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2006 J. Phys.: Condens. Matter 18 S653

(http://iopscience.iop.org/0953-8984/18/18/S11)

INSTITUTE OF PHYSICS PUBLISHING JOURNAL OF PHYSICS: CONDENSED MATTER

J. Phys.: Condens. Matter **18** (2006) S653–S663 doi:10.1088/0953-8984/18/18/S11

Extending chromosomal DNA in microstructures using electroosmotic flow

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Received 30 July 2005, in final form 20 September 2005
Published 19 April 2006
Online at stacks.iop.org/JPhysCM/18/S653

Abstract

The extension of chromosomal DNA is a key technique in high-resolution gene location such as extended fibre fluorescent *in situ* hybridization. In this paper, we propose a new micro-device designed for on-chip integration of three functions, (1) positioning of cells, (2) extending chromosomal DNA from the cells, and (3) anchoring the extended DNA fibres. The device has a flow chamber equipped with (a) multi-phase electrodes to create electroosmotic flow (EOF) of variable direction, (b) a set of micro-fabricated pocket-like structures for cell positioning, and (c) a micro-pillar array whose surface is chemically modified to anchor DNA fibres. First, unidirectional EOF is induced, by which cells are carried into the micro-pockets and positioned, one cell at each pocket. After rupturing the cells, chromosomal DNA from each cell is hydrodynamically extended by EOF. When the EOF direction is rotated through 90°, the fibres are made to contact with the pillars to be anchored. Because the velocity of the EOF is controllable, the breakage of DNA during the process can be minimized. Thus immobilized DNA fibres are suspended a few microns above the surface, allowing free access of probe molecules.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Locating specific genes on a chromosome is a basic technique for genomic studies [1, 2]. Fluorescent *in situ* hybridization (FISH) is a commonly used method for this purpose, whose principle is the hybridization of DNA base-pairs [3, 4]; a fluorescence-labelled oligonucleotide probe, having the sequence complementary to the gene to be detected, is prepared and allowed to hybridize with the DNA, whereby the location of the gene can readily be determined by an observation under a fluorescence microscope. FISH has found its application in the sequencing

project [1, 2], in the diagnosis of genomic abnormalities [5–10], and in the observation of the structure and the movement of chromosomes in the nucleus [11–15].

The resolution of FISH depends upon the conformation of the sample DNA. It is 1–5 Mbp (mega base pairs) for a closely condensed chromosome [16], and 1–5 kbp (kilo base pairs) if DNA is extended to a straight fibre [17]. The limitation is due to the optical resolution of a microscope, and with the use of a scanning near-field optical microscope, a resolution as high as 50 bp (base pairs) has been reported [18, 19].

A variety of techniques have been developed for the extension of chromosomal DNA, including the methods based on hydrodynamic shear in a molten agarose [5], pressure-driven flow [20] and a moving meniscus [21, 22]. However, they have such problems as:

(a) In extending DNA, the applied force must be large enough to overcome its entropic elasticity [23–26], yet must not exceed the mechanical strength of the DNA fibre (about 100–300 pN [25, 27]). In all above techniques, however, the force is not well controllable, and the extending condition is determined rather empirically. The mechanical breakage during the extension, as well as in the preparation process, limits the size of extendable DNA length to several hundred kbp [10].
(b) The locations of the extended DNA are random, and may overlap each other, which might prevent the observation of each single extended DNA fibre.
(c) In the moving meniscus method, DNA is adsorbed on the solid surface as the meniscus recedes and dries. The adsorption lowers the accessibility of probe molecules, and also can cause molecular deformations or steric hindrances. As a result, the yield of probe binding, which is a key factor of FISH efficiency, becomes substantially low [28]. On the other hand, in the pressure-driven flow method, DNA fibres are maintained in wet condition; however, this method must be used in combination with a method to anchor extended DNA to prevent back-coiling when the flow is stopped.

In our previous work, we have demonstrated the use of electroosmotic flow (EOF) for a controllable extension of chromosomal DNA [29], but its positioning and anchoring without steric hindrance remained as problems. In this paper, we propose a method and a device for the extension and the anchoring of DNA based on micro-fabricated structures combined with EOF of variable direction.

2. Principle

2.1. Electroosmotic flow for DNA extension and cell transport

EOF is a liquid flow induced by an externally applied electric field. In the case of a glass substrate immersed in a water solution, as is the case of this paper, its surface is negatively charged due to the dissociation of silanol groups, to which positive ions in the solution are attracted to form a Debye layer. The thickness of the Debye layer is about 1 nm for physiological solution and about 10 nm for 1 mM saline solution [29]. When an external electric field parallel to the surface is applied, the positive ions in this very thin layer migrate towards the negative electrode, thus causing a liquid flow. As a result, the velocity profile, starting from $v = 0$ on the wall, has a steep gradient within the layer, and becomes constant in the bulk water, as depicted in figure 1(a).

EOF has found wide applications in lab-on-a-chip [30–32] such as on-chip capillary electrophoresis, where the nearly constant velocity profile is effectively utilized in transporting liquid plugs. In this paper, we make use of another advantage of the velocity profile for DNA extension. Suppose a coiled DNA is anchored on the substrate at one of its ends as depicted in

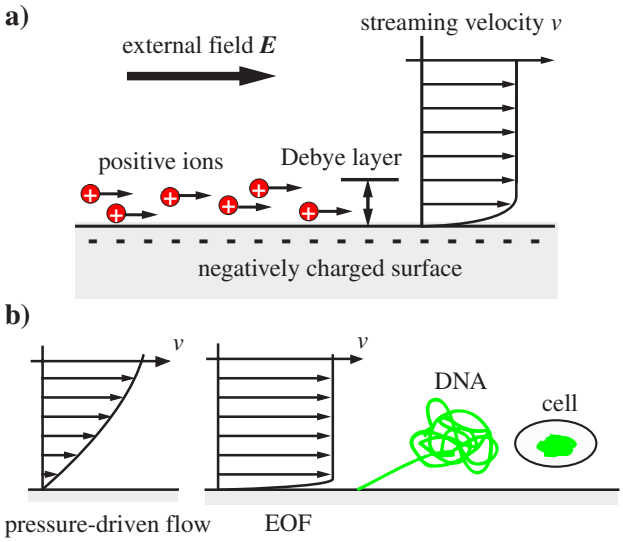

Figure 1. Electroosmotic flow (EOF). The mechanism of EOF (a) and velocity profile difference between EOF and pressure-driven flow (b).

figure 1(b), and is to be extended by a flow. EOF gives constant and controllable hydrodynamic drag regardless of the vertical position of the coil. This is in contrast to the case of pressure-driven flow where the velocity profile is parabolic, and the drag may be too small to stretch DNA coils near the surface, while too large to exceed the mechanical strength of the fibre if the coil is apart from the surface. For the same reason, EOF enables transportation of cells with a constant velocity irrespective of its elevation from the substrate.

Not only the magnitude, but also the direction of the flow can be controlled with the use of EOF. By arranging multiple sets of electrodes on a substrate and switching the voltage between them, two-dimensional motion control of a particle on a substrate, or sweeping DNA fibre within the horizontal plane, should be possible. Simplicity and no need for mechanically moving parts are other advantages of the EOF-based manipulations.

2.2. The structure and the function of the proposed device

The device for the extension of chromosomal DNA we propose in this paper is schematically depicted in figure 2. It is designed to perform three functions on a chip: (a) cell positioning, (b) extending DNA fibres out of the cell, and (c) anchoring the DNA fibres. It consists of four electrodes, A to D, and a set of micro-pillars and micro-pockets. The procedure is as follows (figure 3).

P1: Cell suspension is fed atop the device, and cells are allowed to settle down to the substrate surface.

P2: The voltage is applied, A positive and C negative. This induces EOF rightwards in this figure, by which cells are carried and go into the micro-pockets. The dimension of the pocket is chosen to accept only one cell, and due to its gradually narrowing structure, the cell is trapped and immobilized, one at each pocket (figure 3(a)).

P3: Free cells outside the pockets are flushed. The substrate is dried once to secure the immobilization of the cells.

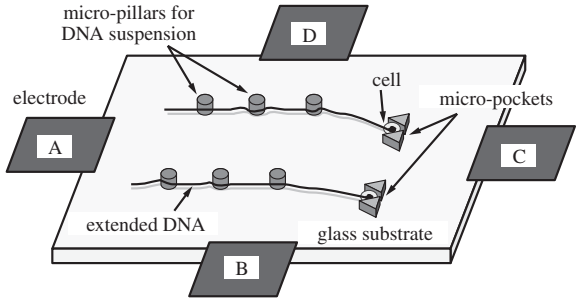

Figure 2. The micro-device for the extension and suspension of DNA.

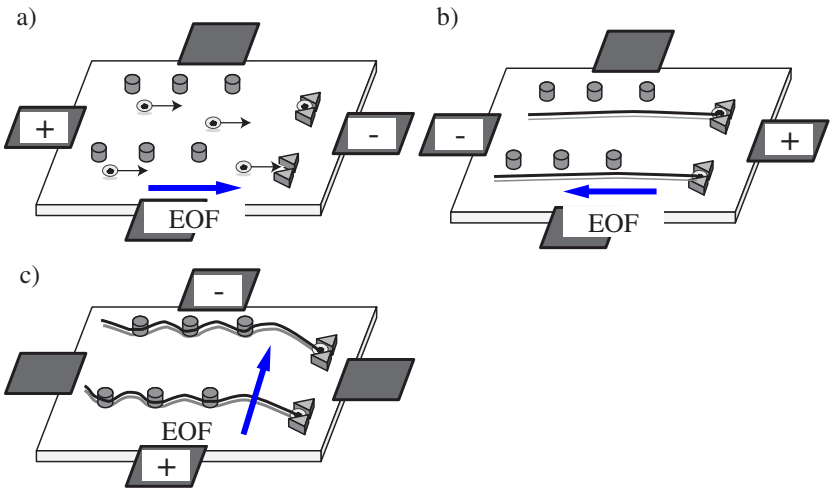

Figure 3. The process of DNA extension in the device. Cell positioning (a), DNA extension (b) and DNA anchoring (c).

P4: By adding enzyme/surfactant solutions, the cells in the pockets are ruptured.

P5: Leftwards EOF is induced by applying voltage, C positive and A negative, and DNA fibres are drawn out of the cells and extended (figure 3(b)).

P6: After the extension is completed, the voltage is switched, B positive and D negative. EOF upwards in the figure is induced, and the DNA fibres are pushed against the micro-pillars whose surface is chemically modified to have positive charges (figure 3(c)). Due to the electrostatic interaction, negatively charged DNA fibre is adsorbed and anchored onto the pillars. This anchoring is intended to prevent back-coiling and to maintain extended DNA when the electric field is turned off.

The device can potentially solve the problems addressed in the introduction in such a way that:

(a) Because DNA is fed to the device as a whole cell, and because the magnitude of EOF is well controllable, the breakage of DNA fibres during the handling and the extension can be minimized.

(b) The extension starts exactly from individual cells at the micro-pockets, in which a complete set of DNA is contained.

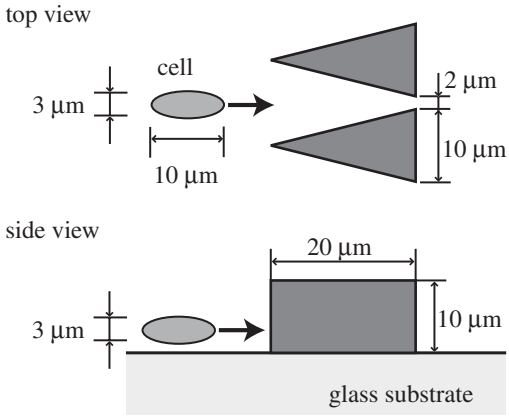

Figure 4. The micro-pocket for cell positioning.

(c) No drying process is involved, and the extended DNA is suspended without contact to the solid surface except at the micro-pillars. Hence better probe binding is expected.

3. Material, fabrication, and method

The cell used throughout our experiments is a fission yeast (S. pombe, wild-type strain 972h$^-$), having three chromosomal DNA, 5.7, 6.4, and 3.5 Mbp (1.6, 1.9 and 1.2 mm in length respectively, assuming the structural constant of B-form DNA, 0.34 nm bp^{-1}). After the enzymatic removal of the cell wall followed by a mild fixation with glutaraldehyde, the membrane is ruptured, and is soaked with surfactants to remove proteins and lipids inside the cell. This pre-treatment makes so called 'caged DNA' as detailed in [29], where roughly purified DNA is confined in the 'cage', i.e. cytoskeleton protein reinforced by the fixation. This is a necessary step to remove cytoplasmic protein such as histone or lipid membrane structures, and to make DNA easily extendable; what we referred to as 'cells' in figures 2 and 3, as well as what we will in later sections of the paper, in fact is this 'caged DNA'. Destruction of the cage after placing it in the micro-pocket is done by adding 2 mg ml^{-1} Pronase (Roche Diagnostics) and 0.5% sodium dodecylsulfate in 50 mM EDTA, 200 mM Tris HCl (pH 7.5).

The caged DNA has an ellipsoidal shape with diameter 3 μm and length 10 μm. It has been observed that DNA drawn out of it by EOF is extended a few microns above the substrate, because the initial position of the extension is elevated by the cage itself. Based on this observation, a micro-pocket as depicted in figure 4 was designed. It consists of a pair of triangular pillars, with the minimum spacing of 2 μm to trap a cell, and an opening of about 10 μm. The height of the pocket is chosen to be 10 μm. The pillar for DNA suspension has the same height, and its diameter is arbitrarily chosen to be 10 μm.

Figure 5 shows the fabrication process.

(a) The structure pattern is defined on an aluminium layer on a glass substrate (52 mm $\times$ 76 mm $\times$ 0.12 mm) with standard photolithography (figure 5(a)).
(b) The thick photo-resist SU-8 25 (Microchem Corp.) is directly spin-coated on the pattern to a thickness of 10 μm, and exposed from the backside of the substrate to transfer the pattern to the resist (figure 5(b)).
(c) Developed (figure 5(c)).

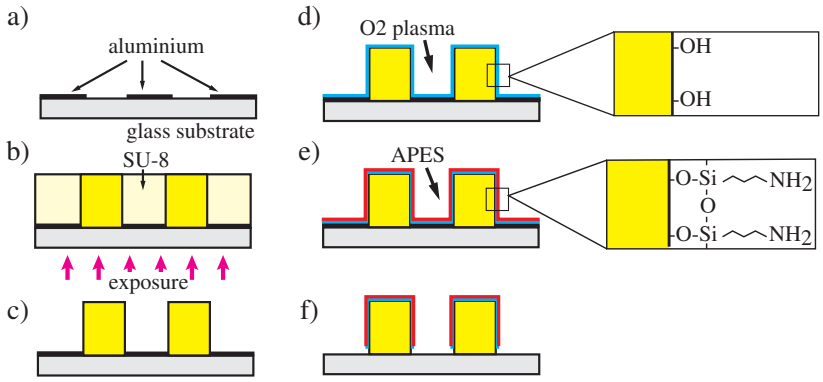

Figure 5. The fabrication process of the microstructure. Aluminium patterning (a), exposure of SU-8 (b), development of SU-8 (c), O_2 plasma treatment to introduce hydroxyl groups on the surface (d), 3-aminopropyltriethoxysilane (APES) coating to introduce amino groups (e) and aluminium etching (f).

(d) Exposed to oxygen plasma in a reactive-ion etcher (RIE-10NR, SAMCO), to create hydroxyl groups on the polymer resist surface as described in [33] (figure 5(d)).
(e) Dipped in 3-aminopropyltriethoxysilane (APES) and baked for 1 h at 120 °C, to bring amino groups on the polymer surface (figure 5(e)).
(f) Finally, aluminium is removed by a wet-etching solution (50% H_2SO_4, 5% CH_3COOH, 1% HNO_3) (figure 5(f)).

This process lifts off amino groups on the bottom substrate, while APES linked on the polymer surface remains unaffected. Amino groups are left at the top and the side of the polymer structure, and are protonated to be positively charged when immersed in water, to act as a binding agent for anchoring negatively charged DNA strands. This selective patterning of pillars with the chemical group is necessary in order to prevent DNA adsorption onto the substrate surface.

To form a flow chamber, a PDMS sheet with a cross-shaped opening is mounted on the substrate, in such a way that the microstructures fit into the centre of the cross (figure 6). It is filled with the cell suspension, but no coverage is placed on the top, and it is open to air. Platinum electrodes are immersed within the bends made at every ends of the cross, to prevent irregular flow near the electrode from entering the central part. The electrodes are connected through a switchboard to a dc voltage supply.

The whole process is observed under an inverted optical microscope (IX-71, Olympus), switchable to the phase-contrast and the fluorescent mode. A fluorescent intercalator dye YO-PRO-1 (Molecular Probes) is added to the final concentration of 0.1 μM to visualize DNA, together with the reducing agent dithiothreitol 1.4 mM to prevent fluorescent bleaching, and the observation is made through a high-sensitivity video camera (SIT, Hamamatsu Photonics).

4. Results and discussion

4.1. Cell positioning

The EOF cell positioning is first demonstrated using an array of micro-pockets as shown in figure 7. It is observed that when a cell carried by EOF goes into a pocket, it blocks the flow through the gap, so that the next incoming cells must go over or around the structure, and thus

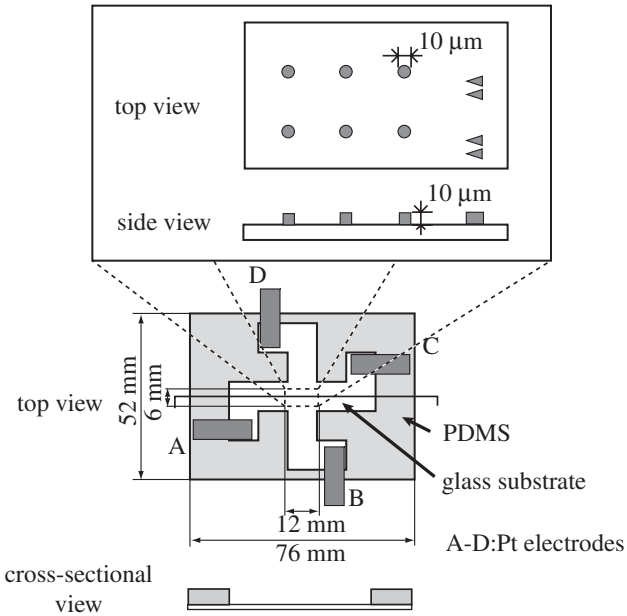

Figure 6. Flow chamber for EOF of variable direction. The insert shows an enlarged view of micro-pillars and micro-pockets.

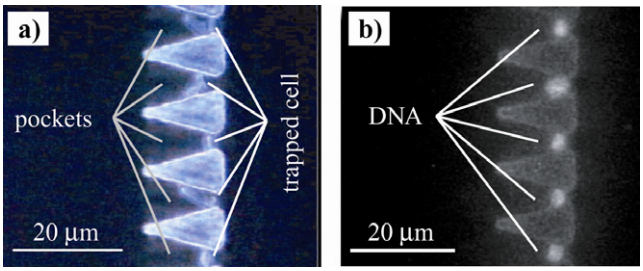

Figure 7. Cells positioned at the pockets. Phase contrast image (a) and fluorescence image (b).

only one cell is trapped at a pocket. Figure 7(a) shows a phase contrast view of micro-pockets and cells, and (b) is a fluorescence image of DNA in the cells, both taken after drying in the process P3 (section 2.2). Thus the starting position for DNA extension is defined.

4.2. DNA extension

Monitoring DNA extension through the camera, the applied voltage is gradually increased. At 30 V, the EOF velocity is about 50 μm s^{-1}, and DNA extension is observed without breakage. As estimated by the focal position of the microscope, the extended DNA is a few microns above the substrate. Figure 8 is a photograph of an extended DNA, as shown by a superposition of several video frames. DNA, drawn by a leftwards EOF out of a cell at the right end of the photograph, is extended to a length of 0.8 mm (2.4 Mbp). The direction of the extension is that of the EOF, indicating that the hydrodynamic force surpasses the electrophoretic force on negatively charged DNA (which should be in the opposite direction). The extended length

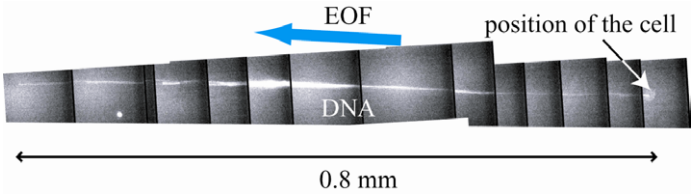

Figure 8. A typical result of DNA extension.

is more than three times longer than the extendable length by the moving meniscus method (0.7 Mbp = 0.24 mm, [10]), and is attributable to the controllability of the EOF to avoid breakage during the extension itself, and also to the protection of DNA from shearing in the 'cage' during the handling process before the extension.

The maximal extended length differs in each experiment, in the range of several hundred microns to around 1 mm. This is presumably due to the fact that (a) the residual proteins, which hamper DNA extension, are not uniform in each experiment, and (b) DNA is anchored in the cell at non-specific locations. In fact, it is not the strength of the EOF that determines the maximal length or the extending speed. In a separate experiment using isolated and well-purified DNA from the same type of cells, EOF extension approximately equal to the full DNA length is observed [34]. It is rather a matter of chemical processing how impurities, such as histone, can be removed from caged DNA, whose optimization is now underway.

There are three chromosomal DNA molecules in the cell, so the fibre observed in figure 8 is likely to be a bundle of multiple DNA fibres. The bundle can be separated, however, using a rotating EOF as shown in figure 9. These video frames are taken moving the microscope view to highlight the DNA fibres, so the position of the cell from which DNA is extended, i.e. the anchoring point of DNA fibre, is different from frame to frame (shown by a white circle in each photograph). In frame 1, a cell is at its right end, and the EOF extends the DNA leftwards. Frame 2 is the instance when the direction of EOF is rotated by 90°, by which DNA is swept downward. 10 s later, the fibres are rotated and aligned along the direction of the EOF (frame 3). When the EOF is rotated again rightwards, the short DNA fibre rotates more quickly than the long one, and these two are separated (frame 4 and 5). However, when the direction of EOF is kept constant for a certain period of time, they all merge into one bundle (frame 6). The separation is often observed when there is a large difference in the lengths of DNA fibres. It is because the angular velocity of rotation ($=u_\theta/r$, where u_θ is the circumferential component of the translational velocity and r is the distance from the anchoring point) is slow for a DNA section distant from the anchoring point. This observation suggests that by arranging several micro-pillars with different distances from the cell positioning micro-pocket, the chromosomal DNA can be separated by length with the use of a rotating EOF.

4.3. DNA suspending

The anchoring and suspension of extended DNA is experimentally demonstrated, using the micro-pillars having amino groups on the surface. Figure 10 shows the result. The pillars having 10 μm diameters are aligned on a straight line with a pitch of 30 μm. In figure 10(a), DNA starts from a cell somewhere outside the view on the right of the photograph, and is extended by the slightly inclined leftwards EOF. Then the direction of EOF is rotated clockwise by 90°, and the fibre is pushed against the pillars (figure 10(b)). In this photograph, the fibre between the pillars is observed to bend upwards by EOF, and the fibre is made to contact along a portion of pillar contour. During this process, anchoring by adsorption occurs due to

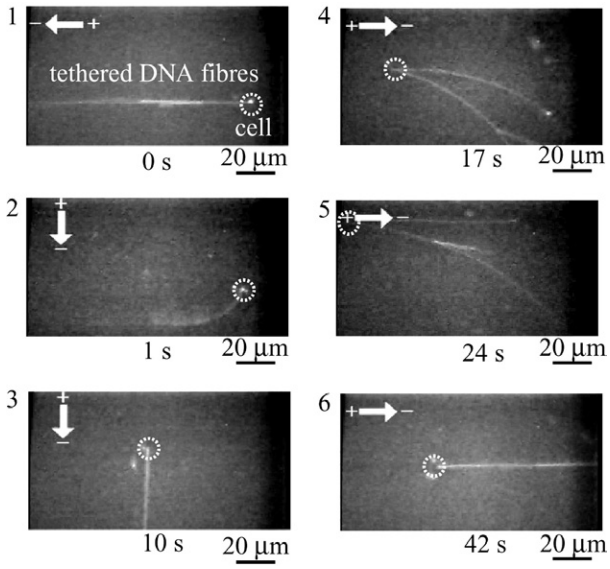

Figure 9. Separating individual DNA by sweeping a bundle in a rotating EOF.

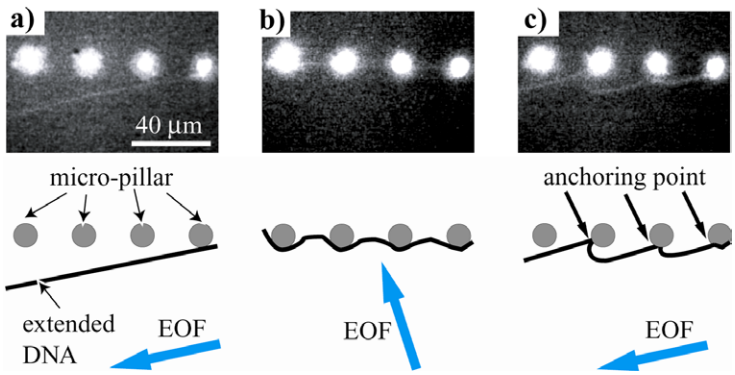

Figure 10. Anchoring of the extended DNA fibre onto micro-pillars.

the electrostatic interaction. When the direction of EOF is reversed (figure 10(c)), the fibre is bent downwards between the pillars, but the anchoring is strong enough to withstand the hydrodynamic drag.

When a DNA fibre suspended in this way is used for FISH, a potential problem is its breakage. As DNA nicks inevitably occur with a certain probability, strand separation with such a suspended form will result in disintegration. This may be solvable by making the pillar pitch small enough that there is only one nick in each interval.

5. Conclusion

A controllable extension of chromosomal DNA from a cell and its anchoring are demonstrated with the use of micro-fabricated structure in combination with electroosmotic flow (EOF). The micro-chip we have developed consists of (a) micro-pockets for the immobilization of

individual cells, (b) micro-pillars to anchor and suspend extended DNA, and (c) a set of electrodes to create EOF of variable direction. Carried by the EOF, the cells are positioned into micro-pockets, one cell at each pocket, and DNA is extended out of cells, again by EOF. Then the direction of EOF is changed to push the extended DNA onto micro-pillars, whose surface is chemically modified to have positively charged chemical groups, and DNA anchoring occurs by the electrostatic interaction. A method of selective modification of the pillar structure is developed, and using the chip, the cell positioning, the extension of DNA fibres up to 2.4 Mbp, and the suspension of the extended DNA, are experimentally demonstrated. DNA suspended in this way is held without contact to the solid surface except at the pillars, allowing free interactions with foreign molecules.

Acknowledgments

We would like to thank Dr Hidehiro Oana of the University of Tokyo and Dr Osamu Kurosawa of Advance Co. for valuable discussions, and Dr Kimihiko Sugaya of the National Institute of Radiological Sciences for providing the fission yeast strain. This work was supported in part by BRAIN (Seiken Kiko) Research and Development Program for New Bio-industry Initiatives, Grant-in-Aid for Scientific Research on Priority Areas (C) 'Genome Science' (nos 14011225, and 15011200) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), and Industrial Technology Research Grant Program in 2003 from New Energy and Industrial Technology Development Organization of Japan (NEDO). Photography masks were fabricated using EB lithography apparatus of VLSI Design and Education Center (VDEC), The University of Tokyo.

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