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Micro vapor bubble jet flow for safe and high-rate fluorescence-activated cell sorting†

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Safe, high-rate and cost-effective cell sorting is important for clinical cell isolation. However, commercial fluorescence-activated cell sorters (FACS) are expensive and prone to aerosol-induced sample contamination. Here we report a microfluidic cell sorter allowing high rate and fully enclosed cell sorting. The sorter chip consists of an array of micro heating hotspots. Pulsed resistive heating in the hotspots produces numerous micro vapor bubbles with short duration, which gives rise to a rapid jet flow for cell sorting. With this method, we demonstrated high sorting rate comparable to commercial FACS and the significant enrichment of rare cancer cells. This vapor bubble based cell sorting method can be a powerful tool for contamination-free and affordable clinical cell sorting such as circulating tumor cell isolation and cancer cell therapy.

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Introduction

Sorting cells based on fluorescent labels is an essential step in numerous research and clinical applications, usually by fluorescence-activated cell sorting (FACS). In FACS, cells are encapsulated in droplets and electrostatically deflected into collection containers depending on measured fluorescence. Although in the past 50 years this sorting method has gradually become more capable, such as up to 100 000 cells per s sorting rate,¹ aerosols generated during FACS are a biosafety concern.^{2,3} Consequently, potential sample cross-contamination is a concern.^{4,5} FACS systems therefore typically need to be operated by highly trained staff in well controlled laboratories. As new diagnostic and treatment methods today demand sterile high throughput cell sorting, such as for rare cell isolation and cell therapy, there is an increasing need for a compact scalable cell sorting method.

Microfluidic cell sorting with closed actuators in disposable chips addresses the limitations discussed above. Previously reported microfluidic sorters typically rely on physical cell characteristics such as cell size, electrical impedance and compressibility to sort cells.^{6,7} These methods are less effective

at discriminating cell samples of minor morphological heterogeneity, such as small circulating tumor cells (CTCs)⁸ or lymphocyte subtypes. Thus, a generic microfluidic FACS platform is required, allowing high-throughput cell sorting compatible with the use of fluorescent labels for biomarkers. The recent developments include cell isolation by electroosmotic flow,⁹ piezoelectric jet flow,¹⁰ vapor bubble jet flow,^{11,12} or optical¹³ and MEMS switches.¹⁴ To date, the development of these technologies has been hampered by the relatively low sorting rate per single sorter, as well as difficulties in multiplexing due to the large sorter footprint and complex assembly method.

In this work we present a fast, compact, electrically actuated vapor bubble jet flow sorter. In contrast to vapor bubble sorters reported in the last decade which use a single vapor bubble by resistive heating^{11,12} or high power laser source,¹⁵ many micro vapor bubbles are simultaneously generated in our approach by resistive heating to generate a powerful and brief jet flow for robust high rate cell sorting. More exactly, we used a resistive metal heater comprising precision hotspots with high local current density areas during resistive heating. As such, the method generates many simultaneous micro vapor bubbles of reproducible volume, quantity and homogeneous lifetime. We first investigated the resistive heating process for the vapor bubble creation and collapse, and characterized the resultant jet flow for rapid cell sorting. Then we demonstrated sorting of fluorescent beads at high rates and the enrichment of rare cells. Finally, we assessed the effect of the sorting approach on cell viability.

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Materials and methods

Cell sorter fabrication and packaging

The cell sorter chip was fabricated on a 200 mm silicon wafer (Fig. S1†). First, 400 nm SiO₂ was deposited on the silicon substrate. Secondly, 400 nm Al was deposited and patterned to form the micro heating elements. To insulate the metal heater structure from liquid in experiments, 1.5 μm SiO₂ was deposited. A second lithography step was applied and SiO₂ was etched to open the bond pads. Subsequently, a photo-patternable adhesive material PA-s321 (JSR micro) was spin-coated to a 30 μm film thickness and patterned by photolithography in order to form the microfluidic channels. In the final step, glass dies with pre-fabricated in- and outlet holes were thermally bonded to the PA-channel by die-to-wafer bonding. The wafer was diced and the dies were packaged onto printed circuit boards.

Cell sorter experimental setup

The cell sorting experiment setup was partially constructed on a Zeiss Axio Examiner upright fluorescence microscope (Fig. 1). For illumination, a single LED light source (M505L3-C4, Thorlabs) was used as stroboscope in the vapor bubble and jet flow characterization measurements (Fig. 2 and 3). In all other experiments, two light sources were combined with a beam combiner and focused through a 10×/0.3 N.A

lens objective. The first light source was always a 532 nm CW laser (Z-laser Z10M18B-F-532-pz, 10 mW) for bead and cell detection. The light was focused in the middle of the microfluidic channel close to the jet flow generator to excite the fluorescently labeled cells for triggering of the sorter. The choice of the second light source was dependent on the exact experiment. For the high rate bead sorting test (Fig. 4, Video S3†), the second light source is another 532 nm CW laser, which was focused in the sorted cell outlet channel. For medium rate bead and cell sorting experiments (Table 1), the second laser was a 532 nm nanosecond laser (Newport Explorer 532-2Y) was used to visualize the sorting path of a bead or cell with a train of stroboscope flashes with a duration of less than 12 ns. This laser spot illuminated the outlet junction including jet flow generator nozzle. The emitted fluorescence was collected by the objective lens and passed a dichroic mirror.

In the fluorescence light collection path, a beam splitter was placed to split the fluorescent light to a CCD camera (Zeiss, AxioCam ICm) to visualize the path of a sorted cell and to two fluorescent color channels with PMTs (Hamamatsu, H10722) for cell detection. The data from the PMTs was sampled and processed on a FPGA (National Instruments USB-7855R). If a fluorescent cell passed the detection laser spot, a signal peak was detected. To filter out noise and small particles, a threshold was set and minimum width for a detection event. When a cell was detected in the selected color

Fig. 1 Schematic diagram of the microfluidic cell sorter including optical detection setup. (a) Photograph of cell sorter chip with schematic of optical detection setup. The optical setup includes two channel fluorescent detection, camera, CW laser and nanosecond laser as stroboscope for cell tracking. (b) Microscopic picture of sorter chip with in- and outlet junction and jet flow generation chamber with micro heating elements. The large and small heaters are design variations of which the small is not used during experiments. Only the large chamber is required for sorting. (c) Illustration of sorting method. Red cells are sorted with upper jet flow generator into lower outlet channel and green cells are flowing straight to waste outlet channel. (d) Scanning electron microscope image of several microheater elements inside JFG chamber.

Fig. 2 Vapor bubble creation and collapse. (a) Stroboscopic images of vapor bubble generation at micro heater. 1 of 32 micro heating elements with 8 vapor bubbles visible, resulting in 256 hotspots per jet flow generator. (b) Vapor bubble lifetime for 10, 30 and 100 μs resistive heating pulse width at 0.5 m s^{-1} flow speed and simulated demanded energy per heating pulse and time needed for the SiO₂-water interface to cool down to 298 K. (c) Simulated spatial temperature distribution of cross setting at aluminum micro heater to SiO₂ passivation layer interface (left) and water to passivation layer (right) at vapor bubble nucleation for 30 μs pulse. (d) Temporal temperature profile for 30 μs pulse at nucleation site of micro metal heater to SiO₂ passivation interface (blue) and SiO₂ passivation layer to water interface (red). Vast overhear was required due to SiO₂ insulation between heater and short pulse. (e) Temperature profile of chip cross section at nucleation site. The temperature within silicon and the metal heater was very homogeneous at every moment. Linear temperature gradient was discovered for the SiO₂ passivation layers due to low heat conduction. The water volume was only locally heated adjacent to the SiO₂ surface during the heating phase. When the heating pulse was turned off, the heat diffused to bulk water and also to the silicon substrate through the SiO₂-Al-SiO₂ layers. The nucleation event was not considered in all thermal simulations.

channel for target cells, a jet flow sorter pulse was fired after a certain delay on the falling edge of the peak (Fig. S2†). When necessary, the FPGA sent out a train of pulses to the nanosecond laser to visualize the cell path during a sorter event to calibrate the proper delay for the sorting pulse. A pressurized pump (Fluigent MFCS EZ) was used for the infusion of liquids. The sheath fluid consisted of DI water for all experiments except for the cell viability test where a PBS solution supplemented with 0.5% BSA was used. The flow rate and cell throughput, measured by the FPGA, were constantly monitored by the user during the experiment. In case of deviation from set points (mostly because of air bubble or bead clusters), the operator adjusted the pump pressure and the sorter setting in order to bring parameters back to set points.

Finite element analysis simulations

We performed two finite element modellings in COMSOL 5.2 Multiphysics. To study the heating of the jet flow generator, we coupled the heat transfer and electric current module in time-dependent simulations. The 3D stack of a single heating element with 8 hotspots is simulated with layer stack shown in Fig. 2e. A diffusive surface boundary condition with diffu-

sivity 0.8 was used for the glass top surface and silicon bottom surface. All other external surfaces were given a thermal insulation boundary condition. The temperature-dependent resistivity of Al was extracted from Desai *et al.*¹⁸ To create the resistive heating effect, an electric voltage with a pulse width of 10, 30 or 100 μs was applied to the total device resistance which included the simulated Al heater resistance and a serially connected temperature-independent peripheral resistance. The pulse width had 1 μs smoothness at both the leading and falling edge, which was similar to the actual driving circuit behavior.

To study the jet flow dynamics, we employed the CFD Module in 3D simulation. A 950 μm long inlet channel and three 315 μm long outlet channels connected to the sorting junction were modelled to provide sufficient room for jet flow visualization and limit the model complexity. We did not include the entire jet flow generation chamber in the model, but only the connection segment to the sorting junction. The CFD study consisted of two steps: first a stationary study and then a time dependent step. The stationary study was used to obtain the steady flow profile with preset pressure at the inlet and outlet ends as boundary condition without any jet flow input. The simulated flow profile was used as initial condition in the time dependent step. In this step, a subsequent

Fig. 3 Sorting event duration investigation with fluidic simulation and experimental verification. (a) Fluidic simulation of multiple bead trajectories for 50 μs jet flow duration and 0.6 m s^{-1} average flow speed from left to right. Particle release interval was 75 μs . Two particles were sorted by the jet flow: the first sorted by the push jet flow to the upper outlet channel and the second sorted by the pull jet flow to the lower outlet channel. (b) Fluidic simulation of jet flow deflected particle flow by 50 μs jet flow duration. The flow was injected reversely from right to left with 0.9 m s^{-1} average cell flow speed. The particle release interval was 7.5 μs . The sequential push and pull jet flow created an S-shape bead deflection. (c) Stroboscopic image of a dye flow with jet flow deflection. The dye flow was injected reversely from the outlet (right) to the inlet (left) of the fluidic chip at 0.9 m s^{-1} average flow speed. The jet flow pulse indicates the S-shape from the sequential push and pull mode. (d) Simulated and measured sorting event duration dependency on flow speed and jet flow duration (T_J). The 30, 50 and 170 jet flow duration resulted from 10, 30 and 100 μs resistive heating pulse, respectively. In addition to the simulated and measured data, the dwelling time at different flow speeds and 30, 50, and 170 μs lines are present as reference.

push and pull jet flow of equal power and duration through the jet flow generation chamber connection segment was applied. To study the jet flow duration, we released particles

upstream using the Particle Tracing module. The jet flow duration was the total time interval of particles which were deflected by the jet flow.

Fig. 4 High rate sorting test. (a) Fluorescent image of 4000 beads per s sorting with sorter off (left) and on (right). The first detection spot before sorting was in the central channel and the second spot after sorting was in the upper sorted outlet channel to count the amount of correctly sorted beads. (b) Jet flow sorter recovery for various bead rates for a mixture of two beads (red) and single type beads (blue). At higher bead rates, coincidence events reduced recovery.

Table 1 Sorting results at various initial bead purities and spiked tumor cells

	Test	Detection rate (events per s)	Purity before sorting	Total events	Recovery	Purity after sorting
~1:1 ratio beads	1	1210	41.7%	63 184	92.7%	86.1%
	2	1060	47.6%	435 916	95.4%	89.1%
	3	1240	41.7%	575 284	70.0%	90.9%
	4	1940	41.7%	811 880	83.1%	95.0%
	5	1830	40%	257 643	81.3%	93.0%
High ratio beads	6	303	0.4%	82 287	94.1%	95.9%
	7	359	0.4%	128 114	95.0%	91.5%
	8	371	0.038%	90 568	94.4%	97.1%
	9	769	0.016%	381 892	94.9%	98.9%
Spiked tumor cells	10	893	0.0033%	3 685 034	90.3%	45.2%
	11	909	0.0051%	3 374 873	86.5%	72.1%

Bead preparation

For jet flow duration and sorter performance analysis, Envys Green fluorescent beads of 3.87 μm diameter were purchased from Bangs Laboratories and Crimson Red fluorescent beads of 10 μm diameter were obtained from Life Technologies. For the sorter performance analysis these beads were mixed in different ratios and run through a 30 μm pre-separation filter (130-041-407, Miltenyi Biotec) to eliminate large clusters.

Characterization of sorting event duration

The jet flow duration was characterized with a reverse flow mode where dye was injected from the middle outlet and buffer through the two side outlets. Jet flow firing and stroboscopic illumination were timed by the user software with pre-set delay. By varying the delay, the deflected dye flow was visualized. The longitudinal length of the deflection was measured in the image and the flow rate was read from the flow meter. From this the deflection duration, representing the jet flow duration, was calculated.

Sorter performance characterization

To assess the sorter performance, the recovery and purity are investigated, where recovery is the fraction of sorted target cells compared to the total counted target cells and purity is the fraction of target cells to the total number of sorted cells. After a target cell or bead passed the detection point, a sorter pulse was fired after a preset delay and a push jet flow deflected the cell to a diverging outlet (Fig. S2†). The resistive heating pulse width was matched to the cell flow rate to be sufficiently long for sorting cells with slightly varying arrival time between detection spot and sorting junction. This variation of flow speed originated from the only lateral focusing of cells in our device. For this reason, a 30 μs resistive heating pulse width was selected for a throughput of around 1000 cells per s and 20 μs for 2000 cells per s and higher. The buffer flow rate was set to match the pulse width and the required inter-cell spacing. For the high speed sorting recovery investigation, a second continuous laser spot is placed inside the sorted outlet to count the amount of sorted beads. The fluorescent signal from these spots is spatially separated

into two PMTs, one detecting cells passing in the main channel before sorting and the other the sorted cells. The ratio of passing cells in both channels indicated the sorter recovery. Sorting is done in bursts to prevent overheating of the heater elements.

For the equal ratio sorting tests 1–5 in Table 1, cells from all three outlets were collected in a 1.5 mL Eppendorf tube. 10 μL liquid was extracted from every tube, placed on a glass slide and imaged with a Zeiss LSM 780 confocal microscope with a 10 $\times$ objective. The purity was calculated as the green bead count divided by the total count of beads from the sorted channel. The recovery was calculated as amount of target green beads divided by the total count of green beads from all three outlets. For the low ratio bead experiments rare sorting tests 6–9 in Table 1, the camera was set to continuous recording without synchronization to the sorting events. In order to visualize a sorting event with cell trajectory, a train of stroboscope flashes illuminated the sorting junction after a sorter event (Fig. 1). Frames were counted manually to extract purity and recovery. The camera frame exposure time was set to 300 ms and inter-frame delay was 450 μs . In other words, there was a 0.15% chance of missing cells in the video. This counting method was more accurate than microscopic bead counting, because it avoided the chance of losing beads in post-sorting bead handling. For the rare sorting tests 10 and 11 in Table 1, the same settings for the camera were used to count sorting events on video. On top of that cells were captured from all outlets in Falcon tubes. The collected cell emulsion was pipetted onto a 2 μm pore size sieve (Sieve-ID Spintube, Innosieve Diagnostics) and all liquid was filtered through it. The remaining cells were stained with Hoechst 33342 to visualize the cell nucleus and counted with the Zeiss LSM 780 confocal microscope.

Cell preparation for MCF-7 cells spiking in PBMC's

Whole blood from a healthy volunteer was drawn into 4 BD Vacutainer tubes (K3EDTA, Becton Dickinson) and subsequently diluted 1:1 with room-temperature PBS. 4 mL aliquots of the diluted blood sample were layered over 3 mL Ficoll-Paque PLUS (GE Healthcare) in 15 mL Falcon tubes and centrifuged in a horizontal rotor at 400 $\times g$ for 35 min at

19 °C. The PBMC fractions were pooled and washed twice at $200 \times g$ for 10 min at 19 °C to remove contaminating platelets. Following the second wash, the resulting cell pellet was re-suspended in 1 mL of PBS supplemented with 0.5% BSA and 2 mM EDTA. The MCF-7 model breast cancer line cells were obtained from ATCC (HTB-22) and maintained in culture in T-75 flasks in RPMI medium 1640 (ThermoFisher Scientific) supplemented with 10% heat-inactivated FBS and 1% PenStrep (ThermoFisher Scientific). Medium was refreshed twice a week. On the day of the experiment, the MCF-7 cells were detached from the flask with Trypsin-EDTA (0.25%, phenol red, ThermoFisher Scientific), washed twice at $300 \times g$ for 5 min at room temperature and re-suspended in 1 mL PBS with 0.5% BSA and 2 mM EDTA. The 1 mL aliquots of PBMC and MCF-7 cell fractions were transferred to fresh 15 mL Falcon tubes and 5 μ L of Vybrant cell-labeling stock solution (ThermoFisher Scientific) was added to each sample (Vybrant DiD to PBMCs and Vybrant Dil to MCF-7 cells). The samples were incubated at 37 °C in the dark for 45 minutes. The labeled cell suspensions were subsequently washed twice, re-suspended in PBS/0.5% BSA/2 mM EDTA and passed through 20 μ m pre-separation filters (Miltenyi Biotec). For experiment 11, the PBMC and MCF-7 cell samples were centrifuged for 5 min at $300 \times g$ and re-suspended in a solution of 1% paraformaldehyde (Affymetrix) in PBS/0.5% BSA/2 mM EDTA to fixate the cells. All samples were kept at 4 °C in the dark until further analysis. Filtered and unfiltered samples were counted with a Scepter™ 2.0 handheld cell counter (EMD Millipore) and concentrations were adjusted to $\sim 5 \times 10^6$ cells per mL for the PBMCs and $\sim 2 \times 10^6$ cells per mL for the MCF-7 cells. Depending on the planned MCF-7 quantity for spiking, the MCF-7 suspension was first diluted in buffer and 20 μ L of the diluted suspension was added into the PBMC sample. The cell mixture was filtered by the pre-separation filters (Miltenyi Biotec) just before loading to the fluidic system.

Blood samples were obtained from healthy donors with written informed consent and the study was approved by the Medical Ethical Committee of University Hospital of the University of Leuven (UZ/KULeuven), Ref. S57599. All experiments were performed in compliance with the relevant local laws and institutional guidelines.

Probabilistic analysis of sorter performance

To assess the performance of our sorter we compared it with theoretical maximum performance of an ideal sorter where cells passage is independent of each other and therefore follows Poisson statistics. This means that the arrival time between cell passages is exponentially distributed. To estimate the fraction of cells that arrive within a certain period we used the cumulative exponential distribution

$$P(t \leq T) = 1 - e^{-\lambda T}$$

where λ is the cell passage rate and T the period. To get an indication of the sorter recovery, the coincidence of target

cells is calculated with λ matching the target cell rate and T the sorter event period. The cell rate is extracted from the slope of the exponential curve of the cumulative inter arrival time plot to prevent errors from doublet cells counted as single cells.

Characterization of monocyte cell viability

PBMCs were extracted as described previously and further purified by magnetic-activated cell sorting (MACS, Miltenyi Biotec). The mononuclear cells were incubated with anti-CD14 microbeads and separated with MS columns (Miltenyi Biotec) on an OctoMACS system (Miltenyi Biotec). The purified monocytes were incubated for 15 min at room temperature in the dark with PE Mouse Anti-Human CD45 (BD Biosciences), as per manufacturer's instructions, and subsequently washed and re-suspended in MACS running buffer. Sorted cells were captured on the 2 μ m pore size sieve by placing the sorter chip outlet tube directly on top of the sieve which was pre-wetted with MACS running buffer. The reference sample consisted of the cells that were left over from the cells to be sorted. Afterwards, the cell-containing sieves were transferred and prepared for cell viability assay, which took approximately 1.5 hours. The sieves containing the collected sorted cells and reference cells were centrifuged and incubated with 5 μ L FITC Annexin V solution and 1 μ L of 100 μ g mL⁻¹ PI solution in 100 μ L of Annexin-binding buffer (FITC Annexin V/Dead Cell Apoptosis Kit with FITC Annexin V and PI, Life Technologies). The sample was incubated 15 minutes at room temperature in the dark. 300 μ L of Annexin-binding buffer was added and the top of the sieve was removed. The sieve was imaged with the Zeiss LSM 780 confocal microscope.

Characterization of U937 cell viability

The U937 monocyte model cell line was obtained from the ATCC and cultured according to manufacturer's specifications. An aliquot of the cultured cells was centrifuged at $300 \times g$ for 5 minutes, washed once and re-suspended in MACS running buffer. Cells were then labeled with PE Mouse Anti-Human CD45 (BD Biosciences), as previously described. After incubation, cells were washed once and run through 30 μ m pre-separation filter (Miltenyi Biotec) to eliminate any aggregates. The sorting experiment was performed as for the monocytes and cells were captured on the 2 μ m pore size sieve, stained with FITC Annexin V and PI and imaged on the Zeiss LSM 780 confocal microscope.

Results

Cell sorting principle and chip architecture

The jet flow cell sorter device (42×18 mm²) is composed of three layers (from bottom to top): a silicon substrate, metal micro heating elements for the vapor bubble generation and the microfluidic channels with different in- and outlets (Fig. 1 and Video S1†). When a sample is loaded, cells flow in *via* the central microfluidic inlet and are laterally focused by

sheath flow from the two side inlet channels. In the main microfluidic channel, a laser excites fluorescently-labeled cells that pass through the optical interrogation spot. The fluorescence signal is captured off-chip using a microscope objective and photo multiplier tubes to detect target cells for sorting before they arrive at the sorting junction (Fig. 1a). There, a jet flow generation (JFG) chamber filled with water resides on the side of the main channel at the crossing of outlets. When a target cell arrives at the JFG chamber, a short powerful electrical pulse is applied to flower shaped aluminum micro heaters inside the JFG chamber generating 256 small and short-lived vapor bubbles at thermally favored hotspots with highest resistive heating power. The collective creation and growth of vapor bubbles induces a liquid jet flow from the inside to the outside of the JFG chamber that quickly pushes cells to the farther outlet channel. After switching off the pulse, the vapor bubbles on the hotspots quickly shrink and finally collapse. While target cells are brought to a diverging channel, other cells go straight ahead to the middle outlet channel. This actuation mechanism creates a compact and enclosed cell sorter in which cells of interest can be collected for downstream analysis.

The cell sorter was fabricated in a standard 200 mm silicon processing line (see Materials and methods as well as Fig. S1†). Its metal heater is composed of 32 micro heating elements (Fig. 1b). Each element is a 400 nm thick Al conductor composed of 8 predefined hotspots (Fig. 1c and d), which sums up to 256 hotspots in total inside the jet flow generation chamber of $800 \times 800 \mu\text{m}$.² A homogeneous $1.5 \mu\text{m}$ thick SiO_2 passivation layer on top of Al isolates the heater from the liquid. On top of SiO_2 , there is a polymer layer forming the $30 \mu\text{m}$ high microfluidic channel which is finally closed with a glass cover. All optical fluorescent cell detection and analysis are performed off-chip. The delay between detection and electrical heating pulse is optimized to create a jet flow precisely when the cells arrive at the sorting junction.

Vapor bubble creation and collapse cycle

Previous attempts of utilizing vapor bubble jet flow for cell sorting have not achieved sufficient sorting rate for practical cell sorting,^{11,12} because of the long time needed for large bubble generation and collapse. Since micro vapor bubble lifetime was found to be proportional to its diameter,^{11,16} smaller micro bubbles are clearly more favorable for high speed cell sorting.

In our method, numerous micro vapor bubbles nucleate synchronously at predefined thermal hotspots in the micro-fabricated metal heater in order to deliver powerful jet flow (Fig. 1d). At each hotspot the conductor makes a sharp angle and is locally narrower ($2.8 \mu\text{m}$). As such, the hotspots are thermally favored for vapor bubble creation, because of the locally much higher current density and thus very high heating rate of $2 \times 10^7 \text{ K s}^{-1}$.

To inspect the vapor bubble lifetime during a resistive heating cycle stroboscopic illumination is used. For a $30 \mu\text{s}$

heating pulse, 8 uniform vapor bubbles emerged at every micro heater between 5 and $10 \mu\text{s}$ and continued to grow until $35 \mu\text{s}$ (Fig. 2a). Once the pulse was switched off, the bubble shrank and collapsed. The vapor bubble lifetime was $50 \mu\text{s}$ and its maximum diameter reached $8 \mu\text{m}$. The current chip design and metal mounting assembly allowed continuous bubble generation up to 4000 firings per s without active cooling. The power consumption of each bubble was $3 \mu\text{J}$, similar to previous reports with comparable vapor bubble size.¹¹ The vapor bubble lifetime and the amount of dissipated heat is proportional to resistive heating pulse width (Fig. 2b). Therefore for high rate cell sorting, a short resistive heating pulse with high heating rate is favored.

To better understand the vapor bubble thermal cycle dependence on chip geometry, the spatial and temporal temperature profile was investigated with finite element modeling (Fig. 2b–e). When vapor bubbles started to emerge between $5\text{--}10 \mu\text{s}$ for a $30 \mu\text{s}$ pulse, the temperature reached approximately 145 to 175°C at SiO_2 –water interface of a hotspot and 325°C in the micro heater (Fig. 2c and d). For the short heating pulse, vast overheat of the micro heaters at the hotspots was required due to the $1.5 \mu\text{m}$ thick SiO_2 insulation between heater and water. The heat absorbed by water during heating phase mostly diffuses back to the bulk silicon substrate through the SiO_2 –Al– SiO_2 layers during cooling phase (Fig. 2e). The low thermal conductivity of the thick glass cover acts as insulator while the high thermal conductivity of silicon facilitates quick heat dissipation to ambient environment.

The temporal simulation indicated that the thermal cycle was dominated by the cooling rate (Fig. 2b and d). In fact, during heating, the high resistive heating pulse was always sufficiently powerful to nucleate bubbles within a few micro seconds and then sustain the bubble growth, whereas the heat dissipation in the passive cooling phase was relatively less efficient. Clearly, a shorter but more powerful resistive heating pulse can effectively shorten the thermal cycle because it injects less total heat into water (Fig. 2b). In summary, the simulations demonstrate that the heating phase can more easily be controlled by adjusting the heating rate, while the cooling phase is determined by the chip architecture, ultimately limiting the sorter event rate.

Jet flow characterization

The ensemble creation of the vapor bubbles produces a pressure pulse in the JFG chamber and consequently a push jet flow into the main channel. The push flow will then sort a passing cell by deflecting its flow trajectory. Likewise, when the heating pulse is turned off, the bubble collapse produces a pull flow from the main channel back into the JFG chamber. Both the push and pull flow are able to sort a passing cell but to opposite outlets (Fig. 3a and Video S2†). The push and pull modes constitute a full cell sorting cycle, and the sorting event duration (T_s) is the sum of the push and pull mode's length in time. Since the cell deflection trajectory

results from the combined longitudinal cell flow and the transverse jet flow, T_s is determined by both the dwelling time (T_D) and the jet flow duration (T_J). The dwelling time T_D is the time needed for a cell to travel over the sorting junction, thus calculated as $T_D = L_D/v$, where L_D is the sorting junction length and v the cell flow speed. The jet flow duration T_J is characterized by the vapor bubble lifetime, as water is incompressible. The push and pull pressure is established as soon as the vapor bubbles emerge and collapse, and induces the push and pull flow as fast as the pressure wave travels inside the JFG chamber at the speed of sound ($<1 \mu\text{s}$ in our device).

We first studied the determination of the sorting event duration by computational fluidic dynamics (CFD) modeling for various cell flow speeds and various jet flow durations (Fig. 3b and d). For particular jet flow speeds (e.g. $170 \mu\text{s}$ jet flow duration), the CFD simulation showed two regimes for the sorting event duration: the cell flow speed determining regime at low speed and the jet flow lifetime determining regime at high flow speed. At low flow speed, the dwelling time T_D is longer than the jet flow duration T_J . The cell's trajectory is deflected by the jet flow when the cell is still traveling along the sorting junction. The sorting event is considered over after the cell has completely passed the sorting junction. Thus, for low flow speeds the sorting event duration is determined by the dwelling time (i.e. $T_s = T_D$ when $T_D > T_J$). By contrast, at high flow speed, the dwelling time is shorter than the jet flow duration. The cell is continuously deflected by the jet flow when it travels over the sorting junction. The sorting is considered over when the jet flow ceases firing. Thus, the jet flow speed determines the sorting event duration for high flow speeds (i.e. $T_s = T_J$ when $T_D < T_J$).

Secondly, we visualized the behavior of dye flow during jet flow firing to verify sorting event duration dependence on dwelling time and flow speed. The dye flow was reversely injected from the middle outlet channel and buffer flow from the two side outlet channels to set up hydrodynamic focusing. As such the full push and pull cycle was visualized downstream by stroboscopic illumination after the jet flow firing (Fig. 3c). The sorting event duration T_s consisting of total push and pull mode duration was measured for jet flow duration T_J of 30, 50 and $170 \mu\text{s}$ with the achievable flow speed range in our setup (Fig. 3d). The measured sorting event durations matched well with the CFD simulation. More exactly, the measured sorting event duration T_s was always equal to the dwelling time T_D up to 0.3 m s^{-1} flow speed, regardless of the difference in jet flow durations, because $T_D > T_J$ in this dwelling time determining regime. At higher flow speed, the sorting event duration for the $170 \mu\text{s}$ jet flow became significantly longer than the other two faster jet flows, because the $170 \mu\text{s}$ jet flow duration is longer than the dwelling time at cell flow speed $>0.3 \text{ m s}^{-1}$. In other words, it has run into the jet flow duration determining regime where $T_s = T_J$. By contrast, the short jet flow duration of 30 and $50 \mu\text{s}$ allowed them to stay inside the cell flow determining regime where $T_s = T_D$.

In summary, the sorting event duration is determined by both the cell dwelling time and the jet flow duration, whichever was the limiting factor. The jet flow duration determines the minimal possible sorting event duration and thus the highest possible sorting rate. The actual sorting event duration is also dependent on the flow speed setting, which allows the sorting event duration to stay either in the flow speed determining regime or the jet flow duration determining regime. The optimal flow speed should be set to allow the dwelling time to equalize the jet flow duration ($T_D = T_J$) to make full use of the entire push or pull jet flow power, i.e. around the cutoff flow speeds between the two regimes (Fig. 3d). The maximum sorting rate is the reciprocal of the sorting event duration. We recorded minimum $75 \mu\text{s}$ sorting event duration at 0.87 m s^{-1} average flow speed for $30 \mu\text{s}$ jet flow duration, equivalent to $13\,333 \text{ cells per s}$ sorting rate. Higher sorting rate is expected with higher flow speeds.

Sorting performance

The performance of the jet flow sorting method was evaluated by investigating the purity and recovery for bead and cell sorting at various throughputs and starting sample concentrations. Purity, a check for the quality of the sorted sample, represented the percentage of target cells out of the total sorted cell population, and recovery, a measure of sorting efficiency, represented the ratio of sorted target cells to total detected target cells.

We first investigated the sorting performance with two types of commercially available Crimson Red and Envy Green fluorescent beads. Three different conditions were examined: high rate sorting of pure green beads to determine the maximum sorting capability (Fig. 4); moderate rate sorting of equally mixed red and green beads (Table 1, test 1–5); and then low rate sorting of rare green beads in abundant red beads (Table 1, test 6–9).

For the high rate sorting condition, a solution of 100% pure green beads was sorted at rates ranging from 2500 to 7000 beads per s (Fig. 4 and Video S3†). Hydrodynamic focusing ensured all beads flowed into the central outlet channel without jet flow sorting, whereas most beads were sorted into the upper channel once sorting was activated (Fig. 4a). At the maximum sorting rate of 7000 beads per s, a 60% recovery was attained (Fig. 4b). At high sorting rate, beads arrived random at the detection spot, and therefore could pass the sorter junction when the previous sorting event was still not finished. These coincidence events, in which cells arrive too closely spaced to each other, were responsible for the reduced recovery.

Next, 5 experiments with green and red beads at approximately 1:1 ratio and 4 experiments at high contrast ratio were performed to investigate the sorter performance with beads rates between 1000 and 2000 beads per s (Fig. 4b and Table 1 test 1–9). This resulted in a sorting recovery between 70.0% and 95.4% and a sorting purity between 86.1% and 95.0%. In order to better understand the cause of the

unsorted beads and sorting impurities, we investigated in more detail the bead detection and sorter firing rate of test 4 (Fig. S3†). Beads arrived randomly at the detection spot, causing the cumulative arrival time between cell passages to follow the negative exponential distribution,¹⁷ generating a coincidence event for approximately 15% of arriving red beads. In these coincidence events, only the first bead was sorted while the following coinciding bead(s) were omitted. The amount of coincidence was in complete agreement with the measured 17% unsorted beads. Meanwhile, the red bead: green bead coincidence was also considered the main cause of impurity. In test 4, 4.3% of the jet flow firings were found to be accompanied by a green bead in the 50 μs wide push mode window. This evidently explained the 95% purity in the efforts of red bead sorting. Therefore, we believe the unsorted beads were due to the statistical random bead arrival time, rather than faulty jet flow firing. This means that the sorter operated close to theoretical maximum efficiency at a period of 200 μs , or 5000 events per seconds.

In order to mimic rare cell sorting, a very low concentration of target red beads was spiked into a very high concentration of green beads, and sorted on-chip at throughputs between 300 to 800 beads per s (experiment 6–9). The resulting recoveries (up to 95%) and purities (up to 98.9%) of the sorted red beads were consistently high. The sorter was capable of enriching the concentration of the sample more than 6000-fold without compromising on the recovery.

Lastly in an even more challenging test with biological samples, the ability of the sorter to enrich spiked tumor cells was investigated (Table 1 test 10–11). MCF-7 human breast adenocarcinoma cells and peripheral blood mononuclear cells (PBMCs) were fluorescently labeled with Vybrant Dil and DiD membrane stains, respectively. The MCF-7 cells were spiked at extreme low concentrations of 250 and 500 cells per ml into a 5×10^6 cells per ml PBMC sample and the mixture was sorted on chip. Sorted cells were captured in a vial, transferred onto silicon micro sieves and imaged by confocal microscopy. Subsequent analysis demonstrated that both samples were enriched 14 000-fold, displaying recoveries of 90.3% and 86.3% and purities of 45% and 72%, respectively. The reduced purity observed was mainly due to difficulties in rare cell harvesting and counting as can be seen in the difference between on chip detected tumor cells and total cells on sieve (Table S1†).

Cell viability analysis of sorted cells

In order to probe the potential impact of fluidic and thermal stress from the rapid jet flow on cell health, a cell viability test was performed. Human peripheral blood monocytes and the human pro-monocytic U937 cell line were fluorescently stained with PE-anti-CD45 antibodies and sorted in the cell sorter chip. The sorted cells were collected on a silicon micro sieve and stained with Annexin V, an early apoptotic cell marker and propidium iodide, a dead cell marker, to assess viability. Imaging of labeled cells revealed a cell viability of

Table 2 Viability of monocytes and U937 cells after sorting

Cell	Sample	Annexin V	PI	Both Annexin V and PI	
				Healthy	
U937	Sorted	5%	1%	11%	83%
	Control	12%	0%	9%	79%
Human monocytes	Sorted	14%	1%	11%	75%
	Control	10%	0%	9%	80%

75% for the sorted monocyte sample, only marginally lower than the 80% viability observed for the unsorted control sample (Table 2). The sorted U937 sample exhibited 83% cell viability, compared to 79% for the non-sorted control cells. These results suggest that sorting cells by rapid jet flow generation has only negligible effect on cell viability.

Discussion

We demonstrated a high speed microfluidic fluorescence cell sorting method using micro vapor bubble jet flow. The precisely fabricated micro heating hotspots allowed the generation of hundreds of small and homogeneous vapor bubbles with short lifetime (down to 30 μs) and hence rapid jet flow. The high sorting rate (more than 4000 cells per s) and the aerosol-free sorting style make the method suitable for applications requiring high speed, bio-safety and affordable sorting.

Several elements in the device were essential for rapid and reliable jet flow generation. The lifetime and lifetime uniformity of the bubble array play a central role in jet flow sorting. The bubble lifetime is mainly dependent on two factors: the bubble size and the heating and cooling efficiency. The bubble size is primarily defined by the hotspot dimension in the metal heater. The small hotspot structure for micro bubbles reported in this paper was located at sharp angled nodes with 2.8 μm narrow conductor. Therefore, the device fabrication precision is highly important to achieve the necessary heater accuracy for the desirable bubble size, size uniformity and ultimately the high jet flow speed. In addition to the bubble size, short bubble lifetime demands high heating and cooling efficiency for the nucleation-participating water layer adjacent to the solid surface. Therefore, the passivation layer was preferably thin for efficient heat transfer, but still thick enough to protect the heater from the aggressive attack due to bubble nucleation and collapse. In our device we used 1.5 μm SiO_2 passivation for the balanced heat transfer and protection, and silicon substrate for the high heat conductivity.

Compared with other microfluidic sorting mechanisms, the reported jet flow sorting principle is a generic cell actuation technique, not dependent on any physical cell characteristics. The jet flow sorter is simple to fabricate, integrate and operate. It does not contain any moving mechanical parts, nor requires any high power laser for bubble creation. Moreover, the compact $<1 \text{ mm}^2$ jet flow generation heater could be easily multiplexed on a single chip to increase throughput with parallel sorting. We believe these are the key enablers

for a practically useful microfluidic fluorescence-activated sorter beyond the efforts in prior art. The obviation of aerosol generation and the potential disposability of the device completely prevents sample contamination. Furthermore, the device structure is much simpler than conventional droplet-based FACS and can be readily mass manufactured by industrial chip production facility. Thus, the bubble jet flow cell sorting method opens the possibility of a compact, affordable, single use yet high throughput cell sorter. This method is well suited for clinical and research cell sorting such as CTC isolation, cell therapy and droplet sorting.

Author contributions

C. L. designed and characterized the jet flow principle; J. L. optimized the cell sorter fabrication process; K. dW. constructed the optical setup; B. M. fabricated the chips with supervision of D. S. T; D. V. contributed to the experimental setup; K. B. contributed to the development of the driver circuit; A. D. prepared the cells for the rare cell sorting tests and cell viability test; K. dW. and A. D. performed the cell viability test; C. L. and K. dW. performed the high speed sorting experiment; L. L. oversaw the project; K. dW. and C. L. wrote the manuscript with contributions and advice from all other authors.

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