

Manipulation of Single DNA Molecules via Lateral Focusing in a PDMS/Glass Microchannel[†]

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In a hybridized microchannel made of poly(dimethylsiloxane) (PDMS) and glass, nonuniform electroosmotic flow (EOF) was created when an electric field was applied. PDMS and glass pieces were bonded at room temperature after oxidization with oxygen plasma. After oxidization, the PDMS surface has a higher zeta (ζ) potential than that of glass, which results in faster EOF at that surface. When the channel has a large aspect ratio (width \gg depth), the EOF can be considered as a two-dimensional gradient shear flow. By using a scanning confocal fluorescence microscope, we demonstrated that negatively charged DNA molecules were focused by the nonuniform EOF and the electric field into a thin layer at the glass surface of about one-fifth of the channel depth. This phenomenon was applied to selectively detect target DNA molecules using evanescent-field excitation. Streptavidin-conjugated quantum dots (QD) were used to selectively bind biotinylated DNA. After applying an electric field, free QD remained randomly distributed within the channel. Nonbiotinylated DNA molecules were focused onto the glass surface, but they were not labeled and thus nonfluorescent. Therefore, the increase in molecule count after application of the electric field can be solely attributed to QD–DNA complexes which were focused onto the glass surface. This method does not require the separation of excessive probes (QD) and can be operated continuously to achieve high throughput.

Introduction

It is well-known that early detection of disease can improve the chances for successful treatment. For example, according to the American Cancer Society, the 5-year relative survival rate for all cancers is 62%. However, for cancers that can be detected at their early stages, the 5-year relative survival rate is about 82%.¹ Many diseases like cancer are caused by genetic alterations in certain genes.² Therefore, genetic analysis provides the opportunity to detect disease-associated genes or even predict diseases before the onset of physical changes in the cells. The past decade has seen significant progress in genomics research. Many disease-related mutations or genetic markers have been identified, and numerous genetic analysis methods have been developed.

We have reported several different high-throughput approaches to detect single target DNA molecules in a capillary or a microchannel.^{3–10} In a recent study, we demonstrated that, by applying a dc field and a Poiseuille flow from cathode to anode, DNA molecules can be focused into a narrow region of several microns around the axis of a capillary.^{8–10} Single-molecule detection with an efficiency of $\sim 95\%$ was achieved.⁹ However, since detection only occurs in a narrow strip, the throughput of that method is limited and the overlap of molecules presents problems in single-molecule counting. To solve this problem, the three-dimensional (3D) Poiseuille flow must be replaced with a two-dimensional (2D) gradient shear flow. According to the focusing mechanism discussed in our previous work,⁸ under a gradient shear flow and an electric field, DNA molecules will be focused onto a thin layer instead of a narrow strip. The imaging capacity of the CCD camera can then

be fully exploited and the throughput will be substantially improved. A typical 2D gradient shear flow can also be obtained by applying differential pressure between the horizontal walls of a rectangular channel, provided that the channel width is much larger than the channel depth so that viscous effects from the vertical walls can be neglected.

During past years, remarkable progress in microfabrication has been made.^{11–16} Microfabricated devices have been used in electrophoretic separation, chemical analysis, and chemical synthesis.^{14–17} Typically, to generate a hydrodynamic flow in a microchannel, a syringe pump or elevated buffer reservoir has to be attached to one end of the microchannel to supply the necessary pressure. In addition to the need for creating a complicated structure, joint leakage often causes serious problems. On the other hand, the profile of electroosmotic flow (EOF) in rectangular channels with different zeta (ζ) potentials at the walls has been characterized by Andreev et al.^{18,19} According to their mathematical model, it is possible to obtain a linear gradient in 2D shear flow, provided that the top and bottom walls have different ζ potentials and the width of the channel is much larger than the depth. Our approach is to induce asymmetric shear flow by using electroosmotic flow instead of hydrodynamic flow.

One flexible and cost-effective material for making microfabricated devices is poly(dimethylsiloxane) (PDMS).¹⁵ The PDMS microchannel has been widely used in electrophoresis. The behavior of EOF in this channel has been extensively studied, although the purpose of most studies is to suppress EOF or to obtain a uniform EOF.^{20–24} In this work, we used a PDMS/glass microchannel to obtain a gradient 2D shear flow. The lateral focusing effect of this device will be characterized. The application of this mode of focusing to sensitive immunoassay will be demonstrated by using streptavidin-conjugated quantum dots (QD) to detect single biotinylated DNA molecules.

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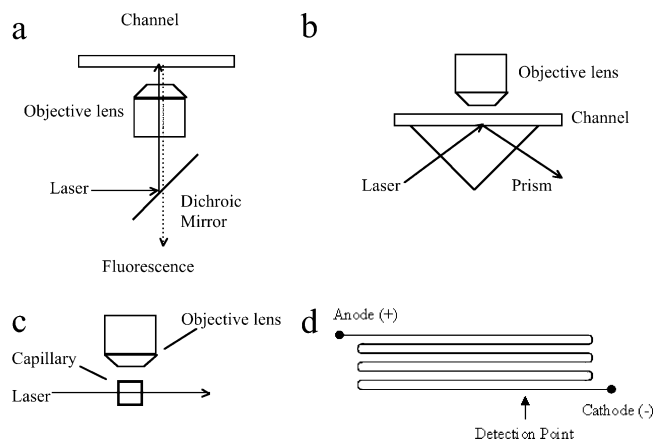


Figure 1. Optical arrangements used in this study. (a) Fluorescence confocal microscopic imaging; (b) evanescent-field fluorescence microscopic imaging; (c) capillary-electrophoresis-based microscopic imaging; (d) pattern of fabricated microchannel.

Experimental Section

Optical Setup. Three different fluorescence-based imaging setups were used in this study. The first setup (Figure 1a) is an epifluorescence microscope (Prairie Technologies, Middleton, WI), which can also be used as a laser-scanning confocal microscope. When operated in the confocal mode, an argon ion laser (488 nm, Melles Griot, Carlsbad, CA) was used to provide the excitation. A dry-type Nikon Plan Fluor objective, 40×0.75 NA, was used both to focus the excitation beam and to collect the fluorescence. This setup was used to obtain the molecular distribution in the microchannel by collecting sectional images in the z -direction.

The second setup (Figure 1b) is a home-built evanescent-field fluorescence microscope, where excitation is based on total internal reflection through a quartz prism. A drop of index-matching oil (type FF, $n = 1.46$, Cargille, Cedar Grove, NJ) was placed between the microchannel and the prism. An evanescent field ~ 100 nm thick was generated at the bottom surface of the channel, which was made of glass in this study. Only molecules within the evanescent field can be excited and detected. A CCD camera (Cascade, Roper Scientific) was mounted on top of a standard upright microscope. A water-cooled argon ion laser (488 nm, Innova-90, Coherent, Santa Clara, CA) provided the excitation beam. A mechanical shutter with shutter controller (Uniblitz, Vincent Associates, Rochester, NY) was used to block the laser beam when the camera was off to reduce photobleaching. Fluorescence was collected by an oil-type objective lens (Zeiss $40 \times$ Plan-Neofluar 1.3 NA). A sequence of frames were acquired for each sample via V++ software (Roper Scientific).

The third setup (Figure 1c) is similar to that reported previously.⁸ Briefly, an intensified charge-coupled device (ICCD) camera (Pentamax, Roper Scientific, Princeton, NJ) was mounted on a Zeiss Axioskop upright microscope. An air-cooled 488-nm argon ion laser (Uniphase, San Jose, CA) provided the excitation beam. By use of a cylindrical lens, the laser beam was focused normal to the square capillary as a thin sheet. Only molecules in the center plane of the capillary (~ 10 μm in thickness) were excited and recorded. A mechanical shutter with shutter controller (Uniblitz) was used to block the laser beam when the camera was off to reduce photobleaching. Fluorescence was collected by a dry-type Zeiss 20×0.75 NA plan Apochromat microscope objective lens. Two 488-nm holographic notch filters with optical density > 6 (Kaiser Optical, Ann Arbor, MI; HNFP) were placed between the objective and

the ICCD to remove scattered light from the excitation beam. The camera was operated in the external synchronization mode with the intensifier disabled open. Data were acquired with the WinView software provided by Roper Scientific. This setup was used to record the migration of single molecules in a square capillary.

Microfabrication. The channel pattern used in this study is shown in Figure 1d. A positive photomask was used to make this pattern on glass. The line width is 300 μm . The spots at both ends have a diameter of 2 mm. The photoresist coated glass plate (Nanofilm, Valley View, OH) was 76×25 mm and 0.25 mm thick. Wet etching technique was employed to create the pattern. The etching time was about 2 min, and the height of the master was about 46 μm . A PDMS microchannel was obtained with SYLGARD(R) 184 Silicone Elastomer Kit (Dow Corning, Midland, MI) according to the manufacturer's instructions. The mixture of base and curing agent was poured onto the channel master and cured at 60 $^{\circ}\text{C}$ for 2 h. The PDMS film was peeled off from the channel master and bonded to a coverslip (0.25 mm) to form an enclosed microchannel after oxygen plasma treatment. Two through holes were drilled in the PDMS sheet at the ends of the channel and served as buffer reservoirs.

Preparation of Biotinylated DNA. The biotinylated λ -DNA Hind III digest (New England Biolabs, Beverly, MA) was separated in 1% Seakem Gold Agarose gel (Cambrex Bio-Science, Rockland, ME) with $1 \times$ TE buffer (pH 8.0). The 23 130 bp band was excised and extracted with QIAEX II Agarose Gel Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The concentration of 23 130 bp biotinylated DNA after extraction was estimated to be 175 pM.

Quantum Dots (QD)–DNA Complex. The 2 μM stock solution of Qdot 605 nm Streptavidin Conjugate (Quantumdot Corp., Hayward, CA) was diluted to 20 pM (concentration of streptavidin) and mixed with 23 130 bp biotinylated DNA. This solution includes 0.4 pM QD and 8 pM 23 130 bp biotinylated DNA and 112 pM nonbiotinylated 23 130 bp DNA. After vortexing, the mixed solution was left for reaction for 1 h. This solution was used without further dilution in this study.

YOYO-Labeled Lambda DNA. λ -DNA (48 502 bp, New England Biolabs) was labeled with YOYO-1 (Molecular Probes, Eugene, OR) at a ratio of 1 dye molecule per 5 bp according to the manufacturer's instructions. Typically, YOYO-1 labeled DNA samples were first prepared as a 200 pM stock solution, incubated at room temperature for at least 1 h, and diluted with corresponding buffer to the desired concentration just prior to the start of the experiment.

Buffer. The buffers used in this study are designated by the ionic strength. The 5 mM pH 8.0 Gly-Gly (Sigma, St. Louis, MO) buffer was prepared by titrating 5 mM NaOH (Sigma) to pH 8.0 with 50 mM Gly-Gly solution. The buffer was then filtered with 0.22 μm filter, and left for photobleaching overnight with a mercury lamp.

Other Reagents. The fluorescent polystyrene microsphere (Sigma) has a diameter of ~ 0.1 μm , and was diluted to 10 ppm with 1 mM Gly-Gly buffer. Other reagents are of analytical grade.

Data Analysis. The images were analyzed on a PC computer with ImageJ, a Java image-processing program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/ij/>). For WinView files, the specific frames were first selected with the "Threshold and Clipping" function of the WinView software and saved as 8

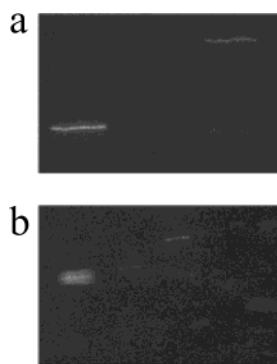


Figure 2. Electroosmotic flow velocity at (a) PDMS and (b) glass surfaces. Length of streaks is the integrated trajectory of microspheres over the exposure time.

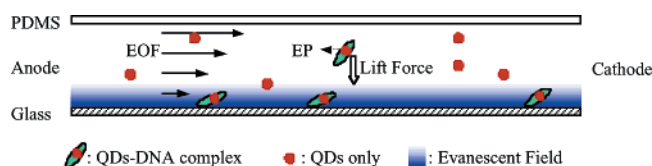


Figure 3. Mechanism of selective lateral focusing of QD-DNA complexes.

bit TIFF files. The 8 bit TIFF files can then be opened and analyzed by ImageJ software. Images from the epifluorescence microscope can be opened directly by ImageJ. Before performing the “Image Analysis” function, the “Smooth” function was performed to reduce the noise and improve the accuracy in particle counting. By using the “Particle Analysis” function of ImageJ, we were able to determine the particle number in each frame, particle area, perimeter, center of mass, etc.

Results and Discussion

Profile of EOF in the PDMS/glass Microchannel. As shown by Andreev’s mathematical model,¹⁹ if the top and bottom surface have different ζ potentials, gradient 2D shear flow should be obtained. Before oxidization, a glass surface usually has a higher ζ potential than that of PDMS because of the presence of silanol groups. However, after exposure of both glass and PDMS to oxygen plasma, the ζ potential of PDMS

increases significantly while that of glass remains almost constant.²⁰ The actual profile of EOF was obtained by using an epifluorescence microscope (Figure 1a) to measure the velocities of 100-nm fluorescent polystyrene microspheres at the glass and at the PDMS surfaces. As discussed in our previous work,⁸ polystyrene microspheres are rigid particles and cannot be deformed and thus cannot migrate laterally. Therefore, they will remain uniformly distributed within the channel and can be tracked at both surfaces. Images of the fluorescent microspheres at the PDMS surface (Figure 2a) and at the glass surface (Figure 2b) were recorded. The streak lengths indicate the migration distances of the microspheres within the exposure time of 850 ms. Since EOF dominates the electrophoretic mobility of the microspheres, the longer streaks imply faster EOF. That is, EOF at the PDMS surface was faster than that at the glass surface.

Lateral Focusing of λ -DNA in PDMS/Glass Channel. As seen in Figure 3, the lateral gradient in EOF velocities (arrows from left to right) will cause nonspherical or deformable particles, such as DNA, to align themselves off axis.^{8–10} Since DNA molecules are driven electrophoretically (EP) from right to left, they experience a lift force due to hydrodynamic drag and will be focused to the bottom (glass) surface. We used YOYO-labeled λ -DNA to characterize the lateral focusing effect in this dual-surface channel. The channel width was 300 μm , and its depth was about 45 μm . The total length of the channel was about 460 mm, and the detection point was about 20 mm from the cathode. The fluorescence confocal microscope (Figure 1a) was used to scan the microchannel slice by slice, with 2.0 μm per slice, starting from the glass surface toward the PDMS surface. Parts a and b of Figure 4 show the images of DNA molecules at positions of 0, 4.0, 8.0, and 12.0 μm from the glass surface before and after applying electric field, respectively. It is obvious that DNA molecules are brought to the glass surface by lateral focusing.

Figure 5 shows that, before applying electric field, DNA molecules are randomly distributed in the channel, but the molecule numbers at the two surfaces were much lower than that at the middle of the channel. This edge effect can be explained by the electrostatic repulsion at the surfaces, which were both highly negatively charged at pH 8.0.²⁵ After the electric field was applied for 1 min, about 95% of molecules

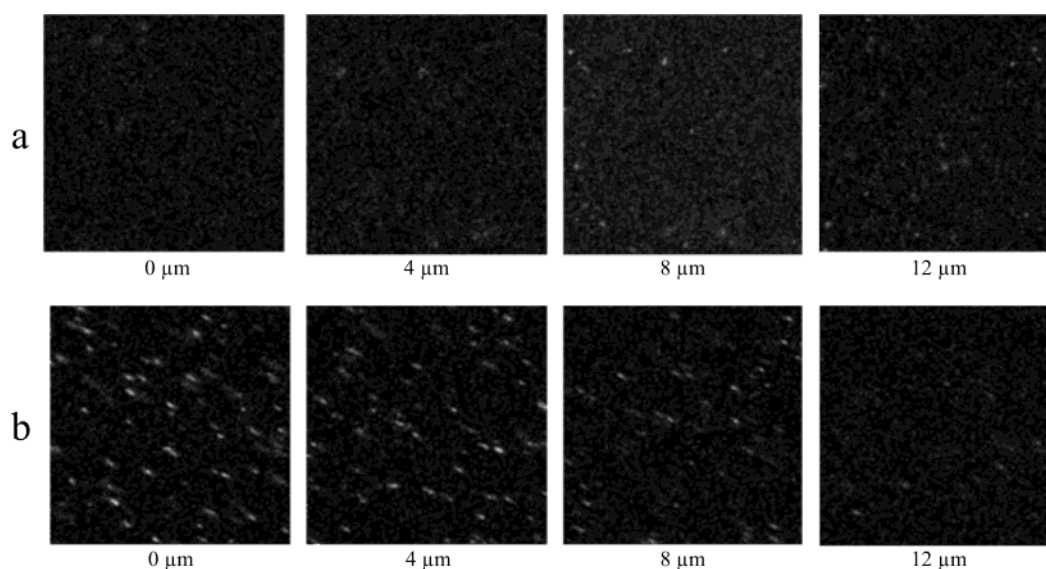


Figure 4. Confocal images of λ -DNA molecules in PDMS/glass microchannel with and without electric field. (a) No electric field: slices at 0, 4, 8, and 12 μm from the glass surface. (b) After applying an electric field of 43.7 V/cm for 1 min: slices at 0, 4, 8, and 12 μm from the glass surface. The sample is 0.3-pM λ -DNA labeled with YOYO-1 at a molecule ratio of 5 bp:1 dye.

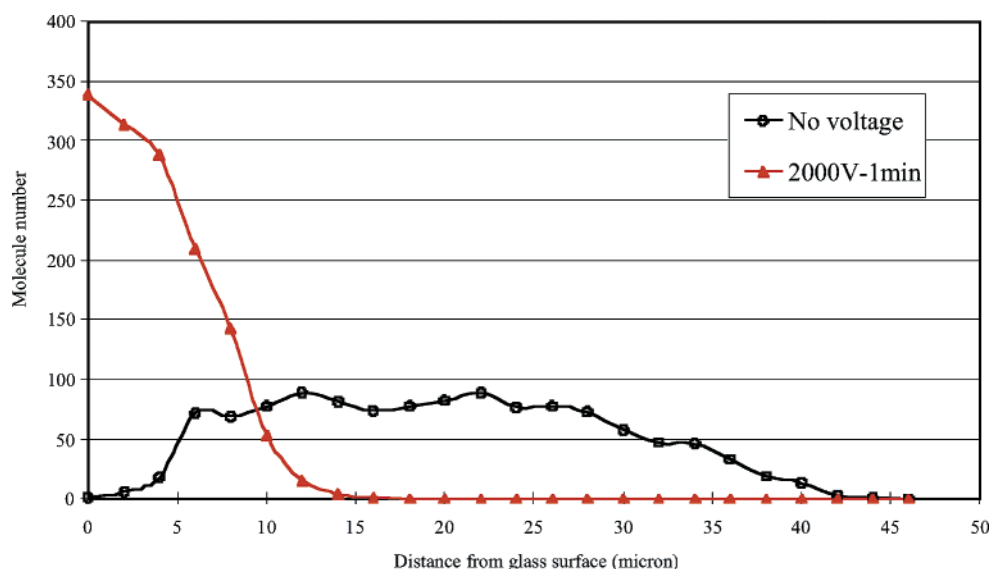


Figure 5. Vertical density profile of λ -DNA molecules in PDMS/glass microchannel with and without electric field.

were focused into an 8- μm layer at the glass surface. It is worth noting that there were 339 molecules found at the glass surface after applying electric field (first slice), compared to 1 molecule before applying the electric field. No DNA molecules were found from the 10th slice (18 μm from the glass surface) to the 24th slice (PDMS surface) after applying the electric field for 1 min. We also note that the DNA molecules were always moving axially along the channel. That is, the increase in number was due to focusing rather than adsorption.⁷

For most single-molecule detection methods, one key issue is the reduction of the detection volume in order to improve the signal-to-noise (S/N) ratio.^{9,26} In evanescent-field fluorescence (Figure 1b), only a $\sim 100\text{-nm}$ layer at the surface will be detected.³ Light scattering is almost completely suppressed, and high S/N ratio can be obtained. However, the molecule detection efficiency (MDE), which is defined as the fraction of molecules in a sample that flow through the detector and are actually detected, is relatively low. For example, for a 40- μm deep channel, even if we neglect electrostatic repulsion at the surfaces and assume that DNA molecules are homogeneously distributed within the channel, the MDE is estimated to be only 0.25%. The actual MDE is even lower than 0.25% due to electrostatic repulsion (Figure 5 without field). For disease diagnosis, this is hardly satisfactory.

The lateral focusing method described above provides a promising solution to improve the MDE. As shown in Figure 5, about 24.8% (339 out of 1367) of the molecules were focused onto the glass surface and can be detected. This is a 99-fold increase in MDE. Our previous results showed that the lateral lift force increases with increasing voltage and shear flow rate.⁸ Therefore, the MDE will be further improved if a higher electric field is applied, which will increase the shear flow rate as well.

Application. In immunoassay, usually a dye-labeled antibody is used to bind the antigen. The antibody–antigen complex is then separated from the free antibody and detected. The separation efficiency of bound/free antibody is critical to the success of immunoassay, especially when the antigen concentration is very low. Here, we demonstrate the application of the lateral focusing phenomenon as described above to perform continuous (homogeneous) immunoassay. The streptavidin-conjugated quantum dots were used to detect biotinylated DNA molecules in a background of nonbiotinylated DNA molecules.

First, we characterized the migration behavior of DNA–QD complexes under gradient shear flow in an electric field by

using a capillary electrophoresis setup (Figure 1c). This method has been well characterized in our previous work.^{8–10} A hydrodynamic (Poiseuille) flow through the capillary can be easily applied by lifting one end of the capillary. A hydrodynamic flow from cathode to anode with a maximum velocity of 0.55 mm/s was applied throughout the experiment, and an electric field of 40 V/cm was applied from the 201st frame in the series of images. The laser power was 15 mW, the exposure time was 40 ms, and the frame rate was 5 Hz. The sample was the 0.4 pM QD–DNA complex solution. The particles from frame 101 to frame 200 (no electric field) and those from frame 401 to frame 500 (after applying electric field for 40 s) were counted using ImageJ software, and the radial distribution of QD–DNA is shown in Figure 6. Since the DNA molecules were not labeled with fluorescent dyes in this experiment, they were not detected unless they were bound to fluorescent QD. As shown in Figure 6, before applying electric field, QD–DNA complexes are randomly distributed within the capillary. However, when an electric field of 40 V/cm was applied for 40 s, the number of DNA–QD complexes at the center region of the capillary increased significantly, implying that the QD–DNA complexes were deformed under shear flow and were thus subjected to lateral lift forces.

As discussed in our previous work, free QD are not subjected to lateral lift force because they are rigid particles and cannot be deformed under shear flow. The fact that QD–DNA complexes and free QD have significantly different migration behavior suggests that we should be able to separate them by using the appropriate shear flow and electric field. We tested the possibility of selective detection of QD–DNA complexes using the PDMS/glass microchannel and evanescent-field fluorescence imaging (Figure 3). For free QD (Figure 7a), the observed particle number was 172 before applying the electric field and 142 after applying an electric field of 30 V/cm for 1 min. There was no major change in particle number after applying electric field in view of molecule counting errors. However, for QD–DNA complexes (Figure 7b), the observed particle number was 35 before applying electric field and 150 after applying an electric field of 30 V/cm for 1 min. The observed particle number increased by more than 4-fold. We then removed the electric field for 5 min and took another image. The observed particle number changed to 37, almost the same as that before applying the electric field. This shows that the

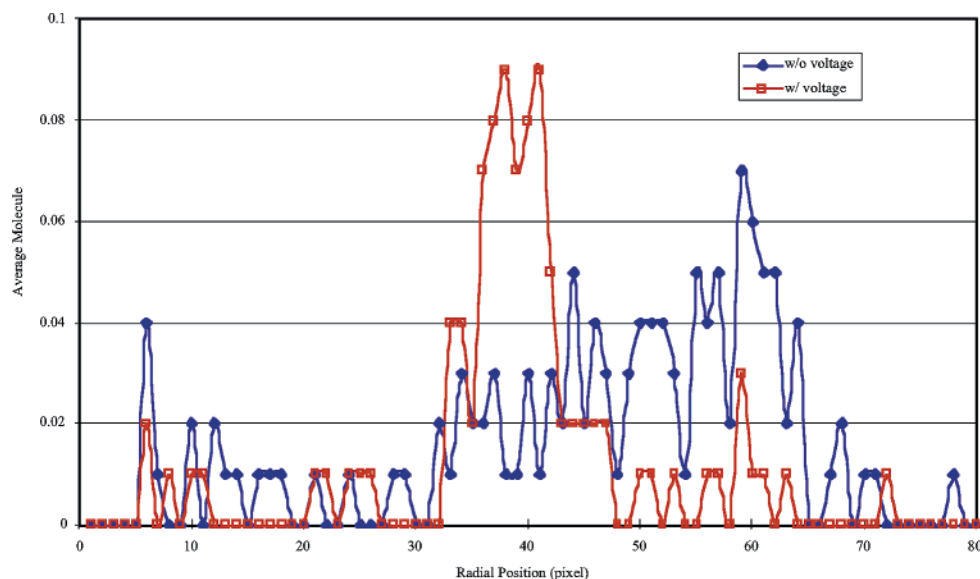


Figure 6. Radial focusing of QD–DNA complexes in a square capillary. Electric field: 40 V/cm. Hydrodynamic flow: 0.55 mm/s at the center of the capillary from cathode to anode.

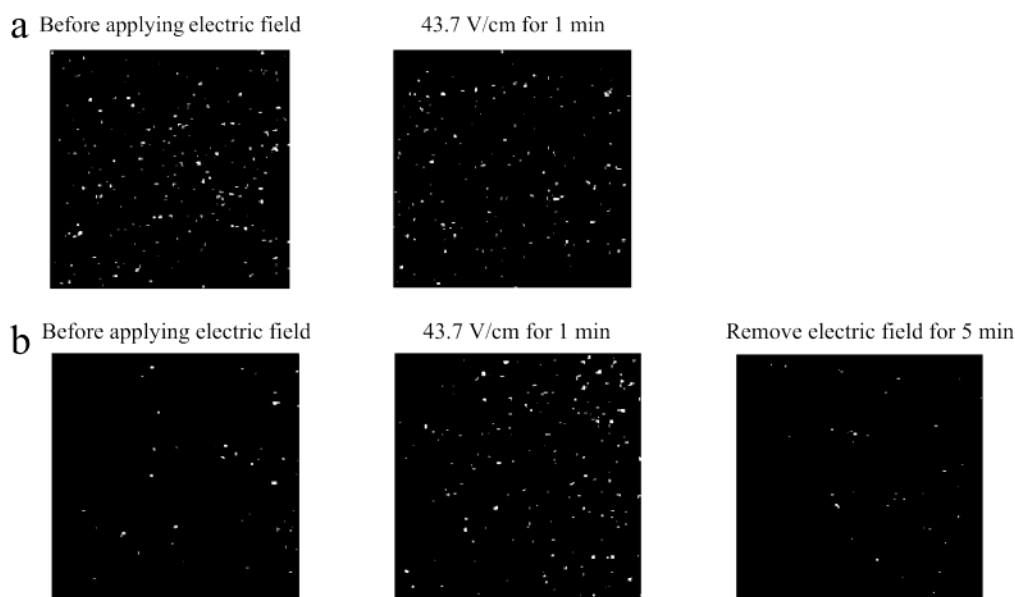


Figure 7. Selective focusing of QD–DNA complexes in a PDMS/glass microchannel. (a) Images of QD at the glass surface using evanescent-field fluorescence microscopy; (b) images of QD–DNA complexes at the glass surface.

density of the QD–DNA complexes equilibrated across the channel during the 5 min period. These results indicate that the increase in particle number could be solely attributed to the existence of biotinylated DNAs, which conjugated with QD and forced them to be focused near the surface. Therefore, the increase in particle number provides quantitative information on biotinylated DNA in the solution without separation of the components.

In immunoassay, usually excess antibody is required to bind the antigen efficiently.⁶ Problems arise when the antigen level is extremely low because the signal from any antibody–antigen complex could be buried in the large background signals from excess antibody. One possible solution is to use the difference in their electrophoretic mobilities for discrimination.⁶ The discrimination based on lateral focusing as shown here should add another factor of 100 (see MDE above) of selectivity toward detecting the rare antigen molecule.

Conclusions

We demonstrated that a simple and cost-effective PDMS/glass microchannel could be used to produce gradient 2D shear flow and that the resulting lateral focusing could be used for immunoassay without bound/free separation. One advantage of this method is that it can be operated continuously, which would be essential for high-throughput screening. Although streptavidin–biotin binding was employed here, extension to any immunoaffinity systems should be possible.⁶ In addition, the approach may be useful for various intracellular species from single cells. For the special case of DNA detection, one can easily use the same streptavidin-conjugated QD to detect specific sequences in moderate-sized DNA fragments (those that are deformable) by hybridizing them to biotinylated primers with the complementary sequences. Since EOF is typically faster than the electrophoretic velocity (except at extremely low pH or high ionic strength), continuous transport of samples from anode to

cathode can be guaranteed. Since the gradient 2D shear flow is created by the imbalance in EOF and no external pressure is required, implementation is greatly simplified. Adaptation to other areas of microfluidics¹⁷ can be foreseen.

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