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Microfabricated Devices for Sizing DNA and Sorting Cells

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ABSTRACT

We have microfabricated devices to size and sort microscopic biological objects, ranging from cells to single molecules of DNA. Sizing is accomplished by fluorescent excitation and detection. The devices are fabricated in a silicone elastomer using a replica method. Single molecules of DNA have been sized to 10% accuracy, and manipulation of *E. Coli* cells has been demonstrated.

Keywords: MEMS, Cell sorting, DNA diagnostics

1. Introduction

There are numerous applications in biology in which one wishes to know the size distribution of DNA fragments in a particular solution. Traditionally, this has been accomplished by measuring mobility with gel electrophoresis, and inferring length from mobility. However, there are several drawbacks to this technique. For medium to large size molecules, resolution is limited to about 10%. It takes anywhere from an hour to days to run a gel, depending on the size of the molecules being sorted. Finally, for extremely large molecules the method fails completely, since the pores of the gel get "gummed up". There are thus several reasons why one would like to find an alternative method to size DNA molecules.

Recently, several new intercalating fluorescent dyes have been developed that virtually only fluoresce when bound to DNA.[1] This permits one to directly measure the length of single DNA molecules by quantitating the emitted fluorescence. This method has been successfully demonstrated in standard flow cytometric systems.[2,3] It has also been used as the basis of a method to measure the apparent length of extended single molecules of DNA with optical microscopy.[4]

We have developed microfabricated devices to size and sort DNA molecules by a direct, fluorescent measurement of the length of the molecules. This method also works for larger objects, and we have demonstrated cell sorting with *E. Coli* cells expressing green fluorescent protein. The microfabricated system has several advantages over traditional flow cytometry, including cost, sensitivity and size. The microfabricated channels allow the use of high numerical aperture optics for light collection. Furthermore, with recent advances in solid state lasers and detectors, we anticipate that a complete system can be made in dimensions smaller than a shoebox.

The devices are fabricated out of a silicone elastomer with a replica method. Using a silicon master as a mold, the fabrication is an easy one step process. Material costs are low, and the devices are disposable. This technique was first suggested by Austin and coworkers at Princeton,[5] building on work by the Whitesides group at Harvard.[6]

There are numerous possible applications for such technology. We anticipate that the DNA analytical system will be used for many routine biological assays, due to its speed and low material requirement. One possible application is DNA fingerprinting. A portable system could be brought to the scene of a crime for direct analysis on small blood samples. Since

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the system does not require PCR, the chance of false signals is reduced. Furthermore, the chance of laboratory error due to contamination and handling will be reduced.

We envision both technologies demonstrated in this paper as stepping stones to building gene cloning "lab on a chip" devices. Small amounts of DNA can be recovered from restriction and ligation reactions, and separated according to size. Performing transfection on the chip allows very small (nanoliter) reaction volumes, so only small amounts of DNA are necessary. The properly transfected cells can then be separated via fluorescent or colorimetric assays in the cell sorting device. Single cells can then be grown into macroscopic amounts necessary for clone libraries, etc.

2. Device Fabrication and Mounting

Negative master devices were fabricated in silicon, and used as molds for the silicone elastomer. Standard contact photolithography techniques were used to pattern the oxide surface of a silicon wafer, which was then etched by reactive ion etch with a C_2F_6/CHF_3 gas mixture. The oxide was then used as a mask for the silicon underneath, which was etched with KOH. The silicone elastomer (General Electric RTV 615) components were mixed together, and pumped in an evacuated chamber for 30 minutes to remove oxygen. The liquid elastomer was then poured on the mold (spin coating was used in cases where the device thickneess needed to be well controlled) and cured in an oven at 95 degrees for two hours. After this, the devices could be peeled from the silicon master and would bond hermetically to glass.

The devices were patterned as shown in figure 1. The large channels had lateral dimensions of 100 microns, which narrowed down to 5 microns at the T junction. The depth of the channels ranged from 2.5 to 20 microns. In early prototypes, we found that due to the large aspect ratio (100 microns in width by 2 microns in depth), some of the elastomer channels would bow and and block themselves by sealing directly to the glass. This was remedied in later versions by adding support pillars to the mask that would prop up the large channels and prevent bowing (see figure 1).

The elastomer is naturally hydrophobic, preventing aqueous solution from entering the channels. We modified the surface of the devices by coating it with a hydrophilic polyurethane (Tyndale Plains Hunter Hydrogel, 0.25% in ethanol) which was allowed to cure for an hour at 95 degrees. After this treatment, the devices were hydrophilic and aqueous solution would easily enter by capillary action. The devices could be cleaned and reused several times if desired.

Devices were mounted in one of two ways. For the DNA sizing devices, a plumbing system of HPLC tubing was devised for sample delivery and recovery. The tubing was glued to a piece of plexiglass with three holes bored through. The device was mounted directly on the opposite face of the plexiglass. Here, the device also functioned as a cover slip for the oil immersion objective used for optical excitation and collection. The overall device thickness was 100 microns in these cases. The plexiglass piece was then attached to the stage of an upright optical microscope. The flow rate was controlled by pressure with a syringe.

For the cell sorting devices, an inverted method was used. Holes were cut in the devices with a razor blade at the input and output portions, forming sample wells. The device was placed on a cover slip or microscope slide, and used in an inverted microscope. Then the aqueous samples were introduced via capillary action, and controlled with electro-osmotic forces. Tin plated copper electrodes were inserted into sample wells for this purpose.

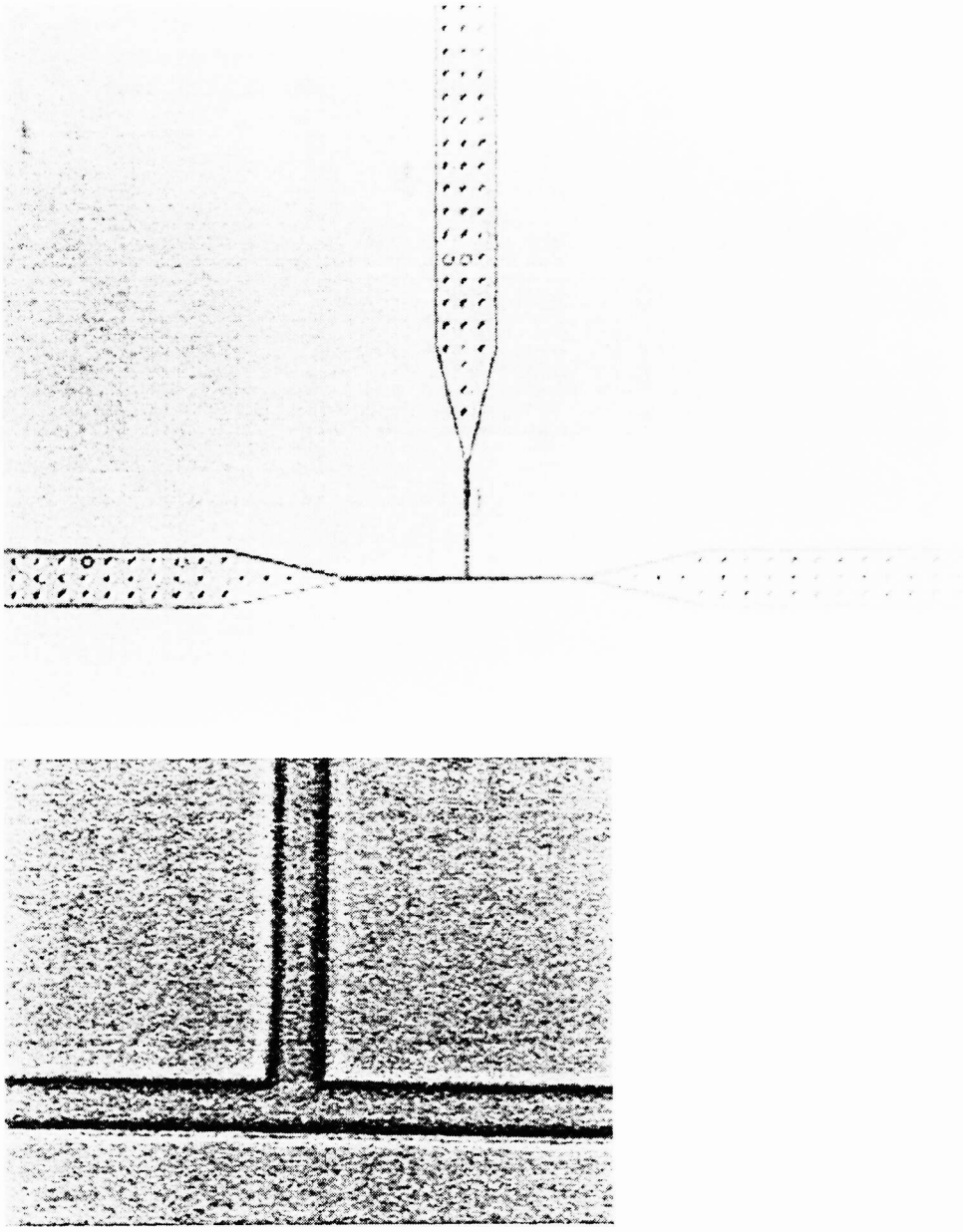

Figure 1. Top: Optical micrograph of an elastomer device. The large channels are 100 microns wide; the channels narrow down to a width of 5 microns at the T-junction. The depth of the channels is 3 microns. The large channels extend approximately 1 cm in length (not shown) for making macroscopic fluidic contacts. Bottom: Higher magnification image of the T-junction. The channels here are 5 microns wide. Note the high fidelity of the replica fabrication process for the straight lines and corners of the channels.

3. Experimental Set-up

For the DNA sorting devices, an upright optical microscope (Olympus) was used. A 10 mW air cooled Argon laser (Uniphase) emitting at 488nm was used for fluorescent excitation. A power level of 3 mW was chosen to be just below the experimentally measured saturation level of the fluorescent dye. The laser was focused through a 60x 1.4NA oil immersion objective, which was also used to collect the emitted fluorescence. Auxiliary lenses were used to adjust the size of the focused spot to approximately 50 microns. The large spot size was chosen to give uniform excitation across the width of the channel. The quality and uniformity of the spot was evaluated by imaging a thin layer of fluorescein in solution with a CCD

camera. The image was digitized and evaluated for symmetry and gaussian shape. Dielectric filters were used to filter laser tube fluorescence (CVI 488 nm line filter) and to reduce background and scattered light from the emitted fluorescence (Chroma D535/50M). A dichroic filter was used to introduce the laser light into the optical train (Chroma 500 DCLP). A schematic diagram of the setup is shown below.

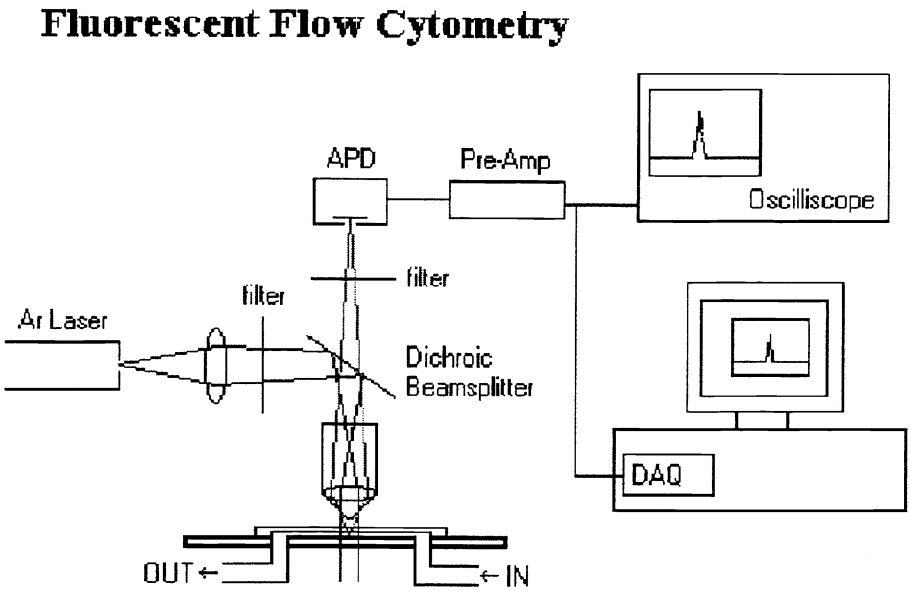

Figure 2: Schematic diagram of DNA sizing experimental apparatus.

Fluorescence was imaged onto a 5 mm avalanche photodiode detector (Advanced Photonics). The detector was cooled to -40 degrees with a two stage thermoelectric cooler (ITI 6320/157/040C), which reduced the dark current of the detector from 50 nA to 90 pA. The detector was biased at 2400 V, giving an estimated gain of 500-1000. The photocurrent was converted to a voltage by a Burr Brown OP128 ultra low noise op amp operating at a gain of 100 mV/nA. The signal was low pass filtered at 1.6 kHz, and digitized at 5 kHz by a National Intruments Lab PC1200 board on a PC running Labview.

We checked the depth of focus of the microscope by centering a 1 micron fluorescent bead in the laser beam. We then plotted the detector output as a function of focal distance, and determined that the signal was essentially flat over a distance of 5 microns. This was then used to design our microfabricated devices; the depth was chosen so that the DNA molecules always remained in the plane of focus of the microscope.

Lambda phage DNA (Gibco) was diluted in buffer (TE with 10 mM NaCl) and stained with the intercalating dye YOYO-1 (Molecular Probes) at a stoichiometry of approximately one dye molecule per 7.5 base pairs. Single molecules of DNA gave measurable pulses whose height corresponded to the length of the molecule. Pulses were collected in large batches and then analyzed off line with home written software for peak detection.

The total interaction region imaged on the detector is approximately 375 femtoliters, smaller than is achievable with standard methods of flow cytometry. This volume is largely determined by the size of the laser spot, and can be reduced further by the use of cylindrical optics.

The cell sorting devices were mounted on an inverted microscope (Zeiss). Electrodes were attached as described above, and a switching box was built to manipulate a potential of 60 Volts between the three channels. Each channel could be independently set for either 60 V, ground, or float. *E. Coli* cells expressing green fluorescent protein were introduced into the devices by capillary action and observed with fluorescence microscopy. Fluorescence was excited with a mercury lamp,

and recorded on videotape with a CCD camera (Hamamatsu). The video images were later digitized and the position of the cells as a function of time was measured.

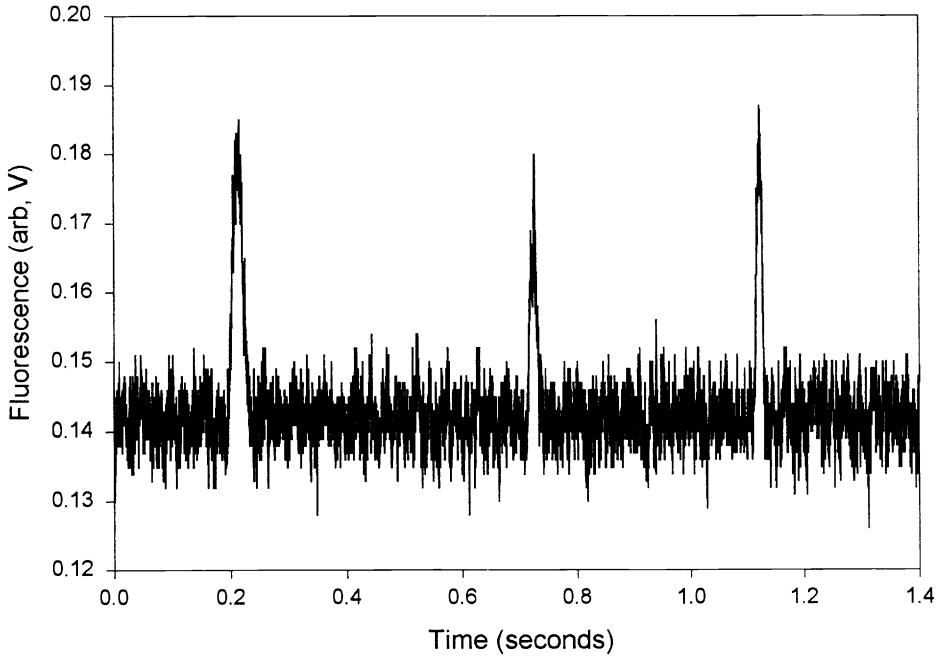

Figure 3: Pulses from single molecules of fluorescently stained lambda DNA as they flow through the device.

4. Results

We took data from a homogenous solution of lambda DNA (48 kbp), a length that is difficult to analyze with standard gel electrophoresis. 619 molecules were analyzed, and the total data acquisition time was 17 minutes. The signal to noise per molecule was approximately 5:1 (individual pulses are shown in figure 3), indicating that the smallest resolvable molecule with our set up is on the order of of 10 kilobase pairs. This is not an intrinsic limit of the method, and we believe that with further improvements to the electronics and analysis we can improve the sensitivity to 1 kilobase pair. We intend to continue testing the device both with smaller molecules such as a lambda digest, and with longer molecules traditionally used in pulsed field gel electrophoresis.

A histogram of the peak heights is shown in figure 4. There is clearly a distribution about a single length value, whose coefficient of variation was calculated to be 10%. This shows that with 20 minutes of data taking and analysis, we are able to size lambda with the same resolution as on a pulsed field gel.

It is worth pointing out the extremely small amount of material used for such an analysis. We used a total of 619 molecules, i.e. on the order of a zeptomole. This was analyzed in a total reagent volume of approximately 6 nanoliters. Again, these are not intrinsic limits of the system and we believer that with further improvements, the reaction volumes can be reduced substantially.

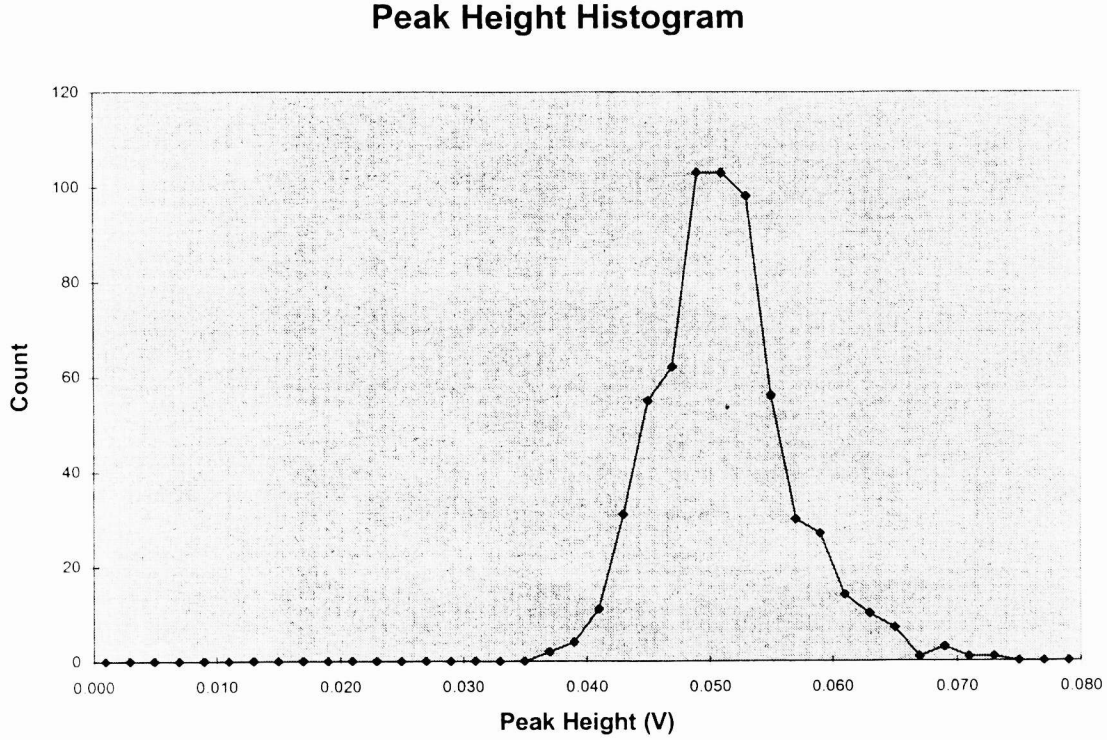

Figure 4: A histogram of pulse heights as measured in figure 3. The heights were measured by taking the maximum value of the peaks; no fitting was done. The single peak in the histogram shows that we are able to measure the length distribution of a single species of DNA with 10% coeffecient of variation.

We have also demonstrated the principle of cell sorting with electro-osmotic manipulation in our devices. *E. Coli* cells were manipulated with electric fields, and switched between one of two possible output channels. A graph of the position versus time of a single *E. Coli* cell as the field is repeatedly reversed is shown in figure 5. The estimated switching time is 50-100 milliseconds, leading to a maximum cell sorting rate of 10-20 Hz. We believe with higher potentials and some device design improvements, we could reach a single channel switching time of 1 millisecond. Then with 100 channels switching in parallel, the overall throughput of the device would be 100 kHz.

5. Conclusion

We have successfully demonstrated DNA sizing and cell sorting in microfabricated devices. With further improvements, we believe that this method will be competitive with the current state of the art in gel electrophoresis, and will find specialized applications in cell sorting, such as lab on a chip. These devices are extremely easy to fabricate from a silicon master, reducing production costs and rendering the devices truly disposable.

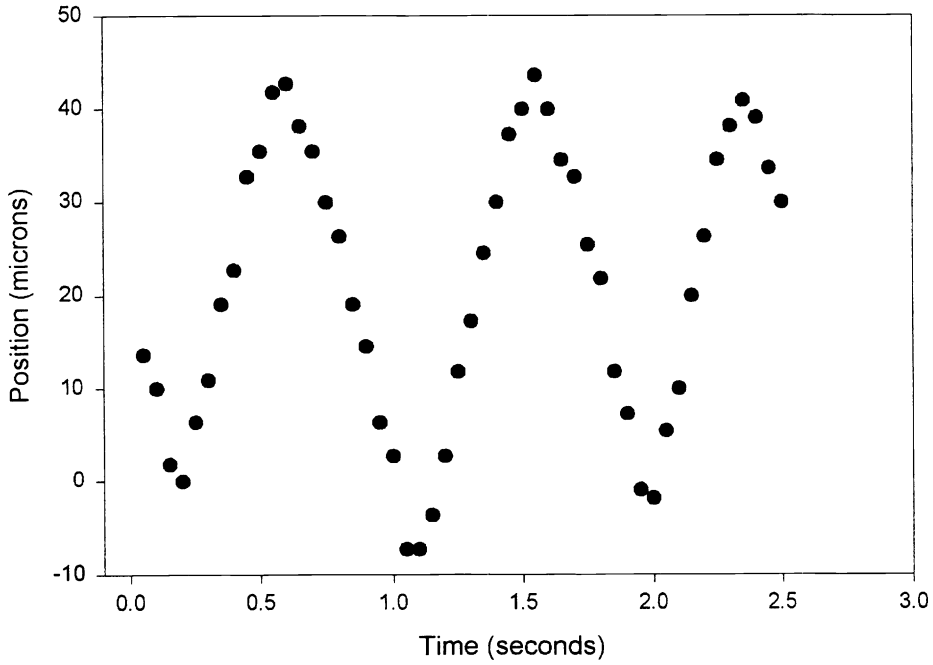

Figure 5: E Coli position versus time as a 60V potential is repeatedly reversed.

6. Acknowledgements

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