An Integrated Microfabricated Cell Sorter

Anne Y. Fu,²,³ Hou-Pu Chou,²,§ Charles Spence,² Frances H. Arnold,³ and Stephen R. Quake*,²

Department of Applied Physics, California Institute of Technology, MC 128-95, Pasadena, California 91125, and Division of Chemistry and Chemical Engineering, California Institute of Technology, MC 210-41, Pasadena, California 91125

We have developed an integrated microfabricated cell sorter using multilayer soft lithography. This integrated cell sorter is incorporated with various microfluidic functionalities, including peristaltic pumps, dampers, switch valves, and input and output wells, to perform cell sorting in a coordinated and automated fashion. The active volume of an actuated valve on this integrated cell sorter can be as small as 1 pL, and the volume of optical interrogation is ~100 fl. Different algorithms of cell manipulation, including cell trapping, were implemented in these devices. We have also demonstrated sorting and recovery of Escherichia coli cells on the chip.

Since the introduction of the first microfabricated electrokinetic analytical device in 1991,¹ much work has been focused on using electrokinetic forces to separate ionic species such as peptides and DNA fragments through capillary electrophoresis.²⁻³ Gradually, it has become apparent that the true potential of microfabricated devices lies in the ability to integrate a complete analysis system “on chip”. Such a complete analysis system in a microfabricated device will need to perform more than just separation and detection. This “lab on a chip” will have to integrate functionalities such as sample handling, mixing, incubation, sorting, transportation, recovery, and automation for reproducibility. Thus, other means of controlling fluid flow within the microfabricated devices have been studied. Dielectrophoresis⁷⁻⁸ and pressure switching⁹ have been used to create valveless switches for separation of particles and cells within microchannels. Spatially fixed temperature zones allow for incubation at various regions of the microchannels.¹⁰ Multiple ports and plugs can dispense different reagents for dilution and enzymatic reactions.¹¹,¹² Advances in developing an integrated analysis system have led to an emergence of chips that can perform enzymatic assays,¹³,¹⁴ immunoassays,¹⁵ polymerase chain reaction,¹⁶ and cell sorting.¹⁷

We previously described our efforts in developing a microfabricated elastomeric cell sorter.¹⁷ Using electrokinetic flow, we demonstrated sample dispensing, interrogation, automation, sorting, and recovery. In this simple device, we separated living Escherichia coli cells expressing green fluorescent protein from nonfluorescent E. coli cells and extracted these cells from the device to regrow on agar plates. We achieved enrichments of 30-fold in a single pass, with 20% recovery of viable cells. The standard “forward” sorting algorithm consists of running the cells from the input channel to the waste channel until a cell’s fluorescence goes above a preset threshold, at which point the voltages are

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¹ To whom correspondence should be addressed: (email) quake@caltech.edu; (fax) (626) 793-8675.
² Department of Applied Physics.
³ Division of Chemistry and Chemical Engineering.
§ Current address: Fluidigm Corp., 7100 Shoreline Court, South San Francisco, CA 94080.
temporarily switched to divert the cell to the collection channel. A novel "reverse" sorting algorithm was also implemented. The cells flow at the highest (scanning) speed from the input to the waste. Upon the detection of a target cell, the flow stops and reverses the cell at a slower (switchable) speed back to the input. When the cell passes through the detection region a second time, it is directed into the collection channel. Then the device can be run at high speed again from the input to the waste. Substantial enrichments of 80–96-fold of micrometer-sized fluorescent beads of different colors were obtained in single runs using both forward and reverse sorting. This reverse sorting is not possible with conventional fluorescence-activated cell sorters (FACS) and could be useful for isolating rare cells or making multiple measurements of a single cell.

Cell sorting has become an indispensable part in the studies of cellular metabolism on a single-cell level. For future applications, these single-cell analyses will demand immediate treatment of cells either before or after sorting with minimal time variation and sample loss. Thus, there is a need for a microfabricated cell sorter that can integrate more functionalities. Electrokinetic flow or direct pressure application alone will not be able to meet these demands.

Multilayer soft lithography is a micromachining technique that exploits the elasticity and the surface chemistry of silicone elastomers in order to create monolithic microvalves within microfabricated devices. This technique 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. fabricated switching valves and peristaltic pumps for sample dispensing and switching. Chou et al. made rotary pumps for mixing and incubation, and Liu et al. demonstrated a rotary PCR device.

Using multilayer soft lithography, we developed a microfabricated elastomeric cell sorter integrated with microvalves and micropumps. Our initial efforts in sorting cells via electrokinetic flow demonstrated that we could sort and recover 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 absorption of any fluidic perturbation (See Figure 1). Other microfluidic functions can be easily integrated for kinetic studies or treatment before or after sorting events. The active areas of these microvalves and micropumps are much smaller than those we previously described in order to accommodate single bacterial cells. The active volume of the smallest valve on this integrated sorter is 1 µL. The new cell sorter also improves buffer compatibility, automation, and cell viability. We have exploited different algorithms of interrogation within this sorter, such as reversible sorting and cell trapping. We have also greatly improved the sorting accuracy and recovery efficiency using this integrated sorter relative to previous work. Finally, we have demonstrated the ability of this sorter to integrate various microfluidic functionalities into one chip to perform a complicated task in an automated fashion.

**EXPERIMENTAL SECTION**

**Device Design and Fabrication.** Fabrication and design of the integrated pressure-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 1, 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 µm in width which narrows down to 20 µm and eventually tapers down to 6 µm 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 mm 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 µm long and 30 µm wide. These valves are 100 µm 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 dimensions of 20, 30, and 50 µm wide. These valves have a valve active area of 20 × 20, 30 × 30, and 50 × 30 µm, respectively. Fabrication details are given below.

**Photore sist Molds.** The control layer and the fluidic layer were cast from two different molds fabricated on silicon wafers. The depth of the fluidic channel was 3.5–5 µm whereas the depth of the control channels was 10–12 µm. All silicon wafers were incubated with hexamethyldisilazane (HMDS) vapor for 2 min before photolithography. For the control channel, the silicon wafer was spin-coated with photore sist (Shipley SJ 5740) at 2000 rpm (ramping time, 15 s; spin time, 60 s). The wafer was then soft-baked in the oven at 90 °C for 60 min. A chrome glass mask was used for photolithography. The wafer was exposed to UV and developed in 20% M icroposit Developer 2401 for 1.5 min.

For the fluidic channel, the silicon wafer was spin-coated with SJ R 5740 at 6000 rpm (ramping time, 15 s; spin time, 60 s). Then

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the wafer was soft-baked in an oven at 90 °C for 60 min. The photolithography process was the same as above. After photolithography, the wafer was placed in an oxygen plasma for 45 min in order to etch a height of 3.5-5 μm of the photoresist.

The heat reflow process of the photoresist was accomplished by hard-baking the wafer on a hot plate at 80 °C for 20 min.

Elastomeric Fabrication. Soft lithography offers an easy way to produce microfabricated devices out of different commercially available silicone elastomers. We have developed two protocols for making the integrated cell sorter. The first protocol uses General Electric (GE) RTV 615 and the second protocol is for Dow Corning Sylgard 184. RTV 615 and Sylgard 184 both are poly(dimethylsiloxane) (PDMS) elastomers cured by cross-linking to silicone hydrides using a proprietary platinum-based catalyst.24 Generally, RTV 615 takes longer time to cure whereas Sylgard 184 is more stiff and chemically inert to acids and bases.

GE RTV 615. For the fluidic layer, General Electric RTV 615 A and B components were mixed at a ratio of 20:1 in a Keyence Hybrid Mixer. This mixture was then spin-coated at 8000 rpm onto the wafer in order to obtain a layer of 10-12 μm thickness. The same thickness can be achieved by adding a diluent, General Electric SF-96, into the mixture (30%/v) and spin-coating at 3000 rpm. This layer was incubated in the oven at 80 °C for 30 min. For the control layer, RTV 615 A and B components were mixed in a ratio of 3:1 and spin-coated at 2000 rpm. After incubating at 80 °C for 20 min, an additional mixture of 10:1 was poured onto the control layer to a thickness of 2 mm. The control layer was then incubated in the 80 °C oven for 1 h. After incubation, the control layer was peeled off from the mold. A 20-gauge Luer stub adaptor was used to drill out air holes for nitrogen injection. The control layer was properly aligned and bonded onto the fluidic layer. The bonded chip was incubated in the oven at 80 °C for 2 h. Fluidic holes were introduced after the incubation. The monolithic chip was sealed with a glass coverslip and incubated in the oven at 80 °C for 3 h. This chip was then ready for sorting.

Dow Corning Sylgard 184. A similar fabrication process uses Dow Corning Sylgard 184. Sylgard 184 A and B components were mixed at a ratio of 20:1 and spin-coated onto the fluidic mold at 5000 rpm. A mixture at the ratio 5:1 was then poured onto the control mold to a thickness of 2 mm. Both were incubated in the oven at 80 °C for 25 min and then bonded together. The bonded chip was incubated in at 80 °C for 1 h.

Figure 1. 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 1-3 act as a peristaltic pump and valves 4 and 5 act as switch valves. The other two pairs of switch valves are not numbered. Holes, labeled as I, C, and W, are the input, collection, and waste wells, respectively. (D) A snapshot of an integrated cell sorter made from GE RTV 615.

Pneumatic Control and Optical Setup. The pneumatic control setup was as previously described.\textsuperscript{20} Five control lines on the cell sorter were connected to the common ports of five external miniature three-way pneumatic switch valves (LHDA1211111H; The Lee Co., West Brook, CT). The normally closed ports on these valves were connected to nitrogen pressure while the normally open ports were connected to vacuum. A National Instruments card (AT-DIO-32HS) digitally controlled the switching of the valves through a fast Zener-Diode circuit. Nitrogen pressure applied to the peristaltic pumps was 60 kPa and to the switch valves was 110 kPa.

The integrated cell sorter was mounted on an inverted microscope with an oil immersion objective (100×, 1.3 NA; Olympus America Inc., Melville, NY) as previously described.\textsuperscript{17} An argon laser (Innova 70, Coherent Inc., Santa Clara, CA) was used as an excitation source. Fluorescence was collected by the objective and projected onto a photomultiplier tube (PMT; Ham-matsu R928, Hamatsu, Bridgewater, NJ) with a custom current-to-voltage amplifier. A National Instrument card (Lab PC1200) digitized any signals from the PMT. A C++ Builder program was written to read signals from Lab PC1200 and digitally control the pneumatic valves through AT-DIO-32HS to automate cell sorting and trapping.

Cell Trapping and Reverse Cell Sorting. The 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. Patterns of 001 and 010 actuated by the AT-DIO-32HS card close valves 1 and 2, respectively (Figure 1). Forward peristalsis was actuated by the pattern 001, 011, 010, 110, 100, 101, whereas reverse peristalsis was actuated by the pattern 101, 110, 110, 010, 011, 001. These two peristalsis patterns alternate each time a cell's fluorescence goes above a preset threshold, trapping the cell near the detection region.

The reverse cell sorting scheme is as follows. With valve 4 as the collection valve (Figure 1), the default flow from the input to the waste was actuated by the pattern 01001, 01101, 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, 01101, 01001 at 10 Hz to slowly bring the cell back to the input channel. Once the cell passed the detection region again, all the valves were closed to stop the flow (11111). Then 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.

RESULTS AND DISCUSSION
Flow Velocity. We characterized the linear flow velocity of the cell sorter using peristaltic pumps at various pumping frequencies. E. coli cells expressing enhanced green fluorescent protein (EGFP) were pumped through the sorter. A 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 (see Figure 3C). The dimensions of the detection region were 32 μm 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. We averaged the widths of these pulses from ~150 cells. The velocity was calculated by dividing the length of the detection region by the average pulse width.

Cells passing through the sorters attained a maximum linear velocity at a pumping rate of 50 Hz (Figure 2). Above this frequency, we started to observe incomplete opening and closing of the valves with each pumping cycle.\textsuperscript{20} 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/s while the Sylgard cell sorter has a maximum of 14 mm/s. From several measurements taken on different devices, we have observed maximum flow velocities ranging from 6 to 17.5 mm/s. This suggests that we may be able to tune the maximum flow velocities of the cell sorters by altering the properties of the elastomer. Adding diluents or mixing different ratios of A and B components 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.

Sorting and Recovery. Cells can be sorted in this integrated device in a variety of ways. Since each valve can be individually controlled in a coordinated fashion, the pumping rate and the valve switching rate can be changed at any time during the course of a sorting event. As mentioned in our previous paper,\textsuperscript{17} both forward and reverse sorting are possible within the microfluidic sorter. Although switching using electrokinetic flow is virtually instantaneous, mechanical compliance in a pressure-switched system is likely to cause the fluid switch speed to become the rate-limiting step in the forward sorting algorithm. For the integrated cell sorter, the rate-limiting step is the intrinsic valve
Table 1. Results of Sorting and Recovery of E. coli Cells Using Four Different Cell Sorters

<table>
<thead>
<tr>
<th>device no.</th>
<th>cells/mL</th>
<th>EGFP fractions</th>
<th>time, h</th>
<th>cells/s</th>
<th>cells sorted</th>
<th>recovery</th>
<th>enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>042800RTV</td>
<td>2.8 x 10^7</td>
<td>4.0 x 10^-2</td>
<td>2.8 x 10^-1</td>
<td>1.8 x 10^-2</td>
<td>2</td>
<td>1.3</td>
<td>9,251</td>
</tr>
<tr>
<td>110300IIRTV</td>
<td>6.3 x 10^7</td>
<td>5.7 x 10^-3</td>
<td>2.0 x 10^-1</td>
<td>4.9 x 10^-3</td>
<td>2</td>
<td>1.1</td>
<td>79,200</td>
</tr>
<tr>
<td>110300IIRTV</td>
<td>2.8 x 10^7</td>
<td>1.1 x 10^-3</td>
<td>9.5 x 10^-2</td>
<td>3.1 x 10^-3</td>
<td>2</td>
<td>26.4</td>
<td>177,333</td>
</tr>
<tr>
<td>110300IIRTV</td>
<td>9.7 x 10^7</td>
<td>2.5 x 10^-3</td>
<td>1.1 x 10^-1</td>
<td>7.7 x 10^-4</td>
<td>3</td>
<td>6</td>
<td>40,922</td>
</tr>
<tr>
<td>110300IISYL</td>
<td>2.6 x 10^7</td>
<td>2.7 x 10^-4</td>
<td>3.4 x 10^-1</td>
<td>1.5 x 10^-2</td>
<td>2</td>
<td>2.16</td>
<td>15,600</td>
</tr>
<tr>
<td>110300IISYL</td>
<td>1.3 x 10^8</td>
<td>4.4 x 10^-4</td>
<td>3.6 x 10^-2</td>
<td>8.0 x 10^-4</td>
<td>3</td>
<td>44</td>
<td>479,381</td>
</tr>
</tbody>
</table>

* Device numbers that end with the characters RTV are made from GE RTV 615 and device numbers that end with the characters SYL are made from Sylgard 184. Cell concentrations at the input well are indicated. Fractions of EGFP at the input, collection, and waste wells are also presented. Recovery was calculated as the percentage of the actual number of EGFP recovered at the collection well divided by the number of positive fluorescent events detected by the device. The enrichment was calculated as the ratio of the fractions of EGFP at the input to the collection wells. The reverse sorting algorithm was used for all runs.

Response time for opening and closing (~5 ms). However, since the fluid is at low Reynolds number and can be reversible, the “reverse” sorting algorithm can be implemented to overcome the intrinsic switching speed of the device.

The reverse sorting algorithm consists of three patterns for a sorting event: the 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 a 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 first closes 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, the default pattern runs again until the next sorting event triggers the reverse pattern.

In a typical run, we separated two populations of E. coli cells, one expressing EGFP and the other expressing p-nitrobenzyl (pNB) esterase. The EGFP cells generate a fluorescence signal which triggers collection. The populations were mixed in ratios of 1:2000 and introduced into the sorter first by nitrogen back flushing mechanism to retrieve the remaining cells inside the collection channel.

The integrated sorter can run without interruption for 5 h 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, this integrated sorter alleviates many issues regarding buffer compatibility, surface chemistry, and cell viability. Different strains of E. coli and different types of bacteria, including magnetotactic bacteria, could be pumped through the integrated cell sorter in their own suspending media. 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 the previous devices that used electrokinetic flow. Although the throughput rate was increased only 2-fold, the recovery yield and the accuracy of sorting improved tremendously. Using the microscope, we observed that the integrated sorter was able to capture most or all of the desired cells into the collection channel, even when they occurred in small numbers. 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 inside the collection channel.

The variability in the results of different runs in Table 1 can be attributed to factors such as initial cell concentration, fraction of EGFP-expressing bacteria at the input, duration of each run, and device fabrication. The reverse sorting algorithm is very efficient when the fraction of EGFP-expressing cells in the sample is small. When sorting rare events, higher throughput and enrichment could be achieved since the sorter can be operated at high speed most of the time. The initial cell concentration is also an important factor for increasing throughput and recovery. As shown from the results of devices 110300IIRTV and 110300IISYL, the recovery yields greatly depend on the number of cells sorted to the collection well. Higher recovery can be achieved if more cells are sorted to the collection well.

**Cell Trapping.** We have also demonstrated a new method of interrogation that consists of trapping a single cell within the detection region. In our previous paper, we introduced a sorting scheme, reverse sorting, that was designed to overcome the intrinsic switching speed of the device and thereby increase the throughput rate when sorting rare events. To further this concept, we devised another algorithm of interrogation by trapping cells within the detection region. A computer algorithm was written 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 3 shows the data recorded by the oscilloscope and 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 (four instances were shown in Figure 3). We have also taken measurements of the mean reverse time for each pumping frequency (Figure 4). The mean reverse time is the time the cell traveled 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 decreases gradually as the pumping frequency increases. This is consistent with the fact that opening and closing of the valves becomes incomplete with increasing pumping frequency.

This method of trapping cells and other bioparticles within a given region inside the sorter opens up new avenues to perform kinetic studies on cells and beads. Multiple time-course measurements of the same cell can be taken to follow the kinetics of a 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 way to investigate kinetics on a single-cell level that cannot be accomplished by commercially available conventional flow cytometers.

CONCLUSION

The integrated cell sorter offers several advantages over the conventional FACS machines. Because the channels in the device are of micrometer dimensions, the volume of interrogation can be precisely controlled to reduce background fluorescence from the media and channel materials. The planar configuration of these devices allows the use of high numerical aperture optics, increasing the sensitivity of the whole system. Due to the simple fabrication process and inexpensive materials, these devices can

Figure 3. Cell trapping. (A) Raw data recorded from the oscilloscope. One cell was trapped within the region of detection by reversing the flow 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 traveled away from the detection region before it turned back. The frequency of the peristaltic pumps was 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) Schematic of the cell trapping algorithm. A cell can be trapped within the detection region (dashed box) by reversing the flow at each detection.
be disposable to eliminate any cross-contamination from previous runs. In addition, the whole cell sorting system is self-contained, unlike the conventional aerosol droplet sorters, allowing for relatively safe sorting of biohazardous materials. Although conventional FACS machines currently achieve higher throughput and recovery, the integrated cell sorter provides an inexpensive, robust, and flexible way to sort and manipulate 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 is not possible with FACS. Sample dispensing and other chemical or enzymatic reactions, such as cell lysis or polymerase chain reaction, can also be carried out downstream immediately after the cell has been sorted. This will enable single-cell studies to be achieved in a more precise and automated fashion. We are presently using this sorter 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. We anticipate that an integrated "lab on a chip" where cell sorting is just the one step of a complete analysis system will be realized soon.

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Figure 4. 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 the direction of the peristaltic pumping has been reversed. The reverse time decreases gradually as the pumping frequency increases. Device 042800RTV was used for these measurements. Nitrogen pressure applied to the peristaltic pumps was at 60 kPa at each frequency.