Elastomeric Microfabricated
Fluorescence-Activated Cell Sorters
(µFACS)

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Introduction

This chapter describes the development of elastomeric microfabricated cell sorters that allow for high sensitivity, no cross-contamination, and lower cost than any conventional FACS. The course of this development depends heavily on two key technologies that have advanced rapidly within the past decade: microfluidics\textsuperscript{32, 19, 38} and soft lithography\textsuperscript{47, 46}. Sorting in the microfabricated cell sorter is accomplished \textit{via} different means of microfluidic control. This confers several advantages over the conventional sorting of aerosol droplets: 1) novel algorithms of sorting and/or cell manipulation can be accomplished, 2) dispensing of reagents and biochemical reactions can occur immediately before or after the sorting event, 3) completely enclosed fluidic devices allow for studies of biohazardous/infectious cells or particles in a safer environment and 4) integration of other technologies can be implemented into the cell sorter. In addition, due to the easy fabrication process and inexpensive materials used in soft lithography, this elastomeric microfabricated cell sorter is affordable to every research laboratory and can be disposable just as a gel in gel-electrophoresis, which eliminates any cross-contamination from previous runs.

Due to the advent of soft lithography, many inexpensive, flexible and microfabricated devices could be designed to replace flow chambers in conventional flow cytometers. Soft lithography is a micromachining technique which uses the process of rapid prototyping and replica molding to fabricate inexpensive elastomeric microfluidic devices with materials such as plastics and polymers\textsuperscript{47}. The elastomeric properties of plastics and polymers allow for an easy fabrication process and cleaning for reuse or disposal. A variety of biological assays can also be carried out due to chemical compatibilities of different plastic materials with different solvents. More accurate sorting of cells can be accomplished since the sorting region is at or immediately after the interrogation point. On-chip chemical processing of cells has been accomplished and can be observed at any spot on the chip before or after sorting. Time-course measurements of a single cell for kinetic studies can be implemented using novel sorting schemes. Furthermore, linear arrays of channels on a single chip, the multiplex system, may be simultaneously detected by an array of photomultiplier tubes (PMT) for multiple analysis of different channels. Multiplexing in these microsystems increases the through-
put rate and allows for synchronous measurements that cannot be done using a conventional FACS (although linear flow velocities of these chips may not attain the high linear flow velocity of a conventional FACS, 10 m/sec). Furthermore, other sophisticated biological assays can also be implemented on-chip due to the simplicity and flexibility of sample handling, mixing, incubation and massive parallelization, such as cell lysis\cite{44}, polymerase chain reaction (PCR)\cite{24}, optical tweezer/cell trapping\cite{1}, and even transformation of cells by electroporation\cite{27} or optoporation\cite{41}. In terms of optical light collection efficiency, these microchips allow for a minimal volume of cell suspension, approximately 100 femtoliters, at the interrogation point. The minimal volume of optical interrogation greatly reduces the amount of background light scatter from the suspending medium and sheath flow, and from the materials of the flow chamber. Due to the planar configuration of these microchannels, higher numerical aperture (N.A.) oil immersion objectives can be used to collect more fluorescence instead of the conventional N.A. = 0.6 dry lens used in both fluidic and aerosol flow chambers. One additional feature for these soft-lithographed microchips is that most of them are disposable due to their inexpensive material. These properties relieved many of the concerns for sterilizing and permanent adsorption of particles onto the flow chambers.

This chapter presents the construction of microfabricated fluorescence-activated cell sorting devices (µFACS) using soft lithography. Sorting of cells were accomplished using electrokinetic flow\cite{16} or with pneumatically actuated microvalves and micropumps\cite{15}. Many future potential applications of µFACS lie in its unique capability of integration into other technologies for measurements of electrical\cite{40}, magnetic\cite{17} and/or other physical cellular properties. Presently, µFACS serves as an inexpensive, robust and powerful tool to perform high throughput screening in various fields, such as directed evolution, digital genetic circuits, microbiology and cell biology of gene expression and regulation. Ultimately, the development of µFACS lays down the foundation of future work in cell sorting and single cell analysis. The vision of a complete integrated lab-on-chip system, where cell sorting is just one of the steps, is now being realized.
An Electrokinetic $\mu$FACS

Within the past decade, high throughput analysis has become an essential part of genetic and biotechnology research. These studies of gene expression and gene evolution often need to analyze up to $10^4$–$10^6$ different species in order to acquire substantial information concerning the genetic and/or evolutionary pathways. Thus new and improved high throughput screening technologies have to be implemented in parallel. Conventional FACS, laser scanning image analysis and silicon microfluidics were all developed to perform high throughput data acquisition in these studies.

The oldest technology, flow cytometry, analyzes and sorts cells in a single profile as they pass through a point of detection in a jet stream. Despite their extensive capabilities of analysis and sorting, these conventional flow cytometers also have major problems of low sensitivity for bacterial cells and DNA, cross-contamination between runs and are mechanically inflexible to work with different cell types and sizes. Hence, to this date, most work in flow cytometry is primarily focused on mammalian cells.

High throughput image analyses of DNA$^{39, 7, 29}$, protein$^{20, 13, 30}$ and cell microarrays$^{35, 48}$ using laser scanning fluorescence microscopy or an ultra-sensitive CCD camera allow up to $10^6$ samples to be detected in a short period of time. Micropipettes or laser tweezers can be used to manipulate a single cell or molecule for further analysis or recovery. Yet, recovery of a population of cells or particles from the arrays is difficult. If further analysis needs to be done, the identities of the individual spots on the arrays must be known a priori.

Many silicon microfluidic devices were also developed to perform high throughput analysis. Electrokinetic forces can be used within microchannels to separate ionic species, such as DNAs and peptides$^{19, 21}$. These DNAs and peptides are stained with fluorescent dyes either before or after separation and are identified as they pass through the detection region. Multiplexing these capillaries allows arrays of samples to be analyzed simultaneously and increases the throughput rate. Large-scale DNA or protein sequencing can be done in this way. This kind of passive separation, however, depends on the inherent differences in the electrophoretic mobility of samples being interrogated. More recently, separation of cells through a microfabricated lattice$^{6}$ or dielectrophoresis$^{1, 36, 34}$ was also done using the differences in their inherent morphology and anatomy. De-
tection of these species occurs only after separation.

Microfabricated silicon devices also created valveless switches as pressure-switching[4] and dielectrophoresis[14] to perform active sorting. Yet the delicate and tedious fabrication process along with the complicated electronic and buffer requirements in dielectrophoresis have inhibited the silicon microfluidic technologies from gaining popularity among biochemists and biologists.

In conjunction with these advancements, an elastomeric microfabricated fluorescence-activated cell sorter (µFACS) was developed to address these needs for high throughput screening tools. Active sorting occurs via electrokinetic flow when the fluorescence of the cell passes a preset threshold. Sorted cells can be recovered at the output wells of the sorter, thus the identities of the cells do not have to be known a priori. Soft lithography offers an easy way to fabricate inexpensive elastomeric microfluidic devices with plastics and polymers instead of silicon. Unlike conventional FACS, this µFACS is easy to operate, highly sensitive for bacteria and DNA, and can be disposable. The flexibility in the design of the flow cell also allows different cell types and sizes to be analyzed.

**Design of an Electrokinetic µFACS**

Adopting from a pioneering work in DNA sizing using an elastomeric microfabricated device[8], a microfluidic flow cell, an optical detection system and electronics were designed and constructed to perform cell sorting. The disposable, soft-lithographed microfabricated flow cell is a silicone elastomer impression of an etched silicon wafer with three channels joined at a T-shaped junction (See Figures 1 and 2).[8, 16]. The dimensions of the channels are 100 µm wide at the wells and narrowing down to about 5–10 µm at the T-junction. Channel depth is 4 µm. The channels are sealed with a glass coverslip. A buffer solution is introduced at the input channel and fills the device by capillary action. The pressure is equalized by adding buffer to the two output ports and then adding a sample containing the cells to the input port. The fluid within the channels is manipulated via electrokinetic flow, which is controlled by three platinum electrodes at the input and output wells. The whole cell sorting device is mounted on an inverted microscope with an oil immersion objective for fluorescence excitation and detection, as shown in Figure 3.
Optical Setup

A schematic of the optical setup for excitation and detection of the microfabricated cell sorter is as follows. The laser beam was collimated to achieve uniform illumination of the samples. A 5 W Argon laser (Coherent Innova 70) was used as an excitation source. For cell sorting, the plasma tube current was set between 10–25 amps with an output power of 100–500 milliwatts at 488 nm. A half waveplate is placed in front of the laser to rotate the polarization of the beam at an angle of 20–60°. This beam is then split by a polarizing cube beam splitter into its $p$-plane and $s$-plane polarized components. By adjusting the angle of the half waveplate, the angle of polarization of the laser beam with respect to the optical axis will differ and thus varying the difference in the intensity of its $p$-plane and $s$-plane components. The $p$-polarized beam, about half the output of the laser, is expanded and collimated by a homemade “telescope”. This beam expander is composed of two lenses in which the first lens has a focal length of 12 mm and the second lens has a focal length of 85 mm. The expanded beam is then directed by a pair of 2-inch steering mirrors into the back of an inverted microscope (Zeiss Axiovert 35). Within the Zeiss microscope, the collimated beam is focused by a pair of lens into the back focal plane of the objective (Olympus Plan Apo 60X, 1.4 NA, oil immersion). The objective again collimates the beam to achieve uniform illumination of the sample.

The fluorescence detection scheme also utilizes the principle of collimating the emitted light. The fluorescence is collected by the objective at infinity focus. The image of the sample is then focused onto an adjustable slit at the sideport of the microscope. This adjustable slit controls the field of detection and the amount of light entering into the PMT. The fluorescence reaches to infinity focus again by a lens with a focal length of 75 mm. A 20/80 beamsplitter images 20% of the collected light into the CCD camera for observation and the rest is directed onto the PMTs. It is straightforward to extend the system to include multiple color fluorescence and light scattering detection, as in conventional FACS machines.

Electronics

A side-on photomultiplier tube (H957-08; Hamamatsu, Bridgewater, NJ) was used as the detector for cell sorting. A transimpedence am-
plifier (OPA128; Burr-Brown, Tucson, AZ) converts the photocurrent to voltage at a gain of $10^7$ V/A. A 15 V DC power supply was built for both the detector and the current-to-voltage preamplifier. The signal was then filtered by an RC low-pass analog filter at 1.6 kHz and then digitized by a NI-Daq card (LAB-PC-1200; National Instruments, Austin, TX) on a personal computer. Running a sorting algorithm in C, the signals were analyzed and appropriate voltages were set on the PC1200 board analog outputs (+5 V to -5 V). These voltages were then amplified by a pair of APEX PA42 (30 V/V) to two platinum electrodes that were inserted one each into the collection and waste wells. The third electrode was ground at the input well. These voltages were set to manipulate the direction of the fluid flow inside the channels.

**Theory of Electrokinetic Flow**

When a potential difference is applied across a microfluidic channel, an electro-osmotic flow is induced. Beads, cells or other particles in the electrolytic solutions are being carried along with electro-osmotic flow. Manipulation of these particles can be accomplished by adjusting the potential difference between two platinum electrodes inserted at the ends of the microchannels. The switching of fluid flow at the T-junction of $\mu$FACS is almost as instantaneous as the switching of the potentials. Electro-osmotic flow is thus a good and easy way to manipulate particles and create valveless switches within any microfluidic device.

According to [3], electrokinetic phenomena arise from forces occurring at mobile electrified interfaces. For example, a potential difference is applied across a glass capillary filled with electrolytic solution. Instead of current flowing through the capillary, the electrolytic solution begins to move within the capillary, resulting in electro-osmotic flow. This simply means that a potential difference has the same effect as a pressure difference within the capillary. Thus the flow velocity, $V$, of electrolytes within a capillary depends on two components, a pressure gradient, $\Delta P$, and an electric field, $X$.

$$V = a_1\Delta P + a_2X$$

So if there is no pressure gradient $\Delta P = 0$, there is still flow, $V = a_2X$, resulting from the electric field, where $a_2$ is the electrophoretic mobility.
Vice versa, one can predict that a current, $i$, can also result from an electric field and a pressure difference within the capillary.

$$i = a_3 \Delta P + a_4 X$$

(2)

So in the absence of an electric field, $X = 0$, a streaming current occurs from the pressure gradient,

$$i = a_3 \Delta P$$

(3)

where $a_3$ = streaming current density. If Equation 3 is divided by the specific conductivity of the electrolytic solution, $\sigma$, then

$$\frac{i}{\sigma} = \frac{a_3 \Delta P}{\sigma} = X_i$$

(4)

where $X_i$ is the electric field of the streaming potential resulting from the application of a pressure difference. Thus, as Equation 5 shows, one can predict that the flow velocity of fluid due to an applied electric field within the capillary is the same as the current resulting from a pressure difference.

$$\left( \frac{i}{\Delta P} \right)_{x=0} = a_3 = a_2 = \left( \frac{V}{X} \right)_{p=0}$$

(5)

From an atomistic view on electrokinetic phenomena, the electrolytic solution consists of many diffuse layers acting as planar electrodes. Each layer is a few angstroms thick and is at a distance, $\chi$, from the wall of the capillary. If a potential difference is applied across the capillary, a layer of charge, $q$, at a distance $\chi$ from the wall of the capillary will experience an electric force $qX$, where $X$ is the electric field. This force will cause the layer to move across the capillary. Yet, this motion will be opposed by the viscous force. Thus when the electrolytic solution reaches a steady gradient, the electric force is exactly equal to the viscous force. The same phenomenon will occur if a pressure gradient occurs within the capillary.

In addition to electrokinetic phenomena, another motion occurs within the electrified capillary, called ion migration. Instead of seeing the electrolytic solution as layers of diffuse charges, individual ions within the solution will move in a specific way in the presence of an electric field. In the absence of any field, ions perform a diffusive, random walk with equal probabilities in all directions. Yet, in the presence of an electric field, these ions will migrate towards
either the positive or negative electrode according to their individual charge and experience collisions with other ions, shielding effects and viscous forces from the medium depending on their distances from the electrodes. Redox reactions inevitably will occur at the interface of electrodes and electrolytic solutions. As a result, ions are being depleted constantly and thus the electro-osmotic mobility of the solution changes over time.\cite{3}

In a more complex system, such as the microfabricated cell sorter, all these electro-kinetic phenomena occur simultaneously and interact with each other. Many experiments were carried out to search for the best conditions for sorting beads and cells. Erratic behaviors and clogging of the beads and cells were often observed due to ion depletion and other unfavorable conditions. Surface treatments of the elastomeric chip and glass coverslips to produce hydrophilicity are described in detail in the next subsection. Different buffer conditions for beads and cells and the fabrication of microelectrodes are also described.

**Surface Chemistry**

In microfabricated chips, where the dimensions of microchannels are comparable to the size of the particles flowing within them, surface chemistry becomes very important. Cells and proteins can nonspecifically adsorb onto any hydrophobic or hydrophilic surface, which may result in clogs or reduce throughput rates. Electro-kinetic phenomena are highly dependent on the ionic strength of the fluid and the surface charge of the capillary. PDMS is inherently hydrophobic, consisting of a Si-O backbone with methyl and vinyl substituents. Its hydrophobicity prevents aqueous solutions, cells and other bioparticles from flowing smoothly inside these microchannels. Clogging of channels due to adsorption of cells was often observed. Thus several methods were developed to render the surfaces of the elastomeric chip and the glass coverslips more hydrophilic.

Three effective methods were developed to make the elastomeric devices more hydrophilic\cite{11}. Coating the surface with polyurethane (Hydrogel RL#153-87, Tyndale; 3% w/v in 95% ethanol and diluted 10X in ethanol) and curing at 90° C for 60 minutes deposits a hydrophilic layer on the surface of the microchannels. Yet, such a coating would seal up channels with depths of 2–3 μm. In addition, the polyurethane coating deteriorates after each use, reducing
the channels’ hydrophilicity and giving inconsistent flow results. A different method exploits the addition of a surfactant, MAKON 6 (Stephan Canada, Longford Mills, Canada; 0.2% v/v) into the mixture of General Electric RTV 615 components followed by curing in an oven as before. MAKON 6 effectively renders the surface of the elastomer hydrophilic, but unexpectedly, it also increases the background fluorescence of the device. Consequently, the third method is to chemically modify the surface of the elastomer by an acid treatment. Immersing the chip in dilute HCl (pH 2.7, 0.0074% in water) for 40 minutes at 60°C will break up the Si-O backbone of the elastomer, modifying the surface to become hydrophilic, consisting of Si-H and Si-OH substituents. This HCl treatment avoids clogging caused by excessive coating, has negligible fluorescence background and does not deteriorate with use.

In order to increase the hydrophilicity of the whole cell sorting device, methods were also sought to lessen the adsorption of beads/cells onto the glass coverslip. Coating the coverslips with various chemicals and treatments had not proven to be successful. Rainex, Bovine Serum Albumin (BSA), and successive multiple ionic polymer layer (SMIL) did not reduce the amount of adsorption of particles onto glass and SMIL even caused cell death. However, cleaning dusts and etching metallic and organic residues off the glass coverslips renders the surface more hydrophilic by exposing its polar silanol surface. Two wash formulas seem to work best: a base wash called RCA and an acid wash for glass surfaces, called Chromerge. RCA treatment is a base wash which consists of 6 parts H₂O, 4 parts NH₄OH, 1 part H₂O₂. Glass coverslips were immersed in a stirred RCA solution at 60°C for 1 hour. Then the coverslips were rinsed and stored in high-purity water for later use. Chromerge (Manostat Corporation, New York, NY) is a chromic acid solution derived from chromium trioxide. Coverslips were immersed in a stirred Chromerge solution for one hour without any heat. Cell adsorption was minimized the most when Chromerge-washed coverslips were used, although hydrophilicity of both RCA-washed and chromerge-washed coverslips were very comparable.

Buffers and Microelectrodes

A good buffer system is critical for the success of electro-osmotic sorting within the chip. There were two major problems encountered in
the search for an ideal buffering system. One was ion depletion and the second was adsorption of cells and beads onto surfaces of glass and PDMS. Ion depletion results from migration of ions to the electrodes, exhausting the amount of ions remaining inside the solution. Erratic movements of beads and cells within the channels were often observed within 30 minutes in the presence of an electric field. Thus, two criteria were used to select the best buffer system: 1) the buffer has to have a run time up to two hours without experiencing heavy ion depletion, and 2) this buffer also minimizes the amount of cell adsorption and maximizes the viability of the cells within an electric field. Various buffers of different ionic strengths such as phosphate buffered saline (PBS), piperazine-N',N'-bis(2-ethane-sulfonic acid) (PIPES), N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) and distilled water were examined. Each buffer was also tested with different salts of various concentrations (CaCl$_2$, MgCl$_2$, NaCl and KCl). In dealing with adsorption, neutral surfactants, such as Triton X-100, Tween 20, MAKON 6, positively charged surfactant, cetyltrimethyl ammonium bromide, and negatively charged surfactants, bovine serum albumin (BSA) and sodium dodecyl sulfate (SDS), were investigated at various concentrations in the buffers to alleviate adsorption. Two buffer systems were discovered which were optimal for these two conditions, one for the carboxylate-modified (CML) beads and the other for HB101 *E. coli* cells. For beads, extra reservoir wells have to be incorporated in addition to the input and the collection wells in order to prevent ion depletion. Although these two buffer systems may not be the absolute best systems for sorting these particles, they are sufficient for carrying out the experiments within a few hours of run time.

Microfabricated electrode pads were sputtered or evaporated onto the surface of glass coverslips in order to prevent electrolysis at the electrode/buffer interface. Metals, such as Au, Ni, Au/Ni, and Pt/Ni, were all evaporated on the surface of the coverslip in a specific pattern using photolithography. Two microelectrodes, placed at either side of the T-junction, were about 200–400 µm apart and about 50–100 nm thick. The cell sorting device was adhered to the surface of the coverslip containing microelectrodes. Yet, at an electric field of 6 V/cm, electrolysis (detected as bubbles from the microelectrodes) and metal plating occurred within the channels. In addition, except for Pt/Ni electrodes, all of the other metals did not adhere well to glass. They can be easily peeled off as the chip detaches from the
glass surface. In the end, platinum wires were used as electrodes instead for the electrokinetic sorting experiment.

**Performance of µFACS**

The performance of any flow cytometer depends on three factors: sensitivity, resolution and measuring rate. Sensitivity, in terms of fluorescence detection, is defined as the minimum number of dye molecules per cell that can be resolved. Resolution is expressed as coefficient of variation for precision in fluorescence detection. The measuring rate for flow cytometers is the number of cells per given time passing through the analysis point without any coincidences. In the case of µFACS, the measuring rate is the fastest time the sorter can respond upon the detection of a desired cell. Thus, three parameters were measured to verify the performance of the microfabricated cell sorter: the coefficient of variance in measurements, the sensitivity of the detection system and the response time in a sorting event.

The coefficient of variance (CV, defined as the standard deviation of the peak divided by the mean for an uniform population of particles) is routinely used as a measure of the system’s precision in fluorescence detection. The CV for this microfabricated cell sorter system ranges from 2% to 5%, measured with LinearFlow Green Flow Cytometry Intensity Calibration Beads (Molecular Probes, Eugene, OR). This discrepancy may depend upon several factors, such as the relative intensity of different calibration beads and the bias settings on the PMT. Specifically, the depth of the channel also affects the CV of the system. A higher CV is obtained when a deeper channel is used due to a greater variation in the position of the beads. Interestingly, different methods of modifying the elastomer to be more hydrophilic also influence the CV. Polyurethane coating of the elastomer yields the best CVs, but the CVs deteriorate with each subsequent use. Although HCl treated channels produce CVs that are 0.5–1% higher than those of the polyurethane coating, their CVs remain consistent with each usage. The MAKON 6 channels give the highest CVs among the three different hydrophilic treatments, about 1–2% higher than the CVs obtained from the HCl treated channels. Regardless, these CVs are already comparable to those obtained from a conventional FACS.

The sensitivity of this system is about 200 dye molecules at 100
events/sec, of YO-YO 1 (quantum yield = 0.52), a DNA intercalating fluorescent dye\textsuperscript{[8]}. This is at least twice as sensitive as the most sensitive flow cytometers commercially available, which can detect about 1000 fluorescein molecules (quantum yield = 0.9) at 1000 events/sec. In addition, an advantage of \( \mu \)FACS is the small detection volume, which in this case is approximately 100 femtoliters, which greatly reduces background fluorescence in the suspension.

The response time of \( \mu \)FACS consists of two parts: 1) time from a signal on the PMT to the actual change in voltage settings of the Pt electrodes and 2) the time from the switching of the voltage settings to actual change of fluid flow at the T-junction. Measurements were taken using wild-type GFP HB101 cells flowing through the detection window to observe the response times of the hardware in \( \mu \)FACS. The time from a signal on the PMT to the actual change in the voltage settings was determined to be between 1.25–1.50 msec, and the actual switching in fluids occurs in less than one millisecond. The whole response time of the device was less than 3 msec for sorting particles electro-osmotically.

The linear flow velocities of GFP expressing \textit{E. coli} cells in \( \mu \)FACS in response to the applied electric field strengths were measured and plotted in Figure 4. A PMT was used to observed the fluorescence from each cell as it passed through the interrogation region. The signal pulses from the cells were recorded by an oscilloscope. The widths of the pulses were calculated as the time the cells took to travel through the interrogation point. The linear flow velocities of the cells were derived and plotted against the applied electric field strengths. As Figure 4 shows, cell velocities escalate with increasing electric field strengths. However, at above a certain electric field strength (greater than the absolute value of -600 V/cm), these cells began to lose viability as indicated by their loss of fluorescence inside the microchannels and at the wells. Thus the fastest velocity that could be attained by \textit{E. coli} cells inside \( \mu \)FACS is \( \sim 3–5 \) mm/sec. Cell viability would be greatly compromised if higher electric field strength is applied.

**Sorting Schemes**

Different algorithms for sorting in the microfluidic device can be implemented by the computer. The standard “forward” sorting algorithm consists of running the cells from the input channel to the
waste channel until a cell’s fluorescence is above a preset threshold, at which point the voltages are temporarily changed to divert the cell to the collection channel. With electrokinetic flow, switching is virtually instantaneous and throughput is limited by the highest voltage that can be applied to the sorter (which also affects the run time through ion depletion effects). In contrast, a pressure-switched scheme does not require high voltages and is more robust for longer runs. However, mechanical compliance in the system is likely to cause the fluid switching speed to become rate-limiting with the “forward” sorting program. Since the fluid is at low Reynolds number and is completely reversible, when trying to separate rare cells one can implement a sorting algorithm that is not limited by the intrinsic switching speed of the device. The cells flow at the highest possible static (non-switching) speed from the input to the waste. When an interesting cell is detected, the flow is stopped. By the time the flow stops, the cell will be past the junction and part way down the waste channel. The system is then run backwards at a slow (switchable) speed from waste to input, and the cell is switched to the collection channel when it passes through the detection region. At that point, the cell is saved and the device can be run at high speed in the forward direction again. This “reverse” sorting method is not possible with standard FACS machines and should be particularly useful for identifying rare cells or making multiple time course measurements of a single cell. Higher throughput rates could be achieved with this algorithm. We first demonstrated reverse sorting with beads using electrokinetic flow and later with cells in the second generation cell sorter which was with integrated valves and pumps. (See Figure 5.)

Decision Making and Sorting Algorithms

Methods of sorting in microfluidic devices are essentially different from the conventional aerosol droplet sorters. In any sorting logic, the detection of each cell first determines whether or not the criteria are met; when these criteria are met, a logic signal is generated to trigger sorting. For conventional aerosol droplet sorters, the detection of the cell usually occurs in a jet stream and the sorting occurs after the stream has been broken up into droplets that contain zero to two cells. Depending on the distance between the interrogation point and the breakoff point, the time lag between these two points may be tens to hundreds of microseconds. For μFACS, detection occurs ~5
μm before the T-junction and sorting is immediately performed in continuous flow. This may allow for more accurate and synchronous sorting (within ten microseconds time frame) which can be critical for time-course measurements or any downstream analysis. In addition, with the reverse sorting algorithm, the detection of the cell actually occurs two to three times more during sorting before the cell is finally shuttled into the collection channel.

For the electrokinetic cell sorter, both forward and reverse sorting algorithms were written in C++. One electrode at the input was at ground and voltage potentials were applied to the other two electrodes at the collection and waste wells. As Figure 6 shows, the arms of the T channel can be considered as three wires with identical resistance (since they have the same dimensions). So the currents from the collection and the waste wells combine at the T-junction and go into the input well.

\[ I_c R = V_c - V_T \; ; \; I_w R = V_w - V_T \; ; \; I_T R = V_T \]  \hspace{1cm} (6)

\[ I_c + I_w = I_T \]  \hspace{1cm} (7)

\[ (V_c - V_T) + (V_w - V_T) = V_T \; since \; I_c R + I_w R = I_T R \]  \hspace{1cm} (8)

\[ V_c + V_w = 3V_T \]  \hspace{1cm} (9)

If the collection channel is set to be “floating”, then \( V_w = V_T \). Ideally, there should not be any current flowing into the “floating” collection channel and hence no electrokinetic flow. This also means that \( V_c = 2V_w \).

**Forward Sorting**

For forward sorting, the voltage potentials are initially set as \( V_c = 2V_w \). So all the electrokinetic flow goes into the waste channel, that is, \( I_c = 0 \). When a cell’s fluorescence reaches above a set threshold voltage, these two voltage potentials switch, that is, \( V_w = 2V_c \). The cell is directed into the collection channel for a certain period of time before the voltage potentials switch back.

**Reverse Sorting**

Similarly, the reverse sorting algorithm is such that the default voltage setting is \( V_c = 2V_w \). When the sorting signal triggers, the cell is slowly reverted back into the detection region again at a tenth
of the original flow rate, that is, $- \frac{1}{10} V_c = - \frac{1}{10} 2V_w$. After the second detection, the cell is slowly directed into the collection channel, $\frac{1}{10} V_w = \frac{1}{10} 2V_c$, before switching back to the default potentials. Unfortunately, due to evaporation and uneven pressure buildup in the two output wells, the voltage potentials have to be readjusted from time to time in order to maintain accurate sorting for both forward and reverse sorting.

**Sorting with Beads and Bacterial Cells**

The use of $\mu$FACS for forward and reverse sorting with electrokinetic flow was demonstrated with fluorescent beads of different emission wavelengths in different ratios and up to 33,000 beads per hour throughput. Extra reservoir wells were incorporated on the outer side of the three wells in order to avoid ion depletion, and platinum electrodes (with the ground electrode in the input well) were inserted into the reservoir wells. The collection wells were filled with buffer and a mixture of red and blue fluorescent beads was injected into the input well in 10–30 $\mu$l aliquots. The optical filter in front of the PMT passed only red fluorescence, allowing selective sorting of red beads. Figure 7 shows a snapshot of a sorting event. Sorting can be performed for as long as three hours with occasional readjustment of the voltage settings. The coefficient of variation in bead intensity was measured to be 1–3% depending on the depth of the channel and the surface treatment of the elastomer.

**Sorting of Beads**

Table 1 shows that a single pass through the $\mu$FACS produced a highly enriched sample of red beads. Whereas the initial concentration of red beads was 7.4%, the collection well held 84% red beads, while the waste well had less than 1%. Similar results were obtained when running in reversible sorting mode when the initial concentration of red beads was lowered to 1% (Table 1). Run times varied from 10 minutes up to 3 hours. With both forward and reverse sorting, enrichments of 80x-97x were obtained in single runs, where the enrichment is defined by the increase in the fractional concentration of red beads.
Sorting of *E. coli* cells

Living *E. coli* cells can also be sorted in µFACS using electrokinetic flow, and that the cells are viable after sorting. Different ratios of wild-type to GFP-expressing *E. coli* cells were introduced into the input well (volume ranges from 10–30 µl of sample); the collection wells were filled with 10–30 µl of buffer with 10^{-5} M sodium dodecyl sulfate (SDS). After inserting the three platinum electrodes into the wells (with the ground electrode in the input well), the voltages were set for forward sorting. After sorting for 2 hours, cells were collected with a pipette and streaked onto LB plates (or other antibiotic-containing plates) and incubated overnight at 37°C for colony counting. We achieved enrichments of 30x with yields of 20%, where the yield is defined by the number of colonies on the plate divided by the number of positive fluorescence events detected in the device. The sorted cells show relatively constant viability in electric fields up to 100 V/cm, corresponding to velocities of 1–3 mm/sec.

The electrokinetic µFACS system offers several advantages over traditional sheath flow methods. Because the channels in the device can be made with micron dimensions, the volume of the interaction region can be precisely controlled, and there is no need for hydrodynamic focusing. As fluid flows continuously through the system, there is no need for droplet formation and a host of challenging technical issues can be sidestepped. Furthermore, no aerosol is formed because the system is entirely self-contained, allowing relatively safe sorting of biohazardous material. The disposability of the sorting devices obviates the need for cleaning and sterilizing the instrument and prevents cross-contamination between samples.

An Integrated µFACS

Cell sorting has become an indispensable part in the studies of cellular metabolism at the single cell level\[^{33, 25}\]. These studies will demand immediate treatment of cells before or after sorting with minimal time variation and sample loss. Thus there is a need for µFACS to integrate more functionalities in order to perform a complete analysis “on-chip”. Electrokinetic flow and/or direct pressure application alone will not be able to meet these demands.

Multilayer soft lithography is a new micromachining technique that exploits the elasticity and the surface chemistry of silicone elast-
omers in order to create monolithic microvalves within microfabricated devices\footnote{43}. Using multilayer soft lithography, \( \mu \)FACS was integrated with microvalves and micropumps \footnote{37}. Initial efforts in sorting cells via electrokinetic flow demonstrated that the sorting and recovery of bacterial cells in microfabricated devices in an automated fashion. However, the electrokinetic sorter suffers from the same drawbacks as all the electrokinetically actuated microfluidic devices such as buffer incompatibilities and frequent change of voltage settings due to ion depletion, pressure imbalance and evaporation. The current cell sorter with microvalves and micropumps is a step closer to the realization of an integrated lab-on-chip. It has incorporated switching valves, dampers and peristaltic pumps for sorting, sample dispensing, flushing, recovery and absorbing of any fluidic perturbation (See Figure 8). Other microfluidic functions can be easily integrated for kinetic studies or treatment before and/or after sorting events. The active areas of these microvalves and micropumps are much smaller than those made by Unger \emph{et al.} \footnote{43} and Chou \emph{et al.} \footnote{9} in order to accommodate the size of a single bacterial cell. The active volume of one valve on this integrated sorter can be as small as one picoliter. The cell sorter also reduces concerns for buffer compatibility, automation and viability of cells. Different algorithms of interrogation within this sorter, such as reversible sorting and cell trapping, were all exploited on these devices. The sorting accuracy and recovery efficiency using this integrated sorter were greatly improved relative to the electrokinetic \( \mu \)FACS. Finally, we have demonstrated the ability of \( \mu \)FACS to integrate various microfluidic functionalities into one chip to perform a complicated task in an automated fashion.

**Design of an Integrated \( \mu \)FACS**

Fabrication and design of the integrated pneumatic-driven cell sorter adopted features from the electrokinetic sorter and novel engineering using multilayer soft lithography. This integrated cell sorter has the shape of a “T” for sorting as in the electrokinetic sorter, but with valves and pumps incorporated. As shown in the schematic layout of Figure 8, the sorter has two layers. The top layer has the control lines for valves and pumps and the bottom layer has the fluidic lines. The fluidic layer has channels of 30 \( \mu \)m in width which narrows down to 20 \( \mu \)m, and eventually tapers down to 6 \( \mu \)m at the T-junction. Supports
are lined up along both sides of the channels for visualization and alignment purposes. Fluidic holes are incorporated at the ends of the “T” for injection of cell samples and buffers. Collection wells of 1 millimeter in diameter at the arms of the “T” are used for recovery. The control layer has distinct functionalities at different regions of the “T” for controlling fluid flow within the fluidic lines. Three valves, acting as a peristaltic pump, have a valve active area that is 80 \( \mu \text{m} \) long and 30 \( \mu \text{m} \) wide. These valves are 100 \( \mu \text{m} \) apart. Three dampers of similar dimensions are placed immediately following the peristaltic pumps to absorb any energy from fluidic perturbations introduced by pumping. Three pairs of switching valves are placed at the arms of the “T”, having the dimensions of 20, 30 and 50 \( \mu \text{m} \) wide. These valves have a valve active area of 20 x 20 \( \mu \text{m} \), 30 x 30 \( \mu \text{m} \) and 50 x 30 \( \mu \text{m} \), respectively. The specific fabrication details of the integrated \( \mu \)FACS are as described\(^{[15]} \).

**Multilayer Soft Lithography**

Multilayer soft lithography is a micromachining technique that is based on rapid prototyping and replica molding methods of soft lithography. A monolithic chip can be made of multiple layers of elastomeric channels, having each layer individually cast from a microfabricated mold. In a typical two-layer system, the bottom layer consists of the fluidic line where the sample will be introduced and interrogated. The top layer has the control line where the valves will be pneumatically actuated. When pressurized air or nitrogen is introduced into the control line, the thin membrane between the two lines is deflected downward and seals off the fluidic line. A valve is created in this way. The simplicity and flexibility in multilayer soft lithography allows for integration of many different operations on the same chip. Unger et al. have fabricated switching valves and peristaltic pumps for sample dispensing and switching\(^{[43]} \), and Chou et al. have made rotary pumps for mixing and incubation\(^{[10, 9]} \).

The process of multilayer soft lithography is divided into two parts: fabrication of silicon molds and fabrication of elastomeric chips. This section discusses the crucial aspects of fabrication in both parts in order to successfully develop multilayer, microfluidic devices that can be pneumatically actuated.

**Silicon Molds**
In multilayer soft lithography, each layer is cast individually from a different microfabricated mold. There are two ways to fabricate molds from silicon wafers for soft lithography. One is to etch into the silicon as mentioned in the previous section, and another is to pattern a thin layer of photoresist onto the silicon wafer. Although an etched mold is permanent and chemically resistant, the rapid prototyping process of patterning photoresist onto silicon wafers allows molds to be made within a few hours. In addition, different photoresists have different light sensitivity, surface chemistry and viscosity. Combinations of these parameters allow the rapid prototyping process to be versatile for making a variety of different dimensions and shapes of the channels. Thus a good choice of photoresist is critical for the success of fabricating a mold of desired dimensions.

The cross-sectional shape of the channels is also a critical factor for the ideal performance of the valves. According to Unger et al., only flow channels with a round cross section are able to close completely as shown in Figure 9\(^{11, 43}\). However, after obtaining the desired thickness from patterning photoresist on the mold, flow channels are usually of a rectangular or a trapezoidal shape due to UV light diffusion and the photolithography process. These flow channels fail to have complete closing of the valves. When under pressure introduced from above, a round-shaped flow channel is able to seal off a section of the channel by flattening completely from the center to the sides of the cross section whereas a trapezoidal-shaped flow channel fails to seal completely from the sides. Thus a further chemical modification of the photoresist after photolithography is needed. If the photoresist (an amorphous polymer) is heated above its glass transition temperature and with sufficient time, it will reflow to the edges and become rounded. This reflow process is used to tailor off any sidewall angles on the photoresist to completely round the flow channels.

The aspect ratio of height over width of the flow channel is also an important issue for the fabrication of valves and pumps. Too high of an aspect ratio will encounter problems in complete closing of valves in terms of geometry and pressure. Usually, the thin valve membrane ruptures first before the valves can be closed completely. Oxygen plasma was used to isotropically etch the photoresist patterned on the silicon wafer. Hence the height of the photoresist can be reduced with dry etch instead of using a higher spin rate. The etching also rounded the flow channel slightly due to the heat that was released.
Further heating at low temperature may be needed to completely round the flow channel. The procedures of fabricating molds for different microfluidic functions, such as valves, peristaltic and rotary pumps, can be found in the following references[43, 15, 9, 28].

Elastomers

Silicone elastomers retains much of the same chemistry as the natural organic polymers. Yet, its unique silicon-oxygen linkage provides much greater stability to high temperature and chemical resistance. The commercial preparation of silicone elastomers is shown in Table 2[26]. First, silica (sand) is reduced to elemental silicon metal. The elemental silicon metal is then ground and reacts with methylchloride in the presence of a copper catalyst at 300° C. The products of this reaction are mono-, di-, and trimethylchlorosilanes (equation 10). These silanes can be purified using fractional distillation. After distillation, the dimethylchlorosilanes\(^1\) are hydrolyzed to form silanols which then condenses to form cyclic silanes and other low molecular weight silanes (equation 11). These low molecular weight silanes are reacted with a base (KOH) to form dimethyl tetramers, a cyclic silane (equation 12). These tetramers are then linearized to become polydimethylsiloxane (PDMS) by an addition of a strong base and a monofunctional silane (equation 13). The monofunctional silane acts as a chain stopper and determines the viscosity of the linearized polymer.

Different organic side chains can be substituted in place of methyl on the silicon-oxygen backbone of PDMS. These substitutions serve to optimize a certain property of the elastomer as required by a specific application. The inclusion of vinyl groups at various concentrations greatly increases the cross-linking efficiency of the polymer and yields elastomers with lower compression set, or more rigidity. The substitution of phenyl groups allows the polymer to be more flexible at low temperatures down to -93° C. The inclusion of trifluoropropyl groups yields elastomers with higher resistance to many harsh chemical environments. An elastomer can be made by mixing different concentrations of these four functional groups, methyl, vinyl, phenyl and trifluoropropyl, in order to meet the demand of

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\(^1\)Dimethylchlorosilanes are used to form linear chains of silicone polymers whereas trimethylchlorosilanes are used to form 3D cross-linking network for rigid, nonelastomeric molding.
different applications.\textsuperscript{[26]}

Curing agents are added for the vulcanization of silicon rubber. Traditionally, curing agents are organic peroxides which can decompose to free radicals and react with the methyl and vinyl groups of silicone polymer. The vinyl groups have a much higher reaction rate and thus have a much higher number of cross-links. The number of cross-links within the polymer determines the final physical profiling of the resulting silicone rubber. An alternative to vulcanization is called an addition cure. An addition cure utilizes a silicone hydride (SiH) as a cross-linking agent to the methylvinyl polymer. In the presence of a platinum catalyst, an addition reaction occurs in which the hydride is cross-linked to the vinyl group of the silicone polymer. This addition reaction occurs without any by-products, such as water, and proceeds quite actively at room temperature (see Figure 10). Thus this type of silicone rubber is known as “room temperature vulcanization” or RTV.

Beginning in the 1990’s, soft lithography has been slowly gaining recognition in fabricating silicone elastomer microfluidic devices for biological and chemical analysis. Many issues concerning the surface chemistry and the elastic properties of the silicon elastomers have been encountered. Especially, in multilayer soft lithography, the composition of PDMS in each layer is different. The fluidic layer has an excess of one component, such as the curing agent, whereas the control layer has an excess of the other component, the silicone polymers. This is to enhance the surface bonding between the two layers to form a monolithic chip (Figure 11).\textsuperscript{[43]} One can design the fluidic layer to be either the top or the bottom layer, depending on the application of interest. The protocols of the elastomeric fabrication for different multilayer devices can be found in the literature\textsuperscript{[43, 15, 9, 28]}. The integrated $\mu$FACS was the first device made using multilayer soft lithography that demonstrated the automation and coordination of different valves and pumps on-chip to perform complex and decision-making functions.

Flow Velocity and Cell Trapping

Several parameters of the integrated sorter were characterized in preparation for sorting. These parameters are the optimum nitrogen pressure applied to the peristaltic pumps, the linear flow velocity of the cell sorter and the mean reverse time. The mean reverse time
is an indirect measurement of the response time of the peristaltic pumps and the actual fluid flow.

The linear flow velocity of fluorescent beads was measured with increasing nitrogen pressure applied to the pneumatic valves. As Figure 12 shows, the flow velocity of the beads at 100 Hz pumping rate increases steadily with increasing nitrogen pressure and then falls drastically to zero at 100 kPa. This is due to the incomplete opening of the valves with too high of an actuation pressure. Thus high actuation pressure may adversely affect the peristaltic pump. Devices from different RTV batches were found to have slightly different optimum pressure. But in most devices, 60 kPa is the optimum pressure for peristaltic pumping. Vacuum pressure is constantly applied at the normally open ports for faster opening of the valves. In addition, the rigid polystyrene beads eventually get stuck on the valve membrane. These beads affect the performance of the peristaltic pumps and cause incomplete closing of the valves. GFP E. coli cells were used instead for the following measurements.

The linear flow velocity of the cell sorter using peristaltic pumps was also measured at various pumping frequencies. Escherichia coli cells expressing enhanced green fluorescent protein (EGFP) were pumped through the sorter. A photomultiplier tube (PMT) was used to observe the fluorescence from each cell as it passed through the detection region. The detection region was near the T-junction at the 6 µm width channel region. The dimensions of the detection region were 32 µm long along the fluidic channel and 20 µm wide. The width of the pulse from each cell detected by the PMT was the time it took for each cell to travel through a distance of 32 µm near the T junction. The widths of these pulses were averaged from about 150 cells. The velocity was calculated from dividing the length of the detection region by the average pulse width.
Cells through the cell sorters attained a maximum linear velocity at a pumping rate of 50 Hz (Figure 13). Above this frequency, incomplete opening and closing of the valves occur with each pumping cycle. In two separate devices, one made of GE RTV 615 and another made of Dow Corning Sylgard 184, the values of maximum flow velocity are different. The RTV cell sorter has a maximum flow velocity of 10 mm/sec while the Sylgard cell sorter has a maximum of 14 mm/sec. From several measurements done on different cell sorters (unpublished data), different maximum flow velocities were observed ranging from 6 mm/sec to 17.5 mm/sec. This suggests that the maximum flow velocities of the cell sorters may be tuned by altering the properties of the elastomer. Adding diluents or mixing different ratios of A and B component of the fluidic layer should allow us to fine-tune the stiffness of the valve membrane, which will affect the minimum closing pressure and the maximum pumping frequency. Changing the dimensions of the fluidic channel will also allow us to tune the flow velocity since different volumes will be moved with each actuation.

A novel method of interrogation was also demonstrated which consists of trapping a single cell within a region of detection. Analogous to reverse sorting for the electrokinetic μFACS we devised an algorithm so that each time the sorter detects a fluorescent cell, it will reverse the direction of peristaltic pumping. Eventually, the cell falls out of the trapping region and flows into the output wells. Figure 14 shows the raw data recorded by the oscilloscope on the pattern of cell trapping. At 10 Hz pumping frequency, a single cell was redirected into the detection region more than 10 times before it fell out of the trap. At a higher pumping frequency of 75 Hz, multiple cell trapping instances were recorded. Measurements of the mean reverse time were taken for each pumping frequency. In Figure 15, the mean reverse time was taken to be the time the cell travelled away from the detection region between the first detection and the second detection when the flow direction is reversed. This was measured as the time between the first and the second pulses read by the PMT. The mean reverse time gradually decreases as the pumping frequency increases. This is consistent with incomplete opening and closing of the valves will occur with increasing pumping frequency.

This novel method of trapping cells and other bioparticles within a given region inside the sorter opens up new avenues to perform enzymatic kinetic studies on cells and beads. Multiple time course
measurements of the same cell can be taken to follow the kinetics of an enzymatic reaction. Sample dispensing can be done before or after the first interrogation and with each successive detection. Cell sorting can still occur after a certain number of detections. Sorted cells can also be redirected back into the sorting region to be sorted again. This enables a new avenues for kinetic studies on a single cell level that cannot be accomplished by commercially available conventional flow cytometers.

**Sorting and Recovery**

Sorting in this integrated cell sorter can be done in a variety of ways. Since each valve can be individually controlled in a coordinated and timely fashion, the pumping rate and the valve switching rate can be changed at any time in the course of a sorting event. However, to overcome the limitation of the switching speed which is delayed by the intrinsic valve response time, a reverse sorting scheme was used to sort cells in this integrated pneumatic-driven sorter.

The reverse sorting algorithm for the valves and pumps is as follows (See Figure 16). Three patterns are generated for a sorting event: default pattern, reverse pattern and recovery pattern. The default pattern pumps the cells into the waste channel at 100 Hz with 60 kPa valve pressure. Once a desired cell is detected, the reverse pumping pattern is generated at 10 Hz pumping frequency to bring the cells slowly back into the detection region to be detected once more. If there is no detection of any desired cell, the flow will reverse until the end of the reverse pattern and then generate the default pattern again. However, if the desired cell is detected again, the recovery pattern will be generated. This pattern will first close all the valves to stop any fluid flow. Then with the waste valve remaining closed, the collection valve is opened and the pump sends the detected cell slowly into the opened collection channel, pumping at 10 Hz for a selected number of cycles. Following this pattern, the default pattern starts again until the next sorting event.

In a typical run, two populations of *E. coli* cells were separated, one expressing EGFP and the other expressing para-nitrobenzyl (pNB) esterase. The EGFP cells generate a fluorescence signal on the PMT, which triggers collection. The populations were mixed in a ratio of 1:2000 and introduced into the sorter first by nitrogen back pressure and then by peristaltic pumping when sorting began. After
3 hours, the cells at the collection and waste wells were retrieved using a pipetman into two microcentrifuge tubes. The contents of each microcentrifuge tube were then divided and spread out on two different antibiotic plates, ampicillin (amp) and tetracycline (tet). Since EGFP expressing cells grow only on amp plates and the pNB esterase expressing cells grow only on tet plates, the fraction of these two cells in each well can be easily counted on the different antibiotic plates. The plates were placed in a 37°C incubator overnight and the colonies were counted. In the longest run, about 480,000 cells were sorted in 3 hours at a rate of 44 cells/sec. The recovery yield is 40% in this run and the enrichment ratio is about 83 fold. The enrichment ratio is calculated as the ratio of the fraction of EGFP in the collection well to the fraction of EGFP in the original mixture at the input well. Overall up to 50% recovery has been obtained, with as high as 90-fold enrichment. A throughput rate of 100,000 cells per hour has also been achieved.

The integrated sorter can run without interruption for 5 hours or more, and can be used numerous times after proper cleaning. One device was used for 6 months continuously with tens of millions of actuations on each valve and pump. Compared with our previous electrokinetic sorter\cite{16}, this integrated sorter alleviates many issues regarding buffer compatibility, surface chemistry and cell viability. Different strains of E. coli cells and different types of bacteria, including magnetotactic bacteria, could be pumped in their own suspending media through the integrated cell sorter. By incorporating valves and pumps to control sorting by pneumatic actuation, the integrated sorter has a better capability of fine-tuning the flow and is less harmful to the cells than electrokinetic flow. Thus, although the throughput rate was increased only twofold, the recovery yield and the accuracy of sorting improved tremendously. Under the observation of the microscope, \( \mu \text{FACS} \) was able to capture most or all of the desired cells into the collection channel even when they occurred in small numbers. Since we are only recovering most of the cells at the wells, we believe that we will be able to reach close to 100% recovery in future devices by incorporating a flushing mechanism to retrieve the remaining cells that are inside the collection channel.

The variability in the results of different runs can be attributed to factors such as initial cell concentration, fraction of EGFP-expressing bacteria at the input, time duration of each run, retrieval using pipetmen and device fabrication. Although conventional FACS machines
still achieve higher throughput, recovery and accuracy of sorting, the integrated µFACS serves as an alternative, inexpensive, robust and easy way for sorting or manipulating single cells. Multiplexing the cell sorting channels can increase the overall throughput and allow for simultaneous measurements of cells in different compartments. Innovative sorting schemes can be implemented on the device to perform time-course measurements on a single cell for kinetic studies, which cannot be done by any conventional FACS. Sample dispensing and other chemical or enzymatic reactions, such as cell lysis and/or polymerase chain reaction, can also be carried out downstream immediately after the cell has been sorted\cite{44,24}. Moreover, a device of similar design has already been fabricated for sorting mammalian cells and other cell types by Fluidigm, Inc., South San Francisco, CA.

**Applications of µFACS to Digital Genetic Circuits**

Genetic networks contain thousands of molecules interacting in various metabolic pathways in order for a cell to maintain proper metabolism. These networks consist of highly branched and interwoven “genetic circuits” that are analogous to complex, interconnected electrical circuits\cite{31}. Recent advances have led to the design of de novo genetic circuits inside *E. coli* cells to function as an oscillator\cite{12} and a toggle switch\cite{18}. Moreover, when an autoregulatory, negative feedback loop was added to a de novo genetic circuit, the noise or the variability in gene expression was dramatically reduced\cite{2}. These findings grant us not only a deeper insight into the regulatory mechanism of natural genetic networks, but a glimpse of how we can design de novo genetic circuits to program a cell or a group of cells to perform computational functions. Weiss *et al*. are laying down a general principle to designing genetic circuits that can implement the digital logic abstraction and thus are capable to program cell behaviors that are complex, predictable and reliable\cite{45}.

Digital logic, that is, physical chemical signals being translated to logical true (HIGH) or false (LOW) signals, can be engineered inside living cells using simple genetic elements, such as promoters, repressors, operator regions and other DNA-binding proteins. Digital genetic circuits would allow us to reduce the inherent stochastic noise with high predictability and reliability. This also requires the presence of adequate noise margins, that is, the ability to produce a
valid logical output signal from a physical input representation that
is marginally valid or imperfect\(^{23}\) — a signal restoration. These
noise margins are critical for tolerating any noise or loss of signals
within the circuit. By measuring the relation between the input and
output signals in a steady state, that is, a transfer function, the gain
and noise margins of a logic gate or a circuit can be calculated. The
“forbidden zone”, which corresponds to valid inputs but invalid out-
puts, can also be mapped. In any circuit design, the gain must be
greater than one and be highly nonlinear.

Moreover, actual biological behaviors of genetic circuits display
variations due to stochastic effects and other systematic fluctuations\(^{2, 12, 45}\).
These circuits are best characterized using flow cytometry to cap-
ture such inherent noise on the single cell level. The rest of this
chapter presents the characterization of a simple genetic inverter,
the \(cI/\lambda P(R-O12)\) genetic inverter\(^{45}\). Using \(\mu\)FACS, we were able to
measure its transfer function and fluctuations. A transfer band of
the inverter, which encompasses actual biological fluctuations, was
also plotted.

The \(cI/\lambda P(R-O12)\) Genetic Inverter

A simple inverter gives LOW output for HIGH input, and HIGH
output for LOW input. The design of the \(cI/\lambda P(R-O12)\) inverter
is described in the work of Dr. Ron Weiss\(^{45}\). The \(\lambda P(R-O12)\) is a
synthetic promoter that lacks the \(O_{R3}\) operator of the wild-type \(\lambda\)
promoter. Repressor dimers of \(cI\), the \(\lambda\) repressor, bind to \(O_{R1}\) and
\(O_{R2}\) almost simultaneously; this cooperative binding could lead to
much higher gains from the drastic change of repression activity over
a small range of repressor concentrations. This also would achieve
a highly nonlinear behavior as required in the circuit for a good
digital performance. Two plasmids were eventually designed and
modified to function as a genetic inverter in \(E. coli\) cells, pINV-112-
R3 and pINV-107mut4. In this circuit, the repressor \(cI\) controls the
output signal, the enhanced yellow fluorescent protein (EYFP), while
it itself is controlled by another repressor, \(lacI\). Under a constitutive
promotor, \(lacI\) is always expressed, repressing the expression of \(cI\).
However, in the presence of an inducer, isopropyl-\(\beta\)-D-thiogalactoside
(IPTG, which inhibits the binding of \(lacI\) to its operator region), \(cI\)
is expressed with enhanced cyan fluorescent protein (ECFP) under the
same promoter. This genetic inverter switches from “HIGH” (high
EYFP fluorescence) to “LOW” (low EYFP fluorescence) output ranges depending on the concentration of the inducer.

The transfer function of this logic gate, that is, the relation between the input and output signals, was then estimated by measuring several points on the curve. The mRNA level of the input protein, cI, represents a input signal in this circuit. By measuring the fluorescence intensities of ECFP in E. coli cells, the actual mRNA signal can be approximated. Similarly, the mRNA level of the output protein, EYFP, represents the output signal. This output signal level can also be approximated by measuring the fluorescence intensities of EYFP in cells. Since EYFP and ECFP are nearly identical in their decay rates and can have equivalent translation rates, the relative levels of these proteins can be normalized in terms of their fluorescence and protein numbers. The relationship between the ECFP and EYFP fluorescence intensities was approximated by comparing the fluorescence values of pINV-102 and pINV-112-R3 cell populations induced at 1000 µM IPTG as they flowed through μFACS. The “absolute” values of their fluorescence intensities were obtained from their mean fluorescence values subtracted by the background noise and then divided by the numbers of cells scanned. The ratio of the fluorescence intensities of ECFP and EYFP can then be estimated from these two “absolute” values. All the figures shown in this section were normalized for the ECFP input and EYFP output signal levels.

Figure 17 shows the μFACS data in the form of histograms of output signals of the inverter at various concentrations of IPTG. At low concentration of 0.1 µM IPTG (LOW input signal), the output EYFP signal levels were HIGH. At high concentration of 1000 µM IPTG (HIGH input signal), the output EYFP signals were LOW. At the forbidden zone of intermediate input signals, 30 µM IPTG, the EYFP output signals varied widely among these cells and thus were invalid. As the graph of the ECFP input and EYFP output signals at various concentrations of IPTG in Figure 18 shows, the transition (or the forbidden zone) between HIGH and LOW states occurred in the range of 10–60 µM IPTG region. This sharp transition clearly displays the highly nonlinear behavior of the circuit due to the mutual amplification of ECFP. The pINV-102 plasmid was constructed by inserting a p(lacIq):lacI region and EYFP into pPROTet.E132 backbone (Clonetech). The pINV-107 plasmid was derived by inserting the p(lac):EYFP region from pINV-102 into the pBR322 backbone (Clonetech) and replacing p(lac) with λP(R-O12).
to the cooperative binding of the repressors.

There were also good noise margins between the HIGH and LOW output signals. Immediately before and after the transition, that is, at 3 and 100 µM IPTG, respectively, the output EYFP fluorescence levels did not overlap, as shown in Figure 19. Thus this inverter was able to restore marginally valid input signals to valid physical logic representations of HIGH or LOW output signals. This is critical for tolerating noise or loss of signals within the circuit. If some of the input signals are lost due to diffusion or interference with the host mechanism, these reduced input signals can still be valid and be restored through digital abstraction. By normalizing their fluorescence intensities, a transfer curve of the ECFP input signals and EYFP output signals is plotted in Figure 20. These results demonstrate that this genetic inverter does exhibit fairly high gain and good noise margins for digital logic computation.

A Transfer Band

The transfer curve in Figure 20 is plotted using the means of the fluorescence intensities of the cell populations induced under different IPTG concentrations. This curve does not, however, describe the fluctuations that occur within biological systems. These fluctuations can be inherent due to stochastic effects, dead or damaged cells, size distribution in addition to the systematic variations from µFACS. Scatter plots of cell populations at different IPTG concentrations of the $cI/\lambda P_{(R-O12)}$ inverter are shown in Figure 21. These plots reflect the distributions of the cells at different input levels. An alternative way introduced by Weiss$^{45}$ to describe the transfer function of a genetic logic gate, the transfer band, is intended to capture such noise in signal levels within biological systems. This band maps out a region, which includes the maximum and minimum values of input and output signal levels, that encompasses these fluctuations. The transfer band of the $cI/\lambda P_{(R-O12)}$ inverter, shown in Figure 22, is enclosed by a pair of transfer functions: one maps to the values of the mean plus one standard deviation of the input and output signals at each IPTG concentration, and the other maps to the values of the mean minus one standard deviation of the input and output signals. This band thus represents $\sim70\%$ of all the cell populations, since it includes all the cells that are within one standard deviation away from the mean value.
Artificial genetic networks will one day enable us to program living cells to perform computational functions and thus behave predictably and reliably. An important aspect of designing complex genetic circuits is having a resource of gates with different DNA-binding proteins and their respective binding regions. Although a number of natural DNA-binding proteins and their reaction kinetics are known, modifications of these proteins and their binding regions are pivotal to correctly program cell behaviors\textsuperscript{12, 45}. Novel genetic and/or protein engineering techniques could be used to fine-tune the existing genetic logic gates and to create novel genetic regulatory elements in order to construct faster and more complex circuits\textsuperscript{23}. Moreover, the transfer curve only describes the static behaviors of the circuit, but not the dynamic behaviors. Hence, in the future, \(\mu\)FACS could be designed to take time-course measurements such that the dynamic behavior of the circuit can also be determined.

**Future Prospects and Conclusion**

Unlike the conventional aerosol flow cytometers, \(\mu\)FACS allows for flexibility in designing different ways of sample dispensing and methods of interrogation. This will enable single cell studies to be achieved in a more precise and automated fashion. The sorter is presently being used as a stand-alone device for molecular evolution and other biological applications. More fluidic functionalities, such as rotary pumps and incubation, can be integrated in the future. The electrokinetic \(\mu\)FACS allows for an easy fabrication process and disposal. The control of electrokinetic flow within these chips can be used for sensitive DNA and peptide studies in the fields of genomics and proteomics\textsuperscript{8}. On the other hand, the integrated \(\mu\)FACS which has a more complicated fabrication process, offers many versatile ways for sample dispensing, cell manipulation and accommodation for different cell types.

A working \(\mu\)FACS system can be assembled for approximately $15,000. Most of this amount represents the cost of the external optics and detectors used to read out the chip, since the cost of the chip itself is negligible. Considerable cost savings can potentially be realized by fabricating the detectors and optical filters directly on the chip. We believe that this will be an important component of future integrated biomedical chip-based systems.
Future developments of the sorter may evolve in various directions. In terms of optics, the sorter can include other optical signals such as light scatter and absorbance measurements. There are also ways for the sorter to measure magnetic and electrical signals. We have demonstrated the feasibility of the sorter to detect and sort magnetotactic bacterial cells\cite{17}. Others also have demonstrated cytometric capacitance measurements on cells in microfluidic devices\cite{40}. In an exploratory experiment, reverse micelles containing \textit{E. coli} cells were sorted on a similar \mu FACS setup\cite{42}. These technologies can all be incorporated into the sorter to make simultaneous measurements of multiple parameters of single cells. In the near future, with the advances of electronics and optics, these sorters could be made into hand-held machines to be readily used in clinics, hospitals, environmental field testing and biological weapon detection.

References


Figure 1: Optical micrograph of the $\mu$FACS device made from GE RTV 615.
Figure 2: SEM image of an etched silicon mold. The etched silicon wafer is fabricated as follows:\textsuperscript{[11]} A \textless 100 \textgreater \ lightly doped silicon wafer with an oxide layer of 200–300 nm thickness was used. After using standard contact photolithography techniques to pattern the photoresist (Shipley SJR 1813) onto the silicon wafer, a mixture of C$_2$F$_6$ and CHF$_3$ gases is used to etch the wafer by reactive ion etch (RIE). RIE etches away the photoresist and the oxide layer, exposing the silicon layer underneath. A wet etch using KOH anisotropically etches deeper into the silicon layer. The final product is an etched silicon wafer that becomes a mold for the silicone microfluidic chip.
Figure 3: A schematic of the setup for µFACS. The cell sorting device is mounted on an inverted microscope (Zeiss Axiovert 35) with an oil immersion objective (Olympus Plan Apo 60X, 1.4 NA). Epi-fluorescent excitation was provided by an Argon ion laser (Coherent Innova 70) for cells and a 100 W mercury lamp for beads. Fluorescence was collected with the same objective and projected onto the cathode of a Hamamatsu H957-08 photomultiplier tube with custom current-to-voltage amplifier. Part of the light can be directed onto a CCD camera for imaging. The detection region is 5–10 µm below the T-junction and has a window of 15 x 5 µm dimension. The window is implemented with a Zeiss adjustable slit. Cells or particles can be directed to either side of the “T” channels depending upon the voltage potential settings. The voltages on the electrodes are provided by a pair of Apex PA42 HV op amps powered by Acopian power supplies. The third electrode is ground. The PMT signal is digitized by the PC, which also controls the high voltage settings via a National Instruments Lab PC1200 card.
Figure 4: A graph of the linear flow velocities of cells in response to the applied electric field strengths inside $\mu$FACS. These data were taken on the same device but on two separate days. The width of the detection volume was 12 $\mu$m.
Figure 5: A schematic of forward and reverse Sorting.
Figure 6: T-channel wire diagram. The arms of the T channel can be considered as three wires with identical resistance since they have the same dimensions.
Figure 7: A snapshot of a sorting event. A red bead (oval-shaped) is being sorted into the right, collection channel. The green beads (round-shaped) in the left, waste channel are stagnant due to a voltage change which directs the electrokinetic flow into the collection channel. Preparation of beads is as follows. Red and blue fluorescent beads (1 μm diameter, Interfacial Dynamics Corporation, Portland, OR) were suspended in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄) with 10% BSA (1mg/mL) and 0.5% Tween 20 in 10:1 blue:red ratio and overall concentration of 1.5%. Fluorescence of the beads was excited by a 100 W mercury lamp with 488DF20 optical filter. A 630DF30 optical filter (Chroma Technology Corp., Brattleboro, VT) was used to select the red fluorescent emission. The μFACS device had 3 x 4 μm channels. A 100:1 blue:red ratio was used for reverse sorting.
Figure 8: A schematic of the integrated cell sorter. This sorter is made of two different layers of elastomeric channels bonded together. (A) The control layer contains lines where pressurized nitrogen and vacuum are introduced to actuate the closing and opening of the valves, respectively. (B) The fluidic layer contains lines where the sample is injected. (C) In this integrated cell sorter, valves 1, 2 and 3 act as a peristaltic pump and valves 4, 5 act as switch valves. Other two pairs of switch valves are not numbered. Holes, labeled as I, C and W, are the input, collection and waste wells respectively. Patterns of 00001 and 00010 actuated by the AT-DIO-32HS card closes valves 1 and 2 respectively, where 0 indicates “valve open” and 1 indicates “valve close”. (D) A snapshot of an integrated cell sorter made from GE RTV 615.
Figure 9: Cross sections of a trapezoidal-shaped and a round-shaped flow channels. Profiles of the channels when they are under actuation pressure. A flow channel with a round-shape cross section is able to completely seal off the channel under pressure. A channel with a trapezoidal-shape cross section will have leakage from the sides.\textsuperscript{[11, 43]}
Figure 10: Room temperature vulcanization: cross-linking reaction of PDMS and its curing agent. In the presence of a platinum catalyst, an addition reaction occurs in which a silicone hydride (SiH) is cross-linked to the vinyl group of the silicone polymer. This reaction occurs without any by-product and proceeds actively at room temperature.
Figure 11: Multilayer soft lithography fabrication. Multilayer soft lithography fabrication creates a monolithic chip of multilayers of channels, having each layer individually cast from a microfabricated mold. When both layers are half-cured, the top layer is peeled off from the mold and bonded to the bottom layer. After the bonded layers are fully cured, a monolithic chip is created.
Figure 12: A plot of linear flow velocities versus applied pressure from the integrated cell sorter. The velocity of the beads increased as the pressure applied to the peristaltic pumping increased. Beads stopped flowing at 100 kPa due to the incomplete opening of the valves at such high pressure.
Figure 13: A linear flow velocity profile from the integrated cell sorter. The velocity of the cells increased as the frequency of peristaltic pumping increased, reaching to a maximum value at a certain frequency. (●) are the flow velocities recorded using an GE RTV 615 cell sorter with 30% SF-96, whereas (○) are the flow velocities from a Sylgard 184 cell sorter. Nitrogen pressure applied to the peristaltic pumps was 60 kPa at each frequency. Each value of the flow velocities is the mean velocity of measurements taken from 150 cells. Some data points were taken on separate days from the same devices.
Figure 14: Cell trapping. A) Raw data recorded from oscilloscope. One cell was trapped within the region of detection by reversing its direction each time it was detected. The difference in the periodicity between each detection may be due to the variation in the distances the cell travelled away from the detection region before it reverted back. The frequency of the peristaltic pumps was at 10 Hz and the nitrogen pressure applied to the valves was at 60 kPa. B) Raw data recorded at 75 Hz pumping frequency. Multiple instances of cells trapping were recorded. C) A schematic of the cell trapping algorithm. A cell can be trapped within the detection region by reversing the flow at each detection. Cell trapping scheme is as follows. A series of 0 and 1 patterns were used to digitally control individual valves on the chip, where 0 and 1 indicate “valve open” and “valve close,” respectively. Forward peristalsis was actuated by the pattern 001, 011, 010, 110, 100, 101, whereas reverse peristaltic was actuated by the pattern 101, 100, 110, 010, 011, 001. These two peristalsis patterns alternate each time a cell’s fluorescence reaches above a preset threshold. Detection region is indicated by the dashed box.
Figure 15: Mean reverse time as a function of the pumping frequency. This is the average time for the cells to flow back into the detection region after reversing the direction of the peristaltic pumping. The reverse time gradually decreases as the pumping frequency increases. These data were used as references in order to calculate the timing for the reverse sorting. The reverse peristaltic pumping pattern was the peristaltic pumping pattern in reverse exactly. Nitrogen pressure applied to the peristaltic pumps was at 60 kPa at each frequency.
Allocate memories as data output buffers

Initialize PCI-1200 and AT-DIO-32HS

Allocate memories as data output buffers

Generate default pattern

Reading signals from PMTs

If signal > set threshold and if ratio > set ratio

Generate first detection pattern

reading signals from PMTs

and if ratio > set ratio

generate second detection pattern

If signal > set threshold

reading signals from PMTs

and if ratio > set ratio

generate third detection pattern

cell count ++

if count = # cells sorted

Generate END pattern

Figure 16: Sorting logic for reverse sorting. Reverse cell sorting scheme is as follows. With valve 4 as the collection valve, the default flow from the input to the waste was actuated by the pattern 01001, 01011, 01010, 01110, 01100, 01101 at 100 Hz. Once a target cell was detected, the reverse flow was actuated by the pattern 01101, 01100, 01110, 01101, 01011, 01001 at 10 Hz to slowly bring the cell back to the input channel. Once the cell passes the detection region again, all the valves were closed to stop the flow, 11111, and then the recovery of the cell was actuated by the pattern 10001, 10011, 10010, 10110, 10100, 10101 at 10 Hz to close the waste valve and direct the cell into the collection channel. The default flow was actuated again once the cell was in the collection channel. A pattern of 11111 was actuated at the end of the run to close all the valves for cell retrieval.
Figure 17: Histograms of EYFP output signals. At 1000 µM IPTG, the output of the inverter exhibited LOW EYFP fluorescence. At 0.1 µM IPTG, the output of the inverter exhibited HIGH EYFP fluorescence. At the forbidden zone of 30 µM IPTG, the EYFP output fluorescence signals were invalid.
Figure 18: ECFP input (red squares) and EYFP output (blue dots) signals at different IPTG concentrations. The forbidden zone, or the sharp transition, is mapped out in the range of 10–100 µM IPTG. The noise margins are immediately before or after the transition, at 3 and 100 µM IPTG, respectively. The “forbidden zone”, where valid inputs result in invalid outputs, is to be avoided. The noise margins, where marginally valid inputs can still result in perfect output representations, are critical for tolerating any noise or loss of signals within the circuit.
Figure 19: The noise margins of the $cI/\lambda_{P(R-O12)}$ inverter. Immediately before and after the transition, at 3 and 100 $\mu$M IPTG, respectively, the inverter can still output valid HIGH or LOW output signals from the marginally valid input signals.
Figure 20: The transfer function of the $cI/\lambda_{P(R-O12)}$ inverter.
Figure 21: Scatter plots of cell populations at different IPTG concentrations of the $cI/\lambda_{P(R-O12)}$ inverter are shown as red dots. Approximately 10,000 cells were measured in each concentration. The populations at 0.1 (black dots) and 1000 µM (green triangles) concentrations are shown on every plot for comparison of HIGH and LOW signal ranges.
Figure 22: The transfer band of the $cI/\lambda_{P(R-O12)}$ inverter is plotted using two transfer functions. One transfer function (red dots) is plotted using the values of the mean plus one standard deviation of the input and output signals at each IPTG concentrations. The other transfer function (green triangles) is plotted using the values of the mean minus one standard deviation at different IPTG concentrations. Some of the minimum values are in the negative range. The mean values of the transfer function are shown (•). The ranges of the HIGH and LOW inputs and outputs are also shown. This transfer band represents $\sim 70\%$ of the cells.
Table 1: Results of beads and E. coli cell sorting. Preparation of E. coli cells for sorting is as follows. The E. coli cells (HB101) expressing GFP were grown at 30°C for 12 hours in LB liquid medium containing ampicillin (1 colony was inoculated into 3 mL medium containing 50 μg/mL of ampicillin). Wild-type E. coli HB101 cells were incubated for 12 hours in LB-only medium. After incubation, HB101 and GFP-expressing HB101 E. coli cells were resuspended into PBS (ionic strength = 0.021) three times and stored at 4°C for sorting. Immediately before sorting, the cells were resuspended again in phosphate buffer (4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄) containing 10⁻⁵ M SDS and diluted to a concentration of 10⁹ cells/mL. The cells were filtered through a 5 μm syringe filter (Millipore Bioscience Inc., Bedford, MA) for elimination of elongated cells. A μFACS device with 10 x 4 μm channels was used. Fluorescence was excited by the 488 nm line of an Argon ion laser (6 mW into the objective), Coherent Innova 70 (Laser Innovations), and the emitted fluorescence was filtered with a 535DF20 filter.
Si + CH₃Cl $\xrightarrow{Cu}$ Me₃SiCl  $\quad$ Me₂SiCl₂  $\quad$ MeSiCl₃  | bp 58°C  bp 70°C  bp 68°C

2Me₂SiCl₂ + 4H₂O $\rightarrow$ 2Me₂Si(OH)₂ + 4HCl  \hspace{1cm} (11)

$HO[(CH₃)_₂SiO]_mH$  $\xrightarrow{KOH}$ Cyclic Dimethyl Tetramer  \hspace{1cm} (12)

Cyclic Dimethyl Tetramer + Chain Stopper  $\xrightarrow{KOH}$ $(Me₂SiO₂)ₙ$ – PDMS  \hspace{1cm} (13)

Table 2: Polydimethylsiloxane manufacturing process²⁶ from silica (sand) to PDMS. See text for details.