

Bacterial chromosome extraction and isolation

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Received 15th August 2002, Accepted 24th October 2002

First published as an Advance Article on the web 7th November 2002

We have used diffusive mixing and dielectrophoretic trapping to lyse *Escherichia coli* cells in a microfabricated environment and trap the *E. coli* chromosome. We characterize the conditions needed for efficient lysis of the cells, and conditions needed for the dielectrophoretic trapping of the chromatin without the trapping of cytoplasmic proteins.

1 Introduction

One of the great promises of microfabrication technologies when applied to biology is the potential to extract and analyze the contents of a *single* cell. The contents of that cell include the chromatin containing the DNA, the RNA components, organelles in the cell and the proteins in the cytoplasm. This is of course an enormous and difficult task which we are far from accomplishing at present. However, some basic steps have been taken towards this goal and in this paper we present some approaches we have made towards the lysis, trapping and purification of single cell chromosomes using the techniques of diffusive mixing and dielectrophoretic trapping.

While the ultimate challenge to the single cell project is a human eukaryotic cell, at this stage of work it is more reasonable to work with prokaryotic cells with their single chromosome, well-defined genome of manageable length and relatively simple cellular content. We chose to start this work with *Escherichia coli* bacterial cells because they are relatively easy to grow and their genome has been extensively studied.^{1,2}

Fig. 1 shows the basic principles of the chip. Lysis of the cells was achieved by rapid diffusional mixing of water with an osmotically stressed cell (a spheroplast) as described below. The mixing region is illustrated in Fig. 1(a). In this figure the *E. coli* (in form described below) is brought into the lysing region using hydrodynamic flow from the right, while the deionized

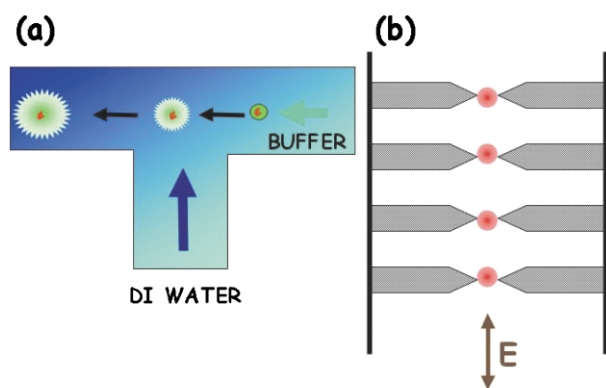


Fig. 1 A schematic of the basic chip operation. (a) The cell lysis region. Spheroplasts are shown as green spheres representing the cytoplasm, while the cell chromosome is shown as a red squiggle. (b) Dielectrophoretic trapping region, where highly polarizable chromatin is trapped in an oscillating electric field E .

water comes in from below. At the T-junction the cells lyse by osmotic shock. Once the cell is lysed the lysate is transported into a region where the chromosome can be separated from the cytoplasm. We then utilized dielectrophoretic trapping at low frequencies to separate the chromatin from the cell debris.

2 Principles

We used two different strains of *E. coli*, the 'wild' type (PS2124) and a green fluorescent protein (GFP)-expressing strain (PS2164). Over-expression of GFP allowed us to easily visualize the cells by epifluorescence microscopy and determine when they had been lysed, and potentially allows us to track the cytoplasmic protein content separately from the DNA.

A difficulty of working with *E. coli* is the peptidoglycan layer between the inner and the outer membranes of the bacteria. The role of the peptidoglycan layer is to enable the cell to withstand osmotic pressure differences between the cytoplasm and the outside solution. This makes the cell very sturdy and difficult to lyse.³ To solve this problem, we pretreat the cells with lysozyme which breaks down this layer.⁴ As a result, the cell is no longer able to maintain its rod-like shape and will deform into a sphere under the positive internal osmotic pressure. The osmotic pressure differential across the membrane in the presence of 50 mM salt is approximately 6 bar.³ The lysozyme-treated cell will thus absorb water, swell, and finally burst. To control lysis, we simply need to control the osmotic conditions. The lysozyme-treated *E. coli* are stored in a high sucrose concentration solution. The sucrose (which is not membrane-permeant) ensures a high osmotic pressure surrounding medium in which the cell will swell but not lyse (spheroplasts). In order to lyse the spheroplasts, we need to decrease the sucrose concentration by diluting the solution with deionized water. A diffusive mixer chip was used for this purpose.

2.1 Diffusive mixer

Since most fluid flow on a microfabricated chip is non-turbulent due to the small Reynolds number,⁵ mixing of fluids in a chip can only occur *via* diffusion. In this paper, the mixer chip in which the cells are lysed consists of a T-shaped channel as shown in Fig. 1. The principle of the diffusive mixer chips has been described elsewhere.⁶ The cells flow in one channel and deionized water flows in another channel. The water merges in

the cell channel. The mixing occurs by diffusion of the water through the cell flow in a characteristic time τ_{mix} given by:

$$\tau_{\text{mix}} = \frac{w_f^2}{D} \quad (1)$$

where D is the diffusion coefficient of water ($2.4 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ at 25°C)⁷ and w_f is the characteristic distance over which diffusion occurs. In this paper, the maximum characteristic distance is $50 \mu\text{m}$ (width of the channel), and therefore the characteristic mixing time is less than 1 s. With a dilute cell solution running in this device, we can lyse single cells and the corresponding chromosomes can be dragged individually in the device for further analysis.

2.2 Dielectrophoretic (DEP) trapping

Dielectrophoresis (DEP) is the movement of a polar or polarizable object in the direction of the electric field gradient.⁸ Dielectrophoretic trapping is the trapping of such objects in high electric field regions.^{9–11} It has been shown in a recent paper¹² that a charged polymer like DNA has a strongly length dependent dielectric response at extremely low frequency, and that by using shaped insulating barriers, very high electric field gradients can be created to selectively trap the chromatin from a lysed cell.

Consider a channel with dielectric constrictions filled with a conductive solution as in Fig. 1(b) (top-view). An electric field can be applied across the chip by means of external electrodes located at the channel extremities. The constrictions squeeze the electric field and polarizable objects will be trapped in the gap between them where the electric field is at a maximum. To a first approximation the total force acting on an object in this chip is:

$$\vec{F} = q\vec{E} + \alpha(\omega)E_z \frac{\partial \vec{E}}{\partial z} \quad (2)$$

where q is the effective charge and $\alpha(\omega)$ is the polarizability of the object at the angular frequency ω . The first term in the force expression corresponds to the electrophoretic force, which tends to move the object in the electric field direction. The second term corresponds to the dielectric force, moving the object in the direction of the field gradient. Note that the first term scales as E while the second scales as E^2 . When a DC field is applied in the chip, there is a competition between the two forces. At low E the electrophoretic force dominates and the object moves across the chip. As the field is increased, dielectrophoretic trapping dominates the electrophoretic force. In the presence of an AC field, the electrophoretic force averages to zero and the trapping occurs at lower voltage than with a DC field. The idea here for DNA purification is to trap the chromosome with an AC field while applying a small DC field to remove the smaller components of the lysate such as proteins, RNA and membrane fragments. The *E. coli* chromosome has a higher polarizability than any other lysate species¹³ and it should be trapped at low AC fields, whereas smaller molecules should be electrophoresed away from the constriction by the DC field.

2.3 Integration

Eventually, we want to combine lysis and DNA purification on a single chip, allowing us to handle small volumes and to work at low Reynolds number in order to avoid chromosome breakage. The *E. coli* chromosome has to be handled with care since it is rather loosely packed compared to human chromosomes with only 10% of the mass consisting of proteins,^{14,15} and can partially unfold depending on the salt concentration.¹⁶ Fig. 2 shows a schematic of the integrated chip. The chip consists of two mixing areas and a dielectrophoretic trapping

region. Cells flow under hydrodynamic flow from the cell entry region to mixing region 1 where they are lysed. Mixing region 2, not used in this experiment, can be used for further clean-up, such as protein hydrolysis or buffer exchange. Either hydrodynamic flow or DC electric fields can be used to transport the genomic material to the dielectrophoretic trap region. A simultaneously applied AC electric field can be used to trap the genomic material in the electrode array.

3 Materials and methods

3.1 Fluorescence microscopy

All experiments presented here were monitored by fluorescence microscopy using the 488 nm line from an Innova 70C argon-krypton laser (Coherent, Santa Clara, CA) and a Nikon high pressure mercury lamp model HB-1010IAF as the excitation source. The fluorescent dyes and sample preparation are described in the sections below. For all experiments, we used a Nikon upright microscope and imaged the samples through a 1 mm thick silicone rubber layer (see section below) except for the final chromosome trapping experiment, where we imaged the chromosome through a glass coverslip with an inverted Nikon microscope. The images were sent to a Sony digital video recorder.

The lysis was monitored using a Nikon 60 \times dry objective with a numerical aperture (N.A.) of 0.7 and a Photon Counting Intensified Charge Coupled Device (PC intensified CCD) (Hamamatsu Corporation, Bridgewater, NJ). The fluorescent filter set was an XF23 cube (Omega, Brattleboro, VT), with a 485 nm excitation wavelength (22 nm bandwidth) and an emission wavelength of 535 nm (35 nm bandwidth).

DEP trapping of the *E. coli* chromosome was visualized through a Nikon 1.4 N.A. 60 \times oil immersion objective using two different filters: the Omega XF23 mentioned above and an Omega XF02 filter (excitation 330 nm, 60 nm bandwidth/emission 400 nm long pass) used for the Hoechst 33342 visualization. A PentaxMax Gen III 512X512 pixels Intensified camera (Roper Scientific, Trenton, NJ) was used. The camera was interfaced to a computer with Winview (Roper Scientific). The staining of bacteria is described in section 3.3.

3.2 Electronics

A bipolar operational power supply model BOP 1000M (Kepco, Flushing, NY) provided the $\pm 1000 \text{ V}$ driving voltage for the

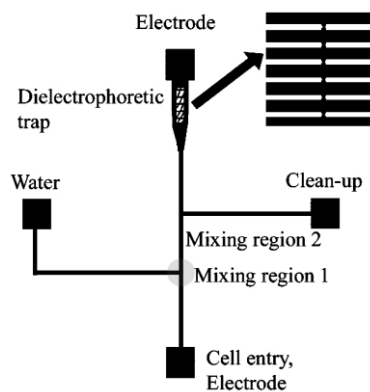


Fig. 2 The combined chip. In our case, the intermediate clean-up region was not used. The cell lysis agent in this paper was simply deionized water, in other cases a more complex lysis agent could be used. The enlarged region in the upper right hand corner shows the dielectrophoretic trapping region in more detail. The vertical spacing between the insulating barriers highlighted by white lines is 20 microns, the horizontal spacing is 10 microns.

DEP experiments. The input to the BOP 1000M was from an HP3325A signal generator (Hewlett Packard, Cambridge, MA) which was connected *via* an HPIB (Hewlett Packard Interface Bus) to a computer running Labview (National Instruments, Austin, TX). External gold electrodes driven by the Kepco BOP were immersed in the liquid droplets at the chip entrances, 1 cm apart from each other.

3.3 *E. coli* spheroplasts

We used two different strains of *E. coli*, the 'wild' type (PS2124) and a GFP-expressing one (PS2164). The latter is isogenic with the wild type except for the plasmid which contains the GFP and β -lactamase genes. *E. coli* expressing GFP (PS2164) was grown at 37 °C overnight in L-broth (Bacto tryptone 10 g l⁻¹, Bacto yeast extract 5 g l⁻¹, sodium chloride 10 g l⁻¹) with 0.125 g l⁻¹ of added ampicillin. Subsequently 100 μ l of the culture was centrifuged, the L-broth was removed and the cells were resuspended in 200 μ l of 50 mM Tris, 10 mM EDTA, 10 mM NaCl, 20 μ g ml⁻¹ lysozyme, 0.58 M sucrose at pH 8. The resulting spheroplasts were then loaded into the chip for lysis.

In the following, we denote 'GFP spheroplasts' as the spheroplasts arising from GFP-expressing *E. coli* and 'wild-type spheroplasts' as the one arising from wild type *E. coli*.

The GFP spheroplasts have free floating GFP in their cytoplasm (excitation: 489 nm, emission: 508 nm), allowing their visualization using fluorescence microscopy. When chromosome visualization was necessary, the DNA was stained with DNA-selective dye Hoechst 33342 (Molecular Probes, Eugene, OR, excitation 350 nm, emission 455 nm) at a final dye concentration of 50 μ M. Wild type spheroplasts were stained with the green nucleic acid dye SYTO 13 (Molecular Probes, OR, excitation 488 nm, emission 509 nm) at 0.3 μ M.

When a nucleic acid stain was used, the anti-oxidant dithiothreitol (DTT) was added to the cell solutions at 0.1 M in order to reduce bleaching. The linear polyacrylamide POP6 (Perkin Elmer, Wellesley, MA) was added to 0.1% to reduce nonspecific cell adhesion as well as electroosmosis.

3.4 Fabrication

A mold for the mixer device was made at the center for Photonics and OptoElectronics Materials (POEM) in Princeton, NJ. The mold was fabricated on a silicon wafer using the epoxy-based negative resist SU-8 (Microchem Corp, Newton, MA) according to standard protocol. The resist was spun on the wafer to a final thickness of 30 μ m. The channels were 50 μ m wide. After a pre-exposure bake, the wafer was exposed together with the mask to UV light (3 mW cm⁻²) for 2 min. After a post exposure bake and development, only the reversed image was left on the surface.

The mixer itself consisted of a silicone cast of the mold described above. We used RTV 615 (General Electric, Waterford, NY) for this purpose. The two-component polydimethylsiloxane (PDMS) chains (RTV 615A) and the curing agent (615B) were mixed at a 9:1 weight ratio. The mixture was degassed, then poured on the mold and cured at 80 °C for 30 min before peeling it off the mold. When necessary, surfactant Contrad (Decon Laboratories Inc., Bryn Mawr, PA) at 5% in water was spun on the mold in order to reduce sticking. The mold was placed on top of a microscope slide with holes corresponding to the channel entrances. The device was mounted on a thick plexiglas plate. Channels drilled in the plexiglas were connected to the holes in the microscope slide with O-rings. The other end of the channels widen to form reservoirs for the solutions. The top of the reservoirs was connected to tubing which were themselves connected to a

pressure regulator. The latter enabled independent air pressure regulation in each reservoir. An oversized stainless steel ring was put on top of the silicone and screwed on the plexiglas, pressing the chip against the plexiglas. The cells inside the chip were imaged from the center of the stainless steel ring, through the silicone layer. Fig. 3 shows a schematic of the chip and the setup.

The combined chip consisted of a silicone rubber cast of structures defined in a silicon wafer. The mask and mold were made at the Cornell Nanofabrication Facility (CNF) (Cornell University, Ithaca, NY). The chrome mask for the mold was made in a pattern generator (GCA/Mann PG3600F). Standard photolithography techniques were used to pattern the photoresist spun on the silicon wafer. The wafer was then etched (30 μ m) with a Plasmatherm SLR-770 ICP Reactive Ion Etcher. The silicone cast was made in the same way as described above. The combined chip pattern is shown on Fig. 2. The chip consisted of two mixing regions and one dielectrophoretic trapping region. The channels are 50 μ m wide and 30 μ m deep. The first mixing region is where the lysis will occur. Two gold electrodes were connected between the top and bottom feed regions (external to the chip). They were used to apply a DC field across the chip to transport the lysate to the DEP region. They were also used to apply an AC field to trap the DNA in the DEP region. Since the electrodes connect the chip's outside reservoirs, we could apply a high electric field at relatively low frequency without gas formation inside the chip. The DEP region is shown in the inset of Fig. 2. The DEP trap consists of series of pairs of 10 μ m wide constrictions separated by a 10 μ m gap. The gap size was chosen to be roughly the same size as the end to end radius of the *E. coli* 4.6 Mbp genome.¹⁷ The chromosome trapping experiment was run without applying pressure. The silicone cast and a glass coverslip were oxygen-plasma treated for one minute, then sealed permanently together.

3.5 Decreasing non-specific adhesion

Cells contain a very complex mixture of proteins, lipids, polysaccharides and nucleic acids, including hydrophobic, hydrophilic, charged and neutral parts. Such a brew of biological objects has a complex interaction with the walls of the microfabricated device, and after lysis, many components can bind irreversibly to walls. To decrease this non-specific adhesion, one can either modify the wall surfaces or the cell solution itself. We found that it was necessary to add Pluronic F-108 (BASF Corporation, Mount Olive, NJ) at 1 g l⁻¹ in the cell

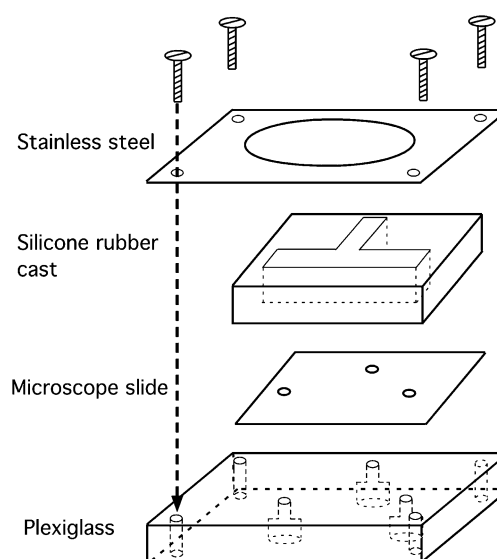


Fig. 3 Schematic of the different layers in the chip.

solution. Pluronic F-108 is a poly(ethylene oxide)/poly(propylene oxide)/poly(ethylene oxide), PEO-PPO-PEO, copolymer. PEO is known to prevent non-specific adsorption of biomolecules on surfaces.^{18,19}

4 Results and discussion

4.1 Lysis

Fig. 4 shows the lysis of GFP spheroplasts in the mixer chip. The channels are highlighted for clarity reasons. The cells flow in from the right, and water flows in from below. The pressures applied at the channel entrances are of the order of 0.1 psi, and were adjusted independently in both channels. We thus controlled the cell flow velocity and were able to temporarily stop it if necessary. There were significant heterogeneities in the cell population. Some cells were more resistant to lysis than others. In that case we had to slow down the cells near the channel intersection so that a sufficient amount of water would diffuse toward the cell. In that way we could observe lysis without moving the objective. In Fig. 4, we can see GFP rapidly diffusing out of the cell, forming a large green ball after lysis while the genomic material remains invisible.

To visualize the genomic DNA, we operated the chip with DNA-stained wild-type spheroplasts. The DNA was stained with SYTO 13 as explained in the previous section. The lysis of one cell, imaging its DNA, is shown in Fig. 5. Since the *E. coli*

chromosome is a compact object, unlike the rapidly diffusing GFP, the DNA does not expand significantly after lysis. This compaction is due to the RNA and DNA-binding proteins which hold the nucleoid together.³

4.2 *E. coli* chromosome dielectrophoretic trapping

We show here that the chip we designed is indeed able to trap chromosomal DNA from lysed cells. GFP containing spheroplasts in which chromosomal DNA was stained with Hoechst 33342 were moved into the chip. Deionized water was used to lyse the cells. After the lysis, we used a DC electric field of 5 V cm^{-1} across the chip to electrophoretically transport the cell lysate towards the DEP region. A 500 V cm^{-1} AC field at 500 Hz was applied in addition to the DC field in order to trap the chromatin. Fig. 6(A) shows the Hoechst 33342 (DNA-selective dye) fluorescent image of the lysate in the DEP region. The DNA is trapped in the gaps between the constrictions. Fig. 6(B) shows an image of the GFP contents at the same chip location and under the same electric field conditions. There is no GFP trapping in the gaps, but GFP is homogeneously distributed throughout the channel (leaving dark areas where the constrictions are located). This shows that the chip can selectively trap the chromosomal DNA without trapping the cytoplasmic GFP of the cell. Of course, there is a large range of frequencies and

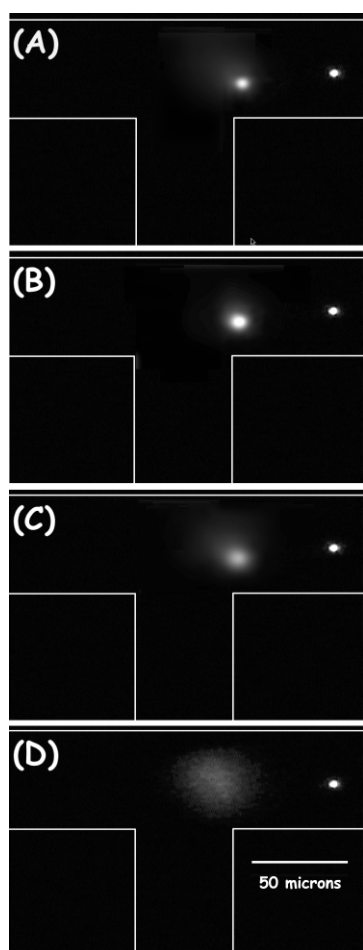


Fig. 4 Time series (from A to D) of GFP fluorescence images of the lysis of a GFP spheroplast by osmotic shock. The channel walls are highlighted as white lines. The spheroplasts come from the right, the water comes from below in the figure. An intact spheroplast is visible on the right of the channel. The total time interval from A to D is one second.

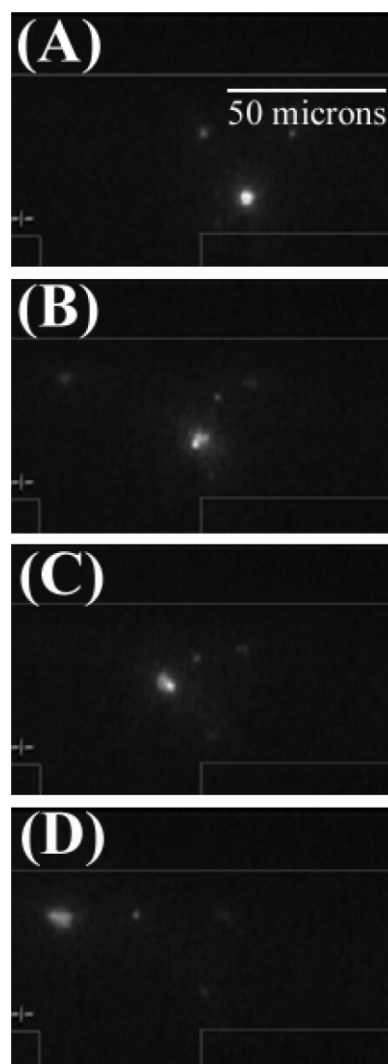


Fig. 5 Time series (from A to D) of chromatin fluorescence images of the lysis of a wild-type spheroplast by osmotic shock. Images are spaced at intervals of 1 second.

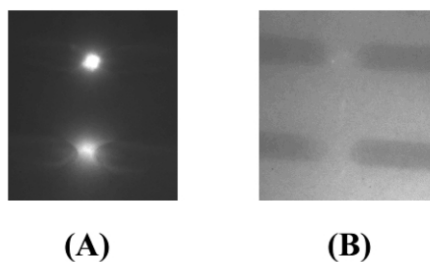


Fig. 6 DEP trapping of the lysate. GFP-spheroplasts were stained with the DNA-selective dye Hoechst 33342 and lysed inside the chip. (A) Hoechst-stained chromosomes trapped by a 500 V cm^{-1} AC field at 500 Hz with an additional 5 V cm^{-1} DC field. (B) In the same location, under the same conditions, the cytoplasmic proteins are not trapped.

voltages that can be explored for selective trapping, and this is just a snap-shot experiment designed to show the basic high selectivity of the DEP trap. The number of chromosomes trapped in each gap in Fig. 6 is estimated to be about five. This number depends on the concentration of spheroplasts in the chip before lysis. We can decrease the number of chromosomes per gap by decreasing the spheroplast concentration. Bleaching prevented us from continuously imaging a single cell from its entry into the chip to the trapping of its chromosome. Therefore, we estimated the number of chromosomes trapped in a gap according to the initial number of cells in the chip and according to the size of the trapped object. Fig. 7 shows single chromosome manipulation in the DEP chip. For that experiment, the cell concentration was lowered so that they would be less numerous than the number of gaps in the chip. Each gap is given a number from 1 to 5. Fig. 7(A) shows the chromatin trapped in gap number 2 with an AC field of 500 V cm^{-1} at 500 Hz. The AC field is then turned off and chromatin is moved in the chip with a DC field of 5 V cm^{-1} as shown in Fig. 7(B), showing the reversibility of the trapping. Finally, the DC field is turned off and the 500 V cm^{-1} 500 Hz AC field is turned on again: the chromatin is trapped again between the closest constrictions (gap number 4) as shown in Fig. 7(C). No GFP was trapped in the gaps in this experiment (data not shown). The time interval between the first and last image is 8 seconds, for a travelled distance of $60 \mu\text{m}$. The chromatin slowed down at the intermediate constrictions, due to the dielectrophoretic force exerted by the DC field, as explained in section 2.2.

5 Conclusion

We have constructed an integrated device which can lyse *E. coli* under controlled conditions, and we have shown that we can transport the *E. coli* chromosome to a dielectrophoretic trapping region where the chromosomes can be selectively held to separate them from lysate fragments. A great deal of work remains to be done in order to fully utilize this technology to allow the lysis, trapping and transport of selected genomic DNA into other integrated structures for further analysis, but we hope that some of the basic principles of single cell lysis and genomic material trapping are presented here.

Acknowledgement

This work was supported by the Defense Advanced Research Projects Agency (Grant MDA972-00-1-0031) and by the

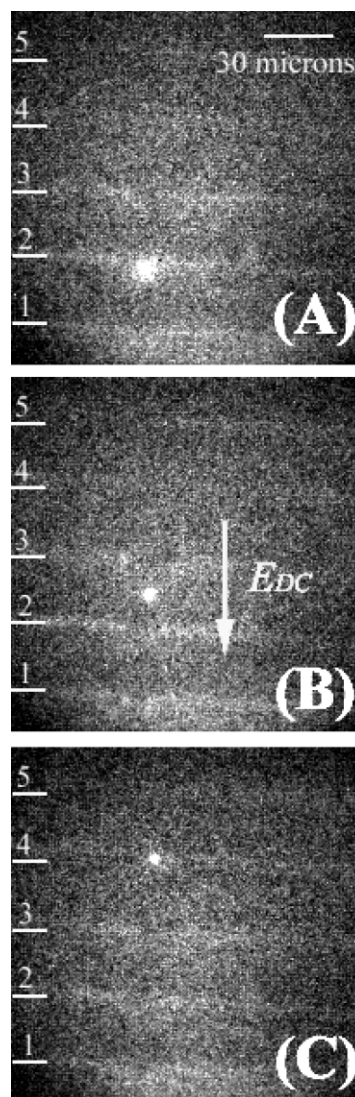


Fig. 7 Single chromosome manipulation. (A) The chromosome is trapped in gap number 2 with a 500 V cm^{-1} 500 Hz AC field. (B) The chromosome is moved by electrophoresis when the AC field is turned off and a 5 V cm^{-1} DC field is applied across the chip. (C) Same conditions as in (A): the chromosome is trapped in gap number 4.

Nanobiotechnology Center, a STC program of the National Science Foundation under Agreement No. ECS-9876771.

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$$R = \sqrt{2pL} = \sqrt{2pNa} = 12 \mu\text{m}$$

where L is the contour length of the DNA molecule, N is the number of base pairs (4 mbp), a is the length of one base pair (0.34 nm) and p is the persistence length of double stranded DNA (50 nm).

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