



Microfluidic Methods for High-Throughput Biological Screening

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Microfluidic Methods for High-Throughput Biological Screening

A dissertation presented

by

Warren Lloyd Ung

to

The John A. Paulson School of Engineering and Applied Sciences

in partial fulfillment of the requirements

for the degree of

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in the subject of

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Microfluidic Methods for High-Throughput Biological Screening

Abstract

Often in biology, rare individuals within a population dominate the population's overall behavior, and we wish to extract those individuals for further analysis. We design a sorting instrument as a flexible platform for the development of novel microfluidic sorting techniques. We demonstrate a microfluidic cell sorter, which screens cells at rates approaching those of commercially-available fluorescence-activated cell sorters. This device incorporates a three-dimensional flow-focusing nozzle with a slanted ceiling groove to enhance the capabilities of a surface acoustic wave (SAW) transducer by harnessing the component of the SAW oriented normal to the plane of the substrate. The device achieves sorting at a rate of 9000 events/s with 54% purity and yields 89% purity, while operating at 1000 events/s; this level of performance approaches that of a FACS operating in its high-purity mode. We also present a rare event sorting technique, which can successfully extract desired droplets from a sample containing nearly a billion droplets. The technique yields pure samples after two rounds. The preliminary round is fast, capable of screening 10 ml of droplets at 100 ml/h, but each sort also captures many other droplets together with the droplet of interest. The second round of sorting enriches the sample to nearly 100% purity, using known designs for high purity drop-by-drop sorting. Thus, we devise a method, which can sort droplets rapidly and achieve high purity of few droplets from samples containing large numbers of droplets. The two disparate approaches to microfluidic sorting use a common platform to create new methods for sorting with biological applications.

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Dedication

I would like to dedicate my thesis to my coming daughter. I hope to instill in you a love of knowledge, a curiosity about how the world works, and a desire to build something great. I also want to thank my wife and our parents for their love and support throughout this whole process.

List of Abbreviations

DAQ	Data acquisition
DNA	Deoxyribonucleic acid
FACS	Fluorescence-activated cell sorting
FEP	Fluorinated ethylene propylene
FPGA	Field-programmable gate array
fps	Frames per second
LED	Light-emitting diode
PMT	Photomultiplier tube
RNA	Ribonucleic acid
PC	Personal computer
PCB	Printed circuit board
PDMS	Poly(dimethylsiloxane)
RF	Radio frequency
SAW	Surface acoustic wave
TTL	Transistor-transistor logic

Sorting Instrument for Microfluidics

Cell sorting is a technique, in which ongoing development is highly interdisciplinary; advancements in engineering and physics are applied to biological applications.¹ In biology, many samples contain mixtures of cells, and only target cells with specific properties within the sample are of interest for a particular experiment; cell sorting extracts these target cells. The purified samples of cells can be analyzed to measure the abundances of specific biomolecules such as DNA, RNA or proteins, or the cells themselves may be desirable because of their phenotype, for example, if they produce products such as antibodies.^{1,2} Microfluidic techniques are now being developed, which offer functionality in addition to cell sorting; microfluidic methods also have specific advantages including rapid prototyping and disposable devices.³⁻⁶ We develop microfluidic approaches that can sort general classes of particles, such as beads, gels, and droplets as well as cells.⁷⁻¹¹ As a foundation for our work in microfluidic sorting, we designed a sorting instrument to serve as the platform for novel microfluidic sorting devices. The sorter uses microscope optics to excite and capture fluorescence from samples flowing through a microfluidic device. If the measured fluorescence indicates that the object passing through the microfluidic device is desirable, the instrument triggers a sorting pulse to retain the object for further processing. The instrument is broadly applicable to microfluidic sorting methods, because the optical system, the microfluidic flow channel, and the sorting apparatus can all be tailored to each application. Here, we provide specific details regarding the system we developed for sorting.

Optics

The optical components of the sorting apparatus are illustrated in Figure 1 below. The apparatus is similar to that detailed in previous publications¹² except that the microscope body is built from modular optical components. Fluorescence is excited by a 473 nm laser (Laserglow Technologies, Toronto, ON)

with 100 mW output. The laser beam is expanded by a beam expander (BE-05-10-A, Thorlabs Inc., Newton, NJ), and steered into the microscope. Excitation light from the laser reflects off the excitation dichroic (FF495-Di03-25x36, Semrock, Inc., Buffalo, NY) and up through the microscope objective (CFI Plan Apochromat Lambda, 10X/0.45NA, Nikon Instruments, Inc., Melville, NY). A cylindrical achromat (ACY254-200-A, Thorlabs Inc.) focuses one axis of the beam into a line in the back aperture of the objective. The same objective focuses the excitation light into a line in the microscope's focal plane and collects the resulting fluorescence emission from the sample. The emitted fluorescence passes through the excitation dichroic, but reflects off the fluorescence dichroic (FF605-Di01-25x36, Semrock, Inc.); the desired fluorescence then passes through a colored glass long pass filter (FGL495, Thorlabs Inc.) and a dielectric band pass filter (FF01-520/44-25, Semrock, Inc.) illuminating the photocathode of a photomultiplier tube (H10723-20, Hamamatsu Photonics K.K., Hamamatsu, Japan), while noise sources of light are attenuated by the filters and do not reach the photocathode. The microscope's field of view is illuminated using an infrared light emitting diode (LED). The infrared light passes through both of the microscope's dichroic filters, and reflects from a turning mirror (CM1-P01, Thorlabs Inc.). The infrared image is focused onto the sensor of a fast camera (HiSpec1, Fastec Imaging, San Diego, CA) by a tube lens (AC254-100-B-ML, Thorlabs Inc.) to enable videos of the sorting process to be recorded at high speed. A Leica manual stage completes the microscope by providing fine adjustment of the sample's position with respect to the optical system.

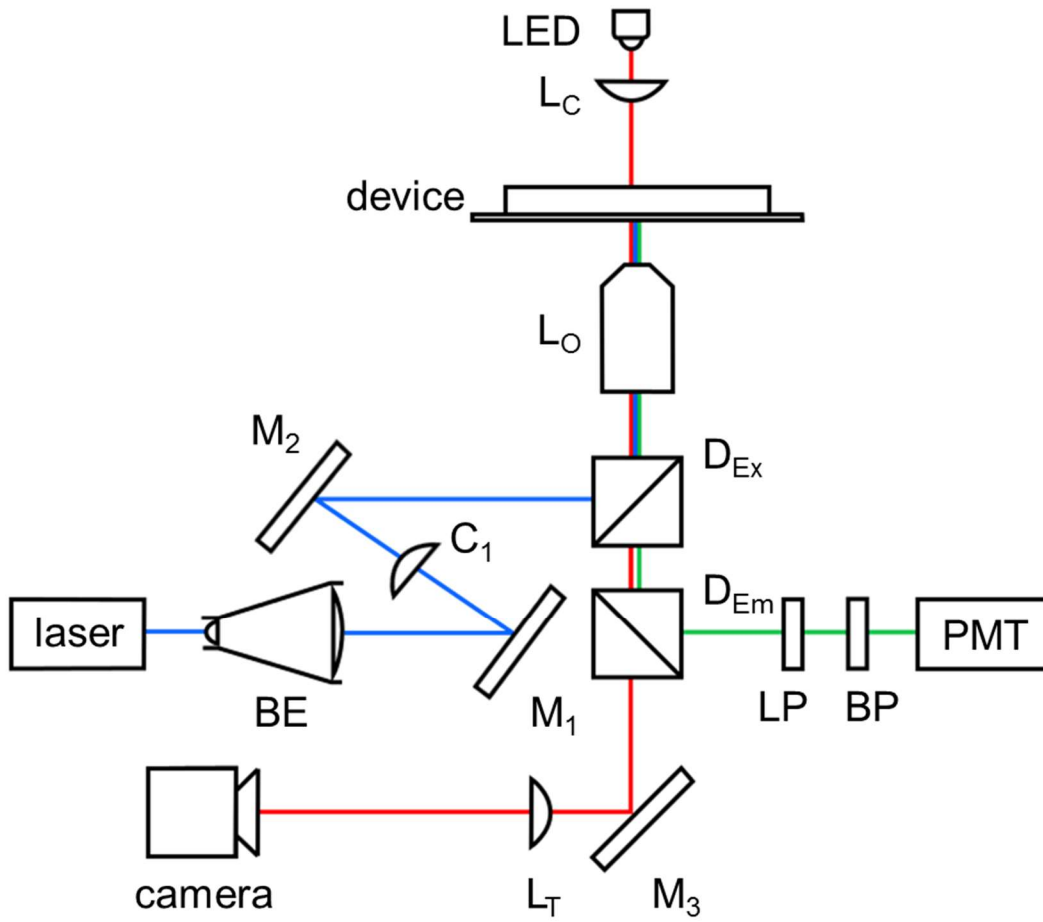


Figure 1: Schematic Diagram of the Sorting Instrument

A schematic representation of the optical system is depicted here. Excitation light (blue) from the laser is shaped by a beam expander (BE) and a cylindrical achromat (C_1) to produce a focused line at the image plane of the objective (L_o). Beam steering mirrors (M_1 and M_2) control the position and orientation of the line in the image plane. A dichroic filter (D_{Ex}) reflects the laser light up through the objective, while the fluorescence emission (green) collected by the objective is transmitted by the filter. The fluorescence then reflects off the emission dichroic (D_{Em}) and passes through two additional filters, a colored glass long pass (LP) and a dielectric band pass (BP), before illuminating the photocathode of a photomultiplier tube (PMT). The microfluidic channel can be imaged in bright field using an infrared LED to illuminate the objective's field of view. The light from the LED is collimated using a lens (L_c), and is collected by the objective after illuminating the sample. The infrared light is transmitted by both dichroic filters and reflects off a turning mirror (M_3). The light is focused into a real image, on the sensor of a fast camera, by the tube lens (L_T). The microfluidic device is placed at the image plane so that the particles flowing through the channel can be detected by the sorting apparatus.

Data Acquisition Hardware and Software

The signals corresponding to sample fluorescence are processed continuously, as they are acquired, to determine whether a sorting pulse should be triggered in real time. The PMT module measures the intensity of light in the accepted wavelength range and generates a voltage proportional to the intensity of the incident light. This voltage is digitized by the data acquisition (DAQ) card (PCIe-7842R, National Instruments Corp., Austin, TX) and analyzed in real time using the on-board field-programmable gate array (FPGA) to extract properties that describe the shape of the fluorescence peak and generate sorting pulses for those peaks with properties that correspond to desirable objects. Sorting pulses are sent to downstream instruments, such as amplifiers, valves or waveform generators, to trigger sorting of micro-scale objects within the microfluidic device. The instrument data is streamed to the associated PC by direct memory access. The user interface on the PC plots the measured data and enables the operator to monitor and change the instrument settings prior to or even during a sort. The DAQ card's FPGA and the PC both run custom code written in LabVIEW (National Instruments Corp.); the code is available from the SEAS Code Repository upon request. The overall system provides the necessary components to achieve high speed sorting based on the measured signal.

The FPGA is an integral part of the system; it performs the peak analysis and sort decision making with performance sufficient for real time analysis. The FPGA, in particular, enables the system to achieve hardware levels of performance, while being reconfigurable for different applications. Moreover, specific knowledge of FPGA programming languages, such as Verilog or VHDL, is not required, because of the provided LabVIEW environment, but low level firmware can be written to further enhance instrument performance. Although alternative implementations for the sorter's electronics exist, none offer the same combination of high performance, low latency, and reconfigurable hardware. The ability to define new functionality for the instrument without needing to re-design the whole system is

particularly well-suited to the research environment, because it enables the sorter to be readily adapted to new kinds of samples and assays.

The sorting pulses generated by the DAQ card trigger sorting within the microfluidic device. However, the sorting pulse is not typically in the exact form necessary to drive sorting via a direct connection to the device; usually, another instrument or transducer is required to convert the signal to the correct voltage level, power level, or frequency for a specific sorting mechanism. The DAQ card can provide either an analog pulse, with ± 10 V range, generated by a 16-bit digital-to-analog converter at up to 30 kHz, or a 3.3 V digital, transistor-transistor logic (TTL) pulse, as a means of interfacing with other instruments. For example, the digital pulse can trigger a high speed camera to capture a movie; if the triggering of the camera is synchronized to the sorting pulse, the camera movie can be used to observe specific sorting events at more than 10,000 fps as they occur. The capability for both analog and digital signal generation ensures the system can employ a wide range of possible sorting mechanisms, such as surface acoustic waves or pneumatic valves, by interfacing with instruments that can amplify and shape the sorting signal.

Conclusion

The microfluidic sorting instrument combines modular, off-the-shelf components into a powerful system tailored for sorting samples at the micro-scale. The design of the hardware and software can be adapted to a wide range of applications in microfluidics. The system can distinguish objects, based on fluorescence, making it compatible with a wide range of fluorescent antibodies, probes, and assays.¹ In combination with a high-speed camera, triggered images of individual sorting events can be captured to elucidate details of the sorting process. The instrument we describe provides a broadly applicable solution for sorting at the micro-scale and it is a key element of the two major sorting methods detailed in the following sections.

Enhancing Surface Acoustic Wave Cell Sorting with a Micro-fabricated Slanted Groove

Introduction

Fluorescence-activated cell sorting (FACS) extracts cells of interest from a mixed population of cells. Since its inception, nearly fifty years ago, FACS has become a fundamental technique in biological and medical science, because it remains the most convenient and widespread method for selecting desired cells based on biological characteristics that can be distinguished with fluorescent probes associated with a specific cellular structure or through the cellular expression of fluorescent proteins.¹ Upon recovery, the enriched population of cells can be analyzed using standard biological assays, such as DNA sequencing or mass spectrometry to reveal characteristics specific to the sorted population, or, if the cells are sufficiently pure, they can seed new cultures of the desired cell type. New technologies based on microfluidics are emerging that complement FACS. Microfluidic devices can sort cells without producing aerosols, whereas FACS generates aerosols;^{1,8,13,14} microfluidic devices are disposable, while the flow cell in FACS requires cleaning and realignment between experiments;¹ and samples are handled with minimal dead volume, reducing the amounts of sample loss, while FACS operates on macroscopic sample volumes.¹⁵ Nevertheless, very few microfluidic FACS devices can compete with commercially available FACS instruments on the basis of event rate and sorting purity. Each of the microfluidic techniques that has potential for cell sorting applications depends on a fast flow or particle actuation mechanism, such as piezoelectric transducers,¹⁶ dielectrophoresis of droplets,¹⁷ or pulsed laser induced cavitation.^{13,18} Surface acoustic waves (SAWs) provide an alternate microfluidic method for rapidly driving flows and directing particle motion. The acoustic waves generated by a SAW transducer can efficiently actuate broad classes of particles including beads and cells, gels and droplets in continuous

flow.^{7,11} Devices based on standing SAWs are used extensively to manipulate cells and particles.^{19–23}

These devices feature exquisite control of each particle's spatial position through dynamic patterning of the underlying acoustic standing wave; a standing SAW device can direct cells into any of five independent collection channels, but it requires long actuation pulses.¹⁹ Traveling wave SAW devices can effect particle motion with sub-millisecond pulses, which makes them well suited to applications requiring high event rates.⁷ However, most SAW sorters only utilize the component of the acoustic wave that is oriented in the plane of the device, despite the fact that the acoustic wave's normal component is several fold greater in magnitude. One particle trapping device demonstrates the use of the normal component of acoustic waves,²⁴ but its design cannot easily be applied to cell sorting at high velocities. Thus, SAW microfluidic devices are promising for cell sorting applications, but there has yet to be a SAW device, which takes advantage of the normal component of the acoustic wave to sort cells rapidly with high purity recovery of the sorted cells.

Here, we demonstrate a microfluidic cell sorter, which screens cells at rates approaching those of commercially-available FACS. This device incorporates a three-dimensional flow-focusing nozzle with a slanted ceiling groove to enhance the capabilities of a SAW transducer by harnessing the component of the SAW oriented normal to the plane of the substrate. We determine the conditions, which maximize device performance, and use these principles to implement a novel cell sorter. The device achieves sorting at a rate of 9000 events/s with 55% purity and yields 90% purity, while operating at 1000 events/s; this level of performance approaches that of a FACS operating in its high-purity mode.

Results and Discussion

When a SAW, traveling along the surface of a substrate, impinges on the flow of liquid within a microfluidic device, it refracts, forming a longitudinal acoustic wave in the liquid; it is this acoustic wave that can deflect cells.²⁵ The angle of refraction for SAW is known as the Rayleigh angle, θ_R , and it

depends on the speed of sound in the liquid, v_l , and the speed of the SAW along the substrate's surface, v_s , according to Snell's law, $\sin\theta_R = v_l/v_s$.^{25,26} In the materials used for SAW microfluidics, the SAW travels along the substrate surface faster than the acoustic wave propagates in the liquid,²⁷ so the Rayleigh angle is small and the refracted wave is directed closer to the substrate's surface normal than to the plane of the substrate. As a result, the normal component of the refracted acoustic wave is larger than the parallel component in general. Most microfluidic devices are fabricated by lithographic techniques, in which a two-dimensional mask defines the shape of the device. Moreover, the devices are assembled such that the plane containing the device's features is parallel to the substrate plane; therefore, most SAW microfluidic techniques utilize single layer devices, in which only the component of the acoustic wave parallel to the substrate is used for particle manipulations. Because the normal component of the SAW is directed away from the substrate surface, multiple layers of lithographic features are required to generate structures with features that vary along the normal direction and, thus, can exploit the normal component of the acoustic wave. A SAW device, which uses multi-layer features to channel the normal component of SAW for sorting, may obtain enhanced sorting performance compared to existing designs, because it channels more of the available power into actuating cells.

We implement a multi-layer device geometry that utilizes the normal component of the acoustic wave for cell sorting applications, illustrated in Figure 2. Here, a tapered interdigital transducer (IDT) can generate surface acoustic waves, which, in turn, can actuate cells. In the tapered IDT design, a range of frequencies can excite SAWs, at different positions along the transducer, because the resonant wavelength, defined by the pitch of the electrodes, varies linearly along the transducer. The slope at which the IDT tapers determines the aperture of the SAW, by limiting the area of the transducer in which a given frequency resonates. The IDT is positioned directly adjacent to the microfluidic device's

sorting channel to increase the amount of power that gets transferred to the liquid, by minimizing the distance the SAW must travel before it refracts into the liquid in the channel, as shown in Figure 2a. The fingers of the IDT are situated beneath an air gap, to prevent the power carried by the SAW from leaking into the device prematurely. The flow channel of the microfluidic device contains micro-fabricated features – a slanted ceiling groove and a vertical flow-focusing nozzle – which enable the device to utilize the normal component of the acoustic wave. Their positions with respect to the IDT and the sorting channel as well as the air gap are shown in Figure 2b. The slanted groove generates a flow with a velocity profile that varies strongly with height;²⁸ within the slanted ceiling groove, fluid flows along the length of the groove, while the flow at the bottom of the sorting channel remains largely unperturbed. Consequently, it is the slanted groove, which harnesses the normal component of the acoustic wave, because the acoustic wave pushes cells to the top of the channel, where they interact with the flow within the groove. Thus, the groove ensures that the height at which a cell passes through the sorting channel determines whether it is discarded or retained. The slanted groove is presented in more detail in Figure 2c. The slanted groove must be paired with a vertical flow-focusing nozzle, to ensure that only target cells interact with the groove. The cell inlet channel of the vertical flow focusing nozzle is fabricated at a lower height than the channels carrying sheath fluid. When the sheath channels intersect the cell inlet, at the entrance to the sorting channel, the sheath fluid focuses the cell flow laterally and vertically into a narrow thread at the bottom of the sorting channel.²⁹ The sheath channels form a Y-shape with the sorting channel, which eliminates stagnation points just after the nozzle.³⁰ The nozzle is offset from the midline of the channel, so that variations in flow rate or other unexpected perturbations will not cause cells to enter the retention channel spuriously. The nozzle geometry is depicted in Figure 2d. In the absence of surface acoustic waves, a particle passing through the device does not interact with the ceiling groove; it transits directly through the sorting channel and out of the device via the waste outlet unperturbed. When a cell of interest is detected, a pulse of SAW is applied,

and the normal component of the resulting acoustic wave pushes the particle to the top of the sorting channel, where advection carries it across the sorting channel; this particle then exits the device through the retention outlet, where it can be recovered. In contrast with previous slanted groove microfluidic devices, which use arrays of slanted grooves to direct flows or particles,^{28,31} here, the interaction of particles with the groove depends on the application of the SAW pulse; the cell is deflected completely into the groove; and a single groove is sufficient to achieve the desired effect. The angle of the slanted groove creates a flow, which ensures that cells continuously flow out of the device as they are sorted, unlike trapping designs, in which the desired particles are pinned by the SAW behind a vertical barrier.²⁴ As a result, the combination of the slanted groove and vertical flow-focusing nozzle offers a device geometry uniquely adapted for rapid sorting of cells.

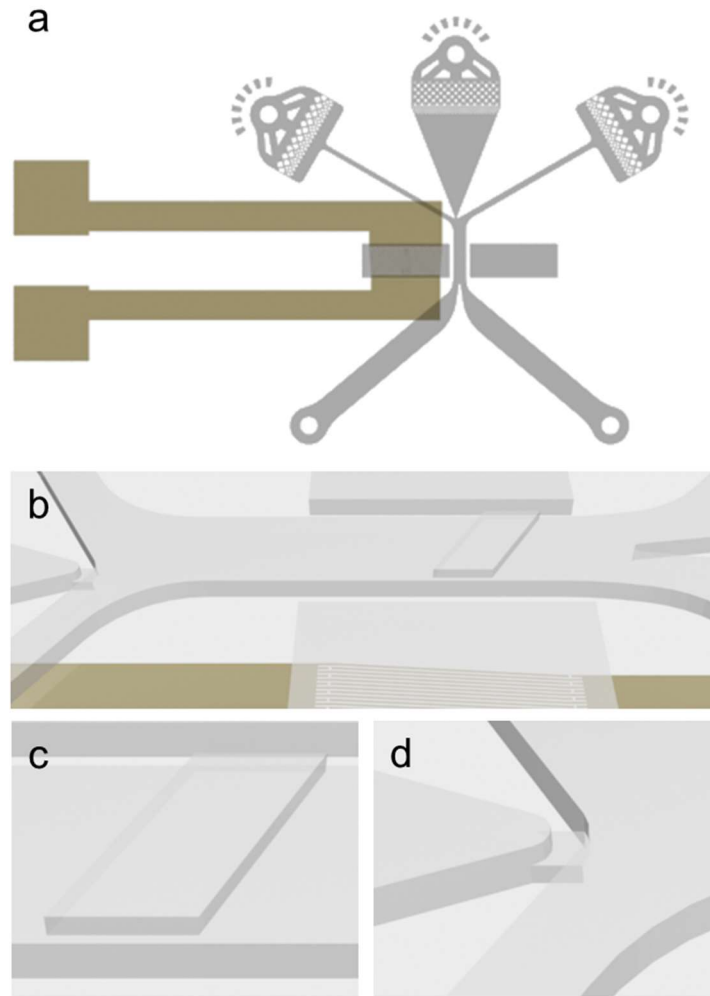


Figure 2: Schematic of the Groove-Enhanced Cell Sorting Design

Here, the relative positions of the flow channel (gray) and interdigital transducer (gold) are illustrated (a). Square metal pads (far left) connect to the fingers of the IDT through bus bars. The flow channel has a cell inlet (upper middle) and two sheath inlets (upper left and right) as well as a waste outlet (lower right) and a retention outlet (lower right). A zoomed in view shows the positions of the flow-focusing nozzle (left), the sorting channel (center), and the slanted groove (above the sorting channel) with respect to the fingers of the tapered IDT (bottom). The flow-focusing nozzle is located where the two sheath flows and the cell inlet meet. The channel bifurcates after the slanted groove. The upper channel leads to the retention outlet, while the lower channel leads to the waste outlet (b). Magnified views of the slanted groove and the nozzle design are provided in (c) and (d) respectively.

We test the capabilities of our slanted groove device for cell sorting applications, by determining the conditions for which an applied SAW pulse consistently succeeds at redirecting a target cell into the retention outlet. As a basis for testing, we use a device made from poly(dimethylsiloxane) (PDMS), with a vertical flow-focusing nozzle that is 50 μm wide and 25 μm tall; a sorting channel that is 250 μm wide and 50 μm tall; and a rectangular groove which is 120 μm wide, rises 25 μm above the sorting channel, and is tilted 60° from the overall direction of flow. The instrument triggers a SAW pulse whenever it detects a cell's fluorescence, and the trajectories of individual cells are captured using a high-speed camera. Each resulting movie is analyzed to extract the trajectory of the target cell; a given sorting event is deemed a success, if the desired cell exits the sorting channel through the retention outlet, or a failure, if the desired cell exits the sorting channel through the waste outlet. Cell tracks showing a non-fluorescent cell proceeding through the sorting channel to the waste outlet and a target cell being successfully pushed into the groove and subsequently traveling along the length of the groove to the retention outlet are shown in Figure 3. For each of the parameters tested, the threshold value for which the device performance becomes consistent is defined as the lowest value for which at least 90% of the measured events are successful. The threshold value for each sorting condition is determined on three independent days of experiment using a total of at least 48 individual events. These experiments assess the robustness and reproducibility of the technique under different conditions.

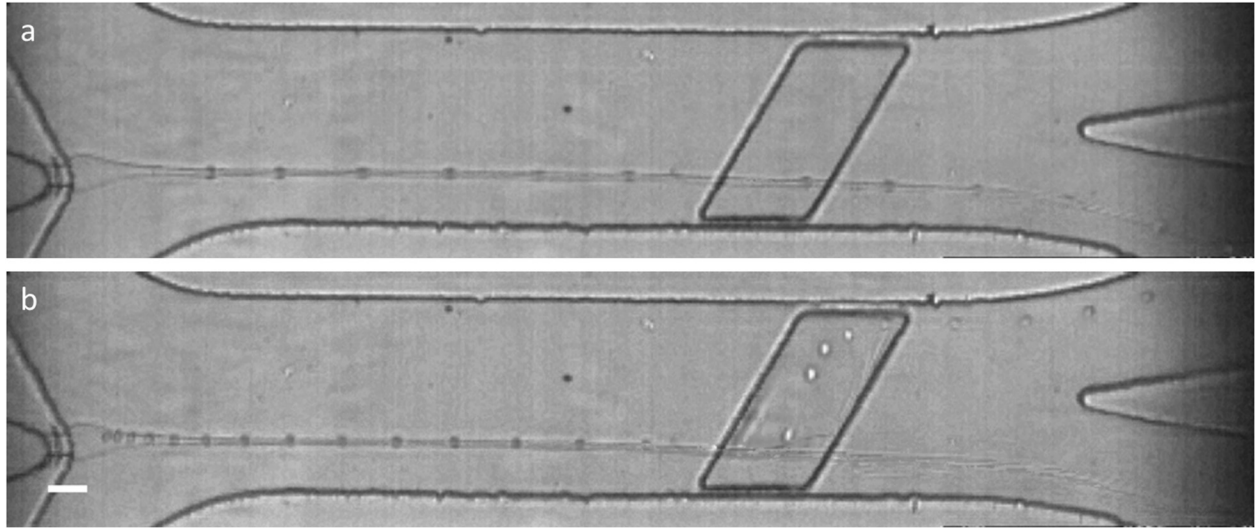


Figure 3: The Slanted Groove Channels Surface Acoustic Wave Actuation

Two single cell tracks are captured using a device with a slanted groove that rises 25 μm above the sorting channel. In one case, no sorting pulse is applied (a), while in the other a RF pulse of 38.26 dBm is applied for 100 μs (b). When no SAW is applied, the cell follows the same trajectory as the bulk of the cell phase fluid. The cell passes through the sorting channel and underneath slanted groove without deflection and exits the device through the waste channel (lower right). When the SAW pulse is applied, the cell is deflected into the slanted groove, where it is carried across the sorting channel by the flow of sheath fluid within the groove. The sorted cells moves laterally more than 150 μm and exits the device through the retention outlet (upper right). The cell phase fluid is visible, because of the index of refraction difference between the sheath phase and the cell phase that contains Optiprep. The cell tracks depicted here are projections of approximately 20 frames taken with a high speed camera at 11,267 fps. The scale bar represents 50 μm .

We determine the minimum length of the SAW pulse required for the slanted groove device to reproducibly actuate cells into the sorting channel for a range of different RF power levels and cell types. As the radio frequency (RF) power used to generate the SAW is increased, shorter pulses provide enough energy to redirect cells into the groove. Cells can be efficiently actuated with pulses as short as 20 μ s. For model adherent and non-adherent cell types, cells are reproducibly deflected into the groove with similar SAW pulse parameters, as shown in Figure 4a. However, one cell line consistently requires less energy for deflection than the other, which suggests that the two cell lines may have inherent differences in their average size or acoustic contrast. Nevertheless, the slanted groove device can actuate both adherent and non-adherent cells and the range of parameters, for which actuation is efficient, is compatible with high-speed sorting applications.

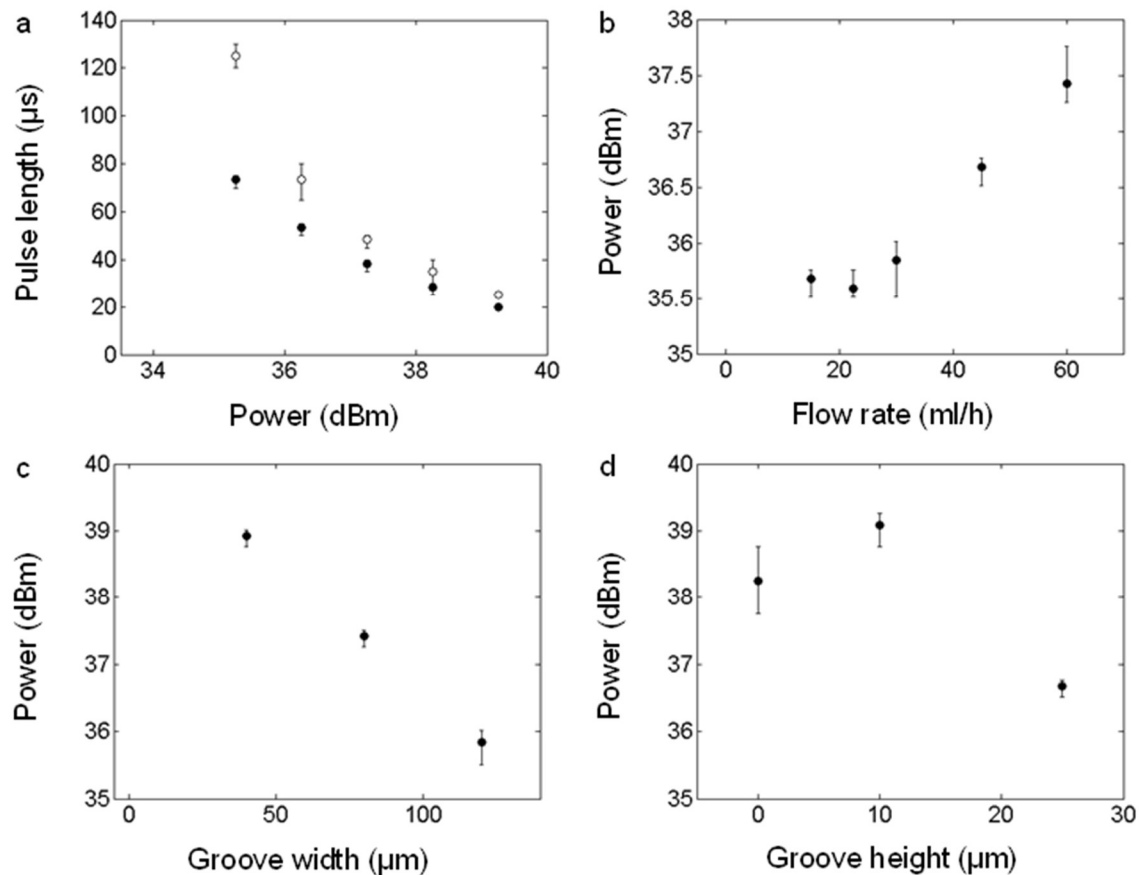


Figure 4: Cell-Sorting Performance of Groove-Enhanced Devices

Sorting devices with slanted grooves reliably actuate cells for a wide range of operating conditions. The symbols on each plot are centered on the mean of the three independent threshold values, while the error bars depict the full range of measured threshold values. For points without visible error bars, the marker size exceeds the extent of the error bars. The length of the pulse necessary to deflect a given cell type increases as the applied RF power decreases. The device actuates both adherent Madin-Darby canine kidney (MDCK; open symbols, ○) cells and non-adherent chronic myelogenous leukemia (K-562; filled symbols, ●) cells with performance levels sufficient to achieve high speed cell sorting (a). As the flow rate is varied, the threshold power required for sorting increases, except at the lower range of flow rates, where it appears there is a minimum amount of power necessary for sorting (b). The threshold power for sorting decreases linearly as the groove is widened (c). The threshold power changes non-monotonically as groove height is increased (d), but sorting takes place with the lowest required power for the tallest tested grooves.

We vary the total flow rate to determine the effect of average flow speed on the sorting process. For each flow rate, the length of the SAW pulse is constant at $50 \mu\text{s}$ and the ratio of cell phase to sheath

flow is also held constant, but we vary the applied power and record the minimum power, for which cells are successfully actuated consistently. At low flow rates, the threshold power and flow rate are essentially uncorrelated, but at higher flow rates, there is a clear correlation between flow rate and applied power, as the cell's deflection becomes limited by the duration of exposure to the acoustic wave pulse. The relationship between the threshold power of the SAW pulse and the overall device flow rate is depicted in Figure 4b. These results clearly show that cells can be redirected by the acoustic wave at a wide range of applied flow rates.

We quantify the impact of groove geometry on cell actuation in our design by varying the groove's width, height, and angle independently. Only one dimension of the groove is changed in each set of experiments and the threshold power required for sorting is measured, while the flow rate and the length of the sorting pulse as well as the other groove dimensions are all kept fixed. As the groove is widened, less power is necessary to cause the cell to interact with the flow within the groove, as demonstrated in Figure 4c below. While it is possible to sort cells using the vertical flow-focusing nozzle without any groove or with a very shallow groove, the best sorting results are attained with grooves fabricated at a height of 25 μm , shown in Figure 4d. Different groove angles show no significant effect on the threshold power required for sorting. There may be a slight variation due to changes in the effective aperture of the groove or because the flow speed is higher in the groove with the lower angle, but these are within the range of measurement error, and have very little effect on the threshold power required for sorting. Our results demonstrate that both the depth and the width of the groove, but not the groove angle, provide geometrical tuning parameters, which can influence the interaction of cells with the groove following SAW actuation.

The characterization experiments reveal a few limitations of the slanted groove device in its current form. Under standard conditions, the SAW cannot push every cell high enough to interact with the

groove reproducibly for power levels below 35 dBm. At flow rates exceeding 60 ml/h, the design of the nozzle becomes limiting, because cells are not effectively confined by the sheath flows from either side. There are also limits on the groove geometry; when the height and width of the groove are increased to 50 μm or 160 μm respectively, cells begin to enter the groove without any applied SAW to the detriment of the purity of the recovered fraction of cells. In spite of these limitations, there are a wide range of conditions for which the slanted groove device provides stable, reliable, and consistent operation, and the design shows promise for cell sorting.

We operate the groove-enhanced cell sorter, under realistic conditions, by sorting fluorescent cells from a mixture of cells. In each experiment, a reference library is prepared with known cell density and fraction of fluorescent K-562 cells. The slanted groove sorter extracts only the fluorescent cells. We operate the device with two different sheath flow rates and two different groove widths, to quantify how these parameters will affect sorter performance. The purified sample is collected and the recovered cells are imaged using a confocal microscope to obtain an independent measurement of cell purity. To elucidate the dependence of purity of the sorted fraction on the event rate at which we operate the sorter, we repeat this process for reference libraries with a range of cell densities at the same sample flow rate. The sorter is able to achieve high purity at low event rates, but the purity decreases, as the concentration of cells increases with a trend that appears linear, as shown in Figure 5. The overall trend fits to a line, which intersects the purity axis at 93% and which has a slope of -4.3% per kilohertz. Although this device uses relatively high levels of SAW power, the viability of the sorted fraction of cells remains high, greater than 96%. These results provide a baseline for the performance of the described sorting prototype; currently, the device can enrich samples at rates nearing 10,000 events/s and can reach purities of 89% at event rates of 1,000 events/s.

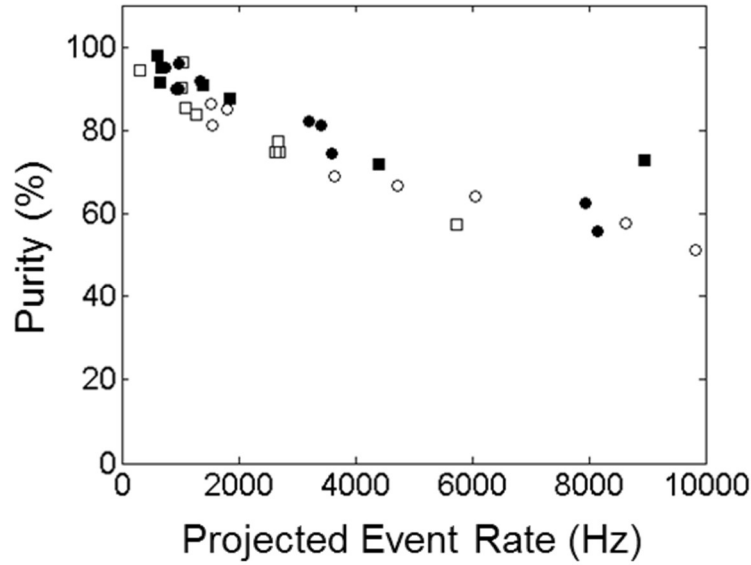


Figure 5: The Dependence of Cell Purity on Event Rate

The purity of each recovered sample is plotted relative to the event rate at which the sample is sorted. Filled symbols (■,●) are used for samples sorted with a 40 μm groove device, while empty symbols (□,○) represent samples sorted with the 80 μm groove. Square symbols (□,■) represent data gathered with total sheath flow rates of 45 ml/h, and circles (○,●) for sheath flow rates of 60 ml/h. All of the data sets follow the same general trend.

At first glance, the entire data set fits adequately to a line, but the fit averages out any effects that arise when operating the device under different flow rate conditions or using different groove widths. To determine whether different flow rate conditions and groove widths have any influence on the recovered sample purity, we bin the data first according to sheath flow rate and then by groove width, and examine the distributions of the residuals – the difference in purity between a given data point and the fit at that point. There is no clear difference between the purity for devices operated with different sheath flow rates, but the devices with a narrow 40 μm groove consistently extract higher purity samples than those with an 80 μm groove; this contrast is evident in the box plots shown in Figure 6. The observation that the narrowest groove provides improved purity suggests that the groove acts as a spatial filter; only cells that enter the groove are carried across the sorting channel to the sorting outlet, and cells only enter the groove, if they are aligned with the groove when the acoustic wave is applied.

This increases the likelihood that only the correct cell enters the groove. This effect offers a unique advantage compared to previous SAW sorting designs, in which the sorting purity can only be increased by changing the design of the SAW transducer or the operating flow rates.

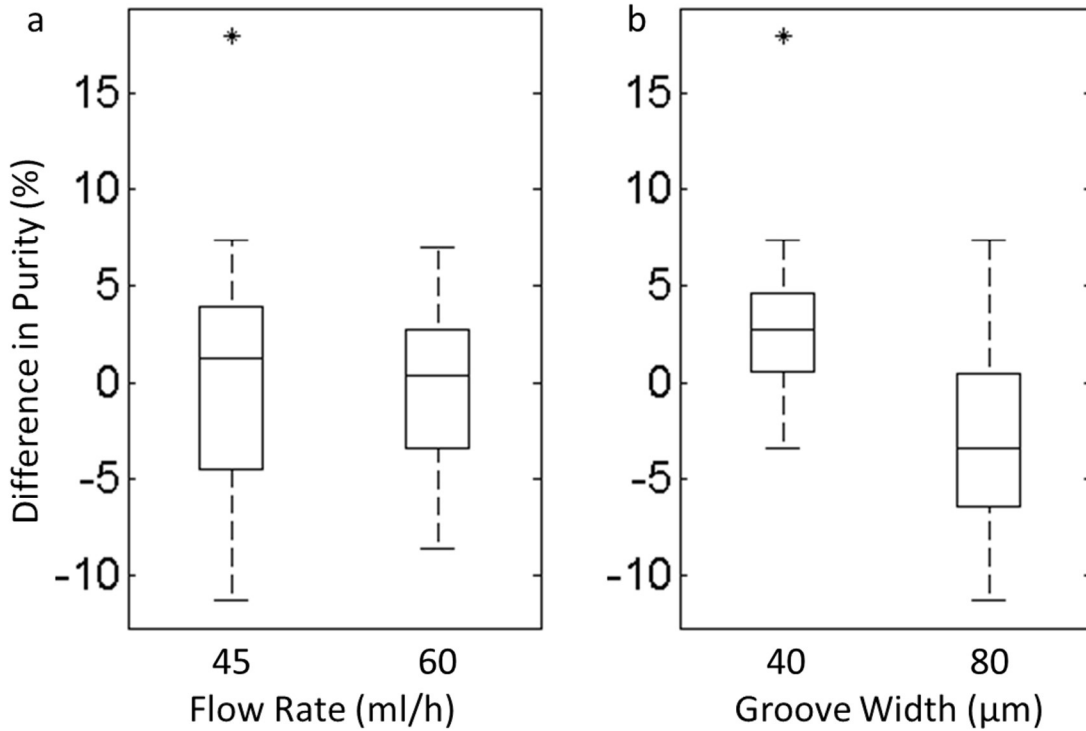


Figure 6: Residuals for Variations in Flow Rate and Groove Width

For each data point, we determine a residual value, the distance on the purity axis from a given data point to the linear fit. The residual data is associated into groups based on the device operating parameters, and distributions of the residuals from the overall trend line are plotted as box and whisker plots for each group. The data are grouped according to the total sheath flow rate (a) and the slanted groove width (b). The median values, represented by the lines in the center of the boxes, are essentially identical for the two different flow rate conditions, while the devices with the narrower groove produce samples that are about 5% more pure than the larger groove on average.

Conclusion

The slanted groove-enhanced cell sorter represents a new class of microfluidic cell sorters using SAWs to sort cells rapidly to high levels of purity. The design features a novel mechanism for channeling the

normal component of the acoustic wave into driving cell motion. The sorter operates at high rates, approaching those of commercial FACS instruments, and also features a high purity mode for recovery of enriched samples. Although alternate approaches exist, slanted groove SAW devices provide several clear routes to enhanced performance, because improvements in the designs of the IDT, the vertical flow-focusing nozzle, and the slanted groove itself can all be tuned and integrated to further enhance overall performance. For example, revising the nozzle design to reduce the spread in cell velocities would lead to increases in the sorting purity; inertial focusing devices could be used to align cells without the need for a multi-layer nozzle design;^{32,33} or fully sheath-less focusing techniques could be employed to eliminate the need for the sheath channels altogether.¹³ Likewise, the IDT design could potentially benefit through the use of a focusing geometry, so that the power density is spread over a wider area, to reduce the chance that the IDT becomes damaged during use. Like other microfluidic cell sorters, the fluid handling region is enclosed and aerosols are not produced by the acoustic waves in the system; therefore, the sorter could find application in screening bio-hazardous samples without the need for additional containment measures.^{1,8,13} The current design is fully disposable, but to completely eliminate the risk of cross-contamination, the IDT also needs to be discarded or sterilized. This issue can be solved by bonding the PDMS flow channel to a PDMS membrane with a micro-fabricated post that channels the SAW from the IDT into the flow channel where it can deflect cells.³⁴ In that design, the PDMS flow channel is fully disposable, while the IDT can be retained, all while maintaining sterile operating conditions. SAW cell sorters are also amenable to parallelization, in which multiple unit cells work together in parallel to produce a single sorter with enhanced aggregate performance. Each SAW unit cell requires only a few components, for example, a voltage-controlled oscillator, an RF switch, and an RF amplifier. By combining tens or hundreds of unit cells, a parallelized instrument could truly achieve unprecedented sorting rates. Moreover, the same SAW device platform is compatible with both cells and droplets,⁸ meaning a single instrument could provide users with both FACS and droplet sorting

capabilities. As a result, we believe the groove-enhanced SAW-actuated cell sorter offers a promising alternative to both traditional FACS instruments and other microfluidic sorters under development.

Experimental

Device Design

Drawings for both IDTs and microfluidic channels are created using AutoCAD (Autodesk, Inc., San Rafael, CA). The spacing of the fingers in the IDT design are chosen such that the resonant frequency varies linearly along the transducer between 161 and 171 MHz.^{34,35} Bus bars on either side of the IDT connect to square pads with 1.5 mm side length, through which external voltages are applied to all the IDT fingers with minimal resistance. Additional markings delimit each transducer so that the IDTs can be cut from the wafer into individual squares with 17.4 mm side length. The design is etched into a chrome mask (Photo-Sciences Inc., Torrance, CA) to ensure that the actual finger widths closely match the designed values. The microfluidic device has three layers, each fabricated using a separate lithographic mask. The first layer contains only the nozzle, because the nozzle is shallower than the rest of the device. The nozzle extends underneath both the cell inlet region and the sorting channel to ensure that the nozzle is insensitive to the alignment of subsequent layers. The nozzle is designed to be nominally 40 μm long, reducing the chance that cells will clog the nozzle. Most of the other features are on the device's second layer, including the air gap for the fingers of the IDT, the sheath and cell inlets, the sorting channel, and the device outlets. The third layer only contains the slanted groove, which is patterned on top of the sorting channel. The groove is drawn 230 μm wide, slightly less than the full sorting channel width, ensuring that even if the groove is slightly misaligned from the sorting channel, the wall of the channel where the acoustic wave encounters the liquid will not be distorted. Distortions of the channel wall could refract the acoustic wave at unexpected angles. Each layer contains at least two sets of alignment marks consisting of an asymmetrical pattern of crosses,³⁶ enabling different layers

to be aligned precisely to the same position. The masks for the individual microfluidic device layers are ordered from CAD/Art Services, Inc. (Bandon, OR) and imaged with a resolution of 25,400 dpi.

Transducer Fabrication

Interdigital transducers are fabricated using a lift-off process, described in the protocol from the Center for Nanoscale Systems at Harvard University.³⁷ The substrates are black lithium niobate wafers (Precision Micro-Optics, LLC, Woburn, MA) with 4 inch diameter and 128° Y-cut. Black lithium niobate is effective in SAW applications and exhibits less pyroelectric effect, making it easier to handle. The wafers are cleaned on the spin coater using acetone, then isopropanol, and spun dry. Residual moisture is removed with a dehydration bake at 180°C for 1 minute. We ease the rate of temperature change by placing the wafer on a hot plate at 115°C for 1 minute just prior to and immediately following baking at 180°C. Resist is dispensed onto the wafer using a disposable dropper. A layer of LOR3A resist (MicroChem, Westborough, MA) is added to the wafer surface; then, the wafer is spun at 4000 rpm to create a layer 300 nm thick. We bake the resist at 180°C for 4 minutes, using the same temperature ramping method as the dehydration bake. We then add a layer of Shipley 1805 (MicroChem, Westborough, MA) and spin that at 4000 rpm. This layer is baked for 1 minute at 115°C. The photoresists are patterned using the IDT chrome mask on a mask aligner (MJB4, Karl Suss, Garching, Germany). We develop the pattern by immersing the wafer in CD-26 developer (Microposit, Austin, TX) for 75 s to form a shadow mask for E-beam deposition. We rinse the wafer clean with water and blow it dry with nitrogen. We clean the exposed surface of the wafer using an oxygen plasma cleaner (SCE106, Anatech, Union City, CA) with 75 W of RF power and an oxygen gas flow rate of 40 sccm for 20 s. We deposit 10 nm of titanium as an adhesion layer, followed by 50 nm of gold using an E-beam evaporator (Denton Vacuum LLC, Moorestown, NJ) to form electrodes on the wafer surface. The photoresist is then lifted off by soaking the wafer in Remover-PG (MicroChem, Westborough, MA) at 80°C for about 60

minutes. We add a layer of Shipley 1813, and bake it at 115°C for 1 minute to form a protective layer. We score the patterned substrates using a dicing saw (Disco DAD321, Tokyo, JPN) to make cuts 250 μm deep in the lithium niobate. The wafer breaks cleanly along the scored lines, yielding up to 21 devices per wafer. The IDTs are cleaned with acetone to remove the protective layer prior to use.

Multi-Layer Soft Lithography

We perform multi-layer lithography to create molds for PDMS replicas as described in Appendix A. Each PDMS replica contains 16 independent devices; the replica is cut into individual flow channels prior to use. We use 0.75 mm diameter holes for the inlets and 1.5mm diameter holes for the outlets. Once the interface holes are formed, individual PDMS flow channels can be mounted into the sample holder.

Surface Acoustic Wave Sorting Apparatus

The sorting apparatus is the one described in the section describing the sorting instrument. Here, the sorting pulse is a TTL signal used to modulate the output of a waveform generator (SMB100A, Rohde & Schwarz, Munich, Germany) through its pulse modulation input. The output of the waveform generator is intensified using a high gain RF amplifier (LZY-22+, Mini-Circuits, Brooklyn, NY). The amplified RF signal drives the tapered IDT to generate the SAWs.

A custom-made sample holder supports the groove enhanced device. A printed circuit board (PCB) connects to the RF amplifier using an MMCX male card edge connector. The PCB is held in place by fixing it to a mechanical base plate using M3 screws. Electrical connections from the PCB to the IDT are created, when pogo pins mounted on the board are pressed into contact with metal pads on the surface of the transducer. An acrylic spacer milled to 3.7mm and laser cut to accommodate the mounting holes ensures that the pins exert enough force to hold the IDT in place and make consistent electrical contact, but not so much force that the substrate cracks under the stress. Each PDMS device is bonded to the substrate using a clamp to create a reversible bond. A 6 mm sheet of acrylic is laser cut to permit fluid

connections to pass through the sheet. The acrylic presses the PDMS onto the substrate using M2 screws to couple the acrylic layer to the baseplate. The PDMS replica contains three sides of the device's flow channel, while the lithium niobate substrate forms the bottom of the flow channel. Once assembled, the entire sample holder fits into the microscope stage.

Slanted Groove Characterization Experiments

Madin Darby canine kidney (MDCK) and human chronic myelogenous leukemia (K-562, ATCC, Manassas, VA) cells are harvested prior to each day's experiments. The MDCKs have fluorescent nuclei, having been stably transfected with green fluorescent protein fused to a nuclear localization sequence, while the K-562 cells are stained by adding calcein AM (Life Technologies, Grand Island, NY) to the cell suspension at a concentration of 1 μ M and incubating the suspension at 37°C for 20 minutes. The cells are re-suspended into injection buffer at between 5 and 10 million cells per ml. Injection buffer consists of 18% Optiprep (D1556, Sigma-Aldrich Co. LLC, St. Louis, MO) by volume, 6 U/ml DNase I (New England Biolabs Inc., Ipswich, MA), 3 μ M magnesium chloride (M4880, Sigma-Aldrich Co. LLC) and 1X phosphate buffered saline (PBS; P3813, Sigma-Aldrich Co. LLC).

Unless otherwise specified, the testing conditions are applied to a device with a slanted groove that is 120 μ m wide, and whose long axis is tilted 60° from the overall direction of flow. We use sheath fluids that consist of 1X PBS. The total sheath flow rate is typically 45 ml/h, with a quarter of the flow coming from the sheath inlet closer to the waste channel and three quarters of the flow from the sheath inlet on the retention side of the device. This ratio of sheath fluid ensures that the cell phase typically exits through the waste channel. The cell phase flow rate is 0.5 ml/h. The frequency of the RF pulse is usually kept constant at 163.1 MHz, but when the groove width is changed, then we vary the frequency to ensure that the SAW actuation aligns with the groove. Prior to testing the sorting performance for each distinct condition, we run control experiments to ensure that cells are not sorted, when the instrument

is triggered and no acoustic wave is applied. Each characterization experiment varies only one element of the standard conditions at a time, and each condition is tested on three independent days of experiment. As mentioned previously, fast movies of individual sorting events are analyzed to determine whether the cell is successfully deflected across the sorting channel into the retention outlet or not.

Slanted Groove Sorting Experiments

As detailed for the characterization experiments, K-562 cells are harvested from culture just prior to conducting the experiment. To create reference libraries of cells, we mix the sample of cells carefully and collect 10% of the cell suspension by volume. This fraction of the cells are stained with calcein AM at 1 μ M for 20 minutes at 37°C, while the remaining cells remain unstained. The two fractions are then combined and the cells are re-suspended in injection buffer at the target cell density.

The cells are sorted using a slanted groove sorting device. We use the standard nozzle geometry and an RF pulse with 38.26 dBm of instantaneous power and 100 μ s duration at 164.1 MHz. The flow rate of the cell suspension is kept constant at 0.5 ml/h. The device is operated with a range of cell densities to test different event rates. Over the entire cell density range, we measure the purity from devices operating at two different sheath flow rates, 45 ml/h and 60 ml/h, and using two different groove widths, 40 μ m and 80 μ m, under the same sorting conditions. Here, the sheath flow is also 1X PBS. The actual rate of fluorescent events is measured by the sorting instrument and the projected total event rate is obtained by dividing this by the measured purity of the initial reference library. We set the thresholds for sorting to ensure that pulses are only applied when we expect only a single fluorescent cell to be present in the channel, by ignoring the lower and upper extremes of fluorescence. In addition, when the sorting rate is high, we further limit the sorting thresholds to set the sorting rate below 500 events/s, reducing the chance that the IDT will be damaged irreparably.

The fluorescence of the cells recovered from the retention outlet is measured using a confocal microscope (SP5, Leica Microsystems Inc., Buffalo Grove, IL). In addition to using calcein to measure the proportion of labelled cells in the recovered sample, DRAQ5 (Life Technologies, Grand Island, NY) is added at a final concentration of 500 nM to label the DNA of all cells present in each sample. To measure cell viability after sorting, we add ethidium homodimer (Life Technologies, Grand Island, NY) to 2 μ M final concentration and incubate the cells for 20 minutes at 37°C. The images are analyzed using a custom Matlab (The Mathworks, Inc., Natick, MA) script to detect fluorescence in the three separate fluorescence channels. The purity of the sorted fraction is determined by determining the ratio of cells labeled with calcein to total number of cells, and the viability is determined as the complement of the ratio of dead cells to total cells.

From Cells to Droplets

The slanted groove cell sorter represents a novel class of microfluidic devices used for cell sorting.

However, cell sorting is a widely-used technique based on a well-developed, mature instrument, the fluorescence-activated cell sorter. At the moment, microfluidic devices cannot match FACS instruments based on performance alone, but they do offer additional novel functionality. In the specific case of microfluidic SAW sorters, this includes the slanted groove's ability to shape flows using micro-fabricated structures as well as features, such as sorting without producing aerosols and deflection of aqueous emulsion droplets in oil. Because of this combination of novel features, a SAW cell sorting instrument can bridge the gap between existing technology and newer microfluidic approaches, by enabling its users to sort cells as usual, while also providing access to new techniques, such as those based on the droplet format. This is only possible, because the design of the instrument is modular, so that different flow channels can be mounted to enable the same system to be tailored for a given cell type or for droplets of water-in-oil without requiring changes to the underlying apparatus.

While cell sorting is a well-established technique, emulsion-based droplet sorting is in its infancy, as a method for biological screening. Droplet sorting is in many ways a more general technique, because each droplet provides an isolated volume in which cells can grow and interact,³⁸ where the products secreted by a cell can remain associated with the secreting cell,^{10,39} or even for completely cell-free assays based on polymerase chain reaction^{40,41} or *in vitro* transcription and translation.^{42,43} Techniques, such as protein engineering for evolution of enzymes^{10,39} and antibody screening,¹² are compatible with the droplet format and may even have specific advantages in droplets. However, droplet sorting instruments have yet to find success on the commercial market. This may be because typical droplet sorting approaches lag FACS in event rate, but it also reflects that many researchers are unaware of the

potential advantages of the droplet format or do not have the tools to take advantage of them. To increase the utility of the droplet format, we developed a new method for rare event sorting with droplets, which should provide new capabilities for droplet sorting, and, in turn, should enable new applications for droplet microfluidics in research and clinical settings; this should help to speed the relevance and adoption of droplets for biological screening.

Rapid Enrichment of Rare Samples with Two-Stage Droplet Sorting

Introduction

Rare target cells can be isolated from mixed samples of cells using sorting techniques. The purified cells can then be grown, sequenced, or otherwise analyzed without the confounding effects of non-target cells. Fluorescence-activated cell sorting (FACS) is the most widely adopted approach to cell sorting; it achieves high purity and single cell resolution.¹ Microfluidic droplet sorting is a complementary technique because droplets can serve as stable compartments for biomolecules and biochemical assays^{40,41} as well as single cells^{12,44} or populations of cells.³⁸ Droplet sorting screens individual droplets, isolating those with desirable characteristics for further processing; target droplets can be extracted at high purity from samples containing tens of millions of droplets in about an hour. Often in biology, rare individuals within a population dominate the population's overall behavior. For example, adult stem cells are rare cells within an organ that play an indispensable role in the organ's growth or recovery from damage by multiplying and differentiating into other types of cells.⁴⁵ Vast sample libraries containing only rare targets arise in clinical samples, in which large volumes of patient samples are being tested for the presence of pathogenic microbes,⁴⁶ circulating tumor cells,⁴⁷ or specific nucleic acids,⁴⁰ and in directed evolution screening, where huge numbers of cells, each expressing a unique variant of a protein, are generated with mutagenesis techniques.^{2,10} In these types of applications, the initial sample may consist of a billion droplets and only contain fewer than ten target droplets; even the fastest droplet sorters would require nearly a day of continuous operation to completely screen such a sample. Fluorescence-activated cell sorters can enrich immense libraries containing few target cells using multiple rounds of screening, in which the instrument's operation is tailored for high screening rate in an initial round and high purity in subsequent rounds; even then the instrument requires several hours to screen a billion cells. A droplet-based method of isolating rare samples rapidly would provide a

powerful alternative to FACS; however, existing droplet sorting designs already operate at their highest screening rate and, therefore, do not benefit from a multi-round approach. A droplet sorting method, which implements a preliminary screening round capable of reducing an immense sample containing only rare target droplets to a level of purity sufficient for existing sorting designs to handle rapidly, would provide a way of achieving droplet-based rare event screening. Because droplet methods can be applied to a range of different sample types, a droplet-based rare event screening technique would enable new applications in biology and medicine that are not feasible with existing techniques.

Here, we present a rare event sorting technique, which can successfully extract desired droplets from a sample containing nearly a billion droplets. The technique yields pure samples after two rounds, and isolates samples based on fluorescence. The first round of sorting uses a design tailored for pre-sorting; pulsed actuation of a solenoid valve captures desired droplets at a rate of nearly 1 million droplets per second, but each valve actuation cycle also carries with it many other droplets. The second round of sorting enriches the sample to nearly 100% purity, using known designs for high purity drop-by-drop sorting. Thus, we devise a method, which can sort droplets rapidly and achieve high purity; the two stages together should be able to achieve an enrichment factor of over 1 million fold from a library containing 1 billion droplets in less than 1 hour.

Results and Discussion

The process of enriching a droplet sample only benefits from a preliminary screening round, if the additional round reduces the overall sample processing time. A preliminary round compensates for any additional handling steps, by screening the bulk of the sample at a much higher rate than a regular droplet sorter can achieve and by greatly reducing the number of droplets that must be screened at the low screening rate necessary for high purity sorting in subsequent rounds. As a result, the advantages of the preliminary screen will be most pronounced, when performing rare event screening, because most

of the sample can be safely discarded and only a few potential target droplets will be subjected to further screening. Our preliminary screening approach attains increases in screening rate, by pressing droplets closer together to pack more droplets into the sorter's detection region simultaneously. The number density of droplets in the channel is increased by flowing the droplets without additional spacer oil and increasing the overall emulsion flow rate to maintain the same average flow velocity. In addition, the detection channel is widened to enable multiple rows of droplets to be interrogated in parallel. The droplets remain stable, even when pressed into contact during flow, because the water-oil interfaces are protected from coalescence by surfactants in the oil phase.⁴⁸ Moreover, because the droplets are stable, the same droplets that are measured in the preliminary screening round can be recovered and analyzed in more depth in subsequent screening rounds. For 25 μm droplets, the pre-screening device can measure a packed emulsion with a sample flow rate of 100 ml/h; this flow rate is about 140 fold greater than that achieved in a serial droplet sorter.¹⁷ Thus, our preliminary screening round offers a screening rate increase of more than two orders of magnitude, because stable droplets can be packed together within the detection channel.

A novel microfluidic device is designed to implement a preliminary sorting, or presorting, round for droplet samples. Droplets enter through the emulsion inlet and flow through the detection channel to the sorting junction. The detection channel is made 500 μm wide to accommodate droplet samples flowing as a densely packed emulsion. The droplets entering the sorting junction encounter flows of fluorinated oil from two sheath inlets on either side of the junction, which press the droplets away from the walls on either side. Downstream, the sorting junction bifurcates into two outlet channels, the waste outlet and the retention outlet. Waste droplets exit the device through the waste outlet, while potential target droplets pass through the retention outlet. The overall device geometry is depicted in Figure 7a. The entire device is fabricated to have 50 μm height. This single layer design simplifies

fabrication of the device molds. The device is shallow to ensure that droplets will pass through the focal plane of the optical system. The optical system of the sorting apparatus is depicted in Figure 1. The presorting device utilizes the same optical system and electronics as a conventional droplet sorting device, since the design of the sorting apparatus accommodates any microfluidic device with the right form factor. A laser line is focused in the image plane of the optical system, spanning the full width of the channel, to detect the fluorescence of all droplets passing through the channel. When a desired droplet is detected, the data acquisition card generates a sorting pulse. This pulse triggers a three-way solenoid valve to switch the emulsion flow from the waste outlet into the sorting outlet; once the flow transitions, the target droplet is directed into the sorting outlet. When the pulse ends, the valve and the emulsion flow revert to their initial states. The normally open port of the three way valve is connected to the waste outlet; the normally closed port is connected to the retention channel; and the valve's common port is left unconnected. The valve connection scheme is shown in Figure 7b. More than a meter of fluorinated ethylene propylene (FEP) tubing is used to connect each valve port to one of the outlets of the microfluidic device, while a short length of tubing leads from the valve to a waste container. The difference in tubing lengths provides a large difference in fluidic resistance, which redirects the bulk of the fluid displaced during actuation of the solenoid valve out the common port, rather than into the microfluidic device.⁴⁹ In addition, the tubing serves as a sample reservoir for the sorted droplets; the sorted droplets are retained within the interface tubing, between the device and the valve, because only a small volume of sample is sorted. There is no risk of the desired droplets mixing with the waste droplets or being lost inside the valve, provided the tubing does not fill up; this is unlikely to happen, when the system is applied to rare event screening. After a round of preliminary sorting, the droplets captured into the tubing can be recovered for further analysis or subjected to further rounds of sorting.

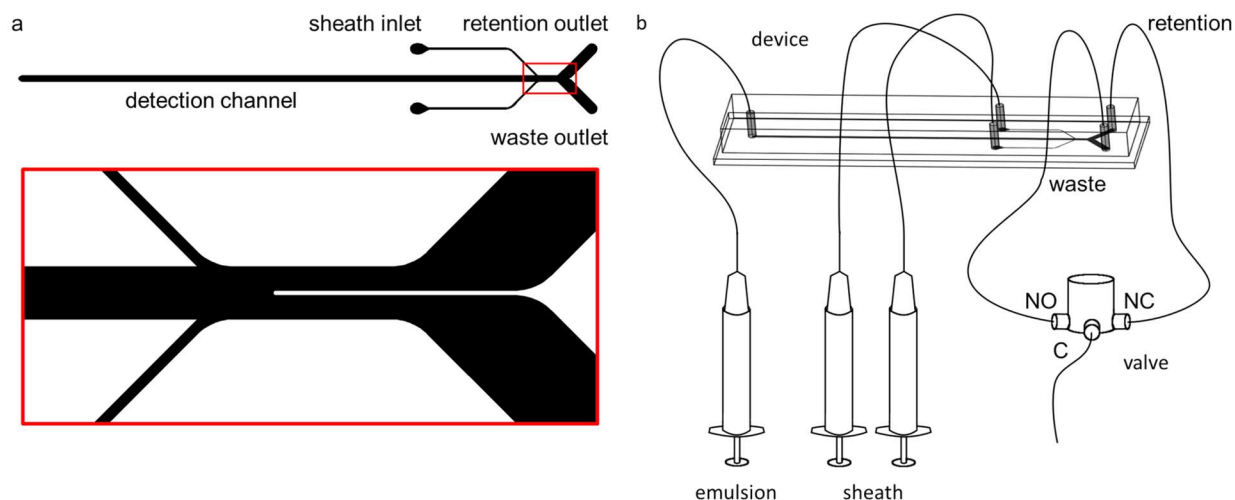


Figure 7: Droplet Sorting Apparatus

A drawing of the flow channel of the microfluidic device is shown in (a). Additional detail of the sorting junction (red rectangle) is shown in the inset. Droplets flow through the detection channel to the sorting junction; they can be diverted into the retention outlet or continue out the waste outlet depending on whether a target droplet is detected. The device's fluidic connections including the connection to the valve are shown in the diagram in (b). The emulsion sample is injected into the detection channel using a syringe. Sheath flows of oil with fluorinated surfactant meet the detection channel at the sorting junction. The channel splits into two outlets, a waste channel (lower right) and a retention channel (upper right), both of which are connected to the solenoid valve. Lengths of fluorinated polymer tubing connect the waste outlet to the valve's normally open port and the retention outlet to the valve's normally closed port. Waste samples exit the device through the valve's common port. The tubing forms a reservoir for the sorted droplets, but the lengths shown here are not to scale.

Valves provide a reliable method of controlling flows and are extensively use in microfluidic applications.^{50,51} There is even one example of a microfluidic method for valve-based sorting of rare cells;⁵² however, this method is intended to be a standalone way of isolating cells for downstream experiments and is not combined with a method for high purity sorting. Despite the existence prior work, our approach to valve-based switching has unique elements, including the way that we connect the valve to achieve sorting with a single external valve at a point inside the device, rather than inside the body of the valve. The use of the interface tubing as a low-volume collection vessel, and the application to rare event sorting of droplets at high speed are also novel. The use of external valves

reduces the complexity of the design of the disposable flow channel. The valve is easily cleaned, because the Teflon valve body never comes in contact with the aqueous sample. In addition, other approaches for fast droplet sorting such as dielectrophoresis and surface acoustic waves are not suited to sort groups of droplets flowing in contact. As a result, valves are one of the only ways that can be expected to consistently handle packed droplets samples at high flow rates. Our valve-based sorting approach uses reliable, commercially available components to achieve flow switching of droplets within the microfluidic device.

We capture the dynamics of the flow switching process, by recording movies of the sorting junction using a high-speed camera. We apply a valve actuation sequence intended to mimic rare event sorting, in which a sorting pulse of 50 ms duration is applied every 10 s. The signal that activates the valve also triggers the camera to ensure that the events are all measured with respect to the rising edge of the sorting pulse. There is a delay of nearly 5 ms between the time at which the electrical signal is sent to actuate the valve and when the flow starts to transition from one outlet into the other, and more than 10 ms elapse before the flow of emulsion completely transitions between the sorting outlets. Although the bulk of the emulsion transitions within tens of milliseconds, because the valve does not seal directly at the point where the channels bifurcate, a small fraction of the flow continues to trickle into the waste outlet until the pressure in this outlet matches the pressure in the flow at the bifurcation. Unlike other cell or droplet sorters, where the sheath flows are used for alignment or detection, here, they only serve to reduce the time the valve takes to switch the flow of droplets. The oil sheath flows ensure that the bulk of the droplets are redirected rapidly by the fast switching dynamics into the retention outlet, while the slow pressure equalization only affects the flow of the oil downstream. The same process happens in reverse, when the valve is switched off, except the falling edge flow transient is more than 20 ms long. A time lapse of a single full switching event is shown in Figure 8 below. The most important

transients, because they determine where sample droplets from upstream will go, are what we term switching transients. Switching transients describe the transition of the flow of emulsion from the detection channel between the downstream outlets, but the high-speed movies also reveal one additional type of transient flow during the switching process; we also observe leakage transients in which flow is displaced from one outlet channel into the other. These leakage transients occur, because the motion of the internal valve element displaces fluid, and a small amount of fluid is pushed into the outlet that is being sealed by the valve, and then exits the device through the other outlet. These leakage transients are comparatively small in terms of duration and flow rate, so we can compensate for them by ensuring that we keep the valve open long enough that the target droplets cannot leak out. Because the flow switching transients are not extremely rapid, the speed of switching sets a significant limitation on the performance of the sorting device, specifically, the number of potential positives sorted with each target droplet and the maximum rate at which positives can be sorted; however, these problems are mitigated to some extent, again because we are looking at samples containing only rare droplets. Our study of the valve's flow switching response suggests that the technique should work for sorting, but that the transient times are long, set by the time it takes for the flow to transition, in addition to the mechanical actuation of the valve.

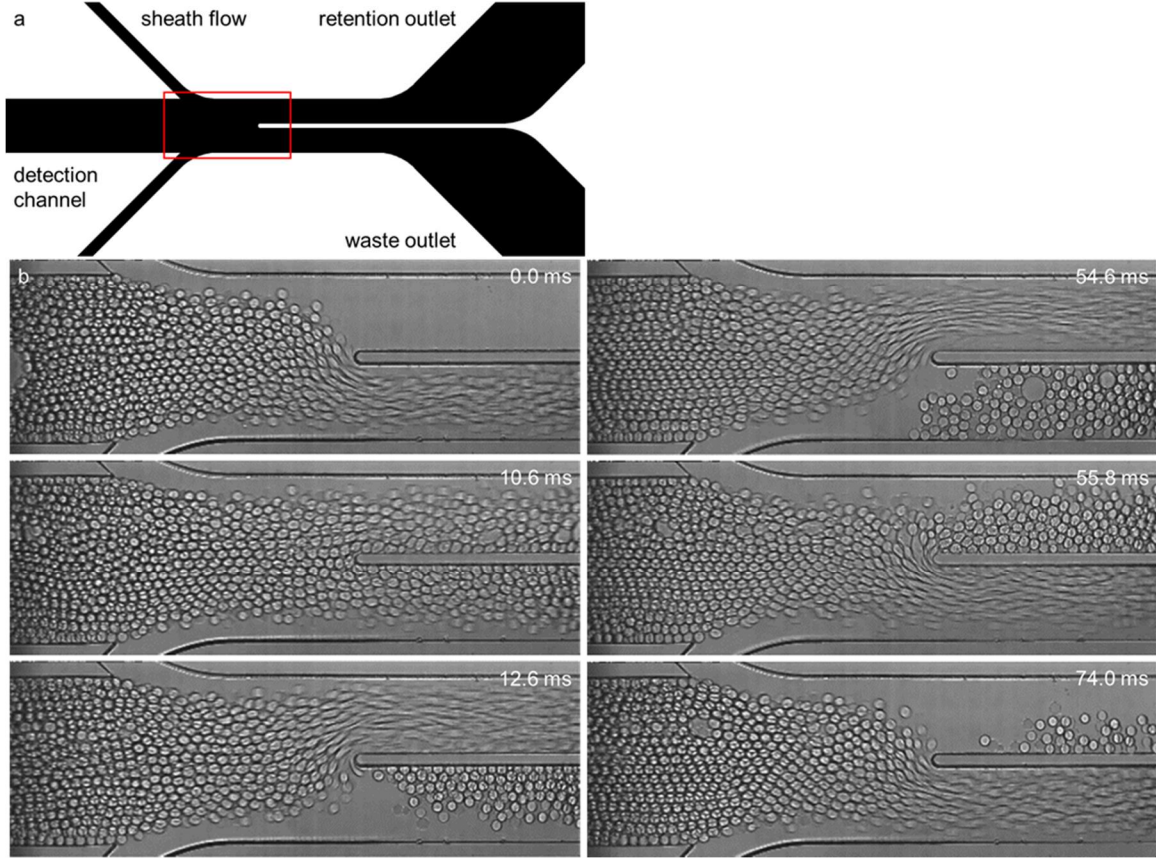


Figure 8: Sorting Dynamics and Flow Transient Behaviour

To determine whether droplet samples containing rare target droplets can be sorted with the valve, we trigger the valve with a pulse length of 50 ms and with 10 s between pulses. The same voltage pulse that activates the valve also triggers the high speed camera to capture a movie of the sorting junction. The region of interest is highlighted with respect to the device (a), by a red rectangle. Here, we observe a single actuation cycle in detail, to observe the flow switching dynamics. Immediately, after the valve is triggered, the flow of droplets leaves the sorting junction through the waste channel (lower right). After just a few milliseconds, we observe a flow transient corresponding with the opening of the valve, the droplets begin to flow into the retention channel (upper right), but droplets from upstream may enter either the waste or the retention channel. After about 12.4 ms, the valve opening transient is complete, all droplets from upstream enter the retention channel, but there is still a small leakage flow of droplets from the waste outlet into the retention outlet. This leakage flow slowly peters out until just before the valve closing transient becomes apparent. Just a few milliseconds later, at 55.8 ms after the trigger, the flow of droplets starts to transition back into the waste channel, marking the start of the closing transient. During this transient phase, a few droplets continue to flow into the retention channel and there is a small amount of flow between the retention and the waste outlets. The flow returns to its original state, with droplets flowing only into the waste channel, after about 74.0 ms.

The major consequence of the length of the switching transient is that the position, at which fluorescence is detected, must be spatially separated from the device's sorting junction by more than a field of view, as shown in Figure 9a. Because the average velocity of droplets passing through the device is greater than 1 m/s, the droplets may travel over a centimeter before the flow switches fully between the outlets. However, not every droplet flows at the average flow velocity; therefore, our design must compensate, not only for the average delay time, but also for the spread in delay times resulting from the dispersion of droplet velocities in the sample flow. We measure this delay for a sample of droplets by placing the detection point 22 mm upstream of the sorting junction, and adding a second optical system to make observations of the sorting junction. Due to physical constraints of the apparatus, this is the minimum separation we can access between the two optical systems. The secondary optical systems is a fluorescence microscope, which uses an LED to excite fluorescence at the sorting junction. The resulting fluorescence is split between a PMT, to determine the droplet arrival time with high temporal resolution, and a high sensitivity camera, to measure the trajectory of the observed droplet. The secondary optical system is depicted in Figure 9b. We create a reference library of droplets of $1:10^8$ and run the droplets through the device, while making measurements with both optical systems. We measure the average arrival time as 28.6 ± 5.0 ms; plots of the delay times are shown in Figure 9c and d. The measured delay times are used to create a valve sequence that should capture all target droplets. Sorting is triggered immediately after the droplet is detected at the detection point, and it is held open for 50 ms. The PMT measurements and a camera frame depicting a single sorting event using the optimized valve sequence are shown in Figure 9e and f respectively. Thus, by characterizing the dispersion of droplet velocities in our system, we are able to compensate for the droplet transit time between the detection point and the sorting junction using an optimized valve sequence.

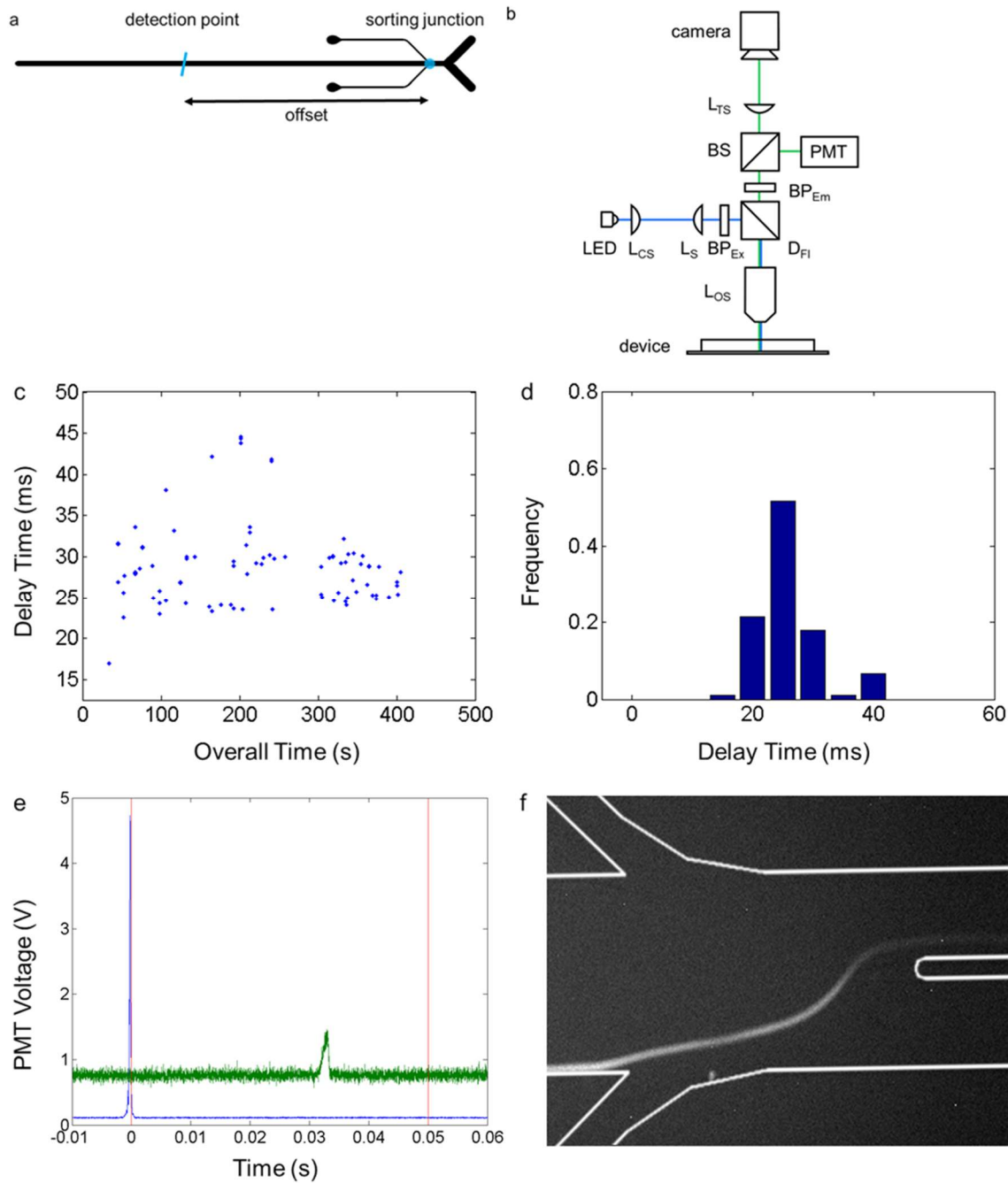


Figure 9: Measuring the Dispersion of Droplet Velocities

The sorting apparatus needs to be focused upstream of the sorting junction (blue circle) at the detection point (blue line) to account for the time required for the flow to switch from the waste channel into the retention channel. We indicate the approximate positions of the detection point and the sorting junction on a schematic of the device (a). We add a secondary optical system focused at the sorting junction for validation purposes (b). The light from a blue LED is collimated with a lens (L_{CS}) and then focused at the back aperture of a second objective

(Figure 9 continued) (L_{OS}) by a shaping lens (L_S). The light is cleaned up by an excitation filter (BP_{Ex}) and reflects off a dichroic filter (D_{FI}). This provides uniform illumination of the sorting junction. The fluorescence from the sorting junction is collected by the objective, is filtered by band pass filter (BP_{Em}) and then split into two equal components by a beam splitter (BS). Half the fluorescence goes to a PMT, which provides fast time measurements of the bright droplets, while the rest of the fluorescence is focused by a tube lens (L_{TS}) onto the sensor of a fluorescence camera, which captures the trajectories of fluorescent droplets. We run a dilute sample of fluorescent droplets through the device and measure the delay time between peaks on the primary and secondary PMTs. We plot the delay time over the course of the experiment (c) and as a histogram (d). We show the PMT data from a triggered valve sorting event using the chosen switching protocol. The primary PMT is shown in blue, the secondary PMT in green, and the applied sorting pulse in red (e). The corresponding camera image shows that actuation of the valve succeeds to deflect the valve into the retention channel (f).

To validate the device, we test its performance by sorting fluorescent droplets from reference mixtures of fluorescent and non-fluorescent droplets. Reference mixtures of 25 μm droplets are created by mixing droplets containing fluorescein with droplets containing PBS at a ratio of 1:10⁸ by volume. We perform triggered sorting experiments, in which the fluorescent droplets are extracted by the actuation of the valve following detection by the PMT. As before, the sorting apparatus is aligned at the detection point, 22 mm upstream of the sorting junction, while the secondary optical system is used to observe the sorting junction. Sorting events are deemed successes or failures based on whether the trajectories of the fluorescent droplets terminate in the retention outlet or the waste outlet respectively. In addition, we ensure that the target droplet does not leak between outlets during the leakage transient by looking at the frames following the initial droplet transit. We did not observe any droplets leaking between channels, when using the optimized valve sequence. We use control experiments to verify that when the valve is not triggered, droplets exit the device through the waste outlet, but when a droplet is detected and an optimized sorting pulse is applied, the desired droplet enters the retention channel, as shown in Figure 10 below. The sorting performance is in excess of 96% for the overall system based on a total of 91 independent sorting events from two independent days of experiment. If we eliminate cases in which the sorting did not succeed, simply because the droplet did not trigger a sorting pulse,

the sorting system has a success rate of nearly 99%. These results suggest that our method for droplet presorting works reliably.

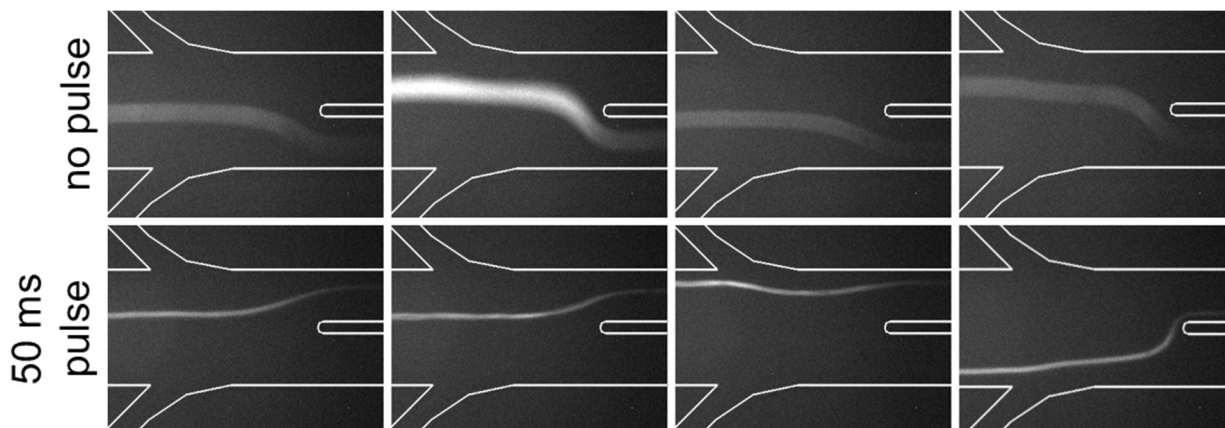


Figure 10: Droplet Streak Imaging for Verifying Triggered Sorting

We use a custom-built fluorescence microscope to image the sorting junction and determine whether target droplets are successfully sorted following triggered actuation of the valve. Droplets flow through the detection channel, where their fluorescence is measured by our sorting apparatus. If a bright droplet is detected, the valve can be actuated to capture the droplets. Here we display four representative streak images from independent sorting events showing that if no pulse is applied to open the valve (top row), the droplets exit the device through the waste outlet (lower right), but if a pulse is applied, the droplets are sorted (bottom row) and instead exit the channel through the retention outlet (upper right).

We measure the purity of the droplets recovered from a preliminary sorting experiment by collecting the droplets and running them through a secondary round of screening in a droplet-by-droplet device and counting both the number of bright droplets and the total number of droplets measured. In this experiment, we use a reference library in which the fluorescein concentration is low, but non-zero in the background droplets, to be able to distinguish them from the oil. Histograms showing the sorted sample in comparison with the positive and negative controls are shown in Figure 11. The purity of the sorted fraction after preliminary screening is approximately $1:1.4 \times 10^5$. This is close to the theoretical limit of the system set by the overall emulsion flow rate, the switching transient of the valve, and the volume of each droplet of 1 in 1.1×10^5 target droplets for our optimized valve sequence with 25 μm droplets.

Even a relatively slow serial droplet sorter can extract each desired droplet from a sample of one hundred thousand droplets in little more than a minute per positive droplet.

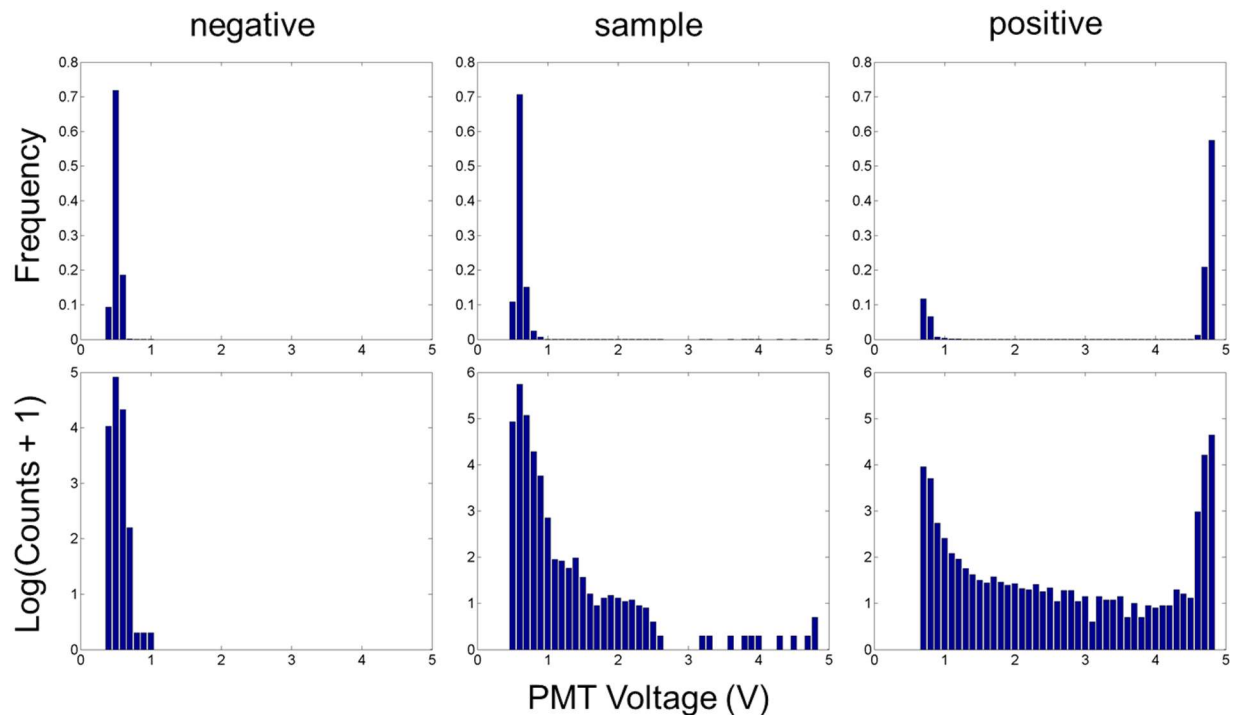


Figure 11: Sample Purity after Initial Sorting Stage

To estimate the purity of the recovered droplets after being sorted by the rare event droplet sorter, we run the droplets collected in the tubing of the retention channel into a droplet-by-droplet device to make fluorescence measurements of the droplets. In this sample, the background droplets have a low concentration of fluorescein, while target droplets have a high concentration of fluorescein. Histograms of fluorescence are plotted for a negative sample (first column), a positive sample (third column) and the actual sample (second column). Here we count the number of droplets that correspond with the positive sample and compare that with the total number of detected droplets to get an estimate of overall purity of $1:1.4 \times 10^5$ in the recovered fraction. We plot the histograms of both the frequency of any given measured peak height and also the log of the absolute counts, to show the overall shape and the distribution of rare events respectively.

Although our proposed presorting device is promising for rare event sorting applications, there are a number of limitations to consider. The biggest limitation of the device is that it cannot enrich samples that have a ratio of target droplets to total droplets that is bigger than the purity ratio for the sorted

fraction of droplets, one in one hundred thousand. For these samples, the droplets exiting the device through the retention outlet will not be significantly more pure than that exiting through the waste outlet, and so the presorting round offers no benefits. Another possible limitation is the volume of the collection tubing. Here, the tubing can hold about 80 target droplets and their associated potential positive droplets, before the two samples start to mix in the body of the valve. In addition, the switching transient is not completely insensitive to the volume fraction of emulsion in the collection tubing is filled. As it fills up, the transient time changes slightly, leading to less efficient sorting. The tubing interface is also limiting, because, occasionally, at the flow rates we apply, the tubing cannot be retained by the compression force of the device's punch holes, and the device begins to leak. The detection system also has at least one substantial limitation. The illumination profile of the laser line is essentially a Gaussian; droplets passing through different parts of the channel will receive different amounts of laser excitation. This reduces the ability of the system to measure the absolute fluorescence of individual droplets in the preliminary round, and creates a slight possibility for dim droplets to go undetected if they pass through the edges of the channel. In spite of all these limitations, our presorting device achieves performance levels that other droplet devices cannot match; it provides a unique capability for droplet-based rare event screening and can be combined with existing droplet sorters to enrich rare droplet samples.

Conclusions

Our novel device implements a droplet based presort. The preliminary round is fast, capable of screening 10 ml of droplets at 100 ml/h; for 25 μm droplets, this means sorting approximately a billion droplets at rates in excess of one million droplets per second. For 10 ml of emulsion, the presort takes less than 10 minutes, and the device sorts with a high success rate, over 96%. Based on the measured level of purity, our first round enrichment factor is about 1000 fold, but theoretically, the first round

enrichment factor could be increased as high as 10,000 fold for the volumes we use. As a result, the designed preliminary screening device provides a means of enriching rare droplet samples to a level, which is easy to handle with serial droplet sorters. The droplets survive reinjection into a droplet-by-droplet sorting device, and are collected with minimal losses. The sorting apparatus features modular flow channels that are applicable to both the valve-based preliminary round and high purity droplet-by-droplet sorting. The additional parts required for the preliminary sorting round are cheap and modular off-the-shelf components, which are readily available. Given the performance level of our preliminary round sorting, we suggest that overall enrichment factors as high as a hundred million can be achieved with a two round sort, based on the published performance of droplet-by-droplet sorting devices. Decreasing the valve switching transient will result in direct improvements in the sorting, by increasing the purity of the sorted fraction. Fast switching solenoid or piezoelectric valves⁵³ could be easily incorporated into the system, but adjustments would need to be made to accommodate differences in the fluidic interface and the valve internal volume. Flattening the illumination profile across the channel and adapting the detection optics to new kinds of assays such as fluorescence polarization or fluorescence concentration will broaden the capability and applicability of our technique. One promising possibility would be to parallelize the sorting by splitting the detection channel and making use of a multi-channel sensor and multiple sorting valves to increase the purity. There are also applications where the preliminary screen can be applied directly. At its most basic, the preliminary screen is a way of concentrating a desired droplet sample from 1 droplet per ml to 10 droplets per μL . In one practical example, the technique could be used to rapidly enrich fluorescent microbes for further analysis on plates, because, while 10 ml is too much volume to dispense onto a plate, microbes can easily be seeded from 100 μL samples. In summary, the development of a preliminary sorter yields a flexible method for the enrichment of rare samples that takes advantage of the strengths of droplet microfluidics and enables further applications in biology and medicine.

Experimental

Microfluidic Device Fabrication

We perform soft lithography to create devices as described in Appendix A. We use 1.5 mm diameter holes for the sorting device to accommodate 1/16" FEP tubing and 0.75 mm diameter holes for the droplet making devices. To screen the recovered droplet samples, we use a serial droplet sorter and make a 1.5 mm hole for the emulsion phase, and 0.75 mm holes for the oil phase and the outlets. After punching interface holes for the tubing, we bond the PDMS slabs to clean 2" x 3" glass slides using oxygen plasma. Droplet making devices are flushed with Aquapel (Aquapel Glass Treatment, Pittsburgh, PA) to create a hydrophobic surface suitable for water-in-oil emulsions.

Rare Event Sorting Apparatus

The bulk of the sorting apparatus is described in the section regarding the microfluidic sorting instrument. We use a 3-way solenoid valve (SV75P61T-2, Valcor Engineering Corp., Springfield, NJ) to sort droplets between two possible outlets. The TTL pulse from the DAQ card opens the valve for the duration of the pulse using an optocoupler circuit with a darlington output stage (PS2506-1-A, California Eastern Laboratories, Santa Clara, CA), which controls whether power from a 24 V supply is delivered to the valve. The valve fluidic connection is described in Figure 7b.

The secondary optical system apparatus is depicted in Figure 9. We use an objective (CFI Plan Fluor, 10X/0.3NA, Nikon Instruments, Inc.) with long working distance on this system so that it can image the device through the PDMS. Fluorescence is excited by an LED (M470L3, Thorlabs Inc.). The blue light is collimated by a lens (AC254-030-A-ML, Thorlabs Inc.) and focused by another lens (AC254-150-A-ML, Thorlabs Inc.) at the back aperture of the objective. The excitation light is cleaned up by a band pass filter (FF01-445/45-25, Semrock, Inc.), it reflects off a dichroic mirror (FF495-Di03-25x36, Semrock, Inc.) and onto the sample. The emitted fluorescence light passes through the dichroic mirror and a band pass

filter (FF01-520/44-25, Semrock, Inc.); the fluorescence light is then split by a beam splitter between two detection systems, a PMT (H10732-20, Hamamatsu Photonics, K. K.) and a fluorescence camera (Retiga EXL, QImaging, Surrey, BC). A tube lens (AC254-150-A-ML, Thorlabs Inc.) is used to focus an image onto the camera sensor. The whole optical system is mounted using a translation stage to an optical extrusion to provide focusing capabilities for the fluorescence microscope. The base of the extrusion is mounted to two translation stages so that the optical system can be aligned to the sorting junction without moving the device out of alignment with the primary optical system. During alignment, a green LED is used to illuminate the sorting junction.

Droplet Reference Library Preparation

Droplets samples are prepared using 25 μm droplet making devices. Positive droplets are made from 350 μM fluorescein salt solution in a single droplet making device. Background droplets of either PBS or 700 nM fluorescein salt are made using a droplet maker with ten flow-focusing junctions in parallel. The channels for pure oil and pure water are connected together for all the parallel devices, but the outlets for each device are independent. The devices are operated with a flow rate ratio of 1:3 oil to aqueous. Each flow focusing junction is capable of making droplets at flow rates of up to 1 ml/h for the aqueous phase and 3 ml/h for the oil phase using syringe pumps.

Reference libraries of droplets are created by serial volume dilution. The dilution steps are at most 1:1000, adding 1 μl of the concentrated sample to 1 ml of background droplets. For all dilutions, we reduce pipetting errors, by using pipettes near their maximum designed volume. Between dilution steps, we mix the sample for about 15 minutes on a rotating wheel mixer to provide thorough mixing.

Droplet Presorting Experiments

The emulsion sample and each sheath flow are driven using syringe pumps at 100 ml/h and 25 ml/h respectively. We start the flow of sheath fluid before the flow of emulsion to ensure that droplets will

not be pushed into the sheath channels. We use 10 ml syringes to hold the emulsion sample and the fluorinated oil used for the sheath. The oil consists of HFE7100 with a low concentration of surfactant, 0.05% by weight; the oil is filtered using 0.2 μm syringe filters. We then use the primary sorting apparatus to sort the desired sample.

For dispersion experiments, which require synchronization between the PMT data and camera data, we synchronize the two optical systems by turning on the LED on the secondary optical system at the same time as the flow of the emulsion sample. The light from the LED that leaks through to the secondary PMT and camera provides enough signal to determine whether the LED is on or off. We can then look for the rising and falling of the background levels in the data to synchronize the timing of the experiment in post processing. Individual droplet delay times are measured using a custom written code in Matlab (The Mathworks, Inc.).

After presorting, we close the valve and disconnect the collection tubing from the device. We then connect the FEP tubing to a luer lock adapter for $\frac{1}{4}$ -28 fittings and attach this to a syringe containing extra oil. The oil can be used to flush the droplets out of the tubing into the device. We then detach the tubing from the valve and connect it to the next device. Secondary rounds of screening use the primary optical system with the long working distance 10X objective. We run the device inverted (PDMS side down) to ensure that droplets float up into the device rather than being lodged inside the collection tubing, reducing the chance of sample loss. We use flow rates of 400 $\mu\text{l/h}$ for the emulsion phase and 4 ml/h for a flow of spacer oil. Droplet cytometry is performed using our sorting apparatus, and the resulting data is analyzed to count the peaks using custom Matlab (The Mathworks, Inc.) code.

Concluding Remarks

In this work, we have detailed two distinct approaches to microfluidic sorting. The two disparate approaches use a common platform to create new methods for sorting with biological applications. In many ways, this embodies the potential of microfluidics. The purpose of microfluidics is not simply to find a single killer application that will legitimize the field. What we are involved in building is the foundation of a new industry, by providing building blocks upon which more sophisticated systems can be based. Numerous fields have benefited immeasurably from development in the field of electronics, including powerful supercomputers, portable laptops and smartphones, and the staggering variety of microchips present ubiquitously in our laboratory instruments. These devices in turn are the result of years of research and development in electronics by various groups and their assembly into products by teams of engineers. We believe that microfluidics has the same kind of transformative potential for applications in healthcare, biology, and chemistry; however, only now are microfluidic devices really finding their way into research instruments. As with electronics, this process requires years of development. We do not claim to have the final solution, but we are grateful to play a role in advancing the field.

Appendix A: Soft Lithography

We perform photolithography to create molds for PDMS replicas, following the recommendations in the manufacturer's data sheet for SU-8 3000 series resist (MicroChem). For each layer, we dispense a small amount of resist onto a silicon wafer. We spin the wafer, typically at 3000 rpm, to create a layer of resist of the desired thickness. We pre-bake each layer at 95°C, until the solvent is fully evaporated, rotating the wafer on the hot plate every 6 minutes. We then align the mask to any underlying features and expose new features using a contact mask aligner (ABM, Scotts Valley, CA). The resist is then baked for 1 minute at 65°C and 5 minutes at 95°C. At this point, additional layers can be added on top of the previous ones. Once all layers are exposed and all baking steps are complete, we develop the features by immersing the wafer in polyethylene glycol monomethyl ether acetate for 5 minutes using an orbital shaker (Roto Mix 8x8, Thermo Fisher, Waltham, MA) to agitate the developer. After development, we rinse the wafer with isopropanol and blow it dry with nitrogen.

The wafer now serves as a mold for creating replicas in PDMS. We mix PDMS (Sylgard 184, Dow-Corning, Midland, MI) base and cross-linker in a 10:1 ratio using a Thinky mixer (AR-100, Thinky Corporation, Tokyo, Japan). The mixer runs in mixing mode for 30 s and degassing mode for another 30 s. We place the mold in a plastic petri dish and pour the uncured PDMS on top. We degas the PDMS for 10 minutes, then place the dish in an oven at 65°C overnight. Once the PDMS is cured, we cut around the edge of the wafer using a scalpel and lift the PDMS replica out of the mold. Interface holes are created with a biopsy punch (Uni-Core, GE Healthcare Life Sciences, Pittsburgh, PA). At this point, the PDMS replicas are ready to be sealed to a substrate and used.

References

- 1 H. M. Shapiro, *Practical Flow Cytometry*, John Wiley & Sons, Inc., 4th edn., 2003.
- 2 E. T. Boder and K. D. Wittrup, *Nat. Biotechnol.*, 1997, **15**, 553–7.
- 3 T. M. Squires and S. R. Quake, *Rev. Mod. Phys.*, 2005, **77**, 977–1026.
- 4 H. A. Stone, A. D. Stroock and A. Ajdari, *Annu. Rev. Fluid Mech.*, 2004, **36**, 381–411.
- 5 T. Thorsen, S. J. Maerkl and S. R. Quake, *Science*, 2002, **298**, 580–584.
- 6 Y. Xia and G. M. Whitesides, *Annu. Rev. Mater. Sci.*, 1998, **28**, 153–184.
- 7 T. Franke, S. Braunmüller, L. Schmid, A. Wixforth and D. A. Weitz, *Lab Chip*, 2010, **10**, 789–794.
- 8 L. Schmid, D. A. Weitz and T. Franke, *Lab Chip*, 2014, **14**, 3710–3718.
- 9 J.-C. Baret, O. J. Miller, V. Taly, M. Ryckelynck, A. El-Harrak, L. Frenz, C. Rick, M. L. Samuels, J. B. Hutchison, J. J. Agresti, D. R. Link, D. A. Weitz and A. D. Griffiths, *Lab Chip*, 2009, **9**, 1850–1858.
- 10 J. J. Agresti, E. Antipov, A. R. Abate, K. Ahn, A. C. Rowat, J.-C. Baret, M. Marquez, A. M. Klibanov, A. D. Griffiths and D. A. Weitz, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 4004–4009.
- 11 T. Franke, A. R. Abate, D. A. Weitz and A. Wixforth, *Lab Chip*, 2009, **9**, 2625–2627.
- 12 L. Mazutis, J. Gilbert, W. L. Ung, D. A. Weitz, A. D. Griffiths and J. A. Heyman, *Nat. Protoc.*, 2013, **8**, 870–91.
- 13 Y. Chen, A. J. Chung, T. H. Wu, M. A. Teitell, D. Di Carlo and P. Y. Chiou, *Small*, 2014, **10**, 1746–1751.
- 14 J. T. Merrill, *Cytometry*, 1981, **1**, 342–345.
- 15 G. M. Whitesides, *Nature*, 2006, **442**, 368–373.
- 16 S. H. Cho, C. H. Chen, F. S. Tsai, J. M. Godin and Y.-H. Lo, *Lab Chip*, 2010, **10**, 1567–1573.
- 17 A. Sciambi and A. R. Abate, *Lab Chip*, 2015, **15**, 47–51.
- 18 Y. Chen, T.-H. Wu, Y.-C. Kung, M. A. Teitell and P.-Y. Chiou, *Analyst*, 2013, **138**, 7308–15.
- 19 X. Ding, S.-C. S. Lin, M. I. Lapsley, S. Li, X. Guo, C. Y. Chan, I.-K. Chiang, L. Wang, J. P. McCoy and T. J. Huang, *Lab Chip*, 2012, **12**, 4228.

- 20 J. Shi, X. Mao, D. Ahmed, A. Colletti and T. J. Huang, *Lab Chip*, 2008, **8**, 221–223.
- 21 J. Shi, S. Yazdi, S.-C. S. Lin, X. Ding, I.-K. Chiang, K. Sharp and T. J. Huang, *Lab Chip*, 2011, **11**, 2319–2324.
- 22 J. Shi, D. Ahmed, X. Mao, S.-C. S. Lin, A. Lawit and T. J. Huang, *Lab Chip*, 2009, **9**, 2890–2895.
- 23 S. Li, X. Ding, F. Guo, Y. Chen, M. I. Lapsley, S. C. S. Lin, L. Wang, J. P. McCoy, C. E. Cameron and T. J. Huang, *Anal. Chem.*, 2013, **85**, 5468–5474.
- 24 D. J. Collins, T. Alan and A. Neild, *Appl. Phys. Lett.*, 2014, **105**.
- 25 X. Ding, P. Li, S.-C. S. Lin, Z. S. Stratton, N. Nama, F. Guo, D. Slotcavage, X. Mao, J. Shi, F. Costanzo and T. J. Huang, *Lab Chip*, 2013, **13**, 3626–49.
- 26 L. Y. Yeo and J. R. Friend, *Biomicrofluidics*, 2009, **3**, 1–23.
- 27 D. Morgan, *Surface Acoustic Wave Filters*, Elsevier Ltd., 2007.
- 28 C.-H. Hsu, D. Di Carlo, C. Chen, D. Irimia and M. Toner, *Lab Chip*, 2008, **8**, 2128–2134.
- 29 S. Chung, S. J. Park, J. K. Kim, C. Chung, D. C. Han and J. K. Chang, *Microsyst. Technol.*, 2003, **9**, 525–533.
- 30 M. B. Romanowsky, A. R. Abate, A. Rotem, C. Holtze and D. A. Weitz, *Lab Chip*, 2012, **12**, 802.
- 31 A. D. Stroock, S. K. W. Dertinger, A. Ajdari, I. Mezic, H. A. Stone and G. M. Whitesides, *Science*, 2002, **295**, 647–651.
- 32 X. Mao, J. R. Waldeisen and T. J. Huang, *Lab Chip*, 2007, **7**, 1260–1262.
- 33 A. A. Nawaz, X. Zhang, X. Mao, J. Rufo, S.-C. S. Lin, F. Guo, Y. Zhao, M. Lapsley, P. Li, J. P. McCoy, S. J. Levine and T. J. Huang, *Lab Chip*, 2014, **14**, 415–23.
- 34 R. W. Rambach, V. Skowronek and T. Franke, *RSC Adv.*, 2014, **4**, 60534–60542.
- 35 L. Schmid and T. Franke, *Lab Chip*, 2013, **13**, 1691–4.
- 36 *Stanford Microfluid. Foundry*, 2015.
- 37 S. Hickman, *Harvard Cent. Nanoscale Syst.*, 2011, 1–10.
- 38 D. F. Jarosz, J. C. Brown, G. A. Walker, M. S. Datta, W. L. Ung, A. K. Lancaster, A. Rotem, A. Chang, G. A. Newby, D. A. Weitz, L. F. Bisson and S. Lindquist, *Cell*, 2014, **158**, 1083–1093.

- 39 R. Ostafe, R. Prodanovic, W. Lloyd Ung, D. A. Weitz and R. Fischer, *Biomicrofluidics*, 2014, **8**, 041102.
- 40 K. A. Heyries, C. Tropini, M. Vaninsberghe, C. Doolin, O. I. Petriv, A. Singhal, K. Leung, C. B. Hughesman and C. L. Hansen, *Nat. Methods*, 2011, **8**, 649–651.
- 41 B. J. Hindson, K. D. Ness, D. A. Masquelier, P. Belgrader, N. J. Heredia, A. J. Makarewicz, I. J. Bright, M. Y. Lucero, A. L. Hiddessen, T. C. Legler, T. K. Kitano, M. R. Hodel, J. F. Petersen, P. W. Wyatt, E. R. Steenblock, P. H. Shah, L. J. Bousse, C. B. Troup, J. C. Mellen, D. K. Wittmann, N. G. Erndt, T. H. Cauley, R. T. Koehler, A. P. So, S. Dube, K. A. Rose, L. Montesclaros, S. Wang, D. P. Stumbo, S. P. Hodges, S. Romine, F. P. Milanovich, H. E. White, J. F. Regan, G. A. Karlin-Neumann, C. M. Hindson, S. Saxonov and B. W. Colston, *Anal. Chem.*, 2011, **83**, 8604–8610.
- 42 L. Mazutis, J.-C. Baret, P. Treacy, Y. Skhiri, A. F. Araghi, M. Ryckelynck, V. Taly and A. D. Griffiths, *Lab Chip*, 2009, **9**, 2902–2908.
- 43 A. Fallah-Araghi, J.-C. Baret, M. Ryckelynck and A. D. Griffiths, *Lab Chip*, 2012, **12**, 882.
- 44 E. Brouzes, M. Medkova, N. Savenelli, D. Marran, M. Twardowski, J. B. Hutchison, J. M. Rothberg, D. R. Link, N. Perrimon and M. L. Samuels, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 14195–14200.
- 45 M. Raff, *Annu. Rev. Cell Dev. Biol.*, 2003, **19**, 1–22.
- 46 D.-K. Kang, M. M. Ali, K. Zhang, S. S. Huang, E. Peterson, M. a. Digman, E. Gratton and W. Zhao, *Nat. Commun.*, 2014, **5**, 5427.
- 47 L. V. Sequist, S. Nagrath, M. Toner, D. A. Haber and T. J. Lynch, *J. Thorac. Oncol.*, 2009, **4**, 281–283.
- 48 C. Holtze, A. C. Rowat, J. J. Agresti, J. B. Hutchison, F. E. Angilè, C. H. J. Schmitz, S. Köster, H. Duan, K. J. Humphry, R. A. Scanga, J. S. Johnson, D. Pisignano and D. A. Weitz, *Lab Chip*, 2008, **8**, 1632–1639.
- 49 K. Churski, J. Michalski and P. Garstecki, *Lab Chip*, 2010, **10**, 512–518.
- 50 M. A. Unger, H. P. Chou, T. Thorsen, A. Scherer and S. R. Quake, *Science*, 2000, **288**, 113–116.
- 51 D. B. Weibel, M. Kruithof, S. Potenta, S. K. Sia, A. Lee and G. M. Whitesides, *Anal. Chem.*, 2005, **77**, 4726–4733.
- 52 A. Wolff, I. R. Perch-Nielsen, U. D. Larsen, P. Friis, G. Goranovic, C. R. Poulsen, J. P. Kutter and P. Telleman, *Lab Chip*, 2003, **3**, 22–27.
- 53 S. Jakiela, P. Debski, B. Dabrowski and P. Garstecki, *Micromachines*, 2014, **5**, 1002–1011.