Chapter 4

Mapping Mammalian 3D Genomes by Micro-C

Elena Slobodyanyuk, Claudia Cattoglio, and Tsung-Han S. Hsieh

Abstract

3D genome mapping aims at connecting the physics of chromatin folding to the underlying biological events, and applications of various chromosomal conformation capture (3C) assays continue to discover critical roles of genome folding in regulating nuclear functions. To interrogate the full spectrum of chromatin folding ranging from the level of nucleosomes to full chromosomes in mammals, we developed an enhanced 3C-based method called Micro-C. The protocol employs Micrococcal nuclease (MNase) to fragment the genome, which overcomes the resolution limit of restriction enzyme-based methods, enabling the estimation of contact frequencies between proximal nucleosomes. Such improvements successfully resolve the fine-scale level of chromatin folding, including enhancer–promoter or promoter–promoter interactions, genic and nucleosomal folding, and boost the signal-to-noise ratio in detecting loops and substructures underlying TADs. In this chapter, we will thoroughly discuss the details of the Micro-C protocol and critical parameters to consider for generating high-quality Micro-C maps.

Key words Micro-C, Chromosomal conformation capture (3C), Hi-C, 3D genome, Loop, TAD, Enhancer–promoter interaction

1 Introduction

Mapping 3D genome organization progresses our understanding of the role of physical chromatin interactions in the context of a functional genome, including the processes of transcription, DNA replication, and DNA repair. Various genome-wide 3D mapping approaches such as Hi-C (Chapter 3), Capture-C (Chapter 5), and HiChIP (Chapter 7) have tackled a variety of biological questions and unveiled many vital links between genome organization and nuclear functions [1]. For example, two levels of chromosome conformation—compartmentalization and local chromatin folding—greatly contribute to the 3D genome structure of interphase chromosomes and are typically superimposed on each other in Hi-C contact maps, due to heterogeneity in cell populations or

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distinct folding processes [2]. A and B compartments, or compartmental domains, correspond to active and inactive chromatin segments, respectively, and appear as a plaid-like pattern in Hi-C contact maps. Transcriptional activity and perhaps the chromatin states in the milieu are likely to be involved in compartment segregation. On the other hand, the loop-extrusion model suggests that the ATP-dependent SMC complex, cohesin, mediates the formation of local chromatin structures, including topologically associating domains (TADs) and loops [3] . Cohesin encloses chromatin loci and extrudes chromatin until paused by CTCF. The extruding chromatin spatially organizes into a self-interacting domain, and the CTCF binding site constitutes the domain boundary insulating interdomain interactions. Stabilization of cohesin at CTCF sites sometimes gives rise to sharp corner peaks in contact maps, which are referred to as loops or loop domains. Importantly, mounting evidence suggests that TADs and loops are functionally linked to stem cell differentiation [4], V(D)J recombination [5], and many developmental processes [6].

Various 3D genome mapping methods differ in their resolution (nucleosome- or restriction-size fragments), genome coverage (all-by-all or point-by-point), and the involvement of a target enrichment step (protein or DNA/RNA pull-down), which determine their detection power for different levels of chromatin structures. Which method to choose depends on the experimental design and the scope of 3D structures of interest. We summarize the key features of these methods and their ideal applications in Table 1.

In the Hi-C protocol [7], chromatin is first cross-linked by formaldehyde and subsequently fragmented with a 4- or 6-cutter restriction enzyme. Religation of fragments yields chimeric DNA readouts in the Hi-C data that capture genomic loci that were crosslinked to one another in vivo. While many factors such as sequencing depth and library complexity impact the effective resolution of a Hi-C data set, a fundamental limit to genomic resolution is the size of the fragments generated before physical interactions are captured via ligation. We developed an enhanced Hi-C method, called Micro-C [8], that interrogates chromosome folding at lengths from single nucleosome to the full genome, which successfully uncovered the fine scale of chromatin structures in budding and fission yeast [9–12], as well as in mouse [13, 14] and human cells [15]. Here we highlight the key aspects of the Micro-C protocol that enable the identification of fine-scale chromatin features, namely, (1) Micro-C uses micrococcal nuclease (MNase) instead of restriction enzymes to fragment the genome into mononucleosomes, which allows the capture of nucleosome and protein interactions and nucleosome-resolution analysis of the chromosomes;

Table 1
Choice of chromosomal conformation capture assay (ligation-based)

	Micro-C (This Chapter)	Hi-C (Chapter 3)	4C (Chapter 2)	5C	Capture-C (Chapter 5)	HiChIP/PLAC-seq/ ChIA-PET (Chapter 7)
Resolution	Single nucleosome (~150 bp)	Depends on restriction enzyme	Depends on restriction enzyme	Depends on restriction enzyme	Depends on restriction enzyme and probe density	Depends on restriction enzyme and antibody
Readout	All-by-All	All-by-All	One-by-All	Multiple-by-Multiple	Targets-by-All	Peaks-by-Peaks
Compartment or compartmental domain	+++	+++	–	+++	–	–
TAD	+++	+++	–	+++	–	–
Nested structures within TAD	+++	+/–	–	++	–	–
Loop/Loop domain (anchored by CTCF/cohesin)	+++	+	++	++	+++	+++
E-P link	+++	+/–	++	++	+++	+++
Gene folding	+++	–	–	–	–	–
Nucleosomal folding	+++	–	–	–	–	–

(2) A dual-cross-linking protocol combining formaldehyde (~2 Å) and the protein-protein crosslinker DSG (disuccinimidyl glutarate; 7.7 Å) or EGS (ethylene glycol bis(succinimidyl succinate); 16.1 Å) substantially increases the signal-to-noise ratio to unmask previously indiscernible structures, such as chromosomally interacting domains (CIDs) in yeast [8] and highly abundant chromatin loops in mammals [14, 15]. The higher resolution of Micro-C improves the capture of the enhancer–promoter connectome that is intimately linked to cell type-specific transcriptional regulation [14].

In this chapter, we will focus on the Micro-C protocol, including: (1) Preparation of cross-linked chromatin from cell culture; (2) Micrococcal nuclease digestion and titration; (3) DNA fragment end-repair; (4) Proximity ligation, removal of unligated ends, and reverse cross-linking; (5) Di-nucleosomal DNA purification; and (6) Micro-C library preparation. Finally, we will briefly introduce the computational analysis of the generated Micro-C data sets.

2 Materials

1. Low-retention tubes and pipette tips (*see* **Note 1**).

2.1 Chromatin Cross-Linking

1. Cell culture medium (without fetal bovine serum), according to cell line used.
2. Trypsin, if using adherent cells, according to cell line used.
3. Formaldehyde cross-linking medium: 1% formaldehyde (FA) in cell culture medium (*see* **Notes 2** and **3**).
4. 1 M and 10 mM Tris–HCl pH 7.5.
5. 1× phosphate-buffered saline (PBS).
6. DSG cross-linking solution: 3 mM disuccinimidyl glutamate (DSG; from 300 mM stock solution in DMSO) in 1× PBS (*see* **Notes 2** and **3**).

2.2 Micrococcal Nuclease Digestion and Titration

1. Micro-C Buffer #1 (MB#1): 10 mM Tris–HCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% (v/v) NP-40, 1× Protease Inhibitor Cocktail (Sigma) (*see* **Note 4**).
2. MNase solution: 20 units/μL micrococcal nuclease stock (Worthington Biochem) in 10 mM Tris–HCl pH 7.5. Stored at −80 °C in 20 μL aliquots; avoid repeated freeze–thaw cycles (*see* **Note 5**). Just before use, thaw an aliquot and prepare a diluted (1 unit/μL) solution in 10 mM Tris–HCl pH 7.5.
3. 500 mM EGTA (*see* **Note 6**).
4. 1× TE buffer: 10 mM Tris–HCl pH 7.5, 1 mM EDTA.
5. Reverse cross-linking solution: 0.5 mg/mL Proteinase K (from a 40 mg/mL stock in 40% glycerol, 1× TE buffer), 1% (w/v) SDS, 0.1 mg/mL RNase A in 1× TE buffer.
6. Phenol–chloroform–isoamyl alcohol (25:24:1, v/v).
7. 100% and 80% ethanol.
8. 3 M sodium acetate pH 5.5.
9. Zymoclean DNA Clean & Concentrator Kit.
10. NanoDrop spectrophotometer.
11. Micro-C Buffer #2 (MB#2): 10 mM Tris–HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$. Filtered through a 0.22 μm filter.

2.3 DNA Fragment End Repair

1. End-chewing master mix: 1.2× NEBuffer 2.1, 2.4 mM ATP, 5.9 mM DTT.
2. 10 units/μL T4 polynucleotide kinase (PNK) (NEB).
3. 5 units/μL DNA polymerase I Klenow fragment (NEB).
4. End-labeling master mix: 1× T4 DNA ligase buffer (NEB), 0.2 mM biotin-14-dATP, 0.2 mM biotin-11-dCTP, 0.2 mM dTTP, 0.2 mM dGTP, 0.1 mg/mL bovine serum albumin (BSA).

5. 500 mM EDTA.
6. Micro-C Buffer #3 (MB#3): 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$. Filtered through a 0.22 μm filter.

2.4 Proximity Ligation, Removal of Unligated Ends, and Reverse Cross-Linking

1. Ligation master mix: 1× T4 DNA ligase buffer (NEB), 0.1 mg/mL BSA, 20 units/μL T4 DNA ligase (NEB).
2. Exonuclease III master mix: 1× NEBuffer 1 (NEB), 5 units/μL exonuclease III (NEB).

2.5 Dinucleosomal DNA Purification

1. 3% TBE NuSieve (Lonza) agarose gel (*see* **Note 7**).
2. Zymoclean Gel DNA Recovery Kit.
3. Qubit dsDNA HS Assay Kit (ThermoFisher).

2.6 Library Preparation

1. End polishing master mix, made from components of the End-It DNA End-Repair Kit (Lucigen): 3.125× End-It buffer, 3.125 mM ATP, 781.25 μM each dNTP, 1/16 volume End-It enzyme mix (i.e., 0.5 μL per 8 μL added to one reaction).
2. Dynabeads MyOne Streptavidin C1 (ThermoFisher).
3. 2× BW buffer: 10 mM Tris–HCl pH 7.5, 2 M NaCl, 1 mM EDTA. Also prepare a 1× stock. Both buffers are filtered through a 0.22 μm filter.
4. 1× TBW buffer: 1× BW, 0.1% (v/v) Tween-20.
5. Magnetic stand for 1.5 mL microcentrifuge tubes.
6. NEBNext Ultra II DNA Library Prep Kit for Illumina, including the End Prep enzyme mix, Illumina adapters and primers, ligation master mix and enhancer, USER enzyme and Q5 master mix.
7. End Prep master mix. Per 30 μL reaction: 3.5 μL End Prep reaction buffer, 1.5 μL End Prep enzyme mix, 25 μL nuclease-free water.
8. AMPure XP beads.
9. Next-generation sequencer (Illumina).

3 Methods

Carry out all procedures on ice, unless otherwise specified. Use low-retention tubes and pipette tips for all samples.

3.1 Preparation of Cross-Linked Chromatin from Cell Culture

1. Culture cells in the recommended conditions (*see* **Note 8**).
2. Trypsinize cells if needed and count cells after inactivating trypsin with media. Harvest cells by centrifugation for 5 min at 850 × *g* at room temperature (*see* **Note 9**).

3. Resuspend cells in formaldehyde cross-linking medium at a concentration of 1×10^6 cells/mL, for a maximum of 30 mL cross-linking medium in a 50 mL tube. First resuspend the pellet using a 1 mL pipette tip, and then add the rest of the media with a larger pipette without touching the cells. Incubate for 10 min at room temperature while nutating (*see* **Note 10**).
4. Add 1 M Tris–HCl pH 7.5 to a final concentration of 375 mM to quench the reaction. For example, add 18 mL 1 M Tris–HCl to a 30 mL sample. Incubate for 5 min at room temperature. Centrifuge for 5 min at 850 × *g* at 4 °C. Aspirate the supernatant.
5. Wash cells twice with cold 1× PBS at a concentration of 1×10^6 cells/mL. For each wash, centrifuge for 5 min at 850 × *g* at 4 °C and aspirate the supernatant.
6. Resuspend the cell pellet in the DSG cross-linking solution at a concentration of 1×10^6 cells/mL. First resuspend the pellet using a 1 mL pipette tip, and then add the rest of the medium with a larger pipette without touching the cells. Incubate for 45 min at room temperature while nutating (*see* **Note 11**).
7. Add 1 M Tris–HCl pH 7.5 to a final concentration of 375 mM to quench the reaction. For example, add 18 mL 1 M Tris–HCl to a 30 mL sample. Incubate for 5 min at room temperature. Centrifuge for 5 min at 850 × *g* at 4 °C. Aspirate the supernatant.
8. Wash cells with cold 1× PBS at a concentration of 1×10^6 cells/mL. For each wash, centrifuge for 5 min at 850 × *g* at 4 °C and aspirate the supernatant.
9. Resuspend the cell pellet in the appropriate volume of 1× PBS to aliquot 1 mL of sample into multiple tubes, at a concentration of 1×10^6 cells/mL (*see* **Note 12**). Centrifuge for 5 min at 850 × *g* at 4 °C and aspirate the supernatant. Snap freeze cell pellets in liquid nitrogen or proceed to the MNase titration. Store frozen pellets at −80 °C (*see* **Note 13**).

3.2 Micrococcal Nuclease Titration (See Note 14)

1. Thaw a cell pellet of 1×10^6 cells and resuspend in complete MB#1 at a concentration of 1×10^6 cells/100 µL. Aliquot 100 µL of cells to a 1.5 mL Eppendorf tube. Incubate for 20 min on ice. Centrifuge for 5 min at 10,000 × *g* at 4 °C. Discard the supernatant using a pipette tip, leaving 10–20 µL behind.
2. Wash the nuclear pellet in complete MB#1 at a concentration of 1×10^6 cells/100 µL. Resuspend the pellet by pipetting up and down. Centrifuge for 5 min at 10,000 × *g* at 4 °C. Discard the supernatant using a pipette tip. Try to remove as much liquid as possible without disturbing the pellet.

3. Resuspend the nuclear pellet in complete MB#1 at a concentration of 1×10^6 cells/100 µL. Resuspend the pellet by pipetting up and down.
4. Add increasing amounts of MNase as follows (*see* **Note 15**).
   (a) 5 units: 5 µL of the 1 unit/µL stock.
   (b) 10 units: 0.5 µL of the 20 units/µL stock.
   (c) 20 units: 1 µL of the 20 units/µL stock.
   (d) 40 units: 2 µL of the 20 units/µL stock.
   (e) 60 units: 3 µL of the 20 units/µL stock.
5. Briefly vortex and spin the tubes. Incubate for 10 min at 37 °C while shaking in a thermomixer at 850–1000 rpm.
6. Transfer the tubes back to ice and stop the reaction by adding 0.8 µL of 500 mM EGTA. Vortex and briefly spin the tubes. Incubate for 10 min at 65 °C. Centrifuge for 5 min at 12,000 × *g* at 4 °C. Discard the supernatant.
7. Resuspend each pellet in 200 µL of reverse cross-linking solution by pipetting up and down. Incubate overnight at 65 °C.
8. Add 200 µL of phenol–chloroform–isoamyl alcohol and vortex for 20 s. Centrifuge for 15 min at 19,800 × *g* at room temperature.
9. Transfer ~200 µL of the aqueous phase to a new 1.5 mL tube and proceed with DNA purification by either ethanol precipitation (**step 10**) or Zymoclean DNA Clean & Concentrator Kit (**step 11**) (*see* **Note 16**).
10. For DNA purification by ethanol precipitation, add 500 µL of room-temperature 100% ethanol and 20 µL of 3 M sodium acetate pH 5.5. Invert the tube to mix and precipitate nucleic acids at −80 °C for at least 1 h. Centrifuge for 15 min at 19,800 × *g* at 4 °C. Discard the ethanol using a pipette and wash the pellet with 1 mL of 80% room-temperature ethanol. Centrifuge for 5 min at 19,800 × *g* at 4 °C. Remove as much ethanol as possible using a pipette. Centrifuge the tube once again briefly and remove as much ethanol residue as possible. Air dry the pellet for 5 min at 37 °C with an open lid. Resuspend the pellet in 15 µL of Zymo Elution Buffer or 10 mM Tris–HCl pH 7.5. Proceed to **step 12**.
11. For DNA purification using the Zymoclean DNA Clean & Concentrator Kit, add 1 mL of DNA Binding Buffer and load onto the column. Wash twice with 400 µL of wash buffer. Elute with 15 µL of Zymo Elution Buffer.
12. Quantify the DNA by NanoDrop (*see* **Note 17**).
13. Check fragment sizes on a 2% regular agarose gel. The desired MNase concentration produces 80–90% nucleosome

monomer: 20–10% dimer ratio. In mammals, the monomer size is ~100 to 175 bp and the dimer size is ~200 to 400 bp (Fig. 1a) (*see* **Notes 18** and **19**).

3.3 Digestion of Cross-Linked Chromatin with Micrococcal Nuclease

1. Resuspend the cell pellet in complete MB#1 at a concentration of 1×10^6 cells/100 μL. Incubate for 20 min on ice. Proceed with centrifugation, washing, and resuspension in complete MB#1 as in "Micrococcal nuclease titration" **steps #1–4**.
2. Add the appropriate amount of MNase, as determined by the MNase titration experiment on the same batch (*see* **Note 19**). Briefly vortex the tubes and incubate for 10 min at 37 °C while shaking in a thermomixer at 850–1000 rpm.
3. Transfer the tubes to ice. Add 0.8 μL of 500 mM EGTA to stop the reaction. Vortex briefly to mix and incubate for 10 min at 65 °C. Centrifuge for 5 min at 10,000 × *g* at 4 °C. Discard the supernatant using a pipette tip.
4. Wash the nuclear pellet in 1 mL of cold MB#2 twice. Centrifuge for 5 min at 10,000 × *g* at 4 °C. Discard the supernatant using a pipette tip. After the second wash, try to remove as much liquid as possible without disturbing the pellet.

3.4 DNA Fragment End Repair

1. Resuspend the nuclear pellet with 50 μL of end chewing master mix. Add 2.5 μL of 10 U/μL T4 PNK, mix well by finger flicking, briefly spin down, and incubate for 15 min at 37 °C while shaking in a thermomixer at 1000 rpm for an interval of 15 s every 3 min (*see* **Note 20**).
2. Add 5 μL of 5 U/μL Klenow fragment to the reaction, mix well by finger flicking, and briefly spin down. Incubate for 15 min at 37 °C while shaking in a thermomixer at 1000 rpm for an interval of 15 s every 3 min (*see* **Note 21**).
3. Add 25 μL of end labeling master mix, mix well by finger flicking, and briefly spin down. Incubate for 45 min at 25 °C while shaking in a thermomixer at 1000 rpm for an interval of 15 s every 3 min.
4. Add 9 μL of 500 mM EDTA, mix well by finger flicking, briefly spin down, and incubate at 65 °C for 20 min without shaking.
5. Centrifuge for 5 min at 10,000 × *g* at 4 °C. Discard the supernatant using a pipette tip. Rinse once with 1 mL of cold MB#3 by pipetting up and down. Centrifuge for 5 min at ~10,000 × *g* at 4 °C. Discard the supernatant using a pipette tip.

3.5 Proximity Ligation, Removal of Unligated Ends, and Reverse Cross-Linking

1. Resuspend the nuclear pellet with 250 μL of ligation master mix, mix well by finger flicking, and briefly spin down. Incubate for at least 2.5 h at 25 °C with slow rotation on an orbital shaker (*see* **Note 22**). Centrifuge for 5 min at 16,000 × *g* at 4 °C. Discard the supernatant using a pipette tip.

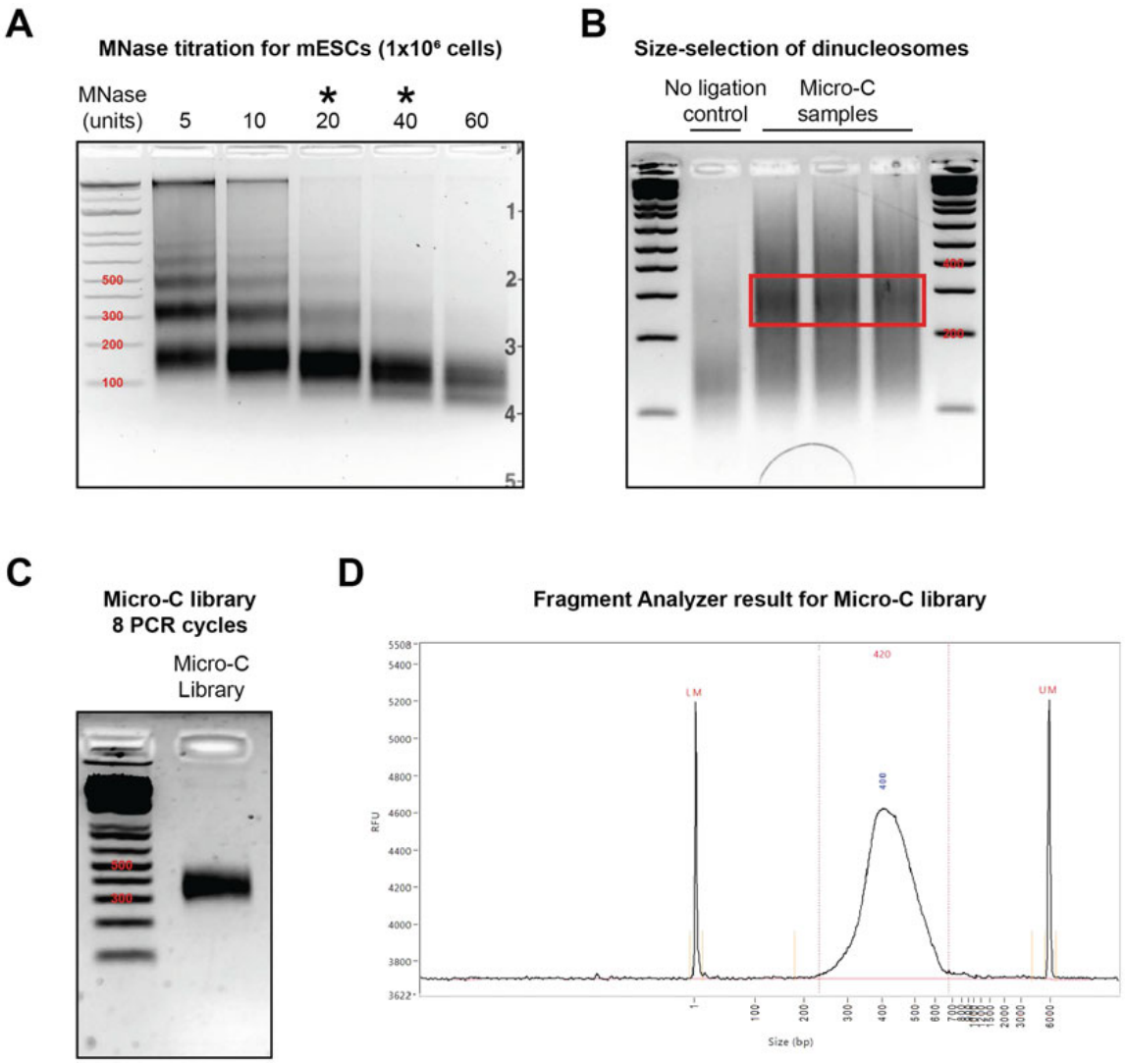

Fig. 1 (**a**) Example of an MNase titration for mouse embryonic stem cells. A million cells were digested by 5, 10, 20, 40, and 60 units of MNase separately for 10 min at 37 °C. After reverse cross-linking and DNA purification, the digestion pattern of DNA was analyzed by running the samples on a 2.5% agarose gel in TBE buffer. The asterisk highlights the optimal conditions for the Micro-C experiment (e.g., 20–40 units). The gel contains 500 ng of the 1-kb plus DNA marker. (**b**) Example of Micro-C samples. Micro-C samples (ligated) and a no-ligation control were analyzed by a 3% NuSieve GTG agarose gel in TBE buffer. The box highlights the size selection of dinucleosomes sized between 200–400 bp. The gel contains 500 ng of the 1-kb plus DNA marker. (**c**) Example of a Micro-C sequencing library. The Micro-C library was amplified with 8 PCR cycles and was subjected to DNA cleanup using the Zymoclean DNA Clean & Concentrator Kit. After pooling the samples with unique barcodes, the library was purified again using AMPure beads at a 0.9× ratio. The sample is ready for next-generation DNA sequencing. Here, to display the size distribution of the Micro-C library, we analyzed the Micro-C library with a 2% agarose gel in TBE buffer. The DNA band should run between 300 and 500 bp (i.e., 300 bp of dinucleosome + 110 bp of the Illumina adapters). There should be no detectable primer/adapter that runs around or lower than 100 bp. The gel contains 100 ng of the 1-kb plus DNA marker. (**d**) Example of a Fragment analyzer result. The Micro-C library shows a clear peak at ~400 bp

2. Resuspend the nuclear pellet with 100 μL of exonuclease III master mix, mix well by finger flicking, and briefly spin down. Incubate for 15 min at 37 °C while shaking at 1000 rpm for an interval of 15 s every 3 min (*see* **Note 23**).
3. Add 150 μL of reverse cross-linking solution and incubate at 65 °C overnight.

3.6 Dinucleosomal DNA Purification

1. Add 250 μL of phenol–chloroform–isoamyl alcohol to the sample. Vortex for 20 s. Centrifuge for 15 min at 19,800 × *g* at room temperature. Transfer the upper layer to a new tube and proceed with DNA purification by either ethanol precipitation (**step 2**) or using the Zymoclean DNA Clean & Concentrator Kit (**step 4**) (*see* **Note 16**).
2. For DNA purification by ethanol precipitation, add 625 μL of room-temperature 100% ethanol and 25 μL of 3 M sodium acetate pH 5.5. Invert the tube to mix and precipitate nucleic acids at −80 °C for at least 1 h. Centrifuge for 15 min at 19,800 × *g* at 4 °C. Discard the ethanol using a pipette and wash the pellet with 1 mL of 80% room-temperature ethanol. Centrifuge for 5 min at 19,800 × *g* at 4 °C. Remove as much ethanol as possible using a pipette. Centrifuge the tube once again briefly and remove as much ethanol residue as possible. Air dry the pellet for 5 min at 37 °C with an open lid. Add 50 μL of 1× TE and incubate at 37 °C for 30 min to dissolve the pellet.
3. Purify DNA again using the Zymoclean DNA Clean & Concentrator Kit (*see* **Note 24**). Add 300 μL of DNA Binding Buffer to 50 μL of DNA and load onto the column. Wash twice with 400 μL of wash buffer. Elute twice, each time with 15 μL of Zymo Elution Buffer for a total volume of 30 μL. Proceed to **step 5**.
4. For DNA purification by Zymoclean DNA Clean & Concentrator Kit alone, add 1.25 mL of DNA Binding Buffer and load onto the column. Wash twice with 400 μL of wash buffer. Elute twice, each time with 15 μL of Zymo Elution Buffer for a total volume of 30 μL.
5. Prepare a 3% TBE NuSieve GTG agarose gel (*see* **Note 7**). Separate nucleosome monomers from dimers by loading two large wells with 20 μL in each well (*see* **Notes 18** and **19**). Cut the bands sized between 250 and 400 bp (Fig. 1b). Avoid cutting below 200 bp to eliminate nucleosome monomers.
6. Use the Zymoclean Gel DNA Recovery Kit to extract DNA from the cut bands. Add 3× volume/weight of Agarose Dissolving Buffer to the cut gel and incubate at 50 °C for 10 min until the gel is completely dissolved. Load onto the column and

wash twice with 400 μL of wash buffer. Elute twice, each time with 9 μL of Elution Buffer for a total volume of 18 μL (*see* **Note 25**).

7. Quantify DNA using the Qubit dsDNA HS assay. There should be at least 50–200 ng of DNA for a high-coverage result (*see* **Note 26**).

3.7 Library Preparation

1. Add 8 μL of end polishing master mix to 17 μL of input DNA. Incubate for 45 min at 25 °C without shaking. Inactivate the enzyme mix by incubating for 10 min at 70 °C.
2. Wash 5 μL of streptavidin beads with 1 mL of 1× TBW (*see* **Note 27**). Mix on a nutator for 2 min, then place beads on a magnetic stand for 30 s. Remove the supernatant with a pipette tip. Resuspend beads in 150 μL of 2× BW. Add 125 μL of water to the DNA sample to bring the volume to 150 μL. Transfer an equal volume (150 μL) of the prewashed streptavidin beads to the sample. Mix the DNA and beads on a nutator for 20 min at room temperature. Centrifuge briefly in a microfuge and add 950 μL of 1× TBW. Invert the tube multiple times to resuspend the beads and incubate for 5 min at 55 °C while shaking at 1200 rpm. Centrifuge briefly in a microfuge and place the tube on the magnetic stand for 30 s. Remove the supernatant and repeat the 1× TBW wash. Centrifuge briefly in a microfuge and place the tube on the magnetic stand for 30 s. Remove the supernatant. Rinse the beads with 500 μL of 10 mM Tris–HCl pH 7.5 without disturbing the beads (*see* **Note 28**). Discard the supernatant and transfer the tubes to ice.
3. Add 30 μL of End Prep master mix and pipette up and down or vortex to resuspend the beads without creating bubbles. Incubate for 30 min at 20 °C while shaking at 1000 rpm for an interval of 15 s every 3 min. Incubate for 30 min at 65 °C to inactivate the enzymes. Transfer samples to ice.
4. Add 3.5 μL of NEB Illumina adapter, 15 μL of Ligation Master Mix, and 0.5 μL of Ligation enhancer sequentially to 30 μL of the sample from the previous step for a final volume of 49 μL. Vortex briefly and incubate for 30 min at 20 °C while shaking at 1000 rpm for an interval of 15 s every 3 min. Add 1.5 μL of the USER enzyme. Incubate for 15 min at 37 °C while shaking at 1000 rpm for an interval of 15 s every 3 min.
5. Add 950 μL of 1× TBW. Invert the tube multiple times to resuspend the beads and incubate for 3 min at 55 °C while shaking at 1000 rpm. Centrifuge briefly in a microfuge and place the tube on the magnetic stand for 30 s. Remove the supernatant and repeat the 1× TBW wash. Centrifuge briefly in a microfuge and place the tube on the magnetic stand for 30 s.

Remove the supernatant. Rinse the beads with 500 μL of 10 mM Tris–HCl pH 7.5 without disturbing the beads. Discard the supernatant. Resuspend the beads in 20 μL of Zymo Elution Buffer (*see* **Note 29**).

6. Take a 1 μL aliquot of beads and transfer to a 200 μL PCR tube. Sequentially add the following reagents on ice: 3.5 μL of nuclease-free water, 5 μL of 2× Q5 PCR Enzyme Mix, 0.25 μL of 10 μM PE1.0 primer, 0.25 μL of PE2.0 primer.
7. Run the following PCR setup:

   Denaturation: 98 °C, 30 s.

   12 cycles: 98 °C, 10 s; 65 °C, 1 min 15 s.

   Final extension: 65 °C, 5 min.

   Hold at 4 °C.
8. Check the library size and estimate library quantity by agarose gel (*see* **Note 30**). Image the gel and calculate the amount of DNA in the sample by using the ladder intensity for comparison. Adjust the number of PCR cycles to yield roughly 50–100 ng of product for sequencing.
9. Assemble full 50 μL PCR reactions by sequentially adding to 200 μL PCR tubes on ice: 19 μL of sample beads, 3.5 μL of nuclease-free water, 25 μL of 2× Q5 PCR Enzyme Mix, 1.25 μL of 10 μM universal primer, 1.25 μL of indexed primer (*see* **Note 31**).
10. Run the following PCR setup, with the minimal number of cycles (**N**) to yield 50–100 ng of PCR product:

    Denaturation: 98 °C, 30 s.

    N cycles: 98 °C, 10 s; 65 °C, 1 min 15 s.

    Final extension: 65 °C, 5 min.

    Hold at 4 °C.
11. Purify DNA with the Zymoclean DNA Clean & Concentrator Kit: Add 250 μL of DNA Binding Buffer to 50 μL of PCR sample. Transfer the tube to a magnetic stand and let the beads attach to the side of the tube. Load the supernatant onto the column. Wash twice with 400 μL of wash buffer. Elute twice, each time with 9 μL of Elution Buffer for a total volume of 18 μL.
12. Quantify the library using the Qubit dsDNA HS Assay Kit (*see* **Note 32**).
13. Pool the indexed samples; indexed samples are typically pooled at a 1:1 molar ratio. Consider using ~1 pmol of total DNA, splitting this amount between indexed samples. For example, you can pool 100 fmol DNA each from 10 samples, or 50 fmol DNA each from 20 samples. Bring the final volume to 50 μL with Zymo Elution Buffer.

14. Resuspend AMPure XP beads thoroughly before pipetting. Add 45 μL of beads (0.9×) to 50 μL of pooled samples. Pipette up and down until fully resuspended. Incubate for 10 min to allow DNA to bind to the beads. Place the tube on the magnetic stand for 5 min, until the solution is clear. Discard the supernatant without disturbing the beads.
15. Add 200 μL of freshly prepared 80% ethanol without disturbing the beads. Leave on the magnetic stand for 30 s. Discard the ethanol without disturbing the beads. Repeat the ethanol wash step. Remove residual ethanol using a pipette tip.
16. Air dry the beads for 5 min, which will avoid overdrying them. Remove the tube from the magnetic stand and add 25 μL of Zymo Elution Buffer for a final DNA concentration of 10–20 nM (i.e., assuming ~25–50% DNA loss during the AMPure cleanup). Thoroughly resuspend the beads by pipetting up and down ~10 times. Incubate for 2 min to allow the DNA to elute off of the beads. Place the tube on the magnetic stand until the solution is clear. Transfer the supernatant to a new 1.5-mL tube.
17. Check samples on an agarose gel or by Fragment Analyzer (Fig. 1c, d) and proceed with Illumina sequencing with the paired-end 50- or 100-bp kit.

3.8 Data Analysis

We use the Linux/MacOS command-line interface (CLI) to process Micro-C data. We recommend using Conda to create a virtual environment for Micro-C analysis.

3.8.1 Preprocessing Micro-C Contact Pairs

1. Install HiC-Pro v.2.11.4 from https://github.com/nservant/HiC-Pro [16].
2. Set up the HiC-Pro configuration file. For example, we used the following settings for Micro-C data in mouse embryonic stem cells.

```
REFERENCE_GENOME = mm10
          BOWTIE2_GLOBAL_OPTIONS = --very-sensitive-local --
reorder --trim5 5 --trim3 5
       MIN_CIS_DIST = 200
       GET_ALL_INTERACTION_CLASSES = 1
       GET_PROCESS_SAM = 0
       RM_SINGLETON = 1
       RM_MULTI = 1
       RM_DUP = 1
       BIN_SIZE = 500 1000 2500 5000 10000
```

3. The output folder contains Micro-C mapping, pairing, and QC statistics, and all unique contacts at base-pair resolution and binned/normalized matrix files.

3.8.2 Convert Tab-Delimited Files to HDF5 (Cool) Format and Normalization

1. Install cooler (https://github.com/mirnylab/cooler) and cooltools (https://github.com/mirnylab/cooltools) packages [17].
2. Build a cool file from the allValidPairs file from the HiC-Pro output:

```
cooler cload pairs -c1 2 -p1 3 -c2 5 -p2 \
      mm10-bin-200bp-bed-file \
      allValidPairs-file \
      output-cool-file
```

3. Build cool files in a different resolution. For example, we are going to build a 1-kb data from 200-bp data:

```
cooler coarsen -k 5 -p 16 \
      input-200bp-cool-file \
      output-1kb-cool-file
```

4. Normalize the data using the matrix balancing method:

```
cooler balance -p 16 input-cool-file
```

   Note that an optimal matrix balancing result may require fine-tuning with the 'MAD-MAX' function. An optimal normalization result is expected to have minimal background noise that could be artificially introduced during the process of matrix balancing.

5. Compute the expected matrix:

```
cooltools compute-expected -p 16 \
      input-cool-files > output-expected-file
```

6. Build multiple-resolution cool files for the HiGlass browser [18]:

```
cooler zoomify -p 16 -balance \
      input-cool-files \
      -o output-mcool-file
```

7. Load multiple-resolution cool files to the HiGlass server by following the tutorial available at https://docs.higlass.io/tutorial.html. Here is an example of Micro-C results (Fig. 2). Publicly available Micro-C data sets are summarized in Table 2.

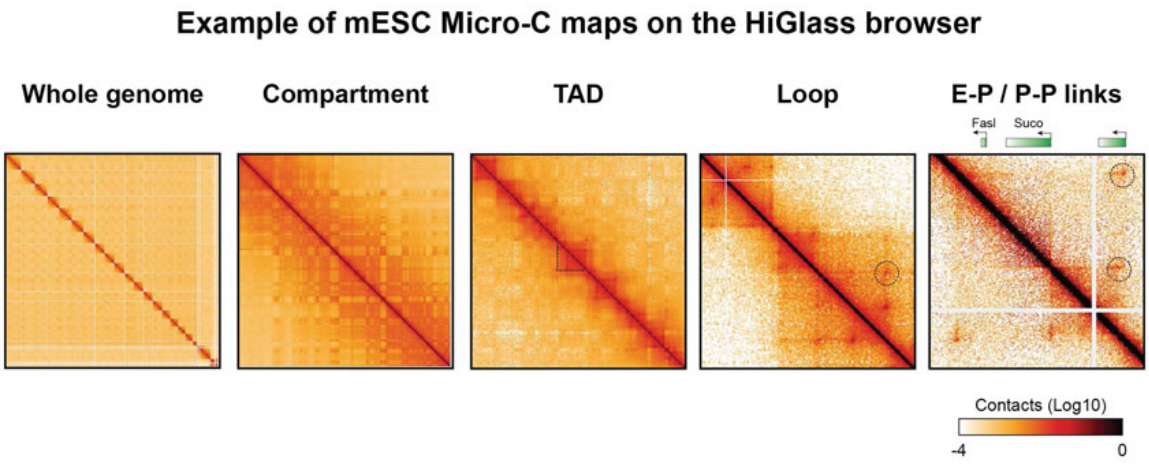

Fig. 2 Example of Micro-C maps on the HiGlass browser. From left to right, the Micro-C maps were zoomed-in from the whole genome scale to a 200-kb region in chr1 with increasing resolution. 3D genome features such as compartments, TADs, loops, and E-P/P-P links are clearly discernible on the maps

4 Notes

1. The quality of low-retention tubes and pipette tips varies with different manufacturers. We tested various brands and found that the 15 mL and 50 mL conical tubes by Eppendorf and the low-retention tips by Rainin perform robustly and consistently to reduce sample loss during Micro-C experiments. Using high-quality supplies is especially important if the amount of input is low, for example, <100,000 cells.
2. We tested a variety of cross-linking conditions in budding yeast [9] and mouse ES cells (Fig. 3), and identified a combination of protein-protein crosslinker DSG (disuccinimidyl glutarate, 7.7 Å) or EGS (ethylene glycol bis(succinimidyl succinate), 16.1 Å) with DNA-protein crosslinker FA (formaldehyde, weaker protein-protein crosslinker) that produces the highest signal-to-noise ratio in Micro-C maps. The irreversible protein-protein crosslinker (DSG or EGS) also prevents loss of cross-linking during the harsher incubation steps (e.g., 65 °C). The usage of DSG or EGS and the order of treating cells with FA and either long crosslinker does not show a significant effect on the results at the scale of chromosome and compartment (Fig. 3). Thus, we choose the combination of FA and DSG as the standard cross-linking procedure for Micro-C.
3. Freshly prepared FA and DSG are critical to achieve the best cross-linking outcomes. We recommend using FA in the ampule packaging and preventing exposure of DSG powder to moisture.

Table 2
Publicly available Micro-C data sets in mammalian cells (last updated 2020/12/20)

Cell type	Genotype/ Treatment	Estimated unique reads	Data links	Lab	References
Mouse embryonic stem cell (JM8.N4)	Wild-type	~2.64 B	GSE1302754DNES14CNC1I	Tjian and Darzacq lab	Hsieh et al. (2020) Mol Cell [14]
Mouse embryonic stem cell (JM8.N4)	Triptolide	~500 M	GSE1302754DNESSY8C22T	Tjian and Darzacq lab	Hsieh et al. (2020) Mol Cell [14]
Mouse embryonic stem cell (JM8.N4)	Flavopiridol	~500 M	GSE1302754DNES7X5GQUR	Tjian and Darzacq lab	Hsieh et al. (2020) Mol Cell [14]
Mouse embryonic stem cell (JM8.N4)	Wild-type	~250 M	GSE126112	Tjian and Darzacq lab	Xie et al. (2020) Nat Methods [19]
Mouse embryonic stem cell (JM8.N4)	CTCF depletion	~225 M	GSE126112	Tjian and Darzacq lab	Xie et al. (2020) Nat Methods [19]
Mouse embryonic stem cell (JM8.N4)	Wild-type	~668 M	GSE123636	Tjian and Darzacq lab	Hansen et al. (2019) Mol Cell [13]
Mouse embryonic stem cell (JM8.N4)	CTCF with RBRi-del	~697 M	GSE123636	Tjian and Darzacq lab	Hansen et al. (2019) Mol Cell [13]
Human embryonic stem cell (H1)	Wild-type	~3.23 B	4DNES21D8SP8	Oliver Rando lab	Krietenstein et al. (2020) Mol Cell [15]
Human foreskin fibroblast (HFFc6)	Wild-type	~4.57 B	4DNESWST3UBH	Oliver Rando lab	Krietenstein et al. (2020) Mol Cell [15]
WTC derived iPS cells	Wild-type	–	4DNESODGV2V24DNESAGG7EUC	Job Deeker lab	–

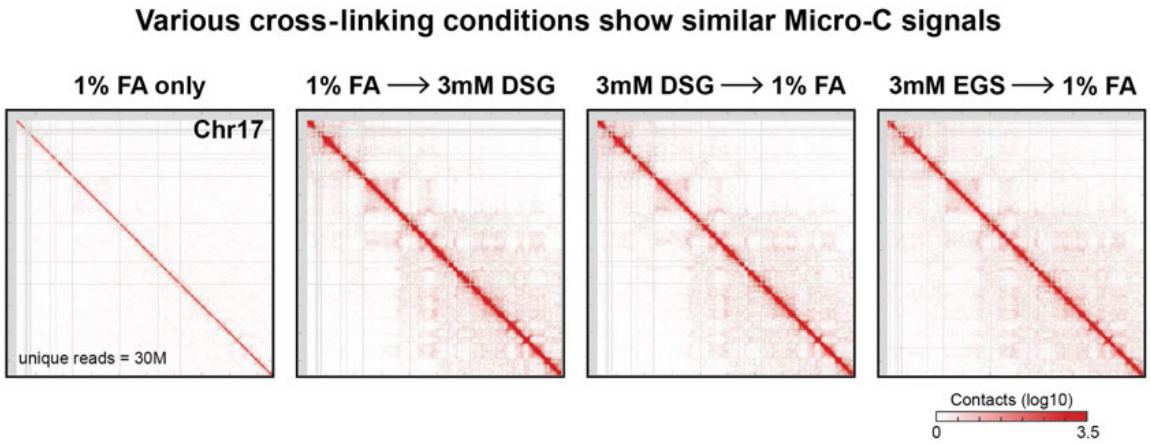

Fig. 3 Example of the effects of different cross-linking protocols. From left to right, cells were crosslinked by (1) 1% formaldehyde (FA) for 10 min at RT; (2) 1% FA for 10 min at RT and then subjected to 3 mM DSG for 40 min at RT after washes; (3) 3 mM DSG for 30 min at RT and then subjected to 1% FA directly in the sample for an additional 10 min at RT; (4) 3 mM EGS for 30 min at RT and then subjected to 1% FA directly in the sample for an additional 10 min at RT. All dual cross-linking protocols produce comparable Micro-C maps at the chromosome and compartment levels regardless of the order of FA and long crosslinkers, while the FA only condition fails to recapitulate the known 3D genome features

4. Prepare a stock without NP-40 or protease inhibitors and filter through a 0.22 μm filter. Add NP-40 and protease inhibitors directly prior to use.
5. Upon first use of the MNase solution, store the aliquot at −20 °C and use another two times at most (maximum three uses per −20 °C aliquot).
6. EGTA is a stronger Ca^{2+} chelator than EDTA.
7. NuSieve GTG agarose is ideal for separating mono- and dinucleosomes, and it yields a higher DNA recovery rate with most gel extraction kits. Typically, a 2.5–3% TBE gel can achieve a clean separation between mono- and dinucleosomes. When making the NuSieve agarose gel, shake at 100 rpm for 10–20 min after adding TBE to the powder to help with dissolving, then microwave as usual.
8. This Micro-C protocol is also compatible with cells sorted from a cell sorter. For this purpose, we prepare ~1–1.5 × 10^7 cells expressing a fluorescent protein (e.g., GFP) growing on a 10 cm^2 culture dish. After cell suspension, PBS wash, cross-linking with 1% formaldehyde, and quenching (*see* Method Subheading 3.1, **steps 2–5**), we resuspend cells with cell medium or PBS and pass the cells through a 35-μm strainer into a sterile 5 mL round-bottom polystyrene FACS tube. We routinely collect one million cells. However, if the targeted population is not abundant, using as few as 100 thousand

cells also produces high-quality Micro-C results. After sorting, proceed to Subheading 3.1, **step 6**. We note that this protocol has not been tested with ex vivo tissues.

9. If your applications or cell lines are sensitive to trypsin treatment, cells can alternatively be crosslinked directly on the dish as follows.
   (a) Wash cells twice with PBS or base culture media (-FBS) at room temperature.
   (b) In the fume hood, add freshly prepared 1% FA crosslinking media (-FBS) and incubate for 10 min at RT while shaking at 50 rpm.
   (c) Add 1 M Tris–HCl pH 7.5 to a final concentration of 375 mM to quench the reaction.
   (d) Wash cells twice with PBS at room temperature.
   (e) Add freshly prepared 3 mM DSG solution and incubate for 40 min at room temperature while shaking at 50 rpm.
   (f) Add 1 M Tris–HCl pH 7.5 to a final concentration of 375 mM to quench the reaction.
   (g) Wash cells twice with PBS at room temperature.
   (h) Scrape off the crosslinked cells, transfer to a low-binding tube, and snap freeze in liquid nitrogen.
10. FBS substantially reduces cross-linking efficiency. Before performing formaldehyde and DSG cross-linking, any FBS residue should be removed by washing cells with PBS.
11. Avoid using glass or plastic surgical pipettes to resuspend cell pellets. Cells easily stick to the pipettes, which results in a drastic loss of cells.
12. We routinely perform Micro-C with 1×10^6 cells. However, in some circumstances or cell types, one may find significant cell loss after DSG cross-linking (i.e., cells stick on the tube or tip). In that case, we recommend starting with 5×10^6 cells. We note that in this protocol, all the reactions are specified for using 1×10^6 cells. If using 5×10^6 cells, we suggest scaling up the volume by 2 for all reactions starting from Method Subheading 3.2.
13. Pellets can be stored at −80 °C for several months.
14. Optimally, every batch of cross-linked cells needs to undergo MNase titration. If not a full titration, perform a "best-guess" digestion and adjust experimental conditions accordingly. For example, when we carry out a new Micro-C experiment with a relatively low amount of input cells that are not amenable to a full titration, we usually run a "best-guess" digestion, that is, using 20 units MNase for 1 M cells. We then adjust the

digestion conditions for the actual Micro-C experiment based on the result of the "best-guess" digestion. Typically, if tri- or tetranucleosomes are clearly visible (an indicator of underdigestion), we will increase the amount of MNase by 1.5×; in contrast, if nucleosome dimers are faint (or invisible) and the majority of DNA is smaller than 100 bp (an indicator of overdigestion), we will reduce the amount of MNase by half. The MNase titration experiment requires good practice to gain an idea of the outcomes of chromatin digestion upon changes in parameters such as the amount of MNase, incubation time, or temperature. If you have a limited availability of your cells of interest, we recommend practicing MNase titration using common culture cells with similar properties.

15. For mouse embryonic stem cells, the optimal amount of MNase is typically around 20 U for 1×10^6 cells.
16. DNA purification by either ethanol precipitation or the Zymoclean DNA Clean & Concentration kit shows indistinguishable results regarding DNA yields and the pattern of the nucleosomal ladder. Purification with the Zymoclean kit saves ~2 h in the protocol.
17. The yield varies by cell type. Typically, one million mESCs with an optimal MNase digestion yields ~2.5 μg of total DNA. A DNA yield lower than 1 μg indicates overdigestion of chromatin. The majority of DNA fragments will run below 100 bp on an agarose gel. We strongly suggest not to use overdigested samples for Micro-C to avoid poor ligation efficiency.
18. For all gels in this protocol, use Orange G gel loading dye, which migrates at <75 bp, and so minimally interferes with the visualization of nucleosomal fragments.
19. A key factor that affects the quality of the Micro-C library is minimizing contamination from unligated dinucleosomes. Since dimers are relatively more abundant than ligated dinucleosomes, they could still remain at a significant amount even after streptavidin purification. We strongly recommend using the digestion conditions that yield 90% monomers and 10% dimers. This reduces the ratio of dimer contamination in the sequencing library while retaining the length of nucleosome ends that are sufficient for ligation.
20. T4 PNK catalyzes the transfer of a phosphate group to the 5′ ends of DNA and removes phosphate groups from the 3′ ends of DNA. This reaction will convert the multiple types of MNase-digested ends to ligatable ends.
21. In the absence of dNTPs, the Klenow Fragment only possesses a 3′–5′ exonuclease activity, creating single-stranded DNA overhangs that can be labeled with biotin in the next step.

22. About 2 to 4 h of incubation for the ligase reaction is sufficient. In our hands, an incubation longer than 4 h is not necessary, as it does not lead to any noticeable improvement in the Micro-C library quality.
23. The exonuclease reaction does not chew off the entire nucleosomal DNA since the crosslinked nucleosome can block the digestion. Thus, extending the exonuclease reaction time from 15 min to 30 min sometimes reduces the ratio of un-ligated dimers in the library.
24. Purifying the ethanol-precipitated DNA with the Zymoclean DNA Clean & Concentrator Kit is critical to remove ethanol and salts that might affect DNA mobility on a gel.
25. The purification of dinucleosomes is the key step to eliminate unwanted contamination from mononucleosomes. The result is much cleaner by gel extraction than by two-sided AmpureXP purification. Using bead-based DNA purification is preferable for lower amounts of DNA.
26. At this point, you may freeze your sample at −20 °C and proceed the following day.
27. The streptavidin beads have a very high capacity, so 5 μL will be more than enough even when starting from 5×10^6 cells.
28. Remove the 10 mM Tris–HCl only once the End Repair mix for the next step is ready. Do not let the beads dry out.
29. At this point, you may freeze your sample at −20 °C and proceed the following day.
30. There is no need to remove streptavidin beads before this step, as they will stay in the well without disturbing DNA migration through the gel.
31. If processing more than one sample, you can choose different indexed primers to barcode each sample and pool samples before sequencing.
32. A high-quality Micro-C library should have ~50–100 ng of total DNA with <12 PCR cycles.

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