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Massively parallel display of genomic DNA fragments by rolling-circle amplification and strand displacement amplification on chip

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ABSTRACT

Massively parallel genomic DNA fragments display on chip plays a key role in the new generation DNA sequencing. Here, we developed a new technology to display the parallel genomic DNA fragment massively based on two-step reaction with Φ29 DNA polymerase. The genomic DNA fragments were firstly amplified by rolling-circle amplification (RCA) reaction in liquid phase, and then amplified further on the chip by the strand displacement of Φ29 DNA polymerase. In our experiments, through DNA colonies produced by two-step amplification reaction T7 genomic DNA fragments are displayed massively and parallely on the chip, which has been verified through hybridizing the probe labeled with fluorescence or extension reaction with fluorescent-dNTP. The significant difference of fluourescence signals between background and displayed DNA fragments could be obtained. Our results show that the method has good reproducibility in experiments, which may be hopeful to serve the high-throughput sequencing.

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1. Introduction

With the dramatic increase of the DNA sequencing in whole genome and the functional genomic studies, such as analyzing the methylation pattern [1], mapping genomic variations [2] and locating genes for disease [3,4], the new generation of DNA sequencing technologies are developing rapidly. In these new generation of DNA sequencing technologies, sequencing reactions usually take place on a chip. One of the main key process [5–7] is to fabricate massively parallel genomic DNA fragment display on a chip, which are usually based on polymerase chain reaction (PCR), including bead clone [8], emulsion-PCR [9], in-gel PCR [10] and solid-phase PCR (bridge-amplification) [11]. Although the above PCR-based methods have been successfully used in commercial high-throughput sequencing systems, they have many limitations, such as high amplification bias and low fidelity in parallel DNA amplification. For example, short fragments tends to be amplified in preference to large one, and some DNA polymerases used in PCR have lower fidelity, such as Taq DNA polymerase. When emulsion-PCR or solid-phase PCR is used to prepare a massively parallel DNA display of a certain genome, a large amount of genome sample and polymerase are needed. This increases the difficulties in sample preparation and sequencing cost. Developing new technology of massively parallel DNA display is therefore needed.

Rolling-circle amplification (RCA) driven by DNA polymerase can replicate the circularized oligonucleotide template with linear kinetics under isothermal conditions. In RCA, a circle of DNA, a short DNA primer (complementary to a portion of the circle), and an enzyme catalyst convert dNTPs to a single-stranded DNA molecule. Φ29 DNA polymerase used widely in RCA reaction has very high fidelity [12], and DNA amplification is accurate.

Lizardi et al. [13], firstly used RCA to do parallel DNA display. In their study, the short DNA primer containing a free 5′ phosphate was bound covalently to the glass surface modified with the reactive amino group. Genomic DNA fragment hybridizing with the primer initiated a ligating reaction, followed with RCA. As the hybridization signal produced only by RCA products was too weak to detect, some procedures had to be performed to obtain detectable signal. Fluorescence-labeled DNP oligonucleotide probe was firstly used to hybridize the single DNA strand of RCA reaction, and then the anti-DNP lgM was added into the reaction system which could bind the DNP site on the oligonucleotide probe. Finally the elongated DNA molecule of RCA was condensed, forming a small globular DNA:lgM aggregate which produced enough fluorescence intensity for detection. This kind of parallel DNA display was not used for the high-throughput sequencing on chips, as genomic DNA fragments could not be amplified in whole process of experiment. Pihlak et al. [14], overcame the weakness of Lizardi's protocol. In their study, the genomic DNA fragments and adaptors were ligated to form circle DNA template which could hybridize the primer bound covalently to the glass surface, then genome DNA fragments

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Table 1
Nucleotide sequences used in this study.

Oligo name	Sequences (5′ → 3′)
Circle-Linker A	Phosphorylation-AGTACGACGAATCTGTATGCTAATGCGGCGTGATGTATTATGCGTATAGT
Circle-Linker B	CTATACGCATAATACATCACGCCGCATTAGCATACAGATTCGTCGTACTT
P1	ATACAGATTCGTCGTACT
P2	NH_2-$(T)_{30}$-TATGCTAATGCGGCGTGA
P3	TAGCATACAGATTCGTCGTACTT
P4	CY3-TAGCATACAGATTCGTCGTACTT

Note: Circle-Linker A was modified with phosphate at 5′-end. Circle-Linker A and Circle-Linker B can be annealed to form linker with single base T overhangs which can be circularized by ligation with the genome DNA fragments with single base A overhangs. P1 is the primer for RCA reaction in liquid phase. P2 modified with amido at 5′-end can be immobilized chemically on chip to hybridize DNA products from RCA. P3 is primer for extension reaction, which can hybridize the single strand products of strand displacement amplification. P4 having the same sequence as P3 but modified with CY3 at 5′-end can also hybridize the products of strand displacement amplification to detect result of massively parallel DNA display on chips.

will be amplified directly by RCA reaction on the chip. But still there are weaknesses in the Pihlak's methods. During the process of ligation, the genome DNA fragments and adaptors are blunt, so the yield of the RCA template is not very high. PCR reaction must be used to obtain enough RCA templates. Moreover signal of RCA products on the chip is not strong enough, so high sensitivity apparatus is needed for detection.

In this study, we developed a two-step amplification method which produced plenty of DNA colonies to realize massively parallel DNA display on chips. In this method, the first step was RCA reaction to amplify the genomic DNA fragments in liquid phase, and then the fragments were amplified further on the chip by the strand displacement of Φ29 DNA polymerase. Massive DNA colonies would appear on the chip. Thus, the strongly detectable signal will be produced, and moreover, no PCR reaction was used in the method.

2. Materials and methods

2.1. Nucleotide sequence and genomic DNA

All nucleotide sequences shown in Table 1 were synthesized by Invitrogen™ (Shanghai, China). T7 phage genome was obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., China.

2.2. Single strand genome-circle preparation

2.2.1. DNA fragmentation

The Eppendorf tube containing 2 μg T7 phage genome and 30 μL sterile water was placed upright, with half of it submerged in the ice, and fragmented by Ultrasonic Cell Crusher (Ningbo Scientz Biotechnology Co., Ltd.) in the model of Ø3 position. So the probe of Ø3 was chosen, and the wattage of the sonicator is not <520. The pattern of sonication was 3 s continuance and 9 s pause. The whole process stood five repeats, 15 min each time. The sonicated DNA was purified by a QIAGEN MinElute Reaction Cleanup Kit (QIAGEN, USA), according to the manufacturer's instructions. The size distribution of the sonicated fragments was determined by 2% agarose gel electrophoresis.

2.2.2. Enzymatic polishing

Sonication generates DNA fragments with a preponderance of frayed ends. Fragments should be blunt-ended and phosphorylated through End-It™ DNA End-Repair Kit (EPICENTRE® Biotechnologies). According to the End-It™ DNA End-Repair Kit protocol, 20 μL of purified, sonicated DNA fragments were combined with 1 μL End-Repair Enzyme Mix, 5 μL End-Repair 10× Buffer, 5 μL 10 mM dNTPs, 5 μL 10 mM ATP, and 15 μL sterile water, incubated in a thermocycler (Peltier Thermal Cycler, MJ Research, Inc, Watertown, MA, USA) at 25 °C for 45 min. The reaction product was purified by a QIAGEN MinElute Reaction Cleanup Kit.

2.2.3. Generating adenine overhangs

Taq DNA polymerase can generate single base 3′ adenine overhangs, which were suitable for TA linking vectors. Polished DNA fragments were diluted to 50 μL with 5 μL 10× Taq Buffer, 3 μL 25 mM $MgCl_2$, 2 μL 10 mM dATP, 3 μL of 5 U/μL Taq DNA polymerase and 17 μL sterile water. The mixture was incubated for 2 h at 70 °C and purified using QIGEN MinElute Reaction Cleanup Kit. The DNA concentration was determined by Thermo Spectronic (Rochester, NY, USA).

2.2.4. Linking vectors

Adaptor sequences were added to the A-ended of the genomic DNA fragments. The 49-base primer sequences as vectors, termed Circle-Linker A and Circle-Linker B, were annealed and diluted to a final concentration of 10 μM. 1 μg A-ended, fragmented genomic DNA was linked with a mixture of 300 pmol of the annealed vector pairs, 1 μL 10× T4 ligase buffer, 1 μL T4 ligase (Fermentas) and 7 μL sterile. The reactant was incubated for 6 h at 4 °C and inactivated at 65 °C for 20 min.

2.2.5. Preparation of single strand genome-circle

After ligation a nick appeared between 5′-end of Circle-Linker B and the adjacent 3′-end of genomic DNA fragment. The single strand circles were formed using exonuclease III to degrade the strand with a nick. Following ligation and inactivation, the reaction mixed with 3 μL 10× NEBuffer 4, 16 μL sterile water, 0.5 μL of 10 U/μL Exonuclease I (New England Biolabs) and 0.5 μL of 10 U/μL Exonuclease III (New England Biolabs), was incubated for 2 h at 25 °C, and inactivated at 80 °C for 20 min. The exonuclease reaction was purified by QIGEN MinElute Reaction Cleanup Kit, and the sample was further diluted to a final concentration of 1 pg/μL.

2.3. Massively parallel genome DNA fragment display on chips

2.3.1. RCA reaction in liquid phase

Mixing 9 μL single strand genome-circle and 1 μL of 10 μM P1, heating to 95 °C for 10 min in a thermal cycler, shutting the thermal cycler off and allowing the mixture to cool slowly back to room temperature over the course of an hour. Then adding another 10 μL solution containing 5 μL sterile water, 2 μL 10× Φ29 DNA polymerase buffer, 0.5 μL 100× BSA, 3 μL 10 mM dNTP, 1 μL Φ29 DNA polymerase (New England Biolabs). The mixture is incubated in 31 °C for 4 h and inactivated at 90 °C for 10 min.

2.3.2. Preparation of slide

Glass surface activation was performed as described by Braslavsky et al. [15]. Slides were sonicated in 2% MICRO-90 soap (Cole–Parmer, Vernon Hills, IL) for 20 min, and then cleaned

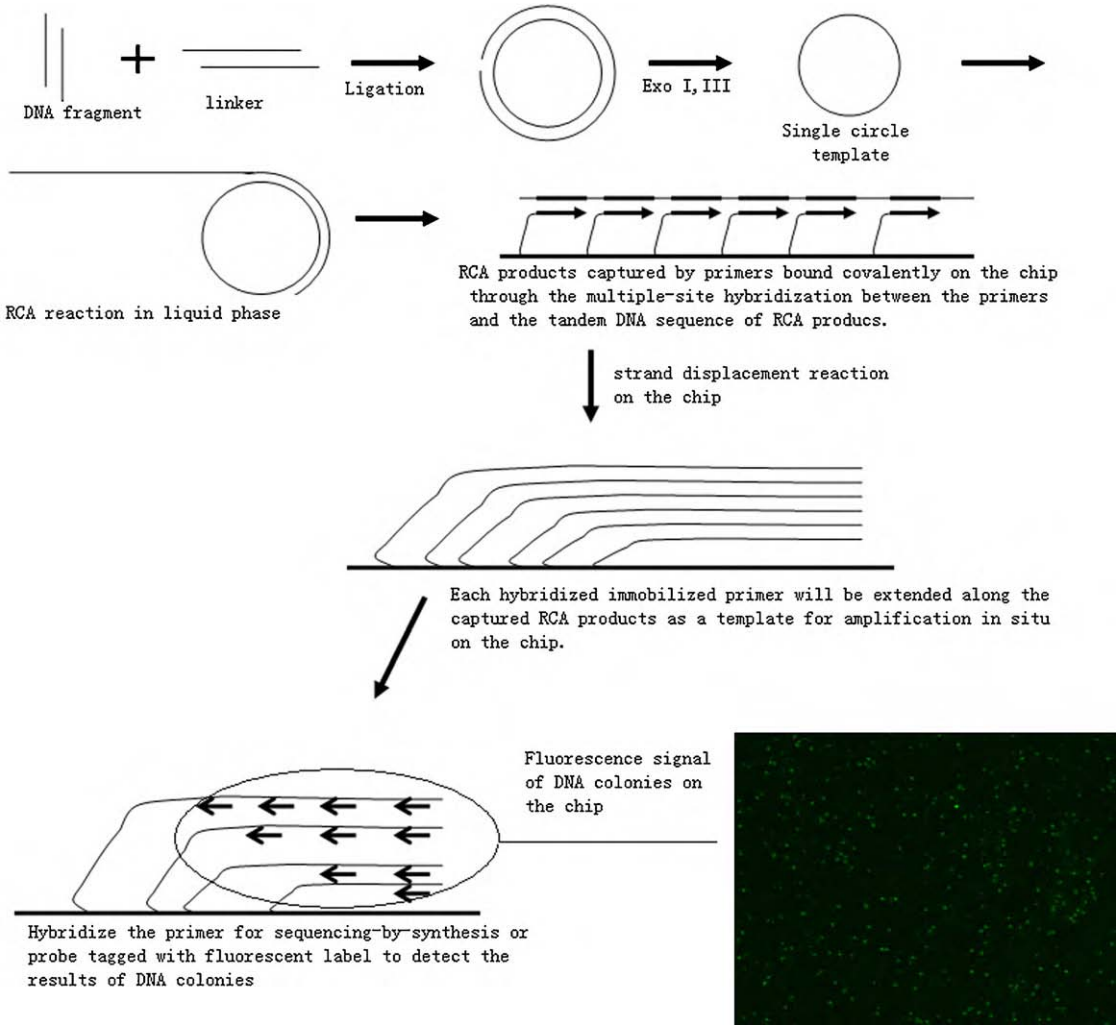

Fig. 1. Schematic strategy of producing random DNA colonies on the slide. Genome DNA fragment and linker produce the single strand circle as template of RCA. Firstly DNA fragment of genome was amplified by RCA in liquid system, secondly the fragment will be amplified further on the chip by the strand displacement of Φ29 DNA polymerase, finally the colonies of genome DNA fragment will be produced on the chip.

by immersion in boiling solution (6:4:1 high-purity H_2O/30% NH_4OH/30% H_2O_2) for 1 h. They were then immersed alternately in 2 mg/mL polyallylamine (positively charged) for 10 min, washed with distilled water, and then 2 mg/mL polyacrylicacid (negatively charged; both from Aldrich) pH 8 for 10 min. The carboxyl groups of the last polyacrylicacid layer prevented the negatively charged labeled nucleotide from binding to the surface of the sample.

10 μL solution containing 100 μM P2, MES buffer and 0.2 mg/μL EDC (dissolved in MES buffer) were placed on the surface of glass slides (already activated above), then the drop was covered with a glass coverslip (15 mm diameter). The slides were incubated at room temperature for 10 min, then glass coverslip was removed by washing with 1 mM Tris-buffer, finally the slides were washed by ultrapure water and blew dry with nitrogen.

2.3.3. RCA product hybridized with the immobilized primers on the chip

5 μL product of RCA in liquid phase was diluted to 20 μL by adding 5 μL ultrapure water and 10 μL hybrid buffer (Biodev-Tech, China). The mixture was placed on the surface of glass slide and the drop was covered with a glass coverslip. At last the slide was incubated in a moist chamber at 42 °C for 8 h.

2.3.4. Strand displacement amplification on chip

When the hybridization was finished, the slides were washed twice with 2× SSC/0.5% SDS for 5 min, 3 min in 0.2× SSC/0.5% SDS, one time for 1 min in ultrapure water and finally blew dry with nitrogen. 10 μL solution containing 1.5 μL sterile water, 2 μL 10× Φ29 DNA polymerase buffer, 0.5 μL 100× BSA, 3 μL 10 mM dNTPs, 4 μL Φ29 DNA polymerase was place on the surface of slide, then the drop was covered with a glass coverslip (15 mm diameter). At last all slides were incubated in a moist chamber at 31 °C for 4 h.

2.3.5. Hybridizing the primer for extension reaction

The slides were washed three times for 5 min in 2× SSC/0.5% SDS at 50 °C, three times for 5 min in 0.2× SSC/0.5% SDS at 40 °C, one time for 2 min in ultrapure water and finally blew dry with nitrogen. 25 μL annealing hybrid buffer containing 2 μM P3 was added and the slides were incubated in a moist chamber for 40 min at 45 °C, followed by washed two times for 5 min in 2× SSC/0.5% SDS, two times for 3 min in 0.2× SSC/0.5% SDS, one time for 2 min in utralpure water and finally blew dry with nitrogen.

A homemade thin PVC-plastic frame (18 mm × 18 mm) was placed on the surface of a slide. After being treated by oxygen plasma, the thin PVC-plastic frame was easy to adhere to the hydrophilic surface of slide stably. Thus, a reaction well was formed.

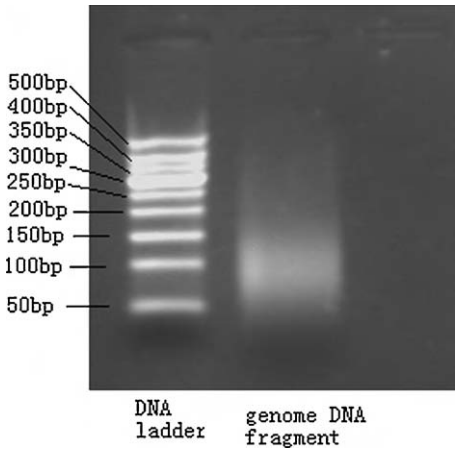

Fig. 2. After the T7 DNA genome is fragmented by ultrasonic, the size distribution of the genome fragments was determined by 2% agarose gel. The size of T7 genome DNA fragment ranges mainly from 50 to 150 bp.

At least 100 μL solution was needed to fill the well. Reagent solutions were poured and pipetted out after use, followed by rinsing with a TE–NaCl buffer. The slides were fixed on the confocal scanner (CapitalBio LuxScan^{TM}-10K(A), China), which will be used to scan the slide after the fluorescence-labeled dNTP incorporation was finished.

In the extension reaction, reaction solution containing 1 μM CY3-dUTP (Amersham Pharmacia), 10 nM klenow fragment polymerase (100 U/mL) in 1× NEB buffer 2 was poured in the reaction well and incubated for 10 min. Having rinsed the well three times with TE–NaCl buffer, the slide was scanned with the confocal scanner to obtain the fluorescence image of first extension.

To obtain the fluorescence image of next extension, the fluorescence signal of previous extension should be quenched, which was performed as described by Aksyonov et al. [16]. By filling the reaction well with the freshly prepared saturated solution of DPI (60 mM in TE–NaCl buffer) and illuminating the slides with laser light in 488 nm wavelength for 2 min, the fluorescence signal of the incorporated fluorescence-labeled dNTP would be destroyed. The well was still rinsed with TE–NaCl buffer again, then the second extension reaction would be carried out, and in the next turn the CY3-dUTP would be replaced by CY3-dATP (from Perkin-Elmer).

2.4. Image scanning and data processing

All images captured by confocal scanner (CapitalBio LuxScan^{TM}-10K(A), China) were analyzed with the software Genepix Pro 3.0 to acquire the average fluorescence intensity of DNA colonies and background. Statistical analyses were conducted using Microsoft Excel.

3. Results and discuss

3.1. Principle of approach

As shown in Fig. 1, the genomic DNA fragments and adaptors were ligated to form circle DNA template, then genomic DNA fragments will be amplified by the RCA reaction in the liquid phase. When the density of primer bound covalently on the surface of chip was high enough, RCA products would be easily captured by the primer on the chip through the multiple-site hybridization between the primer and the tandem DNA sequence of RCA products. Thus, each hybridized immobilized primers can be extended along the captured RCA products as a template for amplification in situ on the chip. This kind of strand displacement amplification

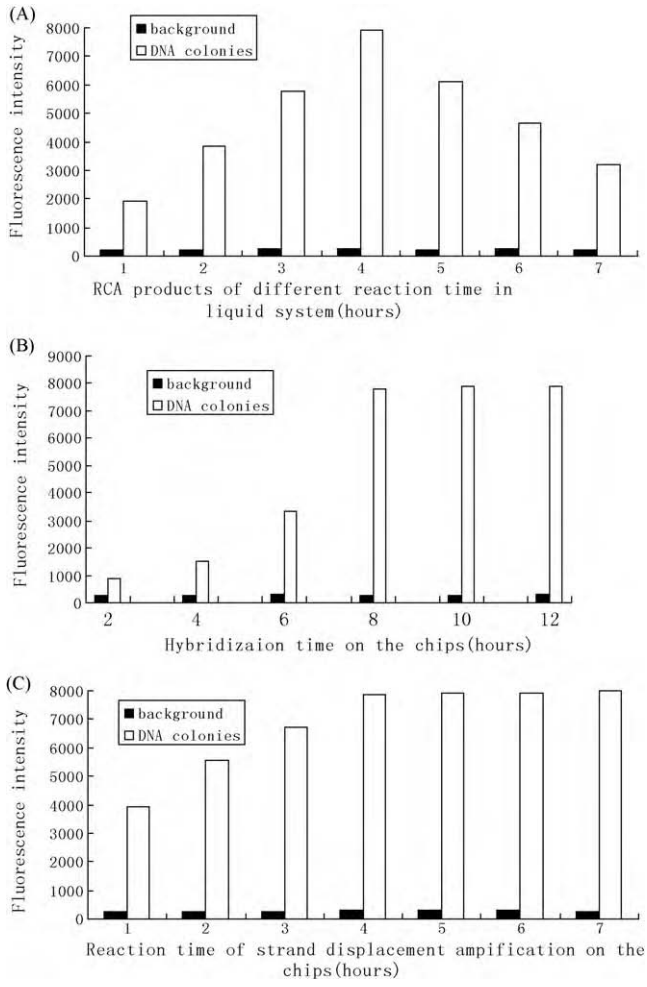

Fig. 3. Experimental conditions affect signal intensity of DNA colonies. (A) Average fluorescence intensity change of DNA colonies versus RCA products of different reaction time in liquid system. The reaction time of these RCA products is 1, 2, 3, 4, 5, 6 and 7 h respectively. The open bars are average fluorescence intensity of DNA colonies on the slide and the filled bars are average fluorescence intensity of background. (B) Average fluorescence intensity change of DNA colonies versus different hybridization time on the chips. RCA products of 4 h reaction time in liquid system to hybridize the primers on different slides was finished in 2, 4, 6, 8, 10 and 12 h respectively. (C) Average fluorescence intensity change of DNA colonies versus different reaction time of strand displacement amplification on the slides. The RCA products of 4 h reaction time hybridized the primers on the different slides about 8 h and the reaction time of strand displacement amplification on the slides was 1, 2, 3, 4, 5, 6 and 7 h respectively.

will produce plenty of DNA colonies to realize massively parallel genomic DNA fragments display on the chip. For parallel display of genomic DNA fragments on chips by RCA reaction, one of the key problems was to detect the fluorescent signal of RCA products. Unlike exponential amplification of PCR, the RCA is linear amplification. It was difficult for a single strand DNA molecule of RCA to produce strongly detectable fluorescent signal when hybridizing the probe labeled with fluorescence or extension reaction with fluorescent-dNTP. Lizardi et al. [13], used lgM to collapse the elongated DNA molecule of RCA, so that the detectable fluorescent signal can be acquired. However, for the collapsed DNA molecule of RCA, it was difficult do continue extension reaction in the sequencing reactions. The DNA colony produced by our protocol was formed by a lot of single strand DNA molecule containing multiple copies of the target genome fragment, and the extension reaction led to strongly detectable signal.

The great feature in our protocol is that the products of strand displacement amplification reaction on chip were single strand

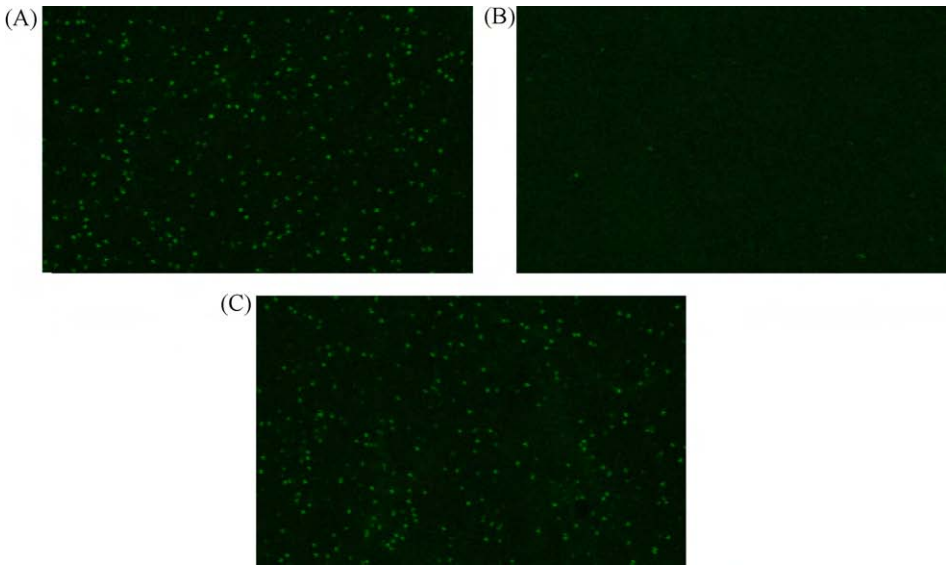

Fig. 4. Fluorescence images were captured by scanning the colonies immobilized on a glass slide. (A) First extension reaction using CY3-dUTP. (B) After destroying the fluorescence of first incorporated CY3-dUTP. (C) Second extension reaction using CY3-dATP.

DNA molecule. Therefore, the products can hybridize the primer or probe directly without any denaturation but only washing processes.

3.2. Genomic DNA fragment and circle preparation

To have enough multiple sites in the RCA products, it was necessary to control the size of genome-circle. Generally, RCA products of small circle template have more multiple sites for hybridization. Circle-Linker A and Circle-Linker B (shown in Table 1) were annealed to form a 49 bp double strand DNA vector. Fig. 2 showed that the size of T7 genome DNA fragment ranges mainly from 50 to 150 bp. So the size of most genome-circle template will be not more than 200 bp, which will be advantageous for RCA products to contain enough multiple sites. Both vector and genomic DNA fragment have sticky end. The vector had a single base "T" overhangs while genomic DNA fragment had a single base "A" overhangs by treated with Taq DNA polymerase. The T–A ligation produced enough single strand genome-circle template for RCA reaction. In this kind of ligation reaction, the amount of genomic DNA in the experiment would be reduced. In our methods, 2 μg T7 phage genome could meet the need of experiment.

3.3. Massively parallel genome DNA fragment display on the chip

The length of DNA produced by RCA would be >70 kb after 40-min incubation [17]; however, the single strand DNA molecule with longer length will be produced in the longer RCA reaction time. The longer RCA product contained more multisite to hybridize, and DNA colonies with stronger signal would be produced by strand displacement amplification reaction on the chip. Fig. 3A shows average fluorescence intensity change of DNA colonies versus RCA products of different reaction time in liquid system. RCA products of different reaction time in liquid system hybrid the primers on the slides at least 10 h, and then strand displacement amplification reaction will take place on the chip to produce DNA colonies which can be detected by Cy3-labeled probe P4. After Cy3-labeled probe P4 hybridized the DNA colonies on the slides, these fluorescence signals could be obtained by a confocal scanner (CapitalBio LuxScan^{TM}-10K(A), China) and analyzed with software Genepix Pro 3.0. From the Fig. 3A, it was found when reaction time of RCA in liquid system last more than 4 h, the signal intensity of DNA colonies on the slides begin to reduce. RCA products of 4 h reaction time showed the best result. Possible explanation for this finding was that the single strand RCA products too lengthy might form some secondary structure to hinder its hybridization on the chip. In the experimental process, the RCA products would be captured by primer on the chip through the multiple-site hybridization between the primer and the RCA products. Only the captured RCA products could be amplified to produce DNA colonies. The multiple-site hybridization between the primer on the chip and the RCA products made hybridization time in this protocol longer than in the general one. Fig. 3B shows average fluorescence intensity of DNA colonies versus different hybridization time on the chips. The hybridization between RCA products and the primers on different slides was finished at different times respectively. The strand displacement amplification reaction would start immediately, and then Cy3-labeled probe P4 was used to hybridize the DNA colonies on the slides, finally the slides were washed and scanned to obtain the fluorescence intensity of major colonies. The result of Fig. 3B showed that the 8 h hybridization between the RCA products and the primers on the slides was able to obtain very strong signals of DNA colonies.

Fig. 3C shows different reaction time of strand displacement amplification on the slides will cause different signal intensity of DNA colonies. The strand displacement amplification of different reaction times took place on different slides respectively. The result demonstrated that when reaction time of strand displacement amplification is more than 4 h, there is no obvious increase in signal intensity. So 4 h reaction on the chip was enough for our protocol.

3.4. Extension reaction on the chip

Fig. 4A showed the result of first extension reaction using CY3-dUTP. It could be found the fluorescence intensity of DNA colonies changed from 7000 to 9579, while that of background changed from 151 to 300. The diameters of major colonies lay between 2 and 3 μm. Fig. 4B showed the result of destroying the fluorescence of first incorporated CY3-dUTP. Compared with the background, the fluorescence signal of DNA colonies could not be shown while the signal intensity of background was not more than 300. Fig. 4C showed the result of second extension reaction using CY3-dATP. Result showed that the fluorescence signal of DNA colonies

Table 2
Results of seven consecutive experiments.

Experiment	Average fluorescence intensity ratio of DNA colonies/background	DNA colonies/mm^2
1	28.73	12,011
2	27.69	11,780
3	26.32	10,967
4	29.37	10,664
5	26.34	11,411
6	30.11	11,311
7	31.06	10,998

appeared again by this turn of extension, and for the fluorescence signals between background and DNA colonies there was still a significant difference. In the further repeated experiment, the great difference of fluorescence intensity between background and DNA colonies could still be observed. All these indicate that DNA colonies produced by RCA and strand displacement amplification are promising to be used for sequencing-by-synthesis on chips.

3.5. Reliability

According to the standard protocol described above, seven consecutive experiments were finished to evaluate the reproducibility of our method. In these experiments, Cy3-labeled probe P4 hybridized the DNA colonies on the slides to produce fluorescence signals. From the results shown in Table 2, it could be found that average fluorescence intensity ratio of DNA colonies/background in each experiment is more than 25 and the number of DNA colonies/mm^2 is more than 10,000. All the data indicated that the method also had good reproducibility.

4. Conclusion

We developed a new method to produce DNA colony on chip for massively parallel genomic DNA fragment display using two-step amplifications. First RCA step was complemented in liquid phase and the second amplification step on the surface of a glass slide. In this method, important catalytic enzyme Φ29 DNA polymerase has been widely used, due to its high fidelity in amplification and isothermal reaction. The amount of polymerase and target genome was small which indicated low cost of the method; this method also had good reproducibility. The result of extension experiments showed that DNA colonies produced by this method were hopeful to be used for the high-throughput DNA sequencing on the chip. It has great potential to increasing the DNA colony density and increasing the fluorescence intensity of DNA colony further.

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