pipette. 

Attention: RT lysis Buffer should be prepared before the experiment. One kind of barcoded oligo dT primer is added to one well individually, which makes all 96-wells carrying different barcoded oligo dT. Carefully note the corresponding PAT-barcode and oligo dT-barcode for each well.

18. Incubate the plate at 55°C for 5 min and immediately put the plate onto a cold block to cool down.

19. Add 2.85 µl RT-mix (0.20 µl SuperScript IV reverse transcriptase (Thermo Fisher), 0.125 µl RNase inhibitor (Takara), 1 µl 5 x SuperScript IV first-strand buffer (Thermo Fisher), 0.25 µl 100 mM DTT, 1 µl 5M Betaine (Sigma), 0.05 µl 100 µM TSO (Table S1) and 0.195 µl PBS, 0.03 µl 1M MgCl₂, and 0.01% Digitonin) to each well. Perform reverse transcription as below: 4°C 2 min, 10°C 2 min, 20°C 2 min, 30°C 2 min, 42°C 10 min, 50°C 2 min 55°C 5 min, and hold at 4°C.

20. Add 5 µl cold Sorting Buffer (PBS supplemented with 2% BSA and 2 mM EDTA) to each well. Combine all cells from 96 wells into a 1.5 ml tube. Wash cells with 1 ml 1% BSA/PBS.

21. Add DAPI(Thermo) to a final concentration of 1.2 µg/ml and stain cells on ice for 15 min.

Redistributing cells and Releasing DNA

22. Filter the cells through a 70 µm cell strainer to remove cell clumps, and sort 20~25 cells into each well of a new 96-well plate which contains 3 µl Lysis Buffer (10 mM Tris-HCl pH 8.5, 0.05 % SDS, 0.1 mg/ml proteinase K) in each well.
23. Incubate the plate at 55°C for 60 min.

24. Add 1 µl 1.8% Triton X-100 together with 1µl 10 mM PMSF to each well, and incubate the plate at 55°C for 5 min to quench SDS.

**Pre-amplification**

25. Add 20 µl PCR mix (5 µl 5 x KAPA HiFi Buffer, 0.5 µl 10 mM dNTP Mix, 0.75 µl 10 µM IS primer (AAGCAGTGGTATCAACGCAGAGT), 0.75 µl 10 µM 3’P2 primer (GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC), 0.25 µl 1 U/µl KAPA HiFi DNA Polymerase (KK2102), 0.5 µl 25 mM MgCl₂ and 12.25 µl ddH₂O) to each well. The PCR enrichment was performed as below: 1 cycle of 72°C 5 min; 1 cycle of 95°C 3 min; 4 cycles of: 98°C 20 s, 65°C for 30 s, 72°C for 5 min; 7 cycles of: 98°C 20 s, 67°C 15 s, 72°C for 5 min; hold at 4°C. Immediately add 0.5 µl total 10 µM connector A/B primer mix(connector A: TCGTCGGCAGCGTCTCCACGC; connector B: GTCTCGTGCCGCTGGCTGTCCC) to each well followed by: 5 cycles of: 98°C 20 s, 67°C 15 s, 72°C for 5 min; 1 cycle of 72°C for 5 min; hold at 4°C.

26. Prepare custom 1:10 diluted AMPure XP beads: Pipette 100 µl original AMPure XP beads to a new tube, put it on magnetic stand, and resuspend beads in 1000 µl AMPure binding buffer (20% PEG 8000, 2.5 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA and 0.05% Tween 20).

27. Add 0.8X (20 µl) custom XP beads to each well and vortex thoroughly. DNA was purified and eluted with 10.5 µl ddH₂O. Transfer 5 µl DNA elution to a new plate for DNA-partition library preparation and 5 µl DNA elution for RNA-partition library preparation.

**DNA-part library preparation**

28. Add 15 µl PCR mix (0.2 µl KAPA HiFi (not HotStrat) DNA Polymerase, 4 µl 5 x KAPA High-GC Buffer, 1 µl 10 mM dNTP Mix, 0.4 µl 25 mM MgCl₂, 8.9 µl ddH₂O and 0.5 µl total 50 µM primer mix (forward: AACTCTTTCCCTACACGACGCTCTTCCGATCTTCTGTCGGCAGCGTCTCCACGC; reverse: GACTGGAGTTTCAGACGTTGCTCTTCCGATCTTCTGTCGGCAGCGTCTCCACGC) to each well. The PCR
enrichment was performed as below: 1 cycle of 95°C 3 min; 8 cycles of: 98°C 20 s, 65°C for 30 s, 72°C for 1 min; 1 cycle of 72°C 5 min; hold at 4°C.

29. Add 2 µl 1:8 diluted ExoI (NEB) to each well and incubate the plate at 37°C for 60 min followed by 72°C for 20 min.

30. Add 10 µl PCR mix (0.2 µl KAPA HiFi HotStart DNA Polymerase, 2 µl 5 x KAPA High-GC Buffer, 0.2 µl 25 mM MgCl₂, 5.6 µl ddH₂O, 1 µl 10 mM Truseq index i5 and 1 µl Truseq index i7) to each well. PCR enrichment was performed as below: 1 cycle of 95°C 3 min; 5 cycles of: 98°C for 20 s, 65°C for 30 s, 72°C for 1 min; 1 cycle of 72°C for 5 min; hold at 4°C.

31. Combine all the PCR product into a 50 ml tube, purify DNA by TIANquick Mini Purification Kit (TIANGEN), and elute DNA in 60 µl ddH₂O.

32. Purify the library once with 0.8X AMPure XP beads.

33. Size selection: Perform gel extraction for the fragments at the size of 400-1,000 bp on 1.5% agarose gel. Finally purify DNA by 0.8 × AMPure XP beads (Beckmann) and elute with 20 µl ddH₂O.

34. Sequence the libraries with paired-end 150-bp reads on Novaseq 6000 platform (Illumina).

RNA-part library preparation

35. Add 2.5 µl tagmentation mix (0.75 µl 10X TAPS-MgCl₂, 0.75 µl DMF and 1 µl Tn5-MEA (25 nM)) to each well, and incubate the plate at 55°C for 5 min in a thermal cycler.

36. Add 1 µl 0.2% SDS and 0.4 µl BSA (NEB) to each well, and incubate the plate at 55°C for 5 min.
37. Add 1 µl 1.8% TX-100 to each well to quench SDS, and incubate the plate at 55°C for 5 min.

38. Add 28.1 µl PCR mix (0.4 µl KAPA HiFi (not HotStart) DNA Polymerase, 8 µl 5 x KAPA High-GC Buffer, 1 µl 10 mM dNTP Mix, 0.8 µl 25 mM MgCl₂ and 17.9 µl H₂O), 1 µl 10 mM Nextera index i5 primer and 1 µl Truseq index i7 primer separately to each well. PCR enrichment was performed as below: 1 cycle of 72°C for 5 min; 1 cycle of 95°C for 3 min; 11 cycles of: 98°C for 20 s, 65°C for 30 s, 72°C for 1 min; 1 cycle of 72°C for 5 min; hold at 4°C.

39. Combine all the PCR product into a 50 ml tube; purify DNA by TIANquick Mini Purification Kit (TIANGEN); elute DNA in 60 µl ddH₂O.

40. Purify the library once with 0.8X (48 µl) AMPure XP beads.

41. Size selection: Perform gel extraction for the fragments at the size of 400-1,000 bp on 1.5% agarose gel. Finally purify DNA by 0.8 × AMPure XP beads (Beckmann) and elute with 20 µl ddH₂O.

42. Sequence the libraries with paired-end 150-bp reads on Novaseq 6000 platform (Illumina).

**Troubleshooting**

**Time Taken**

**Day 1:**

- **Cell treatment (0.5 h)**
- **Binding cells to Concanavalin A beads (1 h)**
- **Binding antibody (4 h)**
- **Binding PAT (1.5 h)**
- **Targeted tagmentation (1.5 h)**
- **Reverse transcription (1 h)**
Redistributing cells and Releasing DNA (1.5 h)

Day 2:

Pre-amplification (3.5 h)

DNA-partition library preparation (step 28-30) (3.5 h)

RNA-partition library preparation (step 35-38) (2 h)

Day 3:

DNA-partition library preparation (step 31-34) and RNA-partition library preparation (step 39-42) (8 h)

Anticipated Results

1. The DNA partition library exhibits good ChIP-qPCR enrichment before sequence.
2. Highly expressed genes can be enriched in pre-amplification products detected by qPCR
3. Size of library fragments should be around 300-1000 bp
4. Sequence results shows good enrichment of positive signals

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