A Microfluidic Device for Physical Extraction of Intracellular Proteins from Bacterial Cells

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Abstract

The analysis of intracellular proteins from bacterial cells typically requires a cell population of ~1000 or more due to the small cell size. Effective combination of concentration and lysis steps is desired for such analysis to be carried out on a microfluidic platform. In here, we report a simple microfluidic device that integrates the capture of the bacterial cells using a microscale bead array and the rapid electrical lysis for the release of intracellular proteins. We study the retention of *Escherichia coli* cells with different concentrations in this type of bead array and the optimal electrical parameters for the electroporative release of intracellular proteins. The bead array can be replaced after each run and this allows the device to be used for multiple times. Our design provides a simple solution to the extraction of intracellular proteins from a bacterial cell population based entirely on physical methods without applying chemical or biological reagents. Our device forms a critical basis for bacterial proteomic studies based on microfluidics.

Introduction

There are numerous assays applied in biological research that are essentially based on analysis of intracellular materials from cells. Conventional assays based on nucleic acids or proteins from cells require at least hundreds to thousands of cells as the starting material. Recently new tools such as capillary electrophoresis and microfluidics have allowed researchers to analyze intracellular contents from a low number of cells [1-9]. Due to powerful amplification techniques such as polymerase chain reaction and detection techniques such as laser-induced fluorescence, the analysis of intracellular nucleic acids and proteins at the single cell level has become routine for mammalian cells [9-13]. However, the analysis of intracellular materials from bacterial cells, especially proteins, still has to be conducted based on a fairly large number of cells. Bacteria are important model organisms to study regulatory networks and protein functions because they have relatively small genomes and low number of protein components compared to eukaryotic cells [14]. Furthermore, there have been established methods for genetic manipulation of some bacteria which make the studies of protein functions attainable. Finally, bacterial proteomic studies provide crucial information on the adaptation networks that are important for bacterial survival and such knowledge is critical for the discovery of antibacterial drugs [15, 16]. For example, a single *Escherichia coli* cell has a length of 2 μm and a diameter of 0.8 μm which yield a volume of ~1 fl [17]. This volume is about three orders of magnitude smaller than that of a typical eukaryotic somatic cell. Analysis of intracellular proteins from bacterial cells typically requires

the gathering of a substantial number of cells in a relative small volume to generate the desired concentration needed for detection. Effective concentration strategies can save valuable time on the enrichment (the selective expansion of the bacterial cell population) typically needed for bacteria detection and more importantly eliminate potential artifacts generated by such expansion. The capture or concentration of bacterial cells in the solution is a nontrivial task due to the small dimensions of the cells. Dielectrophoresis and electrokinetic techniques have been applied to concentrate bacterial cells on microfluidic chips [18-20]. Fabricated or membrane structures were also used for capturing bacterial cells by having feature sizes comparable to or smaller than the cell dimensions [21, 22]. On the other hand, there have been a number of lysis methods developed for the disruption of bacterial cell membrane using mechanical [23-25], chemical [9, 26], thermal [27, 28], and electrical [29-31] means. However, there have been very few reports focusing on the integration of capture and lysis of bacterial cells for analysis of intracellular contents [32].

In this work, we demonstrate a simple microfluidic device that can serve as a basic unit operation for the capture and analysis of bacterial cells. Microscale silica beads (~3.0 or 4.8 μm in the diameter) are packed in a microfluidic channel and the array of beads provides microscale matrix that filters *E. coli* cells in the solution. The packing and release of the microscale beads are achieved using a mechanical valve fabricated by multilayer soft lithography [33]. A subsequent electrical pulse rapidly lyses the cells and releases intracellular proteins from the cells. The green fluorescent protein (GFP) released by GFP expressing *E. coli* cells is detected using laser-induced fluorescence in the downstream of the channel. The bead array here is a temporary structure that can be removed by opening the valve. These features make our device a simple and versatile tool that can be easily incorporated in a microfluidic system for applications involving analysis of proteins from bacterial cells.

Experimental Section

Microchip fabrication

The multilayer microfluidic chips based on Polydimethylsiloxane (PDMS) (General Electric Silicones RTV 615, MG Chemicals, Toronto, ON, Canada) with pneumatic valves were fabricated using multilayer soft lithography with substantial modifications [33]. The microfluidic structures for the fluidic and control layers were designed using FreeHand MX (Macromedia, San Francisco, CA) and then printed out separately on transparencies with a resolution of 5080 dpi. The transparencies were used as the photomasks for the fabrication of two masters on silicon wafers. The positive photoresist AZ 9260 (Clariant, Somerville, NJ) was used for fabricating the fluidic channel master and the negative photoresist SU-8 2025 (MicroChem, Newton, MA) was used for fabricating the control channel master. The depth of the fluidic channels (the thickness of the SU-8 2025) was ~12 μm, measured by a profilometer (Sloan Dektak3 ST). The depth of the control channels was ~58 μm. The PDMS prepolymer mixture consisting of A and B with the mass ratio of 10:1 was used for the fabrication of both the fluidic and control layers. The control layer had a thickness of ~5 mm. The fluidic layer was created by spinning PDMS prepolymer mixture on the fluidic master at 1200 rpm for 35 s which yielded a thickness of 93 μm. This determined that the membrane between the control and fluidic channels had a thickness of 81 μm. The two PDMS layers were firstly cured at 80 ºC for 20 min before the channel surface of the control channel and the upper surface of the fluid channel were oxidized using a Tesla coil (Kimble/Kontes, Vineland, NJ) in atmosphere. The oxidized PDMS surfaces were immediately aligned and brought into contact and then heated in the oven at 80 ºC for 2 hr to form a two-layer PDMS chip. The access holes were punched in the PDMS chip. A glass slide was cleaned in a

basic solution (H_2O: NH_4OH (27%): H_2O_2 (30%) = 5:1:1, volumetric ratio) at 85 °C for 1 hr, rinsed with DI water and then blown dry. The PDMS chip and the glass slide were oxidized using the Tesla coil in atmosphere before they were brought into contact immediately and heated at 80 ºC for another 2 hr to form a closed microchip.

Bacterial culture and preparation

GFP-expressing *E. coli* cells (transformed by pQBI T7-GFP plasmid, Qbiogene, Irvine, CA) were used in the experiments. The bacterial cells were cultured in Luria-Bertani (LB) broth (BIO 101 Systems, Irvine, CA) with 50 μg/ml of ampicillin (Amresco Inc., Solon, OH) at 37 ºC for 18 hr. The density of the cells after culture was ~1×10^9/ml, determined using plate count. The bacterial culture with the volume of 1 ml was centrifuged and then the supernatant was removed. The cell pellet was resuspended in 1 ml 20 mM phosphate buffer (Na_2HPO_4+NaH_2PO_4, pH=7.5). The resulting suspension was directly used or diluted by the phosphate buffer to form densities between 10^3 and 10^{11} cells/ml and then flowed into the device driven by an infusion pump (PHD, Harvard Apparatus, Holliston, MA).

Microchip operation

The device was mounted on an inverted fluorescence microscope (IX71, Olympus, Melville, NY). The layout and the setup of the microchip are shown in Figure 1. The control channel was filled with water to prevent the leakage of air into the fluidic channel. The valve was actuated by pressurizing the control channel using a fast-response solenoid valve (ASCO Scientific). A pressure regulator with a pressure meter was used to adjust the pressure in order to retain the beads by partially closing the channel. The solenoid valve was automated by a LabVIEW program. For the accumulation of silica beads with the diameter of 4.8 or 3.0 μm (Bangs lab, Fishers, IN), the pressure of around 16 psi was needed to partially close the valve with the bead suspension having a constant flow rate of 1 μl/min. We accumulated a bead array with a length of ~ 600 μm and then we switched the inlet solution to bacterial suspension with the same flow rate (1 μl/min) while the valve was kept partially closed during all time.

The detection of the released GFP from bacterial cells after electrical lysis was carried out by focusing a laser focal volume in the downstream of the bead array (~300 μm away from bead array) using a 60X dry objective (NA=0.70). A 488 nm air-cool argon ion laser (Spectra-Physics, Mountain View, CA) was used in the experiments. The excitation and emission were filtered by a 505DCLP dichroic beamsplitter (Chroma Technology Corp., Rockingham, VT). The fluorescence from the released GFP was collected by the same objective and filtered by a D535/40 emission filter (Chroma Technology Corp., Rockingham, VT) before the fluorescent signal was collected by a R9220 photomultiplier tube (PMT) (Hamamatsu Inc, Bridgewater, NJ) biased at the voltage of –1100 V. The photocurrent from the PMT was amplified and filtered by a Low Noise Current Preamplifier (SR570, Standard Research System, Sunnyvale, California). The amplified current was converted to voltage and then digitalized by a PCI data acquisition card (PCI-6254, National Instruments, Austin, TX). The electrical pulses (3 s pulses with 10 s intervals between the pulses) for cell lysis was generated by a high voltage power supply (1AA12P30, Ultravolt, Ronkonkoma, NY) controlled by a LabView program. The fluorescent signal from the released GFP was collected while the pulses were applied.

The fluorescent and optical images were taken using ORCA-285 CCD camera (Hamamatsu, Bridgewater, NJ). A 100-W mercury lamp was applied to generate the excitation and the fluorescence was filtered by a filter cube consisting of exciter HQ480/40, emitter

HQ535/50 and beam splitter Q505lp (Chroma Technology, Rockingham, VT).

Results and discussion

In this work, we created an array of microscale beads in a microfluidic channel by pinching the PDMS channel using a mechanical valve made by multilayer soft lithography with modifications [9, 33]. When we made the master for the fluidic layer, we intentionally avoided the reflow of the photoresist under high temperature. Such reflow was critical for producing the round cross-sectional profile of the fluidic channel so that the complete closure of the channel by such a valve is possible [33]. In our application, we had only the center part of the PDMS membrane in contact with the glass bottom when we actuated the valve due to the square cross section of the fluidic channel (as shown in the inset image of Figure 1). The partial closure of the valve allowed the flow of the fluid and the retention of the beads simultaneously. The advantage of creating the array using the mechanical valve is that the bead array can be formed and removed at will by partially closing or completely opening the valve. Such design allows the device to have multiple purposes other than the capture and analysis of bacterial cells. When the valve was pinched under a pressure of 16 psi, the beads with 4.8 μm diameter were retained by the partially closed valve with a flow rate of 1 μl/min in the channel and accumulated to form a column. Due to the depth and the cross section of the channel (~200 μm × 12 μm), 2~3 layers of the beads were packed in the channel. Assuming the beads are closely packed, the gaps between the beads (potentially allowing the passage of spheres with diameters of 0.75 to 1.25 μm, depending on the fashion that the beads are packed) would allow possible passage of *E. coli* cells (averagely 0.8 μm in the diameter of the rod shape) with difficulty created by the trapping in the gaps.

We tested the capture of *E. coli* cells from a solution with a density of ~1×10^3 cells/ml (diluted by 10^6 times from the original culture broth) at a flow rate of 1 μl/min through the channel. We observed that the cells were all captured by the bead array. The cells penetrated deeper into the array as time progressed and the cells were distributed through the array with a fairly uniform density (Figure 2a). With the low concentration of the cells in the bead array, the number of the fluorescent spots roughly corresponded to the number of cells. The plot of the number of the cells over time (Figure 2b) indicates that the capture of the cells was close to 100%.

We further increased the density of *E. coli* cells to 1×10^7 cells/ml. The images in Figure 3a show that the bead array became heavily populated with *E. coli* cells over the time. We were able to estimate the cell concentration in the array based on the calibration of the fluorescence intensity against fluorescent images from bacterial suspension with known concentrations (Supporting Information Figure S1). The plot of the number of cells against the time in Figure 3b shows that the array was able to concentrate the cells by a factor of 10^4 within 40 min. Based on our data, the 4.8 μm bead array with the cross section of 200 μm×12 μm can accommodate up to ~1×10^5 cells/100 μm length.

The retention of the *E. coli* cells is due to the physical trapping in the microscale structures in the bead matrix when the gaps in the 4.8 μm-diameter-bead matrix (>0.75 μm) were slightly larger than the cell size. The dynamic movement of *E. coli* cells in the matrix was observed while the overall occupancy by the cells gradually increased over time (Supporting Information Movie S1). This mechanism is purely physical and very different from commonly used immunoseparation based on antigen-antibody interaction [34, 35]. Since the identity of the

bacterium in proteomic studies is often known, our approach offers a simple alternative for bacteria capturing and also avoids interference with the results due to introducing an antibody.

Since the size of the gaps between the beads affects the retention of the cells, we also applied silica beads with the diameter of 3.0 μm. The gaps between the beads in this case will allow the entry of particles with a cross-sectional diameter from 0.46 to 0.71 μm. As expected, due to the smaller gaps between the beads, we observed that *E. coli* cells were stopped at the edge without penetrating into the bead array (Supporting Information Figure S2). The bacterial cell layer accumulated over time and such cell layer apparently did not block the flow of the solution. The cells in solution were captured with an efficiency of 100%. The result indicates that 3.0 μm bead array may be useful for applications requiring very high efficiency collection of bacterial cells from the solution. On the other hand, the disadvantage of 3.0 μm bead array compared to 4.8 μm bead array is that it requires a higher pressure to drive the flow due to the array's higher volumetric occupancy and this demands higher performance of the multilayer valve.

The microfluidic bead array provides an ideal platform for the incorporation of rapid lysis methods such as electrical lysis after cell capture. Electrical lysis is generally considered to be the most rapid lysis method and does not require chemical or biological reagents which may interfere with subsequent assays. Electrical lysis of bacterial cells has been applied to flowing cells by us and other researchers [30, 31]. However, in these settings it was difficult to generate cell lysate with substantial concentration for proteomic analysis since the flowing cells could not be easily concentrated. In this study, we accumulated GFP-expressing *E. coli* cells with an original concentration of ~10^7 cells/ml concentrated in the 4.8 μm bead array for around 40 min. We focused a laser in the downstream of the bead array and applied periodic electrical pulses (3 s duration for each pulse with 10 s intervals between pulses) in the channel with different field intensities after such cell capture. We were able to detect fluorescence bursts after the pulses when the field intensity was higher than 1000 V/cm (Figure 4). This threshold is similar to what we observed for electrical lysis of flowing cells [31]. The fluorescence burst indicates the release of intracellular materials (GFP and other intracellular materials generating autofluorescence). Such release was mostly finished within the first electrical pulse when the field intensity was at 1250 V/cm and occurred slower (within the first 6-7 pulses) when the field intensity was at 1000 V/cm. These results reveal that when combined with electrical lysis, the device can produce cell lysate from bacterial cells after the capture of cells using the bead array. The release of intracellular GFP was also witnessed by fluorescent images before and after the lysis and the movie taken during the process of the pulse application (Supporting Information Figure S3 and Movie S2).

One important feature of the device is its reusability after cell lysis. The bead array together with lysed cells can be flushed out when the mechanical valve is open after one run. The bead array can be formed again and the device can be reused after that. We observed that too many electrical pulses at high intensity (e.g. >20 pulses with each lasting 3s at 1000 V/cm) could create dents in the PDMS membrane (Supporting Information Figure S4). The damage at 1250 V/cm was visually similar. This effect of the electrical field on PDMS has been reported previously [36]. The dents decreased the mechanical strength of the valve and led to higher pressure required for the actuation of the valve. This problem can be solved by having multiple valves in the channel and using the valves one by one, when more than 20-30 runs are desired on one device.

Conclusions

In this study, we demonstrate using microscale bead arrays to capture *E. coli* cells. The bead array (formed by silica beads of 3.0 or 4.8 μm diameter) is created using a pneumatic valve which also allows the removal of the structure after operation. The bead array provides the matrix needed for capturing *E. coli* cells in the solution. Electrical lysis is also incorporated to produce rapid release of intracellular proteins from captured cells. Both the bacteria capturing and the lysis are carried out based on physical methods. The approach is simple and eliminates the application of chemical and biological reagents which may alter the results in proteomic studies.

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Supporting Information Available

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Figure Captions

Figure 1. A schematic of the microfluidic device for intracellular analysis of bacterial cells. The most important parts of the device included the microscale bead array and a two-layer pneumatic valve that formed or removed the bead array. The width and length of the fluidic channel were 200 μm and 10 mm, respectively. The width of the control channel was 200 μm. The inset image shows the formed bead array by partially closing the valve.

Figure 2. The capture of *E. coli* cells at the concentration of ~1×10^3 cells/ml by the bead array. The bead array was formed by silica beads with the diameter of 4.8 μm. (a) The optical image of the bead array and the fluorescent images of captured cells over time (at 30, 60, and 90 min). (b) The number of cells captured over time.

Figure 3. The capture of *E. coli* cells at the concentration of ~1×10^7 cells/ml by the bead array. The bead array was formed by silica beads with the diameter of 4.8 μm. (a) The optical image of the bead array and the fluorescent images of captured cells over time (at 2, 10 and 60 min). (b) The variation in the cell concentration in the bead array observed over time. The cell

concentration at different times (indicated by the horizontal lines) was estimated by comparing the fluorescence intensity of the images to those of the images with known concentrations (Figure S1 in Supporting Information).

Figure 4. The fluorescence intensity detected in the downstream of the bead array under different lysis field intensities when the series of electrical pulses were applied. Each trace was generated by a fresh bead array (formed by 4.8 μm beads) and captured cells (~1×10^7 cells/ml solution flowing for around 40 min). The pulse sequence consisted of 15 pulses of 3s duration with 10s intervals in between for pulse intensities at 500, 750, and 1000 V/cm. The pulse sequence consisted of 5 pulses of the same pattern at 1250 V/cm.

Figures

Figure 1

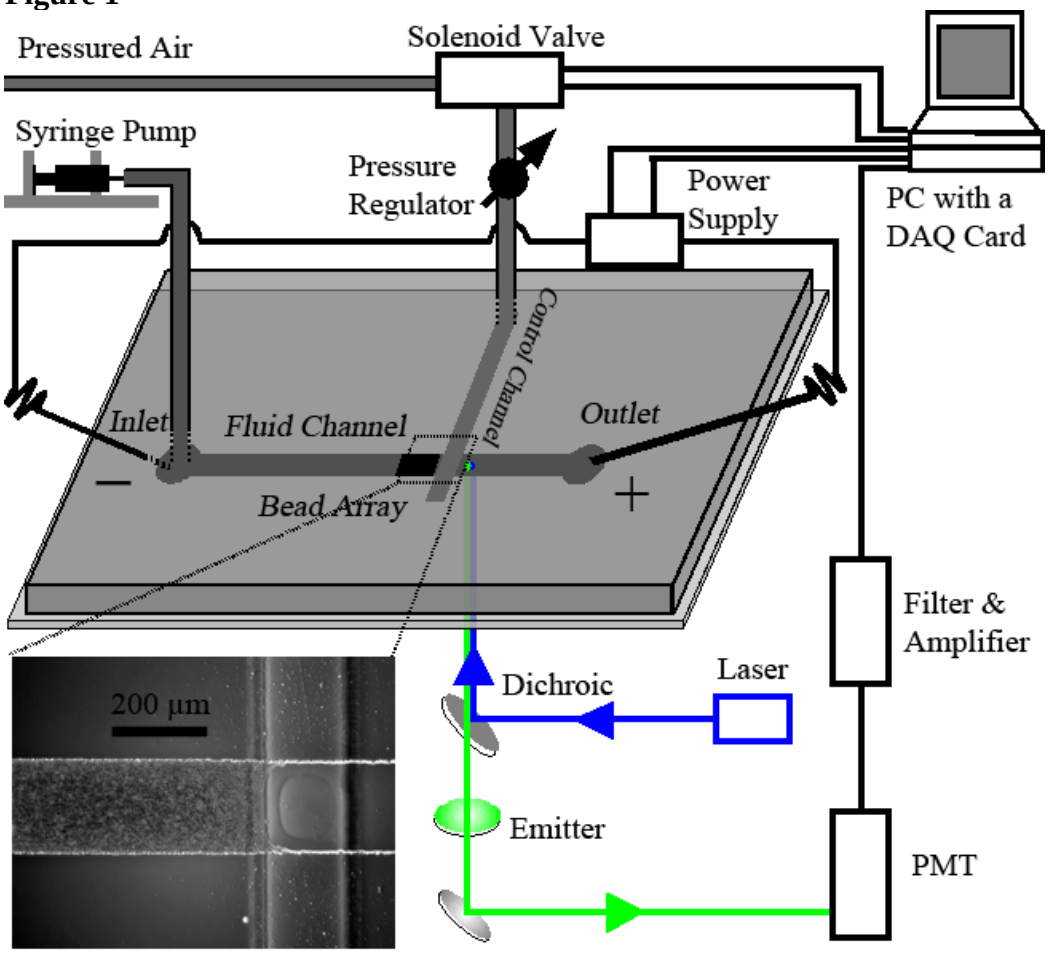
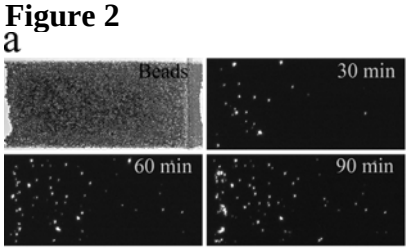


Figure 2



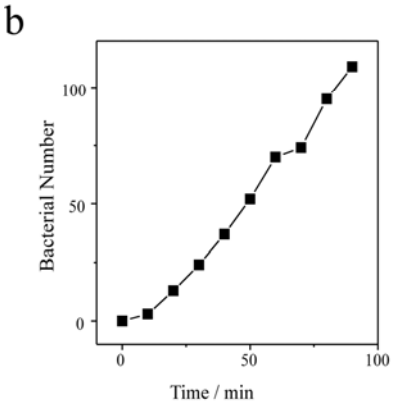


Figure 3

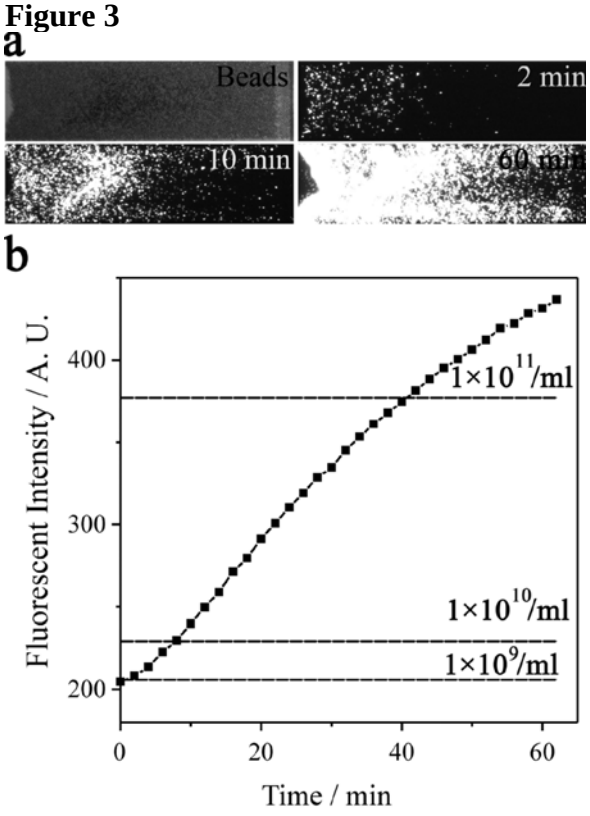


Figure 4