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Quantification of bacterial cells based on autofluorescence on a microfluidic platform

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Abstract

Bacterial counts provide important information during the processes such as pathogen detection and hygiene inspection and these processes are critical for public health and food/pharmaceutical production. In this study, we demonstrate the quantification of the number of bacterial cells based on the autofluorescence from the cell lysate on a microfluidic chip. We tested three model pathogenic bacteria (*Listeria monocytogenes* F4244, *Salmonella* Enteritidis PT1 and *Escherichia coli* O157:H7 EDL 933). In the experiment, a plug of ~150 pL containing lysate from 240 to 4100 cells was injected into a microfluidic channel with downstream laser-induced fluorescence detection under electrophoresis conditions. We found that the autofluorescence intensity increased with the number of cells almost linearly for all three bacteria. The autofluorescence remained a single peak when the cell lysate contained a mixture of different bacterial species. We also demonstrate a simple microfluidic device that integrates entrapment and electrical lysis of bacterial cells with fluorescence detection. Such a device can carry out the quantification of bacterial cells based on lysate autofluorescence without off-chip procedures. This study offers a simple and fast solution to on-chip quantification of bacterial cells without labeling. We believe that the method can be extended to other bacterial species.

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1. Introduction

Rapid methods for determining the number of total bacterial cells are increasingly important in areas such as public health, food technology, water treatment and pharmaceutical industries. There have been a number of classical methods developed over the years for the enumeration of bacterial cells [1]. The most straightforward method is to count the number of bacterial cells under high magnification on special microscope slides known as “counting chambers”. Such a direct count will give a total cell count which includes both live and dead cells. Coulter counters and flow cytometers have been used to detect the number of bacterial cells in suspension, based on the scattering of light and the difference in the conductivity between the bacterium and the

suspending liquid, respectively [2,3]. The most common method for the enumeration of viable bacterial cells is plate count, which obtains the bacterial number by growing the single viable cells separated from one another in space on an agar medium and then counting the macroscopically visible colonies. Plate count is by far the most sensitive method for counting viable bacterial cells. However, the process is time-consuming since the growth can take hours to days.

There has been an increasing trend of applying microfluidic chips as portable tools for bacteria detection due to reduced sample volume (nano- to picoliters), rapid speed, and high level of integration and automation [4–12]. However, only a few reports focused on the quantification of bacterial cells based on microfluidic chips. A membrane filter microchip was demonstrated for capturing bacterial cells followed by visualization and counting [13]. Flow cytometric approach was also applied to the counting of bacterial cells [14–16]. Fluorescent staining was required in all these microfluidics-based bacterial counting.

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Cells of most organisms exhibit a natural fluorescence, commonly called autofluorescence [17]. Autofluorescence can be generated by a range of metabolites and structural components including flavins, NAD(P)H, and lipofuscins. The autofluorescence spectra are often broad and the emission can cover most of the visible wavelength range. In this study, we explore detecting autofluorescence from bacterial cell lysate for quantitative determination of the cell number on a microfluidic chip. Autofluorescence from bacterial cells does not require labeling and it is ubiquitous in virtually all of the bacterial species. When the bacterial cell number is beyond the limit of detection, our method can yield rapid and quantitative results. We demonstrate that laser-induced autofluorescence can be quantitatively detected when a plug containing cell lysate from 240 to 4100 cells is injected into a microfluidic channel. Moreover, we also demonstrate a microfluidic device containing a microbead array for the capture of bacterial cells and subsequent release of cell lysate based on electrical lysis. This simple device offers the capacity of obtaining bacterial count directly from cell suspension.

2. Materials and methods

2.1. Bacteria culture and off-chip lysis

Three pathogenic bacterial species *Listeria monocytogenes* F4244, *Salmonella enterica* serovar Enteritidis PT1 and *Escherichia coli* O157:H7 EDL 933 were used in our experiments. Bacterial strains were grown in Brain Heart Infusion broth (BHI, Difco Laboratories, Spark, MD, USA) at 37 °C in a shaker incubator (New Brunswick, Edison, NJ, USA) at the rate of 150 rpm. After 18-h growth the concentrations of bacterial cells were enumerated using the standard plate count method. The concentrations in the culture broths of *L. monocytogenes* F4244, *Salmonella* Enteritidis PT1 and *E. coli* O157:H7 EDL 933 were 1.8×10^9 , 1.9×10^9 and 1.0×10^9 cells/mL, respectively. Then the bacterial suspensions with the volume of 8 mL were centrifuged (13,000 rpm for 30 s) and the supernatant was discarded. The pellets were resuspended in 0.5 mL 20 mM phosphate buffered saline (PBS, pH 7.0). The suspension containing bacterial cells was sonicated for eight cycles (30 s/cycle with 30 s of stoppage in between) in a Sonifier 150D (Branson, Danbury, CT, USA) and then centrifuged at the rate of 13,000 rpm for 10 min. The supernatant was diluted in electrophoretic running buffer (20 mM sodium phosphate, $\text{Na}_2\text{HPO}_4 + \text{NaH}_2\text{PO}_4$, pH 7.5) by various times for the use in the measurement of autofluorescence.

2.2. Microchip fabrication

Polydimethylsiloxane (PDMS)/glass microfluidic chips were fabricated using the standard soft lithography method [18]. The microscale channels were first designed using the FreeHand MX (Macromedia, San Francisco, CA, USA) software and then printed out on a transparency with a resolution of 5080 dpi. The transparency was then used as the photomask in photolithography for the fabrication of a master on a silicon wafer. For the electrophoresis chip in Fig. 1, we used negative photoresist

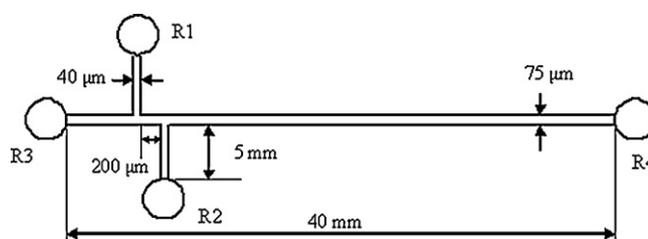


Fig. 1. The schematic of the electrophoresis microchip. R1, sample reservoir; R2, sample waste reservoir; R3, buffer reservoir; R4, waste reservoir. The dimensions of the microchannels are shown in the figure.

SU-8 2005 (MicroChem, Newton, MA, USA). The depth of the microchannels, which was equal to the thickness of the photoresist, was 10 μm measured by a Sloan Dektak3 ST profilometer. PDMS (General Electric Silicones, Toronto, Canada) prepolymer mixture consisting of monomer (RTV 615 A) and curing agent (RTV 615 B) with the mass ratio of 10:1 was poured on the SU-8/silicon wafer master and then cured at the temperature of 80 °C for 1.5 h. The peeled PDMS stamp (~ 5 mm thick) with structures of the microchannels at the surface was then punched to generate four access holes to the channels as reservoirs. A glass slide was pre-cleaned in ($\text{H}_2\text{O} : \text{NH}_4\text{OH}$ (27%): H_2O_2 (30%) = 5:1:1, volumetric ratio) solution with stirring at around 75 °C for an hour and then rinsed with DI water and dried. The PDMS chip and the glass slide were oxidized using a Tesla coil (Kimble/Kontes, Vineland, NJ, USA) in atmosphere and then immediately put into contact to form the PDMS/glass microchip. For the microbead chip in Fig. 5, we applied two different photoresists (SU-8 2002 and AZ 9260, Clariant, Somerville, NJ, USA) to create two depths (3 and 15 μm , respectively) on the master. We applied photolithography twice and aligned the two sets of structures during the process. The difference in the photoresist thickness will translate into the variation in the channel depth, which is critical for retaining the microscale beads in the channel as shown in Fig. 5.

2.3. Microchip operation

The electrophoresis microchip in Fig. 1 consists of one horizontal separation channel (40 mm long and 75 μm wide) and two vertical channels (5 mm long and 40 μm wide) and a double T structure at their intersections. The depth of the channels is 10 μm . We used the double T structure to inject a plug of the cell lysate sample into the separation channel [18,19]. The distance between the two vertical channels was 200 μm which determined a plug size of ~ 150 pL. The microchip was mounted on the stage of an inverted fluorescence microscope (Olympus IX-71, Melville, NY, USA). Four platinum wires were inserted in the four reservoirs as inert electrodes. The microchannels were first conditioned with 50 mM NaOH solution for 15 min and then 20 mM phosphate buffer (pH 7.5) electrophoresis buffer for 20 min. The sample solution (bacterial lysate of different concentrations) was driven from the sample reservoir (R1) to the waste reservoir (R2) by the injection voltage (provided by a high voltage power supply, UltraVolt, Ronkonkoma, NY, USA) to get into double T section. The injection voltage between

R1 and R2 was then switched off and the separation voltage between reservoirs R3 and R4 was switched on to establish a field in the separation channel for electrophoretic separation. The injected plug flowed toward R4 under electrophoretic force and was detected by laser-induced fluorescence at 1 cm or 2.5 cm away from the injection point.

The integrated capture and lysis of bacterial cells was carried out in the microbead chip in Fig. 5. As shown in Fig. 5, to first form the bead array in the microchannel, a suspension of silica beads ($D \sim 4.8 \mu\text{m}$, Bangs lab, Fishers, IN, USA) with a concentration of 1% (volumetric ratio) flowed into the channel driven by a syringe pump (PHD, Harvard Apparatus, Holliston, MA, USA) and got accumulated at the point where the channel depth changed from 15 to 3 μm . After creating a bead array with a length of $\sim 400 \mu\text{m}$, *E. coli* cells with different concentrations flowed into the channel for a defined duration and got retained by the bead array. The number of cells retained in the bead array was estimated based on the cell concentration and the duration assuming a 100% retention rate. We then applied five electrical pulses (with a duration of 2 s each and 18 s intervals in between) of 2000 V/cm generated by a power supply controlled by a LabVIEW program and a PCI-6254 data acquisition card (National Instruments, Austin, TX, USA). Laser-induced fluorescence was detected during the process at ~ 1 mm downstream from the bead array.

2.4. Laser-induced fluorescence detection

Laser-induced fluorescence detection was applied to the detection of autofluorescence from the cells. An air-cool argon ion laser (488 and 514 nm, Spectra-Physics, Mountain View, CA, USA) was used in the experiments. A 10LF10-488 band-pass filter (Newport, Irvine, CA, USA) was used to spectrally filter the laser to yield the 488 nm line. A neutral density filter (Newport) was applied to adjust the power of laser to ~ 3 mW before it entered the microscope. The laser beam was introduced into the microscope and reflected by a 505DCLP dichroic beam-splitter (Chroma Technology, Rockingham, VT, USA) before it was focused by a 60 \times (NA = 0.7) dry objective into the separation channel. The emission fluorescence light was collected by the same objective, and filtered via a D535/40 emission filter (Chroma Technology) before detected by a R9220 photomultiplier (PMT) (Hamamatsu Inc., Bridgewater, NJ, USA) biased at 1100 V. The current coming from PMT was filtered and amplified through a SR570 Low Noise Current Preamplifier (Standard Research System, Sunnyvale, CA, USA) with the parameters set at low noise mode, low-pass filter, 1 Hz cutoff frequency and 100 $\mu\text{A/V}$ sensitivity. The electric current was converted into voltage which was then digitalized by a PCI-6254 data acquisition card (National Instruments, Austin, TX, USA) in a personal computer. During electrophoresis, a LabVIEW program collected the digitalized signal at the frequency of 10 Hz.

3. Results and discussion

We conducted our experiments on three common pathogenic bacterial species: *L. monocytogenes*, *Salmonella* Enteritidis and

E. coli O157:H7. Among the bacterial pathogens, *Salmonella* has been responsible for the largest number of outbreaks and *L. monocytogenes* has been responsible for most of the deaths [20]. *E. coli* O157:H7 is also considered a serious food borne pathogen which has been recently implicated in multistate outbreaks involving fresh produce and green leafy vegetables in 2005. US Department of Agriculture estimated the cost associated with *Salmonella* and *E. coli* O157:H7 related illness to be around 2.4 billion and 444 million dollars in 2006, respectively. Thus, the detection of these pathogens in a rapid and timely fashion in contaminated food is required for the control of the outbreaks. Therefore, we chose to use *Salmonella* Enteritidis, *L. monocytogenes* and *E. coli* O157:H7 in our experiments, due to the serious public health threat and economic burden associated with these pathogens.

We first established that the laser-induced autofluorescence strongly correlated with the cell number. The bacterial cells (a single species or a mixture of three) were lysed by sonication. With the standard plate count method, the concentrations in the culture broths of *L. monocytogenes*, *Salmonella* Enteritidis and *E. coli* O157:H7 were determined to be 1.8×10^9 , 1.9×10^9 and 1.0×10^9 cells/mL, respectively. The autofluorescence from the cell lysate was analyzed using a microfluidic chip with a typical layout for conducting electrophoresis (shown in Fig. 1). The cell lysate was flown into the vertical channel before a plug of the cell lysate (~ 150 pL) was injected into the horizontal channel and detected by laser-induced fluorescence. The numbers of bacterial cells in the plugs were calculated based on the concentrations of the inlet cell lysate solution and the plug size (~ 150 pL). Fig. 2 shows that there is a single peak when the cell lysate is detected at 1 cm from the injection. The peak intensity continued to increase with cell numbers for all three bacterial species. The limit of detection was determined to be as low as 200–400 cells for all three bacteria. As described above, the conventional plate count and the microfluidic flow cytometry approaches offer

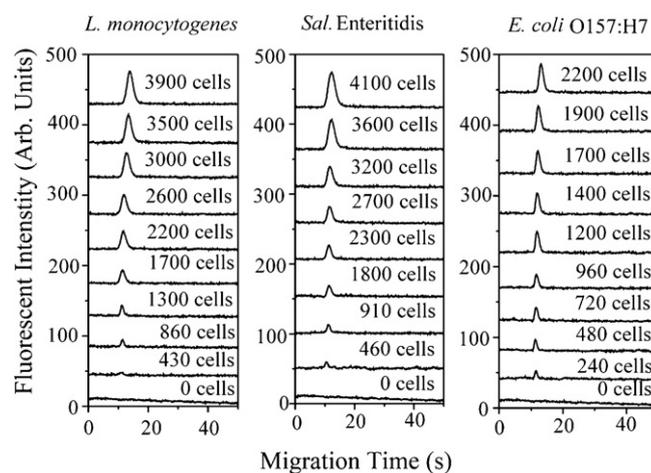


Fig. 2. Electropherograms of lysate of *Listeria monocytogenes* F4244, *Salmonella* Enteritidis PT1 and *Escherichia coli* O157:H7 EDL 933 with different cell numbers (indicated on the curves) obtained using the electrophoresis chip. Experimental parameters: field strength of separation electric field, 300 V/cm; field strength of injection electric field, 300 V/cm; injection time, 60 s; detection point, 10 mm from the injection point.

higher sensitivity than our approach by providing the capability of detecting single cells. However, compared to plate count, our approach provides much more rapid quantification by saving time needed for colony formation. Furthermore, compared to flow cytometric approach, our method eliminates the labeling step and the cell capturing step promises a much less analysis time than that needed for single cell handling.

Based on the excitation (488 nm) and emission (~535 nm) spectra, the autofluorescence we detected was possibly originated from a category of molecular species related to flavins [17]. Flavins are ubiquitous coenzymatic redox carriers in the metabolism of most organisms and excited by 488 nm with the emission around 540–560 nm. The autofluorescence can arise from riboflavin (vitamin B2) and its derivatives, various flavin coenzymes, and flavoproteins [21,22]. However, it is hard to rule out contribution from other autofluorescent species since most of these molecules have broad excitation and emission spectra.

We also tested the cell lysate from a mixture of all three bacterial species (Fig. 3). We again observed only one single peak. By moving the detection point further from the injection (from 1 cm away to 2.5 cm away), the single peak still did not separate into multiple peaks. This suggests that the autofluorescent materials from all three bacterial species are from very similar origins and cannot be easily separated. The peak intensity generated from the mixed cell lysate was approximately equal to the summation of the peaks from all single species. The presence of multiple bacterial species is quite common in real-world sample. The fact that the autofluorescence remains one single peak in the presence of a bacterial mixture makes this approach convenient for quantification of multiple bacterial species when their identities are not known. This allows us to estimate the total bacteria quantity in the samples. Such estimation is often important for rapid hygiene testing. When specificity is desired for the

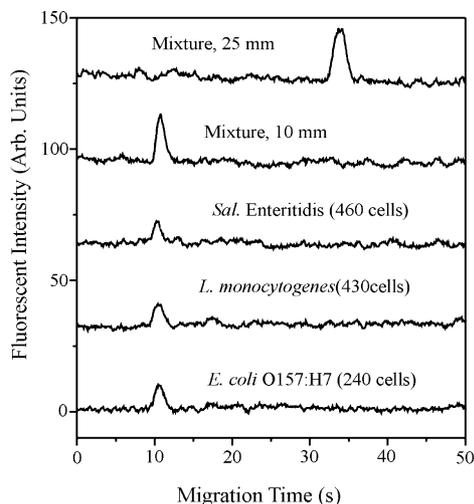


Fig. 3. Electropherograms of lysate of *L. monocytogenes* F4244, *Salmonella* Enteritidis PT1 and *E. coli* O157:H7 EDL933, and their mixture obtained using the electrophoresis chip. The mixture was detected at either 10 or 25 mm downstream from the injection point as indicated in the figure. The lysate of the single species was detected at 10 mm from the injection point. Experimental parameters: field strength of separation electric field, 300 V/cm; field strength of injection electric field, 300 V/cm; injection time, 60 s.

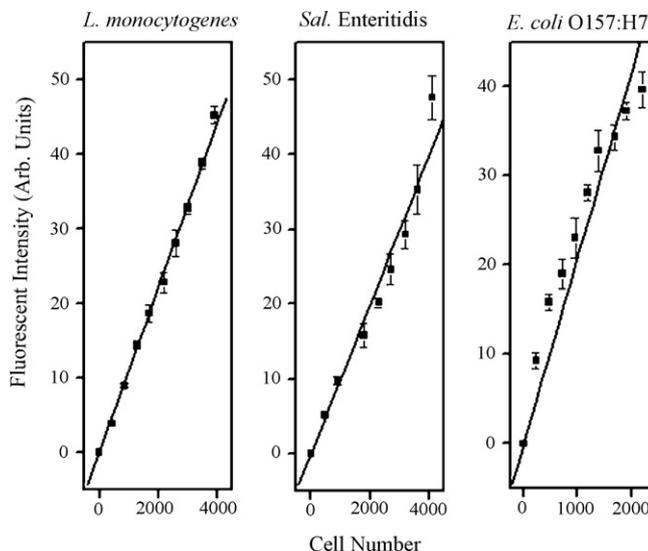


Fig. 4. Relationship between the fluorescent intensity and the number of bacterial cells for all three bacteria. Experimental conditions are the same as in Fig. 2. We add linear trend lines that pass through (0,0) to the data.

detection, antibodies can be immobilized to the surface of the beads for immunoseparation based on antigen–antibody interaction [23,24]. In that case, a packed bed of larger beads than those used in this study should be applied to allow the passage of bacterial cells that are not targeted.

Fig. 4 shows the relationship between the fluorescent signal and the numbers of cells detected for all three bacteria (*L. monocytogenes*, *Salmonella* Enteritidis and *E. coli* O157:H7). The fluorescence intensity increases mostly linearly with the number of cells (in the range of 240–4100) in all three cases. There is some difference in the fluorescence intensity per cell among different species (*L. monocytogenes*: *Sal. Enteritidis*: *E. coli* = 1.1:1:2.1 in the fluorescence intensity per cell based on the slopes of the linear trend lines). This means when the quantification is carried out without knowing the identity (or identities) of the bacterial species, the approach will still give estimation of the order of magnitude of the number of the cells. Such information will allow us to calculate back the number of bacterial cells in the original sample.

It would be ideal to quantify the number of bacterial cells on a microfluidic chip by working with bacterial cell suspension directly. It is not trivial to integrate the cell capture and lysis on chip due to the small size of bacterial cells. In Fig. 5, we show a microfluidic device that contains a microbead array for the capture of bacterial cells. The beads ($D \sim 4.8 \mu\text{m}$) formed a packed bed with gaps inside having dimensions similar to those of bacterial cells (the gaps only allow the passage of spheres with diameters smaller than 0.75–1.25 μm , depending on the fashion that the beads are packed). The packed bed worked as a filter to capture the cells from the solution. The microfluidic channel had two different depths along the length. The microscale silica beads were retained in the channel due to the channel depth variation (Fig. 5B). The bead array captured *E. coli* cells when they flowed through the device. *E. coli* cells were accumulated at the edge of the bead array as shown in the inset image of Fig. 5C.

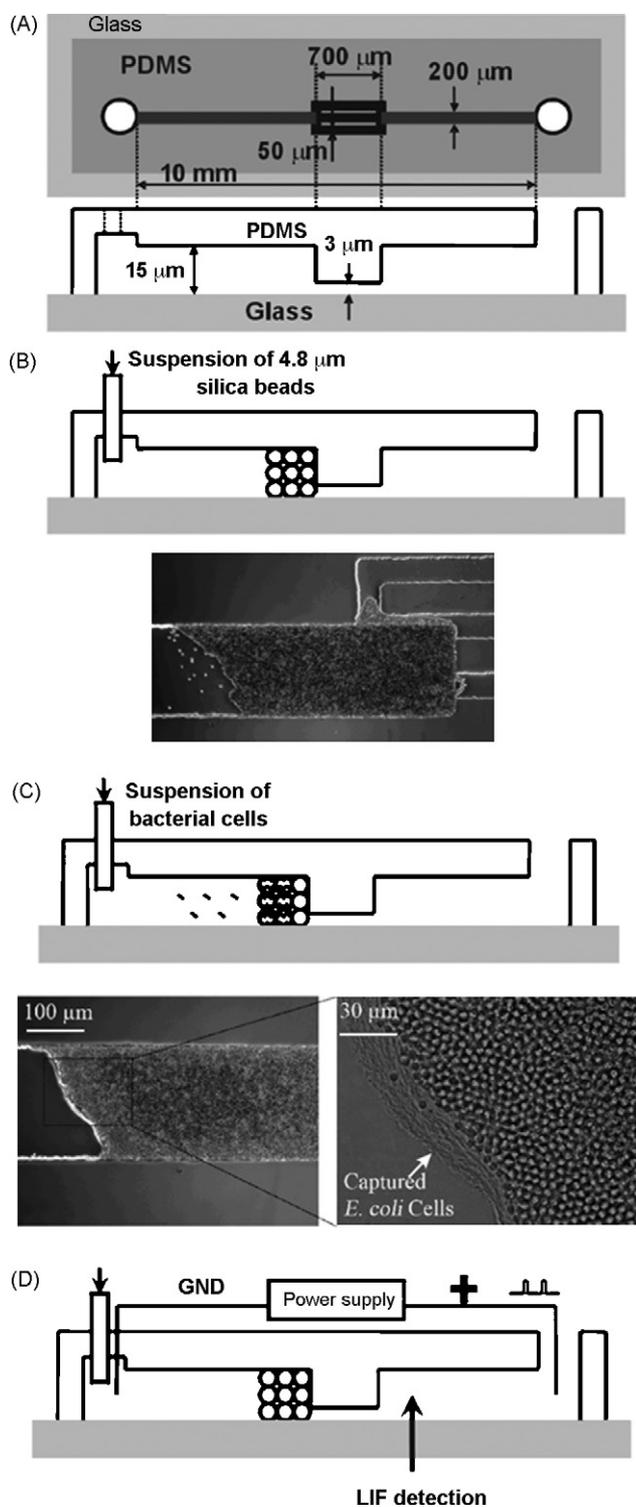


Fig. 5. The microbead chip for the capture and lysis of bacterial cells. (A) The overview and cross-sectional view of the microfluidic chip. (B) The accumulation of microscale silica beads in the device shown both in the schematic and in the image. (C) The capture of *E. coli* cells using the device shown both in the schematic and in the image. (D) The application of electrical lysis and laser-induced fluorescence detection for bacterial count.

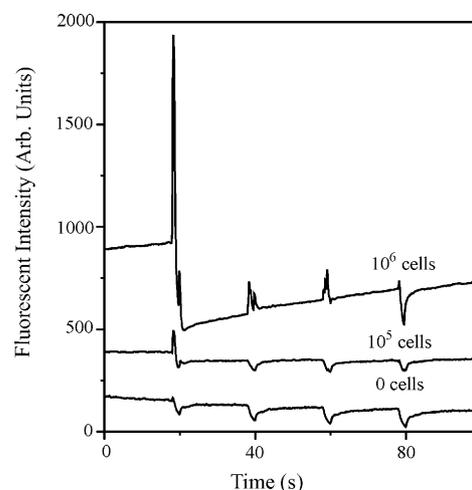


Fig. 6. The fluorescent intensity variation over time measured during the application of electrical pulses. The *E. coli* concentrations in the inlet stream were $1 \times 10^8/\text{mL}$ and $1 \times 10^7/\text{mL}$ to accumulate 1×10^6 and 1×10^5 cells, respectively, by flowing for 10 min at a flow rate of $1 \mu\text{L}/\text{min}$. Five electrical pulses ($\sim 2000 \text{ V}/\text{cm}$) of 2 s each with 18 s intervals in between were applied to lyse the cells.

We observed that after forming the cell layer at the edge within the first 20–30 s, the cells were captured essentially at 100%. We were able to accumulate 1×10^5 and 1×10^6 cells (assuming that the cells were captured 100%). High-intensity electrical pulses ($\sim 2000 \text{ V}/\text{cm}$) were then applied to lyse the cells and release the intracellular contents [10]. In Fig. 6, we were able to detect laser-induced autofluorescence intensity generated by the cell lysate. Most of the lysate was produced during the first pulse. The fluorescent intensity increased with the number of *E. coli* cells. The sensitivity yielded by the integrated device (in Fig. 5) was substantially lower than that of the simpler electrophoresis device (Fig. 1) (by a factor of ~ 20 , based on fluorescence intensity per cell). There were possibly a couple of factors contributing to the lower sensitivity. First, without special treatment of the surface, the silica beads in the case possibly adsorbed significant amount of cell lysate. In principle, the signal will be substantially boosted by coating the silica surface to suppress the adsorption, taking advantage of the rich chemistry associated with silica surface [25–27]. Alternatively, decreasing the number of beads will also increase the sensitivity provided that it does not impair the capturing efficiency. Second, it is possible that the parameters of electroporation may not be optimized for complete release of the intracellular materials. For example, longer pulse duration will likely improve the sensitivity.

The design of the integrated device involves a number of parameters that need to be balanced. A long bead array allows high efficiency for the capturing of bacterial cells. However, a long bead array is also associated with high pressure required for driving the flow which may demand high mechanical strength of the PDMS device and tubing connections. Furthermore, as we indicated above, untreated bead surface in a long bead array may also increase the adsorption of the intracellular materials and decrease the sensitivity. The dimensions of the channel may also affect the sensitivity. In general a channel with a small cross-sectional area tends to make the cells highly concentrated

and yield a high sensitivity. However, a small channel may also increase the pressure needed for driving the flow with the presence of the bead array. Similarly, a high flow rate is desired for fast collection of cells, only when the pressure needed to generate it is not damaging to the integrity of the device.

4. Conclusions

In this study, we demonstrate the rapid quantification of the number of bacterial cells on a microfluidic platform based on the autofluorescence intensity from cell lysate. We demonstrate the method on three model pathogenic bacterial species; *L. monocytogenes*, *Salmonella* Enteritidis and *E. coli* O157:H7. Moreover, we show a simple microfluidic device that incorporates the capture and electrical lysis of bacterial cells using an array of silica beads. Our approach offers a straightforward solution to the quantification of bacterial cells in micro-total analysis systems without labeling. The method and the device may have important applications to pathogen detection, hygiene monitoring of food products, and pharmaceutical production.

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