Single-cell joint profiling of open chromatin and transcriptome by Paired-seq

Chenxu Zhu[1], Zhaoning Wang[1], and Bing Ren[1,2,*]

[1]Ludwig Institute for Cancer Research, La Jolla, California, USA

[2]Center for Epigenomics, Department of Cellular and Molecular Medicine, Institute of Genomic Medicine, Moores Cancer Center, University of California San Diego, School of Medicine, La Jolla, California, USA

[*]Correspondence: biren@health.ucsd.edu

Running Head: Single-cell co-assay of open chromatin and RNA

Abstract

Simultaneous detection of chromatin accessibility and transcription from the same cells promises to greatly facilitate the dissection of cell-type-specific gene regulatory programs in complex tissues. Paired-seq enables joint analysis of open chromatin and nuclear transcriptome from up to a million cells in parallel. It achieves ultra-high throughput single cell multiomics with the use of a combinatorial barcoding strategy involving sequential ligation of multiplexed DNA barcodes to chromatin DNA fragments and reverse transcription products, followed by high throughput DNA sequencing of the resulting DNA libraries and deconvolution of single cell multiomic maps based on cell-specific barcodes.

Key words

Paired-seq, single-cell multiomics, chromatin accessibility, gene expression, epigenome

1 Introduction

Cis-regulation elements (CREs) play a fundamental role in gene regulation. In eukaryotic cells, binding of transcription regulators to CREs leads to depletion of nucleosome and hypersensitivity to nucleases (such as DNase I or Micrococcal nucleases) and Tn5 transposases [1-3]. Methods exploring the hyper sensitivity of active CREs have been developed to map these sequences in the genome, including DNase I hypersensitive sites sequencing (DNase-seq)[4], micrococcal nuclease digestion with deep sequencing (MNase-seq)[5], FAIRE-seq[6], and assay for transposase-accessible chromatin using sequencing (ATAC-seq)[7][8]. The advancement of single-cell chromatin accessibility assays using droplet-based or combinatorial barcoding strategies [9-14] has enabled deconvolution of cell type-specific transcriptional programs from mixed cell populations and primary tissues [15]. However, measuring individual molecular modalities one at a time in single cells does not permit a full view of the gene regulatory process in complex tissues and pathogenesis[16,17]. Co-assay of gene expression together with DNA methylation[18], histone modification[19], chromatin accessibility[20], or high-order chromatin conformation[21], can lead to a better understanding of cell-type-specific gene regulatory programs and enable a better assessment of the role of epigenome in transcriptional regulation of each gene. Several methods have now been reported to enable joint analysis of nuclear transcriptome and accessible chromatin in individually isolated cells[22], or thousands of single cells with plate-based combinatorial indexing[20] and droplet-based barcoding[23].

Paired-seq is a scalable single-cell technology that can assay gene expression and chromatin accessibility for up to a million single cells in parallel[24] with the use of a ligation-based combinatorial indexing strategy[25]. It begins with fragmentation of open chromatin by the Tn5

transposases followed by reverse transcription of nuclear mRNA by reverse transcriptase. DNA barcodes are then subsequently ligated *in situ* to the chromatin fragments and reverse transcription products (cDNA) in each nucleus through a mix-and-polling scheme in 96-well plates. Following nuclei lysis, the chromatin DNA and cDNA are amplified, and then split into two separate libraries corresponding to each molecular modality for next-generation DNA sequencing (Fig. 1). The entire Paired-seq procedure not including DNA sequencing spans two days. With a reasonable sequencing depth (number of sequenced reads per nuclei: 25,000 for DNA and 50,000 for RNA), Paired-seq can generate single-cell multiomics profiles with ~5,000 unique tagmentation loci and ~10,000 unique transcripts per nucleus.

[Fig 1 near here]

2 Materials

2.1 Reagents preparation

1. Tn5 protein were purified according to ref [26]
2. RT primers (Supplementary Table1)
3. Tn5 barcodes (Supplementary Table1)
4. Barcode oligos (Supplementary Table1)
5. Tris-HCl, pH 7.5 (Invitrogen, Cat#15567027)
6. NaCl (Sigma, Cat#S7653)
7. Glycerol (Sigma, Cat#G5516)
8. DTT (Sigma, Cat#D9779)
9. 200 µL thin wall PCR tubes (USA Scientific, Cat#1402-3900)
10. 1.5 mL low-bind tubes (Eppendorf, Cat#022431021)
11. 15 mL tubes (Sigma, Cat# CLS430791)
12. 96-well low-bind PCR plate (Eppendorf, Cat# 0030129512)
13. Sterile Reagent reservoir (Corning Costar, Cat#07200127)
14. Thermocycler (Bio-Rad, T100)

2.2 Nuclei isolation

1. Douncing buffer (DB) (1.5 mL per sample)

Reagents	Stock concentration	Volume	Final Concentration
Sucrose (Sigma #S7903)	1 M	0.375 mL	250 mM
KCl (Sigma #P9333)	2 M	18.8 µL	25 mM
$MgCl_2$ (Sigma #63069)	1 M	7.5 µL	5 mM
Tris-HCl, pH 7.5 (Invitrogen #15567027)	1 M	15 µL	10 mM
DTT	1M	1.5 µL	1 mM

Protease Inhibitor (Roche #4693132001)	50X	30 µL	1 X
SUPERase IN (Invitrogen #AM2696)	20 U/µL	37.5 µL	0.5 U/µL
RNase OUT (Invitrogen #10777019)	40 U/µL	18.8 µL	0.5 U/µL
H_2O	NA	996 µL	NA

2. Nuclei isolation buffer (NIB) (1 mL per sample)

Reagents	Stock concentration	Volume	Final Concentration
IGEPAL CA-630 (Sigma #I8896)	10%	20 µL	0.2 %
BSA in DPBS (Sigma #A1595)	10%	0.5 mL	5 %
Protease Inhibitor	50X	20 µL	1 X
SUPERase IN	20 U/µL	25 µL	0.5 U/µL
RNase OUT	40 U/µL	12.5 µL	0.5 U/µL
DPBS (Gibco #14190136)	1 X	422.5 µL	NA

3. 5% Triton-X100 (diluted from Sigma, Cat#T9284)

4. Dounce tissue grinder set (1.0 mL) (KIMBLE, Cat# DWK885300-0001)

5. Celltrics filters (30 µm) (Sysmex Cat#04-0042-2316)

6. Axygen Maximum Recovery tube (Corning Cat# MCT-150-L-C)

7. TC20 Cell Counter (BioRad)

8. 1.5 mL low-bind tubes (Eppendorf Cat# 022431021)

<u>2.3 Chromatin tagmentation</u>

1. 10 mM PitStop2 (Millipore, Cat#SML1169)

2. 2X Tagmentation Buffer (10 mL, store at 4 °C)

Reagents	Stock concentration	Volume	Final Concentration
Tris-Ac, pH 7.5 (Sigma, Cat#93337)	1 M	660 µL	66 mM
KAc (Sigma, Cat#P5708)	3 M	440 µL	132 mM

$MgAc_2$ (Sigma, Cat#M2545)	1 M	200 µL	20 mM
DMF (Millipore, DX1730)	NA	3,200 µL	32%
Ultrapure H_2O	1 X	5,500 µL	NA

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95 3. Tagmentation Mix

Reagents	Stock concentration	Volume
2X Tagmentation Buffer	2X	66 µL
RNase OUT	40 U/µL	3.3 µL
SUPERase IN	20 U/µL	6.6 µL
Proteinase Inhibitor cocktail	50 X	2.7 µL
PitStop2 (Sigma, Cat#SML1169)	10 mM	1 µL
Ultrapure H_2O		36.6 µL

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97 4. 40 mM EDTA (diluted from Invitrogen, Cat#AM9261)

98 5. Loaded Tn5 (Step 3.1-3)

99 6. ThermoMixer (Eppendorf ThermoMixer R)

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101 2.4 Reverse transcription

102 1. NEBuffer 3.1 (NEB, Cat#B7203S)

103 2. Maxima H minus reverse transcriptase (Invitrogen, Cat#EP0751)

104 3. 5% Triton-X100 (diluted from Sigma, Cat# T9284)

105 4. RT Mix

Reagents	Stock concentration	Volume
5X RT Buffer (with Maxima H minus reverse transcriptase)	5X	52.8 µL
PBS	1X	52.8 µL
dNTP	10 mM	13.2 µL
RNase OUT	40 U/µL	1.65 µL
SUPERase IN	20 U/µL	3.3 µL
Ultrapure H_2O	NA	61 µL

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5. Thermocycler (Bio-Rad, T100).

2.5 Adding DNA barcodes

1. Ligation Mix

Reagents	Stock concentration	Volume
T4 DNA Ligase Buffer (NEB, Cat#B0202S)	10X	500 µL
BSA (NEB, Cat#B9000S)	20 mg/mL	50 µL
NEBuffer 3.1 (NEB, Cat#B7203S)	10 X	100 µL
Ultrapure H_2O	NA	2,250 µL

2. R02 Blocking Solution (see Step 3.1-6)

3. R03 Termination Solution (see Step 3.1-6)

4. T4 DNA Ligase (NEB, Cat#M0202L)

5. R02 Barcoding Working Plate (Step 3.1-4)

6. R03 Barcoding Working Plate (Step 3.1-4)

7. Proteinase K (NEB, Cat#P8107S)

8. SPRI beads (Beckman Coulter, Cat#B23319)

9. 80% EtOH

10. 200 µL thin wall PCR tubes or 96-well PCR plate

11. Eppendorf ThermoMixer

12. PCR plate film (Bio-Rad, Microseal B, Cat#MSB1001)

2.6 Library pre-amplification

1. Terminal Transferase (NEB, Cat#M0315S)

2. 1 mM dCTP (NEB, Cat#N0446S)

3. Anchor Mix (15 µL per sample)

Reagents	Stock concentration	Volume
5X KAPA reaction buffer	5X	6 µL
dNTP	10 mM	0.6 µL
Anchor-FokI-GH (Supplementary Table1)	10 µM	0.6 µL
Ultrapure H_2O	NA	7.2 µL
KAPA HiFI HS (KAPA, Cat#KK2502)	NA	0.6 µL

3. Preamp Mix (20 µL per sample)

Reagents	Stock concentration	Volume
5X KAPA reaction buffer	5X	4 µL
dNTP	10 mM	0.5 µL
PA-F (Supplementary Table1)	10 µM	2 µL
PA-R (Supplementary Table1)	10 µM	2 µL
Ultrapure H_2O	NA	11 µL
KAPA HiFI HS (KAPA, Cat#KK2502)	NA	0.5 µL

4. Qubit dsDNA HS Assay Kit (Invitrogen Q32854)

5. SPRI beads (Beckman)

6. Qubit (ThermoFisher Scientific, Cat#Q33239)

7. 200 µL thin wall PCR tubes

8. Thermocycler

2.7 Library splitting

1. FokI (NEB, Cat#R0109S)

2. NotI-HF (NEB, Cat#R3189)

3. SbfI-HF (NEB, Cat#R3642)

4. Adaptor Mix (see Step 3.1-5)

5. Nextera XT DNA library preparation kit (Illumina, Cat#FC-131-1024)

6. SPRI beads (Beckman)

7. 200 µL thin wall PCR tubes

8. Thermocycler

9. Magnetic separation rack (Bel-Art, Cat#F19900-0003)

2.8 Library amplification

1. Illumina TruSeq i7 index primers (Supplementary Table1)

2. Illumina TruSeq i5 index primers (Supplementary Table1)

3. Illumina Nextera i5 index primers (Supplementary Table1)

4. NEBNext 2X HiFi PCR master mix (NEB, Cat#M0541S)

5. KAPA qPCR quantification kit for Illumina (KAPA, Cat#KK4923/4933/4943/4953/4973)

6. SPRI beads

7. 200 µL thin wall PCR tubes

8. Thermocycler

9. Magnetic separation rack

10. Agilent Tapestation (Agilent 4200)

2.9 Sequencing and data preprocessing

1. Illumina Sequencer: HiSeq 2500/4000, NextSeq 550/2000 and NovaSeq 6000 were tested compatible with Paired-seq libraries.

2. Computation resources: a server with 16 cores and 128 B RAM or above is recommended; storage space dependents on the number of cells analyzed, typically 1TB of storage is needed for analysis of 100 k cells.

3 Methods

3.1 Reagents preparation

1. All oligo DNA sequences in this section are listed in Supplementary Table1. To prepare the RT primer mix, mix 12.5 µL of barcoded T15VN primer (RNA_#XX_RE, 100 µM), 12.5 µL barcoded N6 primer (RNA_#XX_NRE, 100 µM) and 75 µL ultrapure nuclease-free water in PCR tubes. Vortex to mix and store at -20 °C.

2. To prepare barcoded Tn5 adaptors, mix 10 µL barcoded Tn5 adaptor (DNA_#XX_RE, 100 µM) and 10 µL pMENTs (100 µM) in PCR tubes. Using a thermocycler, heat the mix at 95 °C for 5 mins and slowly cool down to 20 °C (0.1 °C/s). Store the annealed adaptors at -20 °C or immediately use for step 3.

3. To prepare barcoded Tn5-complex, add 5 µL of barcoded annealed Tn5 adaptors (from step 2) to 1.5 mL low-bind tubes. Add 35 µL 0.5 mg/mL unloaded Tn5 protein to each tube and pipette to mix 5 times. Then vortex to mix for 3-5 s and spin-down quickly. Incubate at room temperature for 30 mins, then transfer to on ice and sit for 5 mins. Store at -20 °C.

4. To prepare R02 and R03 barcode plates, add 6 µL of R02 or R03 barcoded oligo (BC Plate#02 or BC Plate#03, 100 µM), 5.5 µL of Linker-R02 or Linker-R03 oligo (100 µM) and 38.5 µL ultrapure nuclease-free water to each well of a low-bind 96-well PCR plate, seal the plate (annealing plate). Heat at 95 °C for 5 mins and slowly cool to 20 °C (0.1 °C/s). Aliquot 10 µL of

annealed barcoded oligos from each well of annealing plate to 4 low-bind 96-well PCR plates (working plates). Store the working plates at -20 °C.

5. To prepare Adaptor Mix: (a) prepare P5-complex (25 µL 100 µM P5-FokI and 25 µL 100 µM P5c-NNDC-FokI) and P5H-complex (25 µL 100 µM P5H-FokI and 25 µL 100 µM P5Hc-NNDC-FokI) in two different tubes; (b) in a thermocycler, heat the mixtures for 5 mins at 95 °C and slowly cool down to 20 °C (-0.1°C/s); (c) mix 15 µL of P5-complex with 45 µL of P5H-complex on ice and pipette to mix, then add 240 µL cold ultrapure water (to dilute from 50 µM to 10 µM) and store at -20 °C.

6. To prepare R02 blocking solution, add 264 µL 100 µM Blocker-R02, 250 µL 10X T4 DNA Ligase Buffer and 486 µL ultrapure water to a 1.5 mL tube and mix. To prepare R03 termination solution, add 264 µL 100 µM Quencher-R02, 500 µL 0.5 M EDTA and 236 µL ultrapure water to a 1.5 mL tube and mix. Both R02 blocking solution and R03 termination solution should be kept on ice for later use.

3.2 Nuclei isolation

1. Preparation of single-cell resuspension is required for nuclei isolation, which has different preferred protocols[27]. Here we take nuclei preparation from frozen mouse brain as an example.

2. For each sample, prepare the 1.5 mL douncing buffer (DB) and 1 mL nuclei isolation buffer (NIB) freshly each time before performing the experiments. Prechill any tubes or tools. Set the centrifuge to 4°C.

3. Washing the douncer with 1mL of ultrapure water. Prechill the dounce and pestle (1 mL) on ice (avoid contamination by placing them on a parafilm or in a tube).

4. Add 0.5 mL of DB into douncer, and add 10 µL 5% Triton X-100.

5. Transfer ~20-50 mg dissected frozen mouse brain tissue directly to the douncer with DB.

6. Apply the loose pestle gently 5-10 times on ice, and avoid introducing bubbles.

7. Apply the tight pestle gently 15-30 times on ice, and avoid introducing bubbles.

8. Filter the single-cell suspension with a 30 µm Celltrics filter into a 1.5 mL Axygen Maximum Recovery tube. Spin down at 1,000 g for 10 mins at 4°C and carefully discard the supernatant.

9. Gently resuspend the cell pellet in 0.5 mL of NIB. Spin down again at 1,000 *g* for 10 mins at 4°C, and discard the supernatant.

10. Gently resuspend the cell pellet in 0.5 mL of NIB and incubate on ice for 5-10 mins. Take out 10 µL to measure the nuclei concentration with the cell counter.

<u>3.3 Chromatin tagmentation</u>

1. Freshly prepare the Tagmentation Mix and keep on ice.

2. Label 12 tubes for tagmentation. Aliquot a total of 1,200 – 2,400 k nuclei into 12 tubes on ice, each tube with 100 - 200 k nuclei. Different samples or replicates can be multiplexed here, differed by their 1st round barcode (sample barcode).

3. Spin down the 12 tubes at 1,000 g for 10 mins at 4°C, and carefully discard the supernatant. Samples should be kept on ice.

4. For each tube, resuspend the nuclei pellet in 9 µL Tagmentation Mix. Add 1 µL of barcoded Tn5 into the corresponding tube.

5. Incubate in a ThermoMixer set at 37°C, 550 r.p.m. for 30 mins.

6. Immediately add 5 µL of 40 mM EDTA and gently pipette to mix. Spin down at 1,000 g for 10 mins at 4°C, and carefully remove all the supernatant. Keep the nuclei on ice and proceed to Step 3.4 Reverse transcription immediately.

3.4 Reverse transcription

1. Freshly prepare the RT mix and keep on ice.

2. Add each of the 4 µL barcoded RT primers into the 12 corresponding 200 µL PCR tubes.

3. Resuspend the 12 tubes of nuclei pellet with 14 µL RT mix and transfer to 12 200 µL PCR tubes from the previous step with barcoded RT primers.

4. Add 2 µL Maxima H minus reverse transcriptase to each tube. Tap to mix and briefly spin-down.

5. Perform the reverse transcription program in a thermocycler using the program set up as below:

Step #	Temperature (°C)	Time
1	50	10 mins
2	8	12 s
	15	45 s
	20	45 s
	30	30 s
	42	2 mins
	50	5 mins; repeat step2 for additional 2 cycles
3	50	10 mins
4	12	hold

6. Transfer the 12 tubes to ice. Keep on ice and pool all nuclei into a 1.5 mL Axygen Maximum recovery tube, add 4.8 µL 5% Triton X-100, tap to mix and quickly spin-down.

7. Centrifuge to pellet the nuclei at 1,000 *g* for 10 mins at 4°C, and carefully discard the supernatant.

8. Resuspend the nuclei in 1 mL 1X NEBuffer 3.1, and proceed to Step 3.5 Nuclei barcoding immediately.

3.5 Adding DNA barcodes

1. Prepare R02 Blocking Solution, R03 Termination Solution and two tubes of Ligation Mix freshly before the experiment.

2. Prewash two 15 mL Corning tubes by rinsing each tube with 0.5 mL 0.1% BSA in PBS, and discard the liquid.

3. Add the nuclei suspension to the 1st Ligation Mix, add 100 µL T4 DNA Ligase and gently mix by pipetting up and down.

4. Transfer the nuclei-Ligation Mix to a reagent reservoir, and distribute 40 µL of the mixture to each of the 96 wells of R02 Barcoding Plate with a multi-channel pipette. Seal the plate with film.

5. Incubate the nuclei-barcode ligation mixture in a ThermoMixer set to 37°C, 300 r.p.m. for 30 mins.

6. Open the seal, add 10 µL of R02 Blocking Solution into each of the 96 wells with multi-channel pipette and re-seal the plate.

7. Continue incubating the nuclei – barcode ligation mixture in a ThermoMixer set to 37°C, 300 r.p.m. for another 30 mins.

8. Pool all nuclei in a reagent reservoir, and transfer the mixture containing the nuclei from the reagent reservoir to a 15 mL tube (prewashed with 0.1% BSA in PBS).

9. Wash the reagent reservoir with 1 mL of PBS and combine to the nuclei mixture.

10. Spin down the nuclei with a swing bucket centrifuge at 1,000 *g* for 10 mins at 4°C, and carefully discard the supernatant.

11. Resuspend the nuclei in 1 mL 1X NEBuffer 3.1.

12. Transfer the nuclei suspension to the 2nd Ligation Mix, add 100 µL T4 DNA Ligase and gently mix by pipetting up and down.

13. Transfer the nuclei-Ligation Mix to a reagent reservoir, and distribute 40 µL of the mixture to each of the 96 wells of R03 Barcoding Plate with a multi-channel pipette. Seal the plate.

14. Incubate the nuclei-barcode ligation mixture in a ThermoMixer set to 37°C, 300 r.p.m. for 30 mins.

15. Open the seal, add 10 µL of R03 Termination Solution into each of the 96 wells with a multi-channel pipette.

16. Immediately pool all nuclei in a reagent reservoir, and transfer the mixture containing the nuclei from the reagent reservoir to a 15 mL tube (prewashed with 0.1% BSA in PBS).

17. Wash the reagent reservoir with 1 mL of PBS and combine with the nuclei mixture.

18. Spin down the nuclei with a swing bucket centrifuge at 1,000 *g* for 10 mins at 4°C, and carefully discard the supernatant.

19. Resuspend the nuclei is 50 µL PBS (nuclei stock suspension). Dilute 1 µL of nuclei with 9 µL of PBS and count the concentration of nuclei.

20. Dilute the nuclei stock suspension to 1k/µL. Aliquot 3 µL of nuclei (total 3k) into 200 µL PCR tubes or 96-well low-bind PCR plates (as sub-libraries).

21. Prepare the lysis mix: (a) calculate the number of sub-libraries that need to be lysed; (b) for each sub-library, the ligation mix contains 18 µL PBS, 3 µL 4M NaCl, 3 µL 10% SDS and 3 µL 20 mg/mL Proteinase K; (c) add and mix the reagents in the order of PBS, NaCl, SDS and Proteinase K.

22. Add 27 µL of lysis mix to each sub-library. Incubate in a ThermoMixer set to 55°C, 550 r.p.m. for 2 h.

23. Cool the lysis mixture to room temperature. Add 30 µL of SPRI beads (1X) into each well and mix. Incubate at room temperature for 5 mins. Prepare 80% EtOH.

25. Place the tubes or plate on a magnetic stand, sit for 5 mins until the liquid becomes clear, and carefully discard the supernatant.

26. Add 150 µL of 80% EtOH into each tube/well, sit for 30 s and discard the supernatant.

27. Repeat step 26 for a total of two washes.

28. Elute the DNA/cDNA with 12.5 µL ultrapure H_2O. The purified DNA/cDNA can be stored at -20°C or can be directly used for Step 3.6 Library pre-amplification.

3.6 Library pre-amplification

1. Add 1.5 µL of 10 X Terminal Transferase Buffer and 0.5 µL of 1 mM dCTP into each sub-library. Close the lid, tap to mix and briefly spin-down.

2. Incubate at 95 °C for 5 mins and immediately chill on ice and sit for another 5 mins.

3. Add 0.5 µL of Terminal Transferase into each tube. Close the lid, tap to mix and briefly spin-down.

4. Incubate at 37 °C for 30 mins, followed by heat inactivating the reaction at 65 °C for 10 mins.

5. Prepare the Anchor Mix freshly. Add 15 µL Anchor Mix into each tube. Close the lid, tap to mix and briefly spin-down.

6. Carry out the reaction in a thermocycler with the program below:

Step #	Temperature (°C)	Time
1	95	3 mins
2	95	15 s
	47	1 min
	68	2 mins
	47	1 min
	68	2 mins; repeat step2 for additional 15 cycles

3	72	10 mins
4	12	hold

7. Prepare the Preamp Mix freshly. Add 20 µL Preamp Mix to each tube and gently mix by pipetting up and down.

8. Carry out the reaction in a thermocycler with the program below:

Step #	Temperature (°C)	Time
1	98	3 mins
2	98	20 s
	65	20 s
	72	2.5 mins; repeat step 2 for additional 10 cycles
3	72	2 mins
4	12	hold

9. Add 10 µL of SPRI beads (0.2x) into each tube and mix. Incubate at room temperature for 5 mins. Prepare 80% EtOH.

10. Place the tubes to a magnetic stand, and let them sit for 5 mins until the liquid becomes clear.

11. Transfer the supernatant into new tubes, add 32.5 µL SPRI beads (0.85X) to each tube and mix. Incubate at room temperature for 5 mins.

12. Place the tubes on a magnetic stand, and let them sit for 5 mins until the liquid becomes clear. Carefully discard the supernatant.

13. Add 150 µL of 80% EtOH into each tube/well, sit for 30 s and discard the supernatant.

14. Repeat step 13 for a total of two washes.

15. Elute the amplification product with 40 µL ultrapure H_2O. The purified product can be stored at -20°C.

16. Quantify the concentration of the amplification product with Qubit.

3.7 Library splitting

1. Divide the purified amplification product into two tubes: 20.5 µL for Tn5 tagmentation-derived DNA library preparation and 17 µL for RNA-derived library preparation.

2. Steps 2-11 are for DNA library preparation. Add 2.5 µL 10X CutSmart Buffer, 1 µL SbfI-HF and 1 µL FokI to each 20.5 µL aliquot of the purified amplification product. Incubate in 37 °C for 1h.

3. Add 31.3 µL SPRI beads (1.25X) to each tube and mix. Incubate at room temperature for 5 mins.

4. Place the tubes on a magnetic stand, and let them sit for 5 mins until the liquid becomes clear. Carefully discard the supernatant.

5. Add 150 µL 80% EtOH into each tube/well, sit for 30 s and discard the supernatant.

6. Repeat step 5 for a total of two washes.

7. Elute the amplification product with 15 µL ultrapure H_2O. The purified product can be stored at -20°C.

8. Add 2 µL 10X T4 DNA Ligase Buffer, 1.5 µL Adaptor mix and 1.5 µL T4 DNA Ligase to each tube from the previous step. Close the lid and tap to mix, and briefly spin-down.

9. Carry out the ligation reaction in a thermocycler with the program as below. Put the tubes to thermocycler immediate after the temperature reached 4 °C:

Step #	Temperature (°C)	Time
1	4	10 mins
2	10	5 mins
3	16	15 mins
4	25	45 mins
5	12	hold

10. Add 25 µL SPRI beads (1.25X) directly to the reaction mixture and mix. Incubate at room temperature for 5 mins and repeat the wash steps as described in steps 4-6.

11. Elute the adaptor-ligated DNA with 21 µL ultrapure H_2O. The purified product can be stored at -20 °C.

12. Steps 12-18 are for RNA library preparation. Add 2 µL 10X CutSmart Buffer and 1 µL NotI-HF into the 17 µL amplification product. Incubate at 37 °C for 1 h.

13. Add 25 µL SPRI beads (1.25X) to each tube and mix. Incubate at room temperature for 5 mins and repeat the wash steps as described in steps 4-6.

14. Elute with 10 µL ultrapure H_2O. The purified product can be store at -20 °C.

15. Use 5 µL of the purified product for tagmentation with Illumina Nextera XT. Add 10 µL Buffer TD and pipette up and down to mix.

16. Add 5 µL of ATM to each tube, pipette 10 times to mix and close the lid, and quickly spin-down.

17. Incubate the mixture in a thermocycler at 55 °C for 5mins, cool to 10 °C and immediately place the tubes on ice.

18. Add 5 µL NT to each well, pipette 10 times to mix, close the lid and incubate at room temperature for 5 mins. Proceed to step 8 of 3.8.

<u>3.8 Library amplification</u>

1. Steps 1-7 are for DNA library amplification. Add 2 µL of Illumina TruSeq i7 index primers, 2 µL Illumina TruSeq i5 index primers and 25 µL NEBNext 2X HiFi PCR mix. Use pipette to mix, close the lid and quick spin-down.

2. Carry out the PCR reaction in a thermocycler with the program as:

Step #	Temperature (°C)	Time
1	98	3 mins
2	98	10 s
	63	30 s
	72	1 min; repeat step 2 for additional 11-13 cycles
3	72	5 mins
4	12	hold

3. Add 42.5 µL SPRI beads (0.85X) to each tube and mix. Incubate at room temperature for 5 mins.

4. Place the tubes on a magnetic stand, and let them sit for 5 mins until the liquid becomes clear. Carefully discard the supernatant.

5. Add 150 µL 80% EtOH into each tube/well, sit for 30 s and discard the supernatant.

6. Repeat step 5 for a total of two washes.

7. Elute the DNA library with 25 µL ultrapure H_2O. The purified library can be stored at -20°C.

8. Steps 8-11 are for RNA library amplification. Add 6 µL ultrapure H_2O, 2 µL of Illumina TruSeq i7 index primers, 2 µL of Illumina Nextera i5 index primers and 15 µL NPM to each tube. Pipette to mix, close the lid and briefly spin-down.

9. Carry out the PCR reaction in a thermocycler with the program as:

Step #	Temperature (°C)	Time
1	72	3 mins
2	95	30 s
3	95	10 s
	55	30 s
	72	1 min; repeat step 2 for additional 11-13 cycles
4	72	5 mins
5	12	hold

10. Purify the RNA library as described in steps 3-6.

11. Elute the RNA library with 25 µL ultrapure H_2O. The purified library can be stored at -20°C.

12. Quantify the concentration of library with KAPA qPCR quantification kit for Illumina. Check the size distribution of libraries with Tapestation.

3.9 Sequencing and data preprocessing

1. DNA and RNA libraries with different combinations of indices can be multiplexed for sequencing.
2. Paired-seq requires at least 50 cycles for Read1 (insert genomic sequences), 6 cycles for Index Read1, 8 cycles for Index Read2 and 100 cycles for Read2 (cellular barcodes) (50+6+8+100).
3. Paired-seq data preprocessing includes (a) extracting three-round barcode sequences from Read2, (b) assigning barcode sequences to individual tube/wells and (c) mapping reads to reference genome and generation of cell-counts matrices.
4. All scripts required for Paired-seq data preprocessing and the analysis steps are available from GitHub (https://github.com/cxzhu/Paired-Tag).

4 Notes

1. All the safe pause points in the protocol are indicated in Fig. 1a.
2. For 96-well barcode plates, standard desalting purification can be used. For Index PCR primers, HPLC purification is required.
3. Native nuclei isolated from snap-frozen tissues or fresh tissue are preferred. Crosslinked nuclei will reduce the complexities for Paired-seq DNA libraries.
4. Nuclei preparation, tagmentation, reverse transcription and combinatorial DNA barcoding must be carried out in a single day, which will take ~8 hrs.

5. The optimal input nuclei number is 1.2 M (or 100, 000 * 12 tubes). Less cell number is acceptable but will result in a lower recovery rate. We can typically recover 200,000 -300, 000 from 1.2 M input nuclei (17%-25%) and 30,000 -50,000 from 500, 000 (41,700 * 12 tubes) input nuclei (6%-10%).

6. Prewash the 15 mL tubes with 0.1% BSA in PBS can reduce nuclei sticking to the tube and increase nuclei recovery rate.

7. During removing supernatants after spin-down steps, remove the liquid as much as possible. The downstream reaction might be interfered by residual buffers, salt or oligos from the previous step (e.g., EDTA after tagmentation in step 3.3.6, and adaptors oligos after nuclei barcoding in step 3.5).

8. During purification of nucleic acids from lysis mixture, make sure to wash out SDS as the residual SDS may inhibit the subsequent reactions.

9. The optimal number of nuclei in each sub-library is ~3,500, which gives 6-10% potential barcode collision rate. A higher number of nuclei in each sub-library will result in higher barcode collision. Using nuclei sorting instead of dilution to aliquot sub-libraries can reduce the potential barcode collision, but will also reduce the recovery rate.

10. To determine the optimal PCR cycles for step 3.8.9, the PCR reaction can be carried out in two steps: (a), perform PCR amplification for 10 cycles and put on ice, take out 0.5 µL of PCR mixture and diluted 1000X, quantify with qPCR and calculated the additional cycles needed to reach 10 nM concentration; (b), perform PCR amplification with the needed additional cycles and purify the amplified products, store in -20°C.

11. After preamplification, typically ~40 – 1,200 ng amplified products can be recovered (~1 ng/µL – 30 ng/µL as measured by Qubit). The yields of parallel processed sub-libraries should be comparable with each other.

12. Paired-seq libraries should have a fragment size distribution of 300-1,000 bp (Fig. 2). If fragments of ~245 bp (adaptors) appears as a significant fraction, try to remove them with an additional round of 0.75X SPRI beads size selection.

[Fig 2 near here]

13. Quantification of libraries must be carried out by qPCR. Tapestation (or Fragment Analyzer) and Qubit analysis tend to overestimate Paired-seq library concentrations.

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Figure legends

Fig.1 Overview of Paired-seq protocol. (**a**) Paired-seq protocol can be finished in two days, pause points are indicated. (**b**) Schematics for library preparation strategy of Paired-seq. Both DNA fragments from Tn5 tagmentation and cDNA were pre-amplified with a TdT-based strategy and then split into two portions. For DNA library, the 2nd adaptor was added by ligation; for RNA library, the 2nd adaptor was added by Tn5 tagmentation.

Fig.2 Representative fragment analysis results of Paired-seq library. (**a**) Tapestation analysis results of a representative Paired-seq library. EL: electronic ladder. (**b**) Fragments size distribution of representative DNA (b) and RNA (c) library of Paired-seq.