# scNanoHi-C: a single-cell long-read concatemer sequencing method to reveal high-order chromatinstructures within individual cells

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**Method Article**

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# Abstract

The high-order three-dimensional (3D) organization of regulatory genomic elements provides a topological basis for gene regulation, but it remains unclear how multiple regulatory elements across the mammalian genome interact within an individual cell. To address this, herein, we developed scNanoHi-C, which applies Nanopore sequencing to explore genome-wide proximal high-order chromatin contacts within individual cells. Evaluation of the method suggested that scNanoHi-C reliably and effectively profiled 3D chromatin structure and distinguished structure subtypes among single cells. This method could also be used to detect genomic variations,including CNVs and SVs, as well as to scaffold the de novo assembly of single-cell genomes. Importantly, our results suggested that extensive high-order chromatin structures exist in active chromatin regions across the genome, and multiway interactions between enhancers and their target promoters were identified within single cells. Altogether, scNanoHi-C offers new opportunities to investigate high-order 3D genome structures at the single-cell level.


# Introduction

scNanoHi-C is a 3C-based method that relies on proximity ligation. Specifically, we incubated with 1% formaldehyde (FA) followed by 1.5 mM disuccinimidyl glutarate (DSG) for cross-linking, which improved the detection of both short-range and long-range chromatin interactions by reducing the noise of random ligation[26]. Then, flexible single-cell whole genome amplification for long read length was conducted. Herein, we designed 24 Tn5 enzyme conjugates and used a low-density Tn5 transposon with the same adapter to randomly fragment genomic DNA from each single cell to improve throughput. By pooling cells tagged with different Tn5 barcodes together, throughput could be controlled by combining different barcodes in subsequent amplification primers. In this way, scNanoHi-C can be flexibly applied to sequence only a few cells or several thousands of cells (up to 24×96) in one PromethION run, allowing us to either obtain more contacts to detect high-resolution genome structures within individual cells or sequence more single cells at a shallower depth in a high-throughput manner. Finally, the amplicons after primer-dimer removal were approximately 3 kb long and were appropriate for sequencing on the Nanopore platform

# Reagents

HBSS (Gibco, cat# 14025076)

16% formaldehyde(Thermo Scientific, cat#28906)

DSG  (Thermo Scientific, cat#20593)

MboI (NEB, cat#R0147M)

Tn5 enzyme (Vazyme, Cat# S601-01)

Ampure XP beads (Beckman, Cat#A63882)

Tks Gflex DNA polymerase (TAKARA, Cat# R060B)

# Equipment

PCR instrument Analytik Jena Biometra TRIO

Vortex mixer Scientific Industries Vortex-GENIE 2

Magnetic stand Diagenode B04000001

# Procedure

### 1. Cross-linking

Cross-linking with formaldehyde (FA)

We resuspended the cells (total: 0.5-1.0 million cells) with 1 mL HBSS (Gibco, cat# 14025076), and added 66.67μL fresh prepared 16% formaldehyde (Thermo Scientific, cat#28906, final 1%). Cells were then incubated for exactly 10 minutes at room temperature (RT) with gentle rotation to perform fixation. FA was quenched by the addition of 200μL 1% BSA in PBS and followed by incubating for 5 min at RT and then incubating at least 15 min on ice to stop the FA cross-linking completely. Cells were centrifuged at 1,000×g for 10 min. After removing the supernatant, cell pellets were washed with PBS once (centrifuged 1,000×g, 10 min), then proceeded immediately to disuccinimidyl glutarate (DSG) cross-linking.

Cross-linking with disuccinimidyl glutarate (DSG)

We resuspended the FA cross-link cells with 995μL PBS and added fresh 5 μL DSG 300 mM (Thermo Scientific, cat#20593, final 1.5mM) and incubated for exactly 40 minutes at RT with gentle rotation to perform fixation. DSG was quenched by the addition of 500μL 1% BSA in PBS and followed by incubating for 5 min at RT to stop the DSG cross-linking completely. Cells were centrifuged for 2,000×g, for 15 min. After removing the supernatant, cell pellets were washed with 0.1% BSA in PBS once (centrifuged 2,000×g, 15 min) and pellets were stored at −80 ˚C.

### 2. Chromosome Conformation Capture

Cell lysis

We combined 250μL of ice-cold lysis buffer (10 mM Tris-HCl, pH 8.0; 10 mM NaCl; 0.2% Igepal CA630; 100× cOmplete proteinase inhibitor), and added lysis buffer to FA-DSG fixed cell pellets and incubated cell suspension on ice for 20 min. Then centrifuged at 2,500×g for 5 min at 4 ˚C and discarded the supernatant.

Remove proteins and endonuclease digestion

We resuspended the cell pellet in 50 μL of 0.5% SDS and shaken at 900 r.p.m. for exactly 62 ˚C, 10 min on the thermomixer, added 145 μL of dd$H_2O$ and 25 μL of 10% (v/v) Triton X-100 to a final concentration of 1% (v/v) to quench the SDS. Cells were gently shaken for 15 minutes at 37 °C. Then added 25 μL of 10×rCutSmart™ Buffer and 20μL 25,000 units/mL of MboI (NEB, cat#R0147M), then rotated at 37°C overnight.

Proximity Ligation

We combined 970μL of ice-cold ligation buffer (100μL 10× T4 DNA ligase buffer, 5μL BSA, 865μL $H_2O$), and added ligation buffer to digestion cells and then added 5μL T4 DNA ligase in each tube. Cells were gently shaken at 300 r.p.m for exactly 4 hours at 16°C on the thermomixer. After ligating, cells were centrifuged at 2,500 × g for 5 minutes at 4°C and removed the supernatant. Then cell nuclei pellets were washed with 0.1%BSA in PBS once.

## 3. FACS of ligated nuclei and lysis of cells

We resuspended the ligated nuclei pellets in 200μL 0.25% BSA in PBS in each tube and added 0.2μL 1,000× DAPI for sorting nuclei. A single nucleus was flow sorted into each well of a 96-well plate containing 2 μL lysis buffer (0.04 μL 1M Tris-HCl (pH 8.0), 0.08μL 500 mM NaCl, 0.03μL 10% Triton X-100, 0.0004μL 0.5 M EDTA, 0.05μL 20mg/mL QIAGEN protease). And then incubated at 50°C, 3h; 70°C, 20 min; 68°C, 45 min; 4°C pause for decrosslinking and digestion. After digestion, 96-well plates were stored at −80 ˚C.

## 4. Amplification of single-cell gDNAs

The amplification method was the first one that could achieve high-throughput long-fragment amplification and was different from the existing technology applied to single-cell Hi-C based on the next-generation sequencing (NGS) platform. We developed the amplification protocol of scNanoHi-C from three aspects. First, we embedded Tn5 transposase with only one adaptor sequence instead of two

different adaptor sequences to reduce short fragment amplification which could recover more original DNA fragments of ligation products. Second, we probed the appropriate concentration of the Tn5 enzyme mixture. The length of amplified fragments can be controlled by adjusting the concentration of the Tn5 enzyme mixture. Third, we originally designed 24 types of conjuncted Tn5 enzyme with 24 bp barcodes (the Tn5 adapter primers as shown in Supplementary Table 10) to improve throughput. After tagmentation, we pooled cells with different Tn5 barcodes together for subsequent amplification to improve genome coverage. It could not only meet the needs of high-throughput to reduce cost (we showed 500 single cells in one sequencing run), but also meet high-resolution needs (we showed 24 single cells in one sequencing run) by changing the combination of Tn5 barcodes and PCR barcodes for high-depth sequencing.

In detail, we add 8 μL of Tn5 tagmentation mixture, containing 2 μL of 5× TAPS_PEG8K (50 mM TAPS-NaOH, pH 8.3, 25 mM $MgCl_2$, 40% PEG8K), 1μL of 0.2 ng/μL conjunct Tn5 enzyme (Vazyme, Cat# S601-01) which has 24 types of barcode and 5μL $H_2O$ in each well. The total 10μL tagmentation mixture reaction was then incubated at 55°C for 10 min. To stop the tagmentation reaction, 2.5 μL of 0.2% SDS was added, and then the reaction was left at room temperature for 5 min. And then, every 24 single cells with different Tn5 barcodes were pooled in one tube and then they were purified with 0.8 volume of Ampure XP beads (Beckman, Cat#A63882) and finally eluted with 50 μL $H_2O$, the purification process was also a volume concentration process. These purified genomic DNAs of cells were then used for amplification. The amplification buffer mixture consists of 2 μL 1. 25U/μL Tks Gflex DNA polymerase (TAKARA, Cat# R060B), 50 μL 2× Gflex PCR buffer,18 μL $H_2O$ and 5 μL 10uM I5-nano PCR primers containing a 24 bp cellular barcode (5'AATGATACGGCGACCACCGAGATCTNNNNNNNNNNNNNNNNTCGGCAGCGTC 3') (Supplementary Table 10 for I5 PCR primers), and every 25 μL of purified genomic DNA was added to 75 μL amplification buffer. The amplification reaction was then carried out as follows. First, 72°C for 3 minutes for gap filling, 98°C for 1 minute, then 20-22 cycles of 98°C for 15s, 60°C for the 30s, and finally 68°C for 5 minutes. The amplification products were then purified twice with 0.5× AMPure XP beads. These purified amplicons were quantified using Qubit, 1-96 products with different PCR barcodes (24-2,304 cells in total) were pooled together and about a total of 0.5μg to 2μg amplicons products were used for further library construction. Next, products were sequenced on one Oxford Nanopore PromethION 48 (Oxford Nanopore Technologies; R9.4.1).

# Troubleshooting

# Time Taken

Cross-linking with formaldehyde (FA): total  45min

Cross-linking with disuccinimidyl glutarate (DSG): total ~60min

Cell lysis: total ~30min

Remove proteins and endonuclease digestion: total overnight 12-16h

Proximity Ligation: total ~5h

## Anticipated Results

The length distribution of fragments after MboI digestion is about 6kb

The length distribution of fragments after proximity ligation (medium) and amplification (right) are about 3kb

## Figures

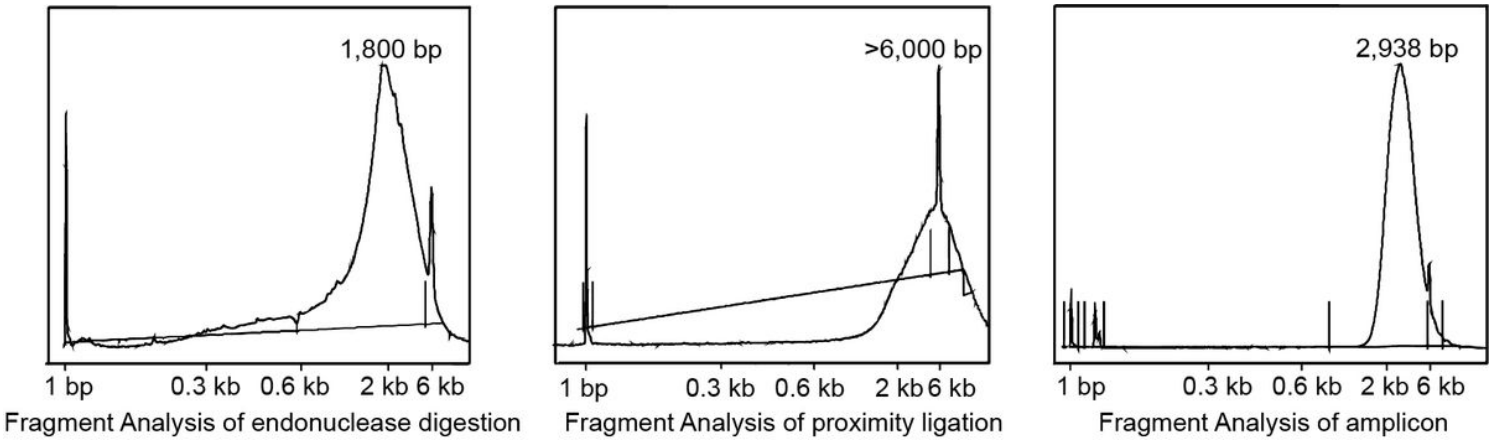


**Figure 1**

Fragment analysis