Flow Cytometry of Escherichia coli on Microfluidic Devices

Maxine A. McClain, Christopher T. Culbertson, Stephen C. Jacobson, and J. Michael Ramsey*
Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-6142

Flow cytometry of the bacterium Escherichia coli was demonstrated on a microfabricated fluidic device (microchip). The channels were coated with poly(dimethylacrylamide) to prevent cell adhesion, and the cells were transported electrophoretically by applying potentials to the fluid reservoirs. The cells were electrophoretically focused at the channel cross and detected by coincident light scattering and fluorescence. The E. coli were labeled with a membrane-permeable nucleic acid stain (Syto15), a membrane-impermeable nucleic acid stain (propidium iodide), or a fluorescein-labeled antibody and counted at rates from 30 to 85 Hz. The observed labeling efficiencies for the dyes and antibody were greater than 94%.

Interest in microfabricated instrumentation for chemical sensing and analysis has grown exponentially over the past decade primarily because these miniature instruments have the potential to provide information rapidly and reliably at low cost. Microfabricated fluidic devices (microchips) fabricated on planar substrates are advantageous for manipulating small sample volumes, rapidly processing materials, and integrating sample pretreatment and separation strategies. The ease with which materials can be manipulated and the ability to fabricate structures with interconnecting channels that have essentially no dead volume contribute to the high performance of these devices. To carry out a complete assay, many different kinds of functional elements can be designed and serially integrated on microchips. These elements include filters, valves, pumps, mixers, reactors, separators, cytometers, and detectors that can be coupled together under computer control to enable the development of a wide range of microchip-based assays. One area of particular interest is the analysis of cells and cell populations, and a rapid screening technique for such assays is flow cytometry.

Only a few examples of cell and particle transport or sorting on microfabricated devices have appeared where hydrodynamic, electroosmotic, and dielectrophoretic forces have been used. We are developing microfabricated fluidic devices for flow cytometry that incorporate electrophoretic (electroosmotic and dielectrophoretic) focusing to spatially confine fluids and particles. Such devices have the potential to significantly reduce the size of flow cytometers and increase the throughput because many devices can be fabricated on a single substrate in parallel. Carryover and contamination can be eliminated on such devices as the microfluidic parts can be fabricated inexpensively so as to be disposable. Finally, rare cell sorting should be easier for the dilution factor in the collection reservoir on a microfabricated flow cytometer can be orders of magnitude smaller than that found for standard commercial flow cytometers.

In this paper, we describe the operation and performance of a microfluidic device for cytometry of Escherichia coli samples labeled with the membrane-permeable stain Syto15, the membrane-impermeable dye propidium iodide (PI), or a fluorescein-labeled polyclonal antibody. The channels of the device have been coated to reduce cell adhesion, and consequently, the focusing was performed electrophoretically without electroosmotic flow. Fluorescent labeling was performed both on- and off-chip, and the labeling efficiencies for all of the fluorescent dyes were determined to be >94%. The cells were continuously transported past the detection window with throughputs of 30–85 Hz.

EXPERIMENTAL SECTION

Microchip Fabrication. The microchips used for the experiments reported below were fabricated using standard photolithographic and wet chemical etching methods. In brief, the channel designs were transferred by UV exposure from a photomask (HTA Photomask; San Jose, CA) to glass substrates coated with...
chromium and positive photoresist (Hoya; Tokyo, Japan). After exposure, the photoresist was developed, the chromium film was etched (CeSO4/HNO3), and the channels were etched into the glass substrate in a dilute circulated HF/NH4F bath. Access holes were ultrasonically drilled (Sonic-Mill; Albuquerque, NM) at the ends of the channels. A glass cover plate (Hoya) was bonded to the etched substrate by hydrolyzing the contact surfaces, bringing them together, and annealing at 500 °C. The reservoirs were pieces of glass tubing (6 mm o.d., 4 mm i.d., 5 mm long) and were epoxied (Epo-tek 353ND; Epoxy Technologies, Inc.; Billerica, MA) to the chip over the drilled channel access holes. A simple cross-shaped channel design was used for all experiments and was similar in design to that reported previously.13 An expanded view of the focusing chamber on this chip is shown in Figure 1. The analysis channel below the focusing chamber (cross intersection) was 50 μm wide at half-depth. The other channels leading to the focusing chamber were 250 μm wide for most of their length but were tapered to only 50 μm at a distance of 500 μm from the chamber. All of the channels were 20 μm deep, and the lengths of the sample, focus 1, focus 2, and waste channels were 7.5, 9, 9, and 20 mm, respectively. The channel widths and depths were measured using a stylus-based surface profiler (P-10, Tencor; Mountain View, CA).

**Chip Coating.** Poly(dimethylacrylamide) (PDMA) was covalently immobilized on the channel walls to minimize cell adsorption.15 The acrylamide coating inhibited electroosmotic flow, but the overall negative charge of the cells allowed electrophoretic transport and manipulation. Before coating, the chip was rinsed sequentially with 1 N sodium hydroxide, water, and methanol. The chip was then rinsed with a 1%(v/v) solution of 3(trimethoxysilyl)propyl methacrylate (Sigma; St. Louis, MO) in methanol for 30 min. The methacrylate solution was flushed from the channels with methanol for 2 h. A 5%(v/v) dimethylacrylamide solution (Aldrich; Milwaukee, WI) in 0.5× TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA pH 8.3, Sigma) was polymerized with 5 μL/mL 10%(w/w) ammonium persulfate (Sigma) and 4 μL/mL of N,N,N,N′,N′-tetramethylmethylenediamine (Sigma). Immediately after mixing, the polymer solution was pulled into the channels, incubated overnight at room temperature, and then rinsed from the chip with 0.5× TBE buffer and water.

**Sample Preparation.** All experiments were performed using the nonpathogenic E. coli strain Y1090 (American Type Culture Collection; Manassas, VA) in log-phase growth. The cells were rod shaped and between 0.7 and 1.5 μm long. The cultures were grown from single colonies in Luria Bertani culture media6 with 50 μg/mL ampicillin (Tissue Culture Grade, Sigma). The cell concentration was determined from the optical density of the culture at 600 nm after it was resuspended in distilled, deionized water (18 M Ω-cm). An aliquot of the suspension was diluted in run buffer and added to the sample reservoir of the chip with a fluorescent dye prior to the experiment. The cells were labeled with three different dyes: a membrane-permeable nucleic acid stain, Syto15 (excitation wavelength, λex = 514 nm; emission wavelength maximum, λem = 546 nm; Molecular Probes; Eugene, OR); a membrane-impermeable nucleic acid stain used to detect nonviable cells, propidium iodide (λex = 514 nm; λem = 617 nm; Molecular Probes); and a fluorescein-labeled polyclonal antibody to E. coli (λex = 488 nm; λem = 520 nm; Fitzgerald Industries International, Inc., Concord, MA).

Syto15 (50 μM) was incubated with the cells in phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM disodium hydrogen phosphate, 1.4 mM monopotassium dihydrogen phosphate, pH 7.4) for 30 min prior to any experiment. The cells were labeled with PI (500 μM) both off- and on-chip. For off-chip labeling, the cells were first subjected to cold shock.17,18 Cold shocking was performed by spinning the cells down, removing the supernatant, and then resuspending the cells in PBS at 0 °C. The suspension was then stored on ice for 1 h. Cell death was verified by visually observing that the cells absorbed the PI prior to placing the cells on the microchip. This method of inducing cell death, although archaic, was used because it minimized the differences in light scattering intensity between live and dead cells. Other methods to kill E. coli, such as fixation in ethanol, acetone, formaldehyde, or gluteraldehyde resulted in weaker scattering and increased cell adhesion to the channel walls. For on-chip labeling, the PI was added to the sample reservoir at the same concentration as was done with the off-chip labeling but without the 30-min incubation. The on-chip labeling relied on the electric field in the chip to compromise the cell membrane, thereby permitting fluorescent labeling.

The fluorescein-labeled antibody to E. coli obtained from Fitzgerald Industries International was optimized for acetone-fixed cells. To fix the E. coli cells, a log-phase culture was spun down at 12 000 units of relative centrifugal force (rcf), resuspended with PBS, spun down, and then resuspended in aceton for 10 min. After fixing, the cells were spun down and resuspended in fresh PBS three times to remove residual acetone from the solution. Nonspecific antibody binding was minimized by blocking the cell sample with a 10%(v/v) solution of normal rabbit serum (Fitzgerald) in PBS for 30 min. The blocked sample was spun down,

![Image](80x563 to 269x752)

**Figure 1.** Image of the focusing chamber of the microchip used for the cytometry experiments. The arrows indicate the direction of cell and anion transport, and the length is proportional to the electric field strength.


RESULTS AND DISCUSSION

As E. coli have a tendency to adhere to native glass surfaces, several different covalent and noncovalent channel wall coatings were tested to eliminate or reduce the cell adhesion, including Sigmacote, Bio-Rad run buffer (148-5032), octadecyltrimethoxysilane (ODTM S), (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-dimethyl-chlorosilane (F13), and PDM A. Of the coatings tested, covalently bound PDM A worked the best. ODTMS and F13 were too hydrophobic for use with aqueous solutions, and Sigmacote and the Bio-Rad run buffer were not effective at preventing cell adsorption. While the PDM A coating did significantly decrease cell adhesion, it did not completely eliminate it; so bovine serum albumin (BSA, 1 mg/mL, Sigma) was added to the buffers. The BSA also reduced cell clumping in solution. In addition to reducing cell adhesion, the PDM A coating minimized electrophoretic flow, requiring the cells to be transported through the channels electrophoretically. The speed at which the cells moved, therefore, was dependent on the magnitude of their charge-to-hydrodynamic drag ratio and the electric field strength in the focusing channel (900–950 V/cm). The charge-to-hydrodynamic drag ratio was affected by both the buffer composition and the physiological state of the cell. The cell velocities for the range of conditions used varied from 2.5 to 4.5 mm/s, as determined by the width at the base of the peaks from the light scattering detector.

Single-point fluorescence (F) and scatter (S)c data were collected from individual Syto15-labeled E. coli cells in log-phase growth as they passed through the observation region (~25 μm downstream of the focusing chamber). Five 60-s runs were made with throughputs ranging from 35 to 64 cells/s. Figure 2A shows an expanded 0.4-s segment from one of these runs. The average baseline width for each detection event was 2.5 ms. Because Syto15 is a membrane-permeable nucleic acid stain, all cells were expected to take up the dye. Coincident scatter and fluorescence detection occurred in ~95% of identified events, while 3.8% of the scatter events were not accompanied by coincident fluorescence events. The scatter events that lacked a coincident fluorescence signal were mostly due (~80%) to unresolved fluorescence peaks (Figure 2B). As seen in Figure 2B, the scatter channel shows two different cells (two separate detection events) while the fluorescence channel indicates one peak with a shoulder (arrow). The counting algorithm did not detect the shoulder as a separate event. Less than 1% of the scatter data was completely unaccompanied by a detectable fluorescence signal. This population was assumed to be noncellular debris. The few fluorescence signals without a corresponding scatter signal (0.75%) may have been due to the presence of a small amount of nucleic acid material in solution which was bound to fluorescent dye but did not generate a corresponding scatter signal.

The intensity distributions for the scatter and fluorescence data are shown in Figure 3. The size of the E. coli cells (0.7–1.5 μm long) places them in the Mie scattering regime8 in which the intensity of the forward scattering signal is proportional to the cross-sectional area of the cell. The variation in scatter signal indicated a variation in cell size, which is expected in log-phase culture. Flow cytometry studies of E. coli in a log-phase growth exhibit similar distributions20 and suggest a large population of recently divided cells of roughly the same size convoluted with an exponentially decaying distribution of larger cells of varied size.

to a 1:25 dilution of antibody stock in PBS. The cells were incubated with the antibody dilution for 2 h, spun down, and resuspended in PBS twice to remove bound antibody and reduce the background fluorescence signal.

Microchip Operation. The cells were focused into a narrow stream as they passed through the cross intersection on the chip in a manner similar to that reported previously.21–13 Potentials were applied at the sample and waste reservoirs using high-voltage power supplies (10A12-P4; Ultravolt; Ronkonkoma, NY) which were independently controlled through a multifunction I/O card (PCI-M1016-XE50, National Instruments; Austin, TX). The focus 1 and 2 reservoirs were grounded. The negatively charged cells were independently controlled through a multifunction I/O card and computer used for voltage control. All data were digitized (2000 Hz sampling frequency) using the same multifunction I/O card and computer used for voltage control. All data were digitized (2000 Hz sampling frequency) using the same multifunction I/O card and computer used for voltage control. All data were digitized (2000 Hz sampling frequency) using the same multifunction I/O card and computer used for voltage control.

Signal Detection. An argon ion laser beam (488 or 514 nm, Omnicrome; Carlsbad, CA) was focused on the channel ~25 μm below the channel cross. The fluorescence emission was collected with a 40× objective (CD-240-M40X; Creative Devices; Neshanic Station, NJ), and focused onto a 350 μm × 400 μm adjustable rectangular spatial filter (74001, Oriel; Stratford, CT).13 The fluorescence and scatter or dual-fluorescence signals were then split using a dichroic mirror. The Syto15 signal was separated from the scatter signal with a 540-nm dichroic mirror (540DRLP-9840; Omega Optical; Brattleboro, VT), passed through a 514-nm notch filter (Kaiser Optical Systems, Ann Arbor, MI) and a 560-nm band-pass filter (560DF40; Omega). The PI signal was separated from the Syto15 signal using a 590-nm dichroic mirror (590DRLP-9926; Omega) and passed through a 635-nm long-pass filter (5130; Oriel). The fluorescein-labeled antibody luminescence was filtered using a 488-nm notch filter (Kaiser) and a 530-nm band-pass filter (530DF20; Omega). An additional 1-mm spatial filter was placed in the path of the scatter signal just prior to the photomultiplier tube (PMT; 77348; Oriel) to reduce spurious noise. Signals were amplified (428-M AN; Keithley, Cleveland, OH) and digitized (2000 Hz sampling frequency) using the same multifunction I/O card and computer used for voltage control. All data analysis was performed using programs written in LabVIEW (National Instruments). Due to the low signal-to-noise ratio of the PI signal in the on-chip labeling experiments, the data were filtered with a first-order Butterworth filter (high-frequency cutoff 350 Hz, low-frequency cutoff 200 Hz). The other experiments were analyzed without any preliminary signal processing.
The fluorescence intensity distribution for the Syto15 was similar to the scatter distribution. The intensity of the fluorescence signal should be proportional to the dye concentration in the cells which, in turn, has been correlated with the amount of nucleic acid in the cell\textsuperscript{20,21}. As such, variation in the amount of DNA and RNA from cell to cell will generate a distribution of fluorescence intensities. The observed variation was, therefore, consistent with that expected for the population of \textit{E. coli} investigated, which contained cells at all stages of cell division.

Knowing that electric fields can affect cell viability\textsuperscript{8,22–24}, a fluorescence-based assay using Syto15 and PI was performed on the chip to ascertain \textit{E. coli} viability after focusing. Syto15 is membrane permeable and labels the DNA and RNA in both live and dead cells. PI is not membrane permeable and only labels dead cells or cells with compromised membranes. For these experiments, the cells were incubated off-chip with Syto15 for 30 min and then added to the sample reservoir on the chip. PI was also added to this reservoir just prior to analysis. Qualitative experiments were performed using an inverted microscope equipped with both video and CCD cameras and showed that most of the cells were viable, i.e., lacked a PI signal, in the wide portions of the sample channel prior to entering the focusing chamber. The cells only began to take up the PI after entering the narrow part of the sample channel 500 \textmu m above the focusing chamber. The cell uptake of PI was caused by the large increase in the electric field from 43 to 183 V/cm which came with the change in resistance per unit length between the wide and narrow sections of the channel.

Cell viability was quantitatively determined by single point detection. Five 60 s runs were made with cell throughputs ranging from 30 to 85 Hz. The PI event count was lower than the Syto15 event count. Only 86\% of the Syto15 events had a corresponding PI event, while 99\% of the PI detection events had a corresponding Syto15 event. These results indicate that >86\% of the \textit{E. coli} cell membranes were electroporated. Successful cell transformation by electroporation generally occurs using high electric fields in short pulses (<10 ms) and usually results in the death of 50–75\% of the cells\textsuperscript{23}. For our experiments, the electric field was continuously applied at levels to induce electroporation. Under such conditions, the cytoplasm will quickly leak out of the cell leading to cell death\textsuperscript{24}. As such, we believe that most of the \textit{E. coli} were killed within a few hundred milliseconds after exposure to the continuous, higher electric field. This is, also, consistent with previous observations reported by Fu et al\textsuperscript{8}.

In these experiments, both PI and Syto were detected simultaneously in the membrane-damaged cells. Although PI has a higher affinity for DNA than does the Syto family of dyes and

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{figure2.png}
\caption{(A) Light scattering (Sc) and fluorescence (F) signals for \textit{E. coli} cells labeled with Syto15 interrogated 25 \textmu m below the focusing chamber. (B) A shorter segment of the same run showing the peaks detected by scattering and fluorescence. The asterisk (*) indicates a peak detected by the peak finding algorithm, and the arrow represents a peak missed by the algorithm. The average cell throughput was 58 Hz.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{figure3.png}
\caption{Histograms of the scatter (Sc) and fluorescence (F) intensities from \textit{E. coli} labeled with Syto15.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{figure4.png}
\caption{Figure 4. (A) Light scattering (Sc) and fluorescence (F) signals for \textit{E. coli} cells labeled with Syto15 interrogated 25 \textmu m below the focusing chamber. (B) A shorter segment of the same run showing the peaks detected by scattering and fluorescence. The asterisk (*) indicates a peak detected by the peak finding algorithm, and the arrow represents a peak missed by the algorithm. The average cell throughput was 58 Hz.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{figure5.png}
\caption{Histograms of the scatter (Sc) and fluorescence (F) intensities from \textit{E. coli} labeled with Syto15.}
\end{figure}

thus tends to displace Syto dyes in the DNA, the extent of the displacement depends on the specific dye used, the relative concentrations of the PI and Syto dyes, and the time between the introduction of the PI and detection. We could not measure the relative concentrations of the dyes in this experiment, but the time between the exposure of the cell to the higher electric field in the narrow part of the channel and observation is only 0.1–0.2 s. This time is not sufficient for the Syto 15 to be completely displaced from the DNA even assuming that the PI concentration relative to the Syto was sufficient to do so. In addition, Syto 15 also labels RNA and thus is not DNA specific as are other dyes such as Syto 9; consequently, the Syto 15 signal from the RNA in the cell should be unaffected by the presence of PI.

Cell viability was also determined for E. coli which had been cold shocked to induce cell death.17,18 The cold-shocked cells were incubated with PI off-chip for 30 min prior to analysis. The cells were examined microscopically and were all seen to exhibit a PI fluorescence signature prior to exposure to any electric field on the chip, indicating that the cold shock was indeed effective in killing the cells. Five 60-s runs were made with cell throughputs ranging from 28 to 36 Hz. The percentage of coincidence detection events between Syto 15 and PI was much higher for the cold-shocked cells than for the cells labeled on-chip. A total of 96% of the Syto 15 detection events had a corresponding PI detection event, while 98% of the PI detection events had a corresponding Syto 15 detection event. The reason for the increase in labeling efficiency was probably the longer incubation time (30 min) for the off-chip-labeled cells after being compromised by cold shock.

The use of a fluorescently labeled antibody specific to E. coli for microchip flow cytometry was also examined. The antibody used was a purified IgG fraction, conjugated to fluorescein by the manufacturer (Fitzgerald Industries International) for fluorescence-based detection. This particular antibody had a higher binding efficiency to cells that had been fixed than with live cells, so the cells were fixed in acetone according to the procedure presented in the Experimental Section. Five 15-s runs were made with cell throughputs ranging from 29 to 43 Hz. Figure 4 shows an expanded 0.4-s segment from one of these runs. The coincidence detection rate between the fluorescence and scatter signals was >95%. Noncoincident scatter and fluorescence signals were very similar at 2.3 and 2.7%, respectively. The strengths of both the scatter and fluorescence signals were slightly lower for this assay than for the other assays reported above because the cell fixation process dehydrated and shriveled the cells causing them to scatter less light than the unfixed cells. In addition, increased adsorption of the fixed cells to the channel walls and antibody dissociation from the cells generated some additional background noise in the scatter and fluorescence, respectively. Even given these considerations, however, excellent signal-to-noise ratios were obtained in both the scatter and fluorescence channels as can be seen in Figure 4. Such immunosassays should also be feasible on live or unfixed cells as demonstrated in the Syto and PI assays reported above if the proper antibody is available. As producing such antibodies to specific strains of bacteria is not difficult, assays on unfixed cells should eliminate any problems associated with using the fixed cells.

In summary, microchip flow cytometry has the potential to be a cost-effective and portable alternative to conventional flow cytometry. The chips used for this paper were robust and, in general, could be run continuously over the course of an experimental session which lasted for a couple of hours. Electrophoretic focusing effectively confined the sample stream for single-cell scatter and fluorescence detection. The system accommodated various cell assays, including viability measurements and immunosassays. While the detection frequencies reported ranged from 30 to 85 Hz, an order of magnitude or two less than is possible with commercial state-of-the-art flow cytometers,5 cell throughputs of over 100 Hz were achieved in some runs. The peak separation at 100 Hz, however, was suboptimal as the cell density was too high. To increase the counting rate at low cell densities, the cell velocity or throughput must be increased. The lack of electroosmotic flow on the chip limits the maximum velocity that we can currently achieve by electrophoretic transport. Pressure-facilitated transport, multiple parallel focusing chambers, or the use of noncoating methods to prevent cell adhesion and allow electroosmotic transport could lead to an increase in cell throughput.

ACKNOWLEDGMENT

Research sponsored by the National Cancer Institute, National Institutes of Health, under subcontract D98CA78858 between the University of California–Irvine and Oak Ridge National Laboratory (ORNL). ORNL is managed and operated by UT-Battelle, LLC under contract DE-AC05-00OR22725 with the U.S. Department of Energy. This research was supported in part by an appointment for M.A.M. to the ORNL Research Associates Program, administered jointly by ORNL and the Oak Ridge Institute for Science and Education. The authors thank Christopher D. Thomas and Shelby M. Orton for fabrication of the microchips, and Linda Foote and Sandra Davern for help with cell culture.

Received for review May 2, 2001. Accepted August 17, 2001.

AC010504V