DNA ANALYSIS BASED ON PHYSICAL MANIPULATION

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ABSTRACT

A novel DNA analysis method based on physical molecular manipulation is proposed and experimentally demonstrated. On a glass substrate is deposited a sacrificial layer, carrier layer and a pair of electrode, onto which DNA solution is fed. By applying high frequency voltage, DNA is stretched straight and aligned, with one of its molecular end anchored on the electrode edge, and immobilized onto the carrier layer. Then using an AFM stylus as a knife, a portion of the DNA together with the carrier layer is cut, and by dissolving the sacrificial layer, recovered onto a membrane filter. Successful recovery is demonstrated using PCR.

INTRODUCTION

In conventional gene sequencing, DNA in water solution is first cut into small fragments using restriction enzymes, and then the base sequence of each fragment is analyzed. In this method, the position of the fragment in the original gene is untraceable. As a result, the total sequence must be inferred using overlaps among each fragment, but this process is not easy to perform especially when there are repetitions in the sequence, and it therefore is a cause of still remaining ambiguity in gene maps.

An inherent problem with such a solution-based method is that the information about 'position' is lost. If DNA is immobilized onto a solid surface and cut, knowing its position on a gene, the construction of a genetic map should be far easier.

The authors have been engaged in the development of DNA manipulation based on microsystems. They have shown that, using high-frequency high-intensity electrostatic field created by microfabricated electrodes, individual DNA molecule can be stretched to the full length, and anchored onto a solid surface, with one of its molecular end in touch with the electrode [1-3]. By using thus aligned DNA and a sharp tool, one should be able to cut at arbitrary position, and obtain fragments from desired positions on a gene.

This paper describes our investigation on DNA analysis based on such physical manipulation of DNA, including the alignment, the cutting, the recovery of cut DNA, and PCR amplification of the obtained fragments.

THE CONCEPT OF DNA ANALYSIS BASED ON PHYSICAL MANIPULATION

The new DNA analysis methodology based on physical molecular manipulation is schematically depicted in Fig.1. The electrode system is just a pair of aluminum electrodes vacuum-evaporated and patterned on a glass substrate. DNA in solution takes randomly-coiled conformation (fig.1 a). When c.a. 1MV/m 1MHz field is applied, DNA is stretched straight and moved towards electrode edge by dielectrophoresis (DEP) [4, 5], and the molecular end in touch with the edge is firmly anchored [2]. The anchoring occurs more easily with a fresh Al surface than with an old (and presumably oxidized)

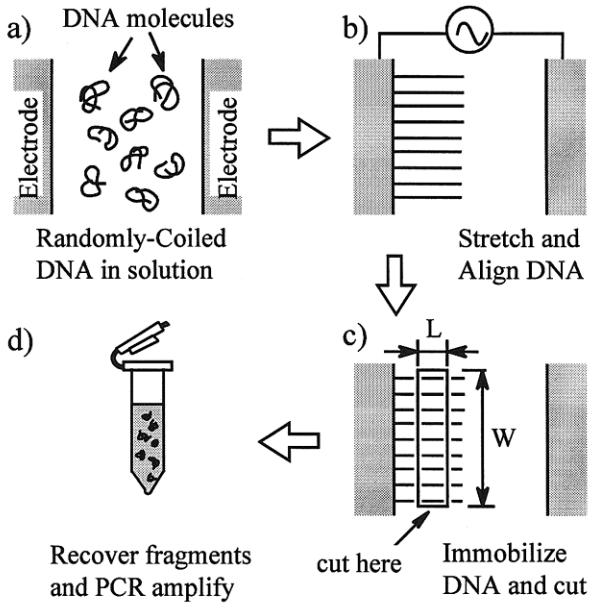


Fig.1 The concept of DNA analysis based on physical manipulation

surface, so we tentatively assume DNA is covalently bonded by an electrochemical reaction. Fig.1 b) schematically shows thus aligned DNA (for simplicity, the strands are shown only on the left electrode, but in reality they are on both electrodes). The electrostatic stretching must be made within a thin water layer sandwiched by the substrate and a cover slip to prevent EHD turbulence produced by the field. So after the DNA alignment, the cover slip must be removed somehow, without disturbing the molecular alignment, to expose aligned DNA and allow an access by a cutting tool. Then using a sharp needle, a desired portion of DNA is cut (fig.1 c), and recovered in a tube. After PCR amplification, conventional DNA analysis method can be used to determine the sequence of the fragment. In this way, one can sequence the 'known' position of the original DNA strand.

Theoretically, PCR amplification can start with a single DNA molecule and one need to pick up only one DNA fragment in fig.1 c). However, in practice, considering molecular damage resulting from fluorescence excitation which is used for visualization of the molecules, or the loss such as adhesion to the tube surface, PCR from one molecule often result in low-fidelity amplification. Our preliminary experiment has shown that 20 molecules are required for reproducible amplification of several kb-sized DNA. The density of DNA obtained by the electrostatic alignment is several molecules per 1 μm of the electrode contour. Therefore, the cutting width W in fig. 1 c) should be several ten μm parallel to obtain adequate number of DNA. The length L is determined by the size of fragment to be cut. The structural constant of DNA is 3kb/μm, so if you want 3kb fragment, L should be 1μm. An advantage of the electrostatic method is that all DNA strands are aligned with one end straight on the electrode edge. Cutting in a square contour as shown in the figure should contain the DNA fragments from the same position in the stretched DNA.

It should be noted that, because DNA is electrically symmetrical, electrostatic orientation of DNA depicted in fig.1 b) is a mixture of one orientation and the other. A method has been proposed to remove one orientation using restriction enzymes [2], but 2-component mixture simply obtained by the electrostatic method will not bring about much difficulty in the sequencing process.

THE STRUCTURE AND THE METHOD USED FOR THE DNA DISSECTION

In order to realize the process of fig.1, methods must be developed to 1) align DNA strands and remove the cover slip without destroying the alignment, 2) cut, 3) remove the fragments from the solid surface, 4) recover with minimal loss.

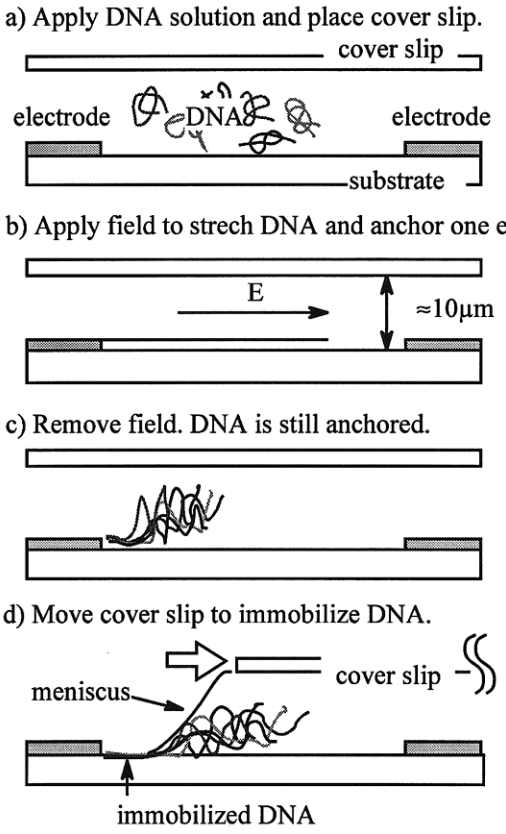


Fig.2 Immobilization of stretched DNA

Immobilization of stretched DNA

Fig.2 depicts the procedure we used for immobilizing stretched DNA. It is a combination of the authors' electrostatic method and the molecular combing developed by Bensimon et al. [6]. DNA solution is fed on the electrode, and a cover slip is placed (fig.2 a). By applying electrostatic field, DNA is stretched and one end is anchored on the electrode edge (fig.2 b). Then the voltage is removed. DNA restores random-coiled conformation because stretching force is no longer there, but the molecular end is still anchored on the electrode edge (fig.2 c). By slowly moving the cover slip parallel to the substrate, evaporation from the open end occurs (fig.2 d), and DNA is stretched again by the meniscus to be immobilized when the surface dries out. The advantage of the method over Bensimon's molecular combing is that molecular ends are right on the electrode edge.

Another method is to add Mg^{++} ions to immobilize DNA on the substrate immediately after electrostatic stretching [2], but we did not adopt this method, because cover slip must be removed slowly parallel to the substrate in any way in order to maintain neat alignment of DNA.

Dissection and recovery of DNA

The most important point in realizing the manipulation-based sequencing may be the handling of dissected fragments. If one just cut DNA as in fig.1 c) and let the fragment diffuse in the solution, one will never be able to collect them again. Our solution to this problem is to use a carrier for handling, on which DNA fragments are attached.

Fig.3 schematically illustrates the method and the device we have developed for this purpose. The structure consists of a glass substrate, onto which a sacrificial layer, a carrier layer, and a pair of electrodes are deposited (fig.3 a). The sample DNA is stretched and positioned on the carrier layer and exposed in air using the method of fig.2. Using an AFM stylus as a cutting knife, DNA is cut at desired location. At this stage, the contact force of the AFM stylus is set low, in order to cut DNA with higher resolution (pre-cutting, fig.3 c). Then the surface is coated by a protective layer, whose function is to prevent the dissected DNA from coming off. The second cutting follows, where the contact force is set stronger, so that the stylus penetrates deeply into the sacrificial layer. Finally, the sacrificial layer is dissolved, and a piece of carrier layer (hereafter called the carrier piece), in which the DNA fragments are embedded, is released and recovered on a membrane filter, to be amplified by PCR.

A special requirement for the process is that all materials used must be compatible with biochemical processes. Use of strong acids or alkaline solution must be avoided. In addition, DNA fragments obtained by the method are inside the carrier piece, sandwiched by the carrier and the protective layer. These layers must be resolved somehow to expose the fragments for PCR. Our choice for the material for the carrier and the protective layer is gelatin, having melting temperature of c.a. 60°C. Positive photo resist is used as the sacrificial layer, which can be resolved by ethanol. Ethanol itself does not affect DNA, and it is conformed by a separate experiment that residual from dissolved positive photo resist does not affect PCR if recovered DNA is washed thoroughly with ethanol.

EXPERIMENTAL

An experimental demonstration of the method is made using sequence-known λ-DNA (48kb, 16μm in length) as the sample.

Principle of the experiment

In order to prove that the desired position in the sample DNA is dissected and recovered, three primers are prepared, as depicted in fig.4 a). They are corresponding

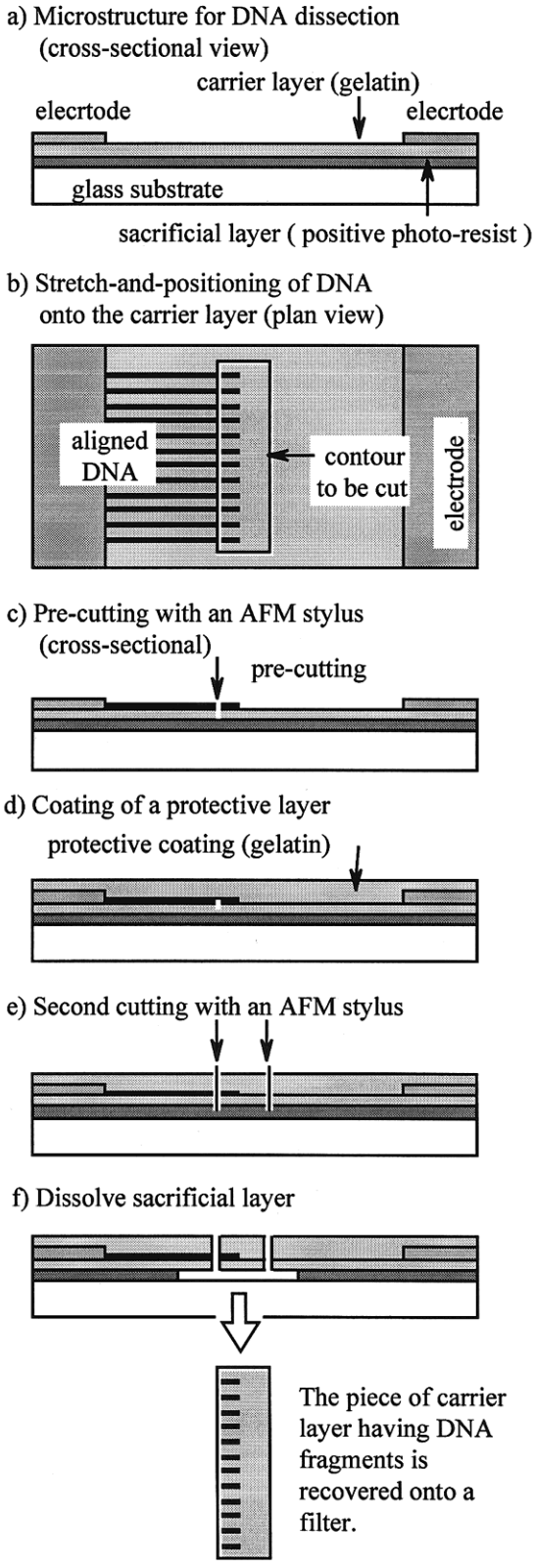


Fig. 3 The process to cut and recover DNA fragments

respectively to the sequences 1) near the left end of λ-DNA (denoted L), 2) near the right end (R), and 3) approximately at the center (C), all about 1kb in length. Because DNA is electrically symmetrical, electrostatic aligning yields a mixture of one orientation and the other, as shown in fig.4 b). If the aligned DNA is cut, say at 3 μm from the end, and successfully recovered, the PCR product should contain the sequence L and R, but not C (fig.4 b). On the other hand, if the sequence C is detected by PCR, it is an indication that unwanted DNA molecules are coming in.

Experimental procedure

76mm × 52mm × 0.17mm^t microscope slide is used as the substrate. Positive photo-resist (S1813, Shipley) of 1μm thickness is spin-coated, and baked at 115°C for 20 minutes, and then irradiated by a high-pressure mercury lamp for 10 minutes. This process is necessary to completely finish photochemical reactions of the resist. Otherwise, when observing DNA under a fluorescent microscope, residual photochemicals yield air bubbles, and the layers are disrupted. Then, as the carrier layer, 3% gelatin (Nitta Gelatin Inc., Japan) solution, stained with fluorescent probe 5-(4,6-dichlorotriazinyl) amino-fluorescein (5-DTAF, Molecular Probes Inc.) of 0.1 μm is spin-coated over the photo-resist layer. The fluorescent labeling is made to facilitate the manipulation of the carrier piece under a microscope. Over the carrier layer is deposited aluminum through a metal mask to pattern a pair of electrodes having 80μm gap. The thickness of the Al is about 50nm.

1μg/ml solution of λ-DNA, fluorescent labeled with YO-PRO-1 (Molecular Probes Inc.) is fed on thus fabricated device, and covered with a 0.17 mm^t cover slip. The solution thickness is about 10 μm. The voltage, 1MHz and 80V across the 80μm gap is applied, and DNA strands are anchored on the electrode edge. The cover slip is removed slowly by pushing it parallel to the substrate at a speed of 500μm/s with a pulse-motor driven linear actuator.

Mechanical cutting of DNA is done using an AFM (BioScope, Digital Instruments), mounted on the stage of an inverted fluorescence microscope (IX70, Olympus) equipped with a high-sensitivity SIT camera (C2400-08, Hamamatsu Photonics) and an image processor (Argus 20, Hamamatsu Photonics) for observation. The use of the inverted microscope enables the monitoring of the AFM stylus through the transparent gelatin (carrier) layer. The stylus used is single crystal silicon (LTAFM, Digital Instruments), with the tip curvature 5 to 20nm and the half cone angle 18°.

The pre-cutting of fig.3 c) is made with the contact force

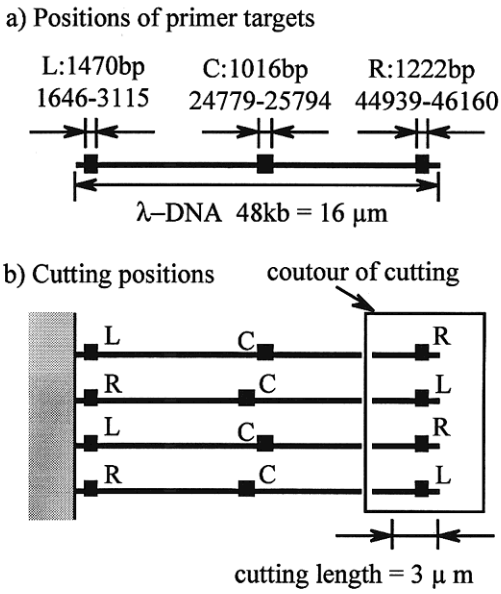


Fig.4 Cutting position and the primer design

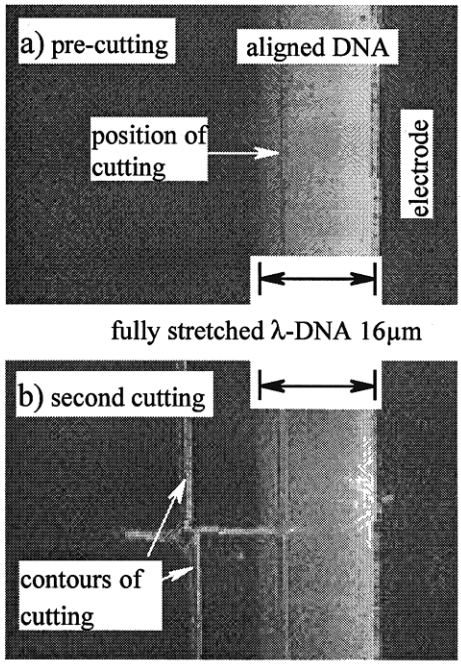


Fig.5 Cutting with an AFM stylus

of the stylus set to 100 nN. Fig.5 a) is a photo of DNA strands stretched to the full length 16μm and aligned on the electrode edge. The strands are so dense that individual molecules are not seen in the photo, but appear as a white belt. The photo clearly shows the cutting of the strands.

After the pre-cutting, fluorescent labeled gelatin as a protective layer is spun in the same way as the carrier

layer. Then the protective layer together with the DNA carrying layer is subject to the second cut (fig.3 e), to cut out the square carrier piece as shown in fig.4 b). This time the contact force is increased to 500 nN so that the tip can penetrate into the sacrificial layer (Fig.5 b).

Finally the sacrificial layer is dissolved using ethanol to recover the carrier piece onto a filter. The filter must retain the carrier piece, while other free DNA fragments, such as these coming off from other areas must be passed. Fabric filters are not suitable, because they are apt to adsorb free DNA. We used the isopore track-etched poly-carbonate membrane filter (TCTP02500, Millipore) having 10μm uniform pores. The pore size is large enough to pass free DNA, still small enough to retain the carrier piece. That the filter does not adsorb free DNA is conformed in a separate experiment. The carrier piece retained on the filter is rinsed well with ethanol to remove residual photo-resist used as the sacrificial layer. The function of the protective layer in fig.3 d) is mainly to prevent the DNA fragment from coming off the carrier piece at this stage.

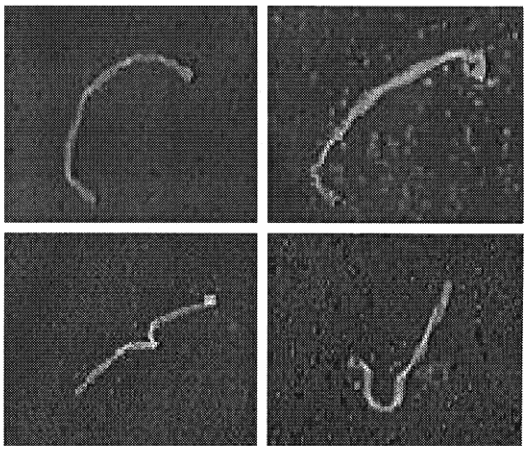
Fig.6 a) shows a carrier piece, cut to c.a. $10\mu m^{L} \times 250\mu m^{W}$, retained on the membrane filter. This observation under a fluorescence microscope is facilitated by the staining of gelatin with the fluorescent dye. Because the carrier piece at this stage is adsorbed on the filter, it was not possible to pick up the piece. So a small area of the filter, having the piece at the center, is manually cut out using a syringe needle (fig.6 b) and put into a test tube. Thus obtained sample contains not only the carrier piece, but also a piece of filter, approximately 200μm × 200μm, but this should not bring contamination, because the filter does not adsorb DNA. The sample is heated to 60°C to melt the gelatin layers, and subjected to PCR assay. The condition for PCR is shown in Table 1.

RESULT AND DISCUSSIONS

Fig.7 shows the electrophoresis of the PCR result. Lane M is the molecular marker. Lane C1 and C2 are positive control, starting from 2000 and 20 λ-DNA molecules respectively. In both lanes, the three bands, from top to bottom L (1470bp), R (1222bp), and C (1016bp) are seen. No distinct difference in brightness is observed between C1 and C2, probably because PCR starting from 2000 molecules is saturated.

Lanes #1 through #6 are PCR of the carrier piece. #1, #4 and #5 clearly show the bands from L and R, and not C. #2 and #3 are relatively dark, but also show L and R only (In #2 and #3, the photo is partially contrast-enhanced to reproduce better when printed). #6 has only one band corresponding to R.

a) carrier pieces on the filter



200μm

b) manual cutting of the filter

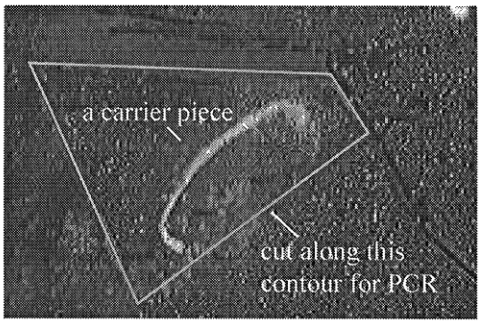


Fig.6 Recovered carrier pieces on a filter

a) PCR Solution

10 × buffer	5 μl
2.5mM dNTP	4 μl
Primer Mix (1pmol/μl each)	4 μl
DNA polymerase (EX Taq, 5U/μl)	0.25μl
Add water to be the total of	50μl

b) 10 × buffer

500 mM	Tris-HCl (pH9.2)
160 mM	$(NH_4)_2SO_4$
17.5 mM	$MgCl_2$
2.5 mg/ml	BSA
200 mM	Trehalose

c) Thermal cycle

96°C	2 min	
94°C	30 sec	35 cycles
65°C	1min	
72°C	1min	
72°C	5min	
4°C	(storage)	

Table 1 PCR conditions

These results demonstrate that the successful mechanical dissection and recovery of DNA are made in all cases #1 through #6.

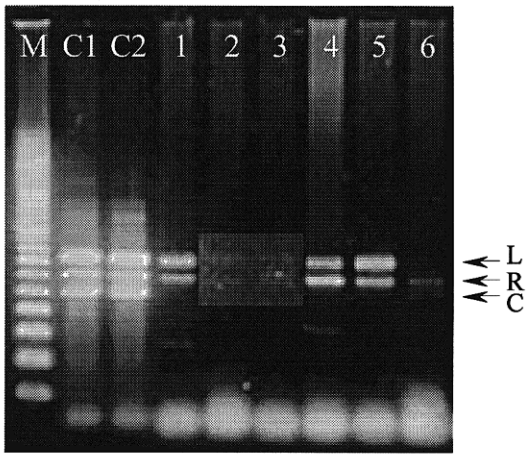
A question about the results of fig.7 are that the bands, especially these in #2, #3 and #6, are relatively dark. There were even the cases when no bands are observed (in about 30% of the trials, data not shown). The width W of the carrier piece we cut out is about 250 μm, and our estimation is at least several DNA strands are immobilized per 1 μm with the electrostatic method. So the piece should carry more than 1000 DNA fragments. But the band of lane C2, starting with 20 molecules, is brighter than that starting with the carrier pieces, #1 - #6. We have conformed in a separate experiment using full length λ-DNA that neither embedding in gelatin, nor residues of positive photo-resist we used for the sacrificial layer, hampers DNA amplification. The fluorescent excitation we extensively used during manipulation processes might be giving damage to DNA.

Another problem we encountered was that, in some cases we did have bands corresponding to C, indicating that unwanted DNA has entered into the carrier piece. Such a phenomenon was more often observed when we applied DNA solution at higher concentration (say 10μg/ml) onto the electrodes. We found that the contamination occurred during spin-coating of the protective layer. DNA strands adhering on the electrode surface was washed away by gelatin solution and confined in the protective layer. The contamination was reduced to a practical level by using lower concentration DNA solution, 1μg/ml, which we used in the experiment of fig.7.

Our present effort is focused for the further reduction of contamination, and also for reducing DNA damage during the physical manipulation.

CONCLUSIONS

A novel DNA analysis method based on physical molecular manipulation is proposed and experimentally demonstrated. In the method, DNA is first stretched to the full length and aligned onto a thin film (carrier layer). Then using AFM stylus as a tool, a desired position of DNA together with the carrier layer is dissected, and recovered onto a membrane filter, to be amplified by PCR. The use of the carrier ensures reliable recovery under a microscope. Experimental demonstration of the method is made, and the bands corresponding to the dissected position are detected by electrophoresis. This result shows the potential of the method for DNA analysis, by which the sequencing preserving the information about position can be realized.



Lane M: marker
Lane C1: λ-DNA (2000 molecules)
Lane C2: λ-DNA (20 molecules)
Lane 1-6: recovered carrier piece
(Lanes 2 and 3 are partially contrast enhanced)

Fig.7 PCR result

ACKNOWLEDGMENTS

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