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Microfluidics-based chromosome conformation capture (3C) technology for examining chromatin organization with a low quantity of cells

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Abstract

Detecting three-dimensional (3D) genome organization in the form of physical interactions between various genomic loci is of great importance for understanding transcriptional regulations and cellular fate. Chromosome Conformation Capture (3C) method is the gold standard for examining chromatin organization, but usually requires a large number of cells ($>10^7$). This hinders studies of scarce tissue samples from animals and patients using the method. Here we developed a microfluidics-based approach for examining chromosome conformation by 3C technology. Critical 3C steps, such as digestion and re-ligation of BAC DNA and cross-linked chromatin, were implemented on a microfluidic chip using a low quantity of cells ($<10^4$). Using this technology, we analysed the chromatin looping interactions in the human β -globin. We envision that our method will provide a powerful tool for low-input analysis of chromosome conformation and epigenetic regulations.

Introduction

A single human genome is about 2 meters long if stretched, thus a high degree of packing is required to fit the genome in a nucleus with a diameter less than 10 μm . This packing is not random, as the 3D organization of the genome is not only important from structural perspective, but also strongly relevant to genomic processes¹⁻³. It has been revealed that genes are regulated by the combined action of many regulatory elements, which can be located at large genomic distances (ten to hundreds of kb) away from the targets⁴⁻⁶. These long-range regulations are mediated by physical interactions between genomic loci that come in close spatial proximity via folding of DNA into chromatin^{4,7-9}. Chromosome conformation capture (3C) methodology has been developed to identify the organization of chromatin by converting physical interactions between elements into specific ligation products that can be quantified by PCR¹⁰⁻¹⁵. In a typical 3C experiments, interacting

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Supporting Information: The Supporting Information is available free of charge on the ACS Publications website. Additional experimental sections on microfluidic device fabrication and operation, BAC DNA purification and control library preparation by conventional method, cell culture and 3C library preparation by conventional method. Supplementary Figure S1, Table S1 and Table S2.

chromatin segments are crosslinked followed by digestion and intramolecular ligation. The ligation products are amplified by PCR using locus-specific primers. Their amounts are then estimated by gel electrophoresis of PCR products, which is proportional to the interaction frequency of two chromatin regions. TaqMan qPCR technology has also been used to provide accurate quantification of ligation products^{11,16}. Variants of 3C assay using sequencing technique to examine ligation products have also been developed for genome-wide contact maps¹⁷⁻²⁰.

Standard 3C-based assays require a large number of cells ($>10^7$ cells) due to material loss and low reaction efficiency, posing a challenge for studies of low-abundance primary samples from lab animals and patients. In addition, 3C methods provide an estimate of the average chromosome conformation in all cells included in the analysis. However, studies (e.g. fluorescence in situ hybridization (FISH)) have shown that even genotypically and phenotypically identical cell populations can exhibit considerable variability in chromosome folding^{21,22}. This heterogeneity within apparently homogeneous population also led to an interest in improving the protocol using fewer cells. A single-cell Hi-C (a variant of the 3C technique) protocol has been reported to study chromosome arrangement in individual cells^{23,24}. However, it still required 10^7 cells or more to conduct.

Microfluidics provides a convenient and powerful platform for manipulating tiny amount of liquid and integrating multiple steps on a small chip. The tiny volume within the microfluidic chip allows the building of high concentrations from limited amounts of samples and reagents (e.g. chromatin, restriction enzyme). Microfluidics allows integration of multiple steps of a complex molecular process on a single chip, which minimizes sample loss due to transfer. It also provides high level of automation and high throughput. Considerable efforts have been made toward genetic²⁵⁻³¹ and more recently epigenetic analysis on microfluidic chips³²⁻³⁹, including microfluidic device for DNA sequencing^{30,31}, microfluidic chromatin immunoprecipitation (ChIP) to study protein-DNA interaction^{33,34}, microfluidic RNA sequencing to determine transcriptome^{36,40}. However, applying microfluidic technology to studies of chromosome conformation has not been reported.

In this study, we developed a microfluidic approach for examining chromatin interactions by 3C method with high sensitivity. Our microfluidics-based 3C assay was able to detect chromosome conformation using less than 10,000 cells, compared to more than 10^7 cells required by conventional 3C technology^{11,41}. The microfluidic device is designed to take advantage of concentration-driven diffusion to replace reagents in the reaction chamber as needed while keeping DNA molecules inside²⁵. Alternating pressure pulses on the two ends of the reaction chamber were used to generate oscillatory movement for sample mixing, which is essential for high efficiency in-nucleus digestion^{33,34}. As a proof-of-concept, we examined the chromatin looping interaction at the human β -globin locus in globin-expressing K562 cells. The chromatin interactions between the β -globin Locus Control Region (LCR) and the γ - δ intergenic region in K562 cells has been well-studied by traditional 3C methods⁴²⁻⁴⁵. BAC DNA can be effectively digested and ligated with our method. On-chip digestion of genome within K562 nuclei showed high efficiency ($>75\%$) for all 3 examined sites. Ligation of digested sample in our microfluidic-based system yielded a 3C library that could be quantified by Taqman qPCR. We show that the chromatin

interaction map revealed by our microfluidic method was similar to that obtained by traditional bulk 3C assay.

Results and Discussion

The principle of chromosome conformation capture (3C) for examining chromatin interaction is illustrated in Fig. 1. Cells are firstly fixed by formaldehyde, which leads to crosslinking of interacting chromatin segments. The subsequent cell lysis allows release of nuclei while keeping information on the spatial organization of chromosomes. Crosslinked chromatin is then cut by restriction enzyme EcoRI, after which DNA ends are linked by T4 DNA ligase. The ligation step is conducted at very low DNA concentration so that the intramolecular ligation of crosslinked fragments occurs to a much higher degree than intermolecular ligation of random fragments. Crosslinking is then reversed by Proteinase K and the ligation products are examined and quantified by Taqman qPCR using specific primers targeting candidate fragments. Since the intramolecular ligation of two elements is largely dependent on their crosslinking status, the amount of each specific ligation product reflects the interaction frequency of the two corresponding fragments.

In order to minimize the influence of primer efficiency on the quantification of chromatin interaction, a PCR control library containing all ligation products of interest in equal amounts needs to be established^{11,41}. Bacterial artificial chromosome (BAC) clones are used. These DNA sequences (usually have size ranging from 150 k-350 k base pairs) are segments of human DNA without 3D conformation. The selected BAC clone should cover the entire genomic region under study with minimal overlapping. This BAC clone(s) is then digested with restriction enzyme and randomly religated by ligase enzyme. In this study, in order to investigate chromatin conformation in human β -globin locus, BAC clone RP11-910P5 (~166 kb, Children's Hospital Oakland Research Institute) covering this region was used as the control library. Interaction frequencies are typically expressed as the ratio of amount of ligation products obtained with crosslinked DNA to the amount of ligation products obtained with BAC library.

A microfluidic device containing a central reaction chamber (0.5 μ l in volume), two side reagent loading chambers (1.3 μ l in volume), and associated connection channels and valves was used for 3C analysis, as shown in Fig. 2. Pneumatic two-layered microvalves (v1-6) were used to control reagent transport into and between chambers. This device was designed to conduct multi-step treatment of DNA by diffusion-based reagent swapping²⁵. We first tested our microfluidic device for microfluidic BAC DNA digestion and ligation. As shown in Fig. 2a, with the valves between the reaction and loading chambers (v3 and v4) closed, purified BAC DNA was introduced into the reaction chamber and digestion solution containing EcoRI enzyme was loaded into the two loading chambers on the sides. After reagent loading, v3 and v4 were opened to allow the digestion reagents to diffuse into reaction chamber for 15 min. Due to the difference in the diffusivity between BAC DNA ($8.5 \times 10^{-14} \text{ m}^2 \text{ s}^{-1}$ for 166 kb BAC DNA, calculated using an empirical equation⁴⁶) and digestion reagents ($8.8 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$ for EcoRI (31 kDa)⁴⁷, $3.0 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ for Triton X-100⁴⁸, $6.6 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ for Tris⁴⁹, $1.0 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ for small ions such as Mg^{2+} , Na^+ and Cl^{-50}), the fast-diffusing digestion reagents entered the reaction chamber with minimal

loss of the slow-diffusing large DNA molecules²⁵. The microfluidic chip was then placed on a 37 °C hotplate for EcoRI digestion for 1 h with all valves closed. After digestion, ligation solution containing T4 ligase (68 kDa, diffusivity of $6.5 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$) was flowed into the loading chambers to replace digestion solution (Fig. 2b). The ligation reagents diffused into the reaction chamber after opening v3 and v4. Due to the small sizes of digested DNA fragments ($< 2 \text{ kb}$, diffusivity $> 2.0 \times 10^{-12} \text{ m}^2 \text{ s}^{-1}$), DNA diffused to occupy the reaction and loading chambers during the same period. The valves between the reaction and loading chambers remained open during ligation reaction (1 h at room temperature). Solution in reaction and loading chambers were collected after ligation reaction. Digested and ligated BAC DNA produced with microfluidic chip (Fig. 2c) and with conventional assay in tube (SI Fig. S1) are analyzed by gel electrophoresis. In both cases, uncut DNA appeared above 10 kb DNA molecular weight marker as a tight bar in the agarose gel. Digested DNA migrated to smear-like pattern in the gel, indicating effective digestion of the DNA. The disappearance of most of the smear-like pattern after ligation is an evidence of good ligation^{12,41}.

Next we examined the chromatin interaction at the human β -globin locus. The human β -globin locus is composed of five genes (ϵ , HBE; $A\gamma$ and $G\gamma$, HBG1 and HBG2; δ , HBD; and β , HBB) and one pseudogene (ψ , HBpsi) located on a short region of chromosome 11. Expression of these genes is regulated by single locus control region (LCR) via its interaction with the individual globin gene promoters. The enhancer-promoter interactions are differentially regulated throughout development and this is important for erythropoiesis^{45,51-53}. In our experiment, human leukemia lymphoblast K562 cells were first crosslinked by fixation and then lysed to extract intact nuclei. ~5000 crosslinked nuclei were flowed into the reaction chamber (0.5 μL in volume) of the microfluidic chip for restriction digestion in order to separate each elements of interest. The operation of the microfluidic chip was similar to BAC DNA on-chip digestion and ligation (Fig. 2). EcoRI in digestion buffer was pumped into the loading chambers with all valves closed. The enzyme entered the reaction chamber by concentration-gradient-driven diffusion after opening the valves while nuclei stayed in the reaction chamber due to their large size. In order to increase nuclei digestion efficiency, an oscillation step was used to improve mixing by moving nuclei back and forth in the chamber. As shown in Fig. 3a, with valves 2 and 5 opened and all other valves closed, the alternating pressure pulses applied on reservoirs 2 and 3 drove the oscillatory movement of nuclei in the reaction chamber³⁴. After oscillatory digestion at 37 °C overnight, the digested sample was collected and analyzed by SybGreen qPCR to determine digestion efficiency. We examined the restriction digestion at 3 sites: HS5, HBG1 and HBB. The digestion efficiencies under different conditions were shown in Fig. 3b. The required cell number as well as the reaction volume in microfluidic chip is 1000 times smaller than that in the tube (conventional method), but showed comparable digestion efficiency. With 4 h incubation, 64.2% HS5, 68.3% HBG1 and 56.2% HBB fragments were digested on microfluidic chip, compared to 73.4% HS5, 72.4% HBG1 and 59.8% HBB digested in tube. With longer reaction time (16 h), the efficiency of restriction enzyme increased both on microfluidic chip and in tube: 71% HS5, 76.9% HBG1 and 76% HBB for microfluidics-based reaction and 74.7% HS5, 77% HBG1 and 78% HBB for in conventional tube reaction. Student's t-test indicates no significant differences between microfluidic

digestion and conventional digestion efficiency with 4 h or 16 h incubation for all investigated sites. Our microfluidic device achieved high EcoRI digestion efficiency (>70% for all sites with 16 h incubation). This highly digested sample is sufficient for subsequent 3C analysis¹¹.

We then used our microfluidic device to examine the interactions between the LCR HS5 (constant fragment) and 6 other sites (candidate interacting fragments) located 30-50 kb downstream in the β -globin locus (including the regions overlapping HBG1 (A γ), HBG2 (G γ) and HBpsi (ψ)) using 1000 cells. Digested sample and ligation reagents were loaded into the reaction chamber. With all valves closed, the microchip was placed on a flat-plate thermal cycler for ligation at 16 °C for 4 h and 20 °C for 0.5 h. Ligated sample was collected and the ligation products were quantified by TaqMan qPCR using specific primers. Ligation products from BAC clone were used as control templates and to generate a standard curve. Fig. 4 shows that the chromatin looping interactions in human leukemia lymphoblast K562 cells, which are known to have high γ expression level⁴². A peak of interaction frequency was found between the LCR HS5 region and HBG1 loci (A γ , located ~41.7 kb downstream), with a value of ~3.6 times of the interaction frequency between HS5 and a site closer to it (~30.7 kb downstream). Since random collisions are likely decreased for sites separated by increasing genomic distances, strong interaction between LCR HS5 and HBG1 indicates the regulation of LCR at the active A γ -globin gene in globin-expressing cells. Frequent interaction between HS5 and HBpsi (located ~47.3 kb downstream) were also identified in K562 cells. In Fig. 4, the chromatin interaction map obtained by our microfluidic system (using 1000 cells, dots in the figure) was consistent with that obtained by conventional 3C method (using 10^7 cells in a tube, circles in the figure). Both also matched well with previous report⁴². Based on this analysis, we showed that our microfluidic 3C technology was effective and sensitive for quantitative identification of chromatin looping interactions.

Materials and Methods

BAC DNA digestion and ligation on the microfluidic chip

Purified BAC DNA in 20 ng/ μ l was flowed into the reaction chamber (0.5 μ l in volume) of the microfluidic chip from reservoir 1 (Fig. 2). Digestion solution made by mixing 22.5 μ l H₂O, 2.5 μ l of 10 $\times$ EcoRI enzyme buffer (1 $\times$ EcoRI buffer: 100 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂ and 0.025% TritonX-100, pH=7.5) and 50 U EcoRI enzyme (New England BioLabs, Cat. No. R0101T) was flown into the two loading chambers (1.3 μ l in volume each) and reservoirs from reservoir 5 and 6. Valve v1 remained open (all other valves were closed) during sample loading. After reagent loading, v3 and 4 were opened to allow the digestion reagent to diffuse into reaction chamber for 15 min. The valves were then closed and the microfluidic chip was placed on a 37 °C hotplate for reaction for 1 h. The digested DNA was either continued with ligation or collected and purified to estimate digestion efficiency. For ligation reaction, the digestion solution in the loading chambers was replaced by ligation solution made by mixing 18 μ l H₂O, 2 μ l 10 $\times$ ligation enzyme buffer (1 $\times$ ligation buffer: 30 mM Tris-HCl, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, pH=7.8) and 4 U T4 ligase enzyme (Promega, Cat. No. M1794) with all valves closed. Ligation reagents

were diffused into the reaction chamber after opening the valves v3 and 4. We kept the valves open during ligation reaction because the small digested DNA fragments would diffuse into loading chambers. The solution in both reaction and loading chambers was collected after reaction for 1 h at room temperature. For collection, all valves were closed except v1 and v6. Ligation products were flushed out of the 3 chambers separately by injecting 10 μ l water through each chamber via reservoirs 5, 1, and 6. The ligated DNA was collected and purified by phenol/chloroform extraction and ethanol precipitation. On-chip digested BAC DNA and on-chip ligated BAC DNA were run on a 0.8% agarose gel to verify digestion and ligation efficiency.

3C library preparation on the microfluidic chip

K562 cells were crosslinked and lysed in a tube as described in conventional method (See Supporting Information). After treatment with 20% SDS and 20% TritonX-100, the reaction solution containing 10^7 nuclei/ml, 0.4% PEG and 0.05% Tween20 was flowed into the reaction chamber (0.5 μ l in volume) of the microfluidic chip from reservoir 1 with v1 opened. The connecting channel between reservoir 2 and the reaction chamber was also filled with the reaction liquid to avoid generation of bubbles during oscillation. A critical step here was that all air bubbles in the reaction chamber needed to be removed to reduce liquid evaporation under 37 °C heating. The hydration channel beneath the reaction chamber was filled with water (under 25 psi pressure) to compensate liquid evaporation in the reaction chamber. The operation of the chip was shown in Fig. 3a. 50 U EcoRI enzyme in 30 μ l 1 $\times$ EcoRI buffer supplemented with 0.5% PEG and 0.1% Tween 20 was flowed into the loading chambers with all valves closed. EcoRI enzyme and other reagents entered into reaction chamber by concentration-gradient-driven diffusion after opening the valves between reaction and loading chambers (valve 3 and 4) for 20 min, while nuclei stayed in the reaction chamber due to their small diffusivity. The digestion was conducted overnight (~16 h) with the microfluidic chip placed on a 37 °C hotplate, valve 2 and 5 opened and all other valves closed. Pressure pulses (2-3 p.s.i. with a duration of 0.5 s) were applied alternately at reservoirs 2 and 3 to enhance mixing of solution in the reaction chamber^{34,54} and facilitate the access of enzyme to nuclei for digestion. The pressure pulses were generated by operating solenoid valves using a DAQ card and LabVIEW program³⁴. After overnight digestion, sample was flowed out of the reaction chamber by 20 μ l PK buffer and collected to a tube containing 500 μ l PK buffer. The digested sample was processed by adding 1 μ l of 10 mg/ml proteinase K (65 °C, 1 h) and then 1 μ l of 1 mg/ml RNase (37 °C, 30 min), followed by purification of DNA by phenol/chloroform extraction and ethanol precipitation. The DNA was finally dissolved in 20 μ l TE buffer.

After digestion, 250 μ l ligation reaction solution was created by mixing 25 μ l digested nuclei ($\sim 4 \times 10^5$), 25 μ l of 10 $\times$ ligase buffer, 12.5 μ l of 20% Triton X-100, 187 μ l H₂O and 0.5 μ l of T4 ligase enzyme (10U). 5 μ l of the solution was used to fill up the reaction chamber (0.6 μ l in volume). ~ 1000 nuclei were remained in the reaction chamber for on chip ligation. The chip with all valves closed was then placed on a flat-plate thermal cycler (Techne, Bibby Scientific) for on-chip ligation at 16 °C for 4 h and 20 °C for 0.5 h. The ligated sample was then flushed out of chip in 20 μ l of PK buffer and collected into a tube containing 1 μ l of 10 mg/ml proteinase K and 500 μ l of PK buffer to reverse crosslinking at 65 °C for 1 h. 1 μ l of

1 mg/ml RNase was then added to the solution and incubated at 37 °C for 30 min. Phenol/chloroform extraction and ethanol precipitation was finally used to purify DNA.

Digestion efficiency determination

SybGreen quantitative PCR was performed to determine the digestion efficiency. Undigested or digested DNA was mixed with iQ™ SYBR PCR Master Mix (Bio-Rad, Cat. No. 1708880), forward and reverse primers (final 0.5 μM each, IDT) and H₂O to reach a total volume of 20 μl. The primers that amplified across restriction sites of interest were listed in SI Table S1. A set of “internal” primers targeting a control region that was not digested were added to establish a reference¹¹. The PCR was performed on a thermal cycler (CFX Connect, Bio-Rad) with following conditions: 3 min at 95 °C for initial denaturation, 45 cycles of 10s at 95 °C, 30s at 62.5 °C and 30s at 72 °C.

Ct values were used to determine the digestion efficiency using the below equation:

$$\text{Digestion efficiency} = \left(100 - \frac{100}{2^{(Ct_I - Ct_C)_D - (Ct_I - Ct_C)_{\text{UND}}}} \right) \times \%$$

Ct_I : Ct value of regions of interest, Ct_C : Ct value of control region

“D” refers to digested sample (the unknown) and “UND” refers to undigested sample (the reference).

3C analysis

TaqMan qPCR was performed to quantitatively analyze each ligation product from K562 cell 3C library using TaqMan probe and primers for detecting interactions between constant fragment LCR HS5 and candidate interacting fragments (see SI Table S2). 10 μl TaqMan PCR solution was generated by mixing 5 μl Quanti Tech Probe PCR Master Mix (Qiagen, Cat. No. 204343), 0.5 μl constant primer (10 μM), 0.5 μl test primer (10 μM), 1 μl TaqMan probe (1.5 μM), 1 μl of ligated DNA sample and 2 μl H₂O. Serial dilution of the control library (ligation products from BAC clone) was used to create standard curves in each qPCR run. The PCR condition was as follows: 95 °C for 15 min, 45 cycles of 95 °C for 10 s and 56 °C for 1 min.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic representation of Chromosome Conformation Capture (3C) method: formaldehyde cross-linking, EcoRI restriction digestion, intramolecular ligation, and Taqman qPCR quantitatively detection of ligation products after reversal of the cross-linking.

Figure 2.

BAC DNA digestion and ligation on a microfluidic chip. (a) Digestion of BAC DNA: load DNA into reaction chamber and digestion solution into side loading chambers; diffuse digestion mix into reaction chamber; digest BAC DNA at 37 °C. (b) Ligation of digested BAC DNA: load ligation solution into loading chambers; diffuse ligation reagents into reaction chamber; ligase fragmentized BAC DNA. (c) Gel analysis: Lane 1, Digested BAC DNA migrates as a smear-like pattern; Lane 2 and Lane 3, DNA ladders; Lane 4, Ligated BAC DNA digestion fragments show disappearance of most of the smear-like mark.

Figure 3.

Digestion of human β -globin locus using conventional and microfluidic method. (a) Digestion of chromatin in nucleoson the microfluidic device. Alternating pressure pulses were applied on reservoirs 2 and 3 for oscillatory digestion. (b) Digestion efficiency at three sites in the β -globin locus (LCR (HS5), HBG1, HBB) evaluated by qPCR. Digestion was performed on a microfluidic chip (microfluidics) or in a tube (conventional) for 4 or 16 h. NS: $p > 0.2$.

Figure 4.

Analysis of the human β -globin locus using conventional and microfluidic 3C method. Chromatin looping interactions between LCR (HS5) and six sites located 30-50 kb downstream in the β -globin locus in K562 cells were analyzed by conventional (dots) or microfluidic (circles) method. The y-axis shows the relative interaction frequencies, and the x-axis indicates the genomic position relative to LCR (HS5). The corresponding locations of LCR (HS5), β -globin genes (HBE, HBG2, HBG1) and pseudogene (HBpsi) are shown below the x-axis. The interaction frequency between HS5 and a site 30 kb downstream was taken as 1 and the other interaction frequency were normalized against this value.