Electronic Supplementary Material (ESI) for Lab on a Chip.
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Efficient cell pairing in droplets using dual-color sorting

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Supplementary material

Materials and Methods

Optical setup

The optical setup (Fig. S2) consisted of an inverted Microscope (Nikon Eclipse) mounted on a vibration-dampening platform (Thorlabs GmbH). 405 nm (20 mW; Dragon Lasers), 488 nm (20 mW; Melles Griot) and 561 nm (20 mW; Dragon Lasers) diode lasers were mounted on the platform. A high speed digital camera (Eosens mini1; Mikrotron) was mounted on top of the microscope camera port to capture the images during droplet production and sorting. The emitted light was separated from the laser beams by a 488/532/638 nm wavelength transmitting dichroic beam splitter (DBS; Semrock Inc.), passed through 450 nm (± 20nm), 520 nm (± 19nm) and 610 nm long pass filters (AHF) and collected in Photomultiplier tubes (Hamamatsu electronics). Data acquisition and control was performed by a PCI-7831R Virtex II FPGA card (National Instruments Corporation) executing a program written in LabVIEW (National Instruments Corporation). The data acquisition rate for the system was 50 kHz for each fluorescence channel.

Microfluidic chip design and fabrication

All chips were designed in AutoCAD. For the droplet generation chip (Fig. 2A), the nozzle was 100 µm × 75 µm (height × width). For the 45 degree sorting chip (Fig.

2B), the size of the restricted sorting channel was 40 μm × 40 μm (height × width) × 475μm (length). The main channels before the restriction channel were 75 um in height and width.

For the collection chip (Fig. 2F), the height of the low layer chamber was 40 μm and the size of the upper layer trap was 100μm in diameter × 100um in height. All microfluidic devices were fabricated using standard soft-lithography [3]. Molds were fabricated on silicon wafers using SU-8 resist (Microchem) and patterned by exposure to 375 nm light through 25400 dpi patterned masks (Suess). A mixture of 90% Polydimethylsiloxane (PDMS) elastomer (Sylgard 184 polymer base; Dow Corning) and 10% (w/w) curing agent (Dow Corning) was poured over the SU-8 molds, degassed and incubated at 65 degree overnight. Polymerized PDMS was peeled off from the mold activated by incubation for 1 min in an oxygen plasma oven (Diemer Femto) and bound to a 50 × 75 × 0.4 mm ITO glass (Delta Technologies). Inlets and outlets were punched using 0.5 mm diameter biopsy punches (Harris Uni-Core) for electrodes and 0.75 mm diameter biopsy punches for the rest. The channels were first flushed by Aquapel (PPG Industries) and, subsequently, by HFE7500 oil (3M).

Cell cultivation and encapsulation

Her2 Hybidoma cells (ATCC® CRL-10463) were grown in complete DMEM medium (Gibco), Jurkat cells (ATCC® TIB-152) were grown in RPMI medium (Gibco), both supplemented with 10% FBS. Hybridoma cells were harvested, stained by Calcein-AM (Lifetechnologies) and Calcein Violet (E-bioscience), respectively, at room temperature for 45 min, washed by PBS twice to remove free dye in the media , and re-suspended in free style media (Gibco) supplemented with 1 mg/ml xanthan gum (Sigma) to prevent cell sedimentation during encapsulation. Subsequently, green and violet cells were mixed equally at a final concentration of 1.5×10^6 cells/ml and injected at a flow rate of 1000 μl/h into the droplet generation chip. Droplets were generated by flow focusing this continuous phase using Novec HFE7500 oil, containing 5% PEG surfactant[3] (custom synthesized at Sigma Aldrich), at a flow rate

of 4000 ul/h. Emulsions were collected in a collection tube (cryotobube, Nunc) which was treated with Aquapel (PPG industries) and, subsequently, rinsed by HFE7500 oil.

Sorting and Imaging

Emulsions were re-injected using an electro-osmotic pump (Nano Fusion Technologies) at a flow rate of about 60 µl/h. Oil with 0.5% and 0.25% of PEG surfactant were loaded in syringes individually and injected by Harvard Apparatus PHD 2000 syringe pumps at a flow rate of 400 µl/h (Fig.2B, (a)&(c)) and 600µl/h (Fig.2B, (d)) respectively . A refilling pump was connected with outlet E (Fig.2B, (e)) to withdraw all of the droplets that did not trigger sorting to the waste syringe at a flow rate of 760 µl/h. Droplet sorting videos were acquired at ~500 frames per second. A customized LabVIEW sorting program was used to control the droplets sorting. The positive droplets were collected in the collection chip (Fig. 2F-H) and the trapping events were monitored on a cell imaging device (CytoMate Inc.). The collection was finished when all of the traps were occupied. Subsequently, the collection chip was rinsed with oil containing 0.25% PEG surfactant to remove un-trapped droplets. Sorting enrichment was determined by automated scanning of the entire collection chip at 10-fold magnification using an inverted fluorescence microscope (Nikon eclipse Ti), equipped with a motorized stage and a Hamamatsu Digital camera. Images were stitched, processed and analyzed using ilastik (ilastik.org) and ImageJ.

Table S1: Sorting results. ND = not detectable

Cell type			Dual color droplets			Single color droplets	Empty droplets
			2 cells	3 cells	≥4 cells	≥1 cell	0 cells
hybridoma cells	Before sorting (PMT data)	Number	379	83	4	3138	ND
		Percentage	10.5%	2.3%	0.1%	87.1%	ND
	Before sorting (collection chip)	Number	5	2	1	57	161
		Percentage	2.2%	0.9%	0.4%	25.2%	71.2%
	After sorting (collection chip)	Number	191	47	5	6	0
		Percentage	76.7%	18.9%	2%	2.4%	0
Jurkat cells	Before sorting (PMT data)	Number	2291	535	69	16868	ND
		Percentage	11.6%	2.7%	0.4%	85.4%	ND
	Before sorting (collection chip)	Number	14	10	2	177	291
		Percentage	2.8%	2.0%	0.4%	35.8%	58.9%
	After sorting (collection chip)	Number	402	32	0	26	4
		Percentage	86.6%	6.9%	0.0%	5.6%	0.9%

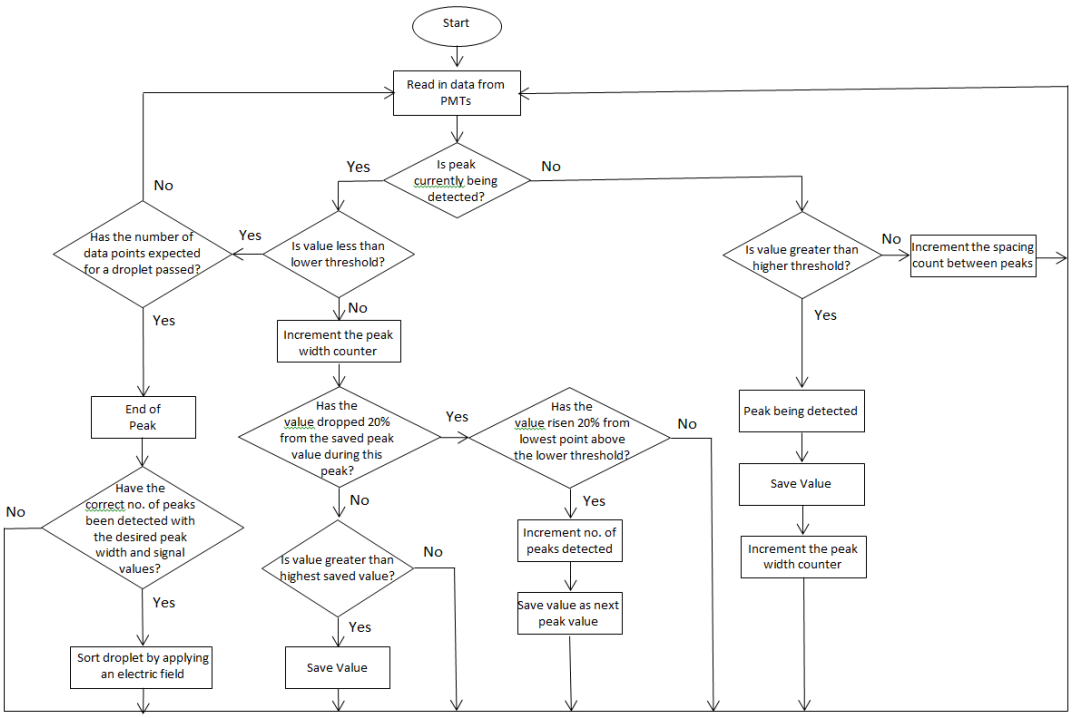

Figure S1. Flowchart summarizing the logic of the LabVIEW control software programmed at EMBL, Heidelberg. This algorithm runs in parallel for both of the PMT channels (one for each colour) and detects peaks in the signal values. This allows cells within droplets to be detected and for a sorting decision to be made for each passing droplet based on the intensity of the signal, the number of peaks detected, the width of the overall peak and the spacing between droplets that contain at least one cell. This software and a user manual can be freely downloaded for academic use at www.merten.embl.de/index.html.

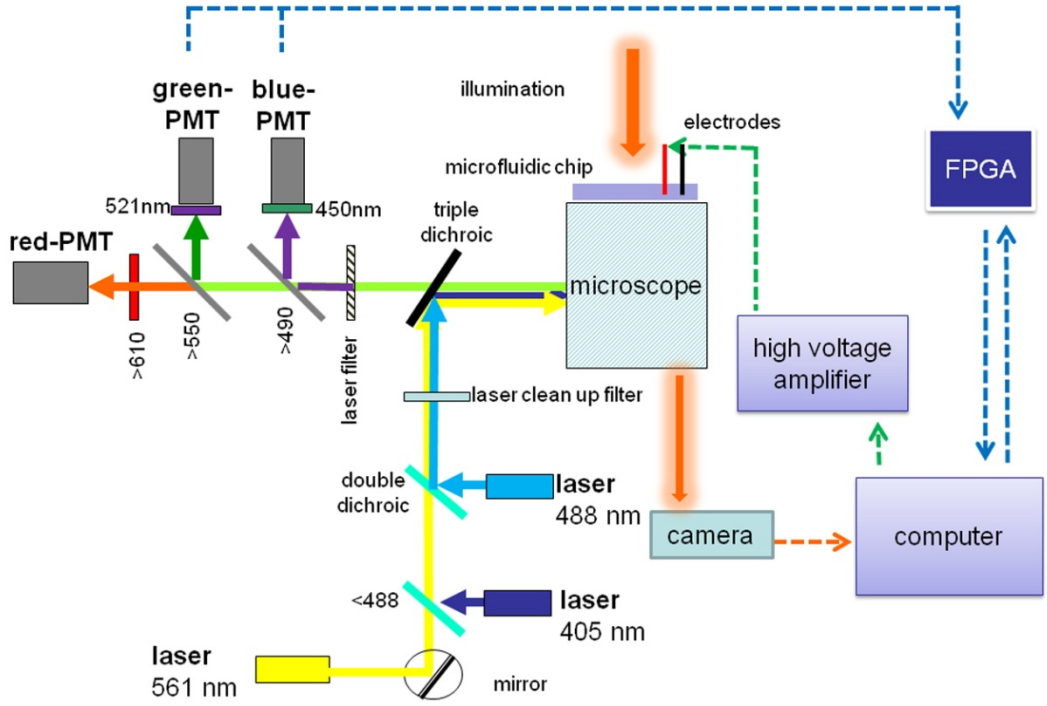

Fig. S2. Schematic of the optical setup. The fluorescence-based sorting setup uses diode lasers with excitation wavelengths of 405 nm (Calcein Violet), 488 nm (Calcein-AM) and 561 nm (optional third laser for assay readouts). Emission signals are detected using PMTs with a 450 nm band-pass filter (blue), a 521 nm band-pass filter (green), and a 610 nm longpass filter (red). Sorting signals are processed using LabVIEW software running on a FPGA card triggering a high voltage amplifier. Imaging is performed using an inverted microscope equipped with a high speed camera.

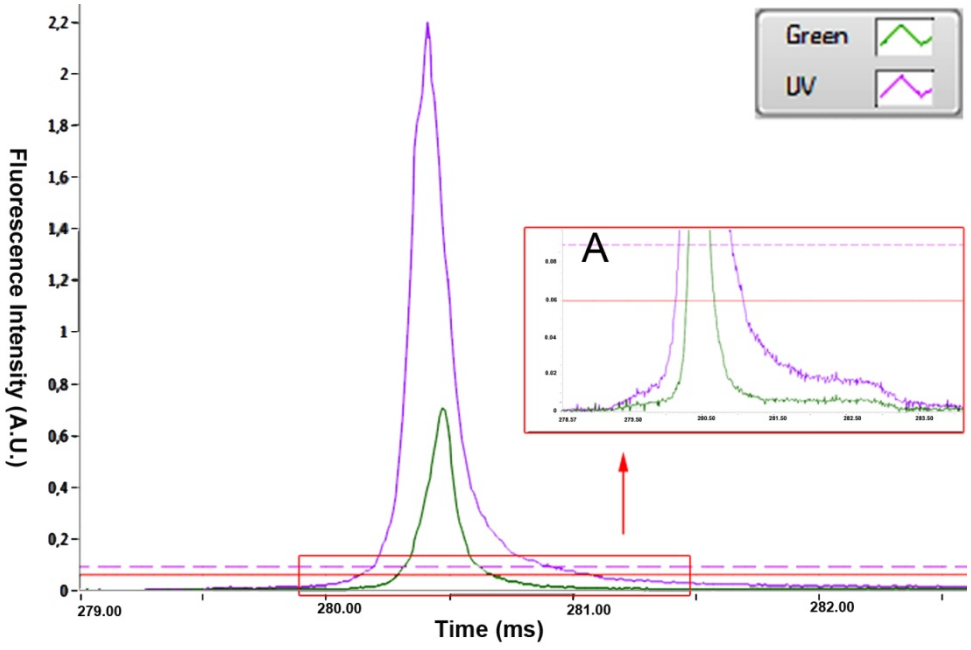

Fig. S3. Example of the signal peaks in one droplet. The zoom in (inset) reveals a jigsaw shape of the signal at low intensity, thus making the use of inflection points for the detection of peaks impossible.

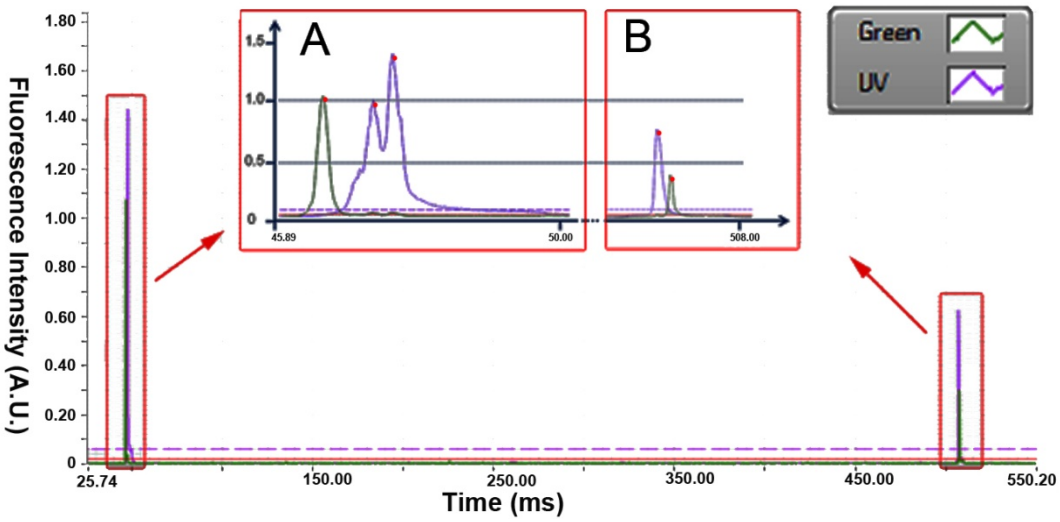

Fig. S4. Signal variation of Calcein-AM and Calcein-violet stained cells inside droplets. (A) Droplet showing one green peak and two overlapping violet peaks, corresponding to a clump of 2 violet cells, with a valley between the two peaks above a value of 0.5 fluorescence units. This value is higher than the green peak of another droplet **(B)** hosting exactly one green and one violet cell. Therefore using static thresholds (solid black lines) is not sufficient to accurately detect the number of encapsulated cells. However, when specifically detecting drops in the fluorescence signal exceeding the maximum noise (red dots), the number of peaks can be correctly determined, independently of the peak intensities.

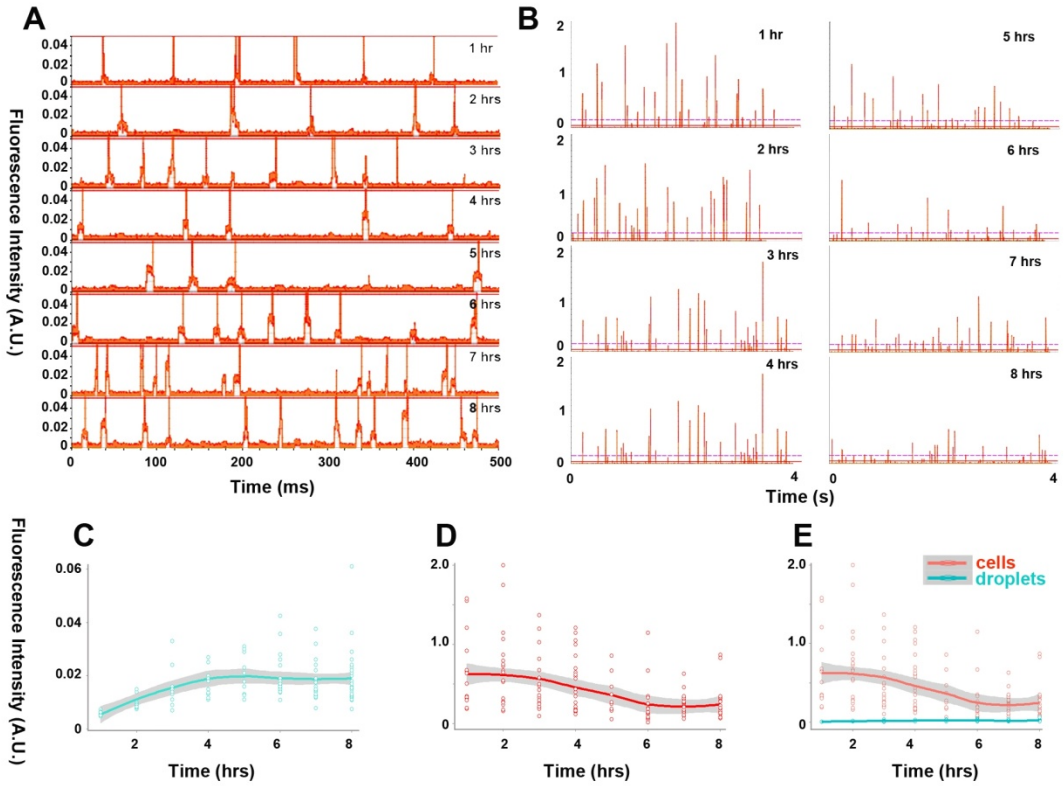

Figure S5. Leakage of Calcein Violet from cells encapsulated into droplets. (A) Zoom in of the fluorescence signals over 8 hours incubation at room temperature. The strongly decreased scale of the Y-axis (from 0 to 0.05 A.U.) allows illustrating the increase in the droplet signal (wide peaks), but requires cropping of the cell signals (narrow subpeaks with intensities as shown in (B)). **(B)** Time course of fluorescence signals of droplets hosting Calcein Violet-stained hybridoma cells. After incubation for the indicated time periods off-chip, the droplets were reinjected into the sorting device and the fluorescence signals were determined in the detection channel using a PMT.. **(C)** Fitted LOESS smoothing line of droplet fluorescence intensities (turquoise line), individual data points (turquoise circles) and confidence (grey shades) of the droplet signals. **(D)** Fitted LOESS smoothing line of cell fluorescence intensities (red line), individual data points (red circles) and confidence bands (grey shades) of the cell signals. **(E)** Intensities of cell and droplet signals plotted at the same scale.

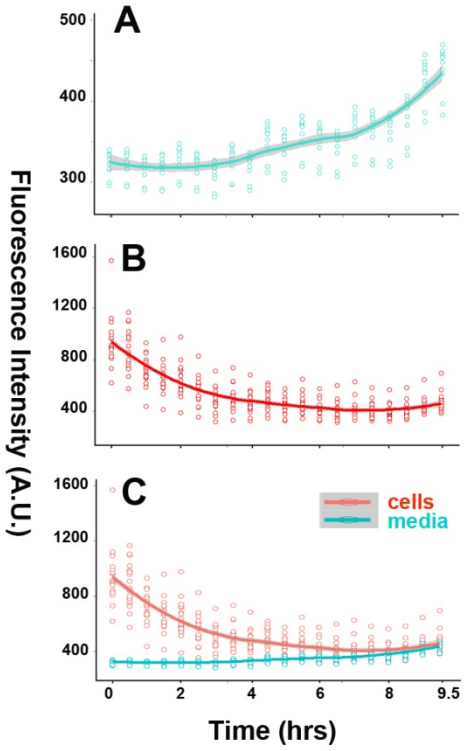

Figure S6. Leakage of Calcein Violet from cells cultivated in glass bottom wells. (A) Calcein Violet-stained hybridoma cells were incubated for the indicated time periods (X-axis) and Fluorescence intensities of the media supernatants over time was determined by imaging (manually selected samples analyzed for their intensity using ImageJ). **(B)** Fluorescence intensities of the calcein violet-stained hybridoma cells over time. **(C)** Intensities of the cell and droplet signals plotted at the same scale. Solid lines = fitted LOESS smoothing line of fluorescence intensities; open circles = individual data points; grey shades = confidence bands.

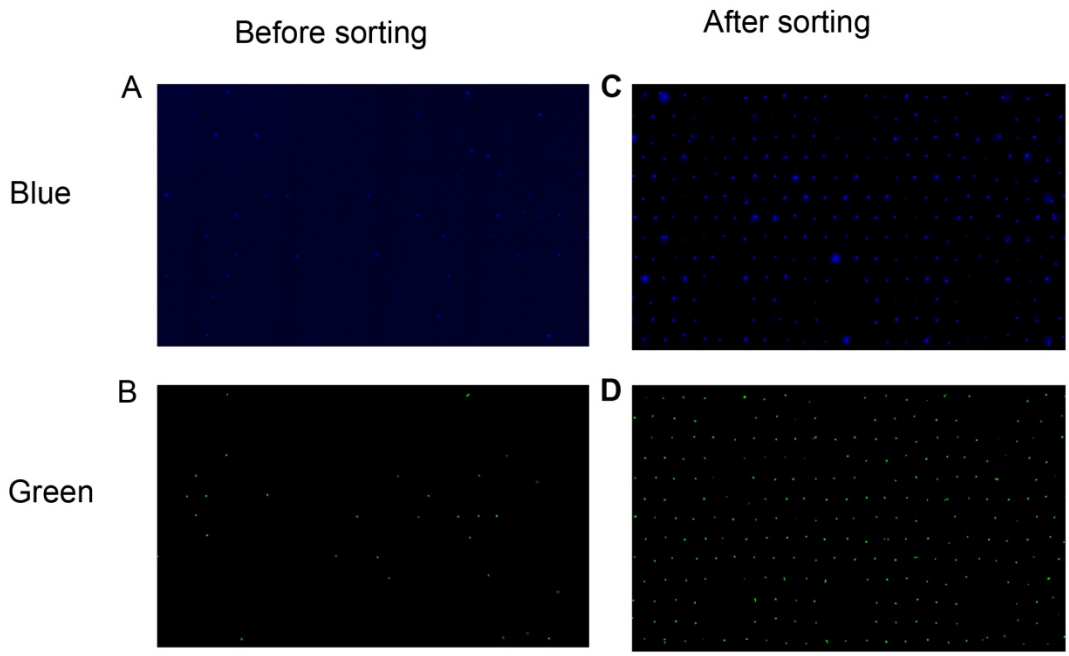

Fig. S7. Efficiency of the sorting process for droplets hosting differently stained Her2 Hybridoma cells. Blue fluorescence of droplets captured in the collection chip before **(A)** and after **(C)** sorting. Green fluorescence of droplets captured in the collection chip before **(B)** and after **(D)** sorting.

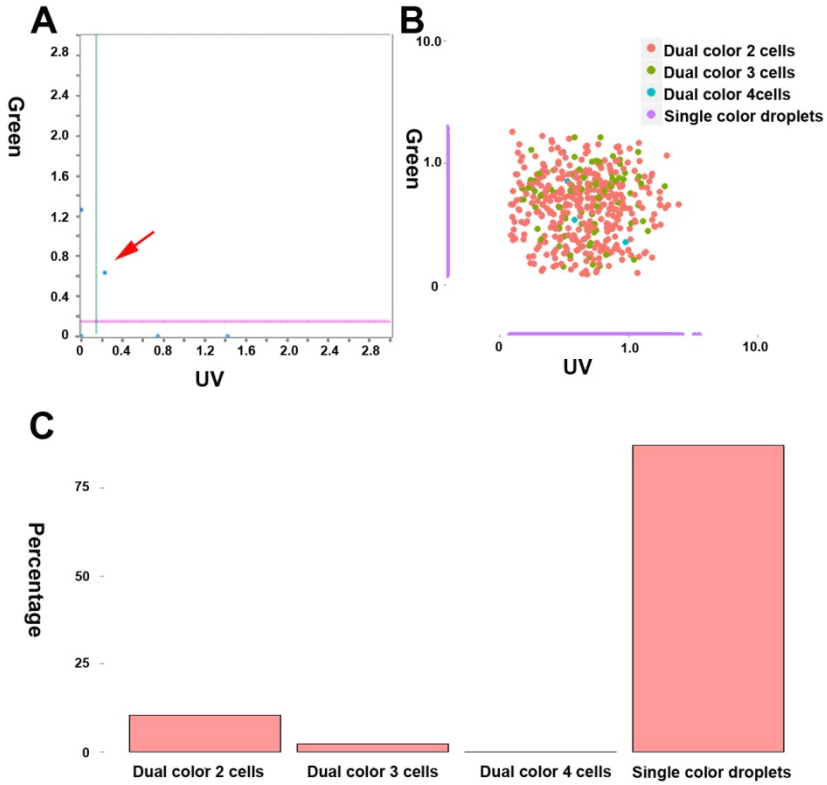

Fig. S8. Fluorescence analysis of the droplets detected by PMT. (A) Two dimensional dot plot of fluorescence signals of droplets. The red arrow indicates one example of the dual color droplet with two cells. **(B)** Dot plot showing violet and green signals of the droplets. **(C)** Droplet occupancy before sorting .

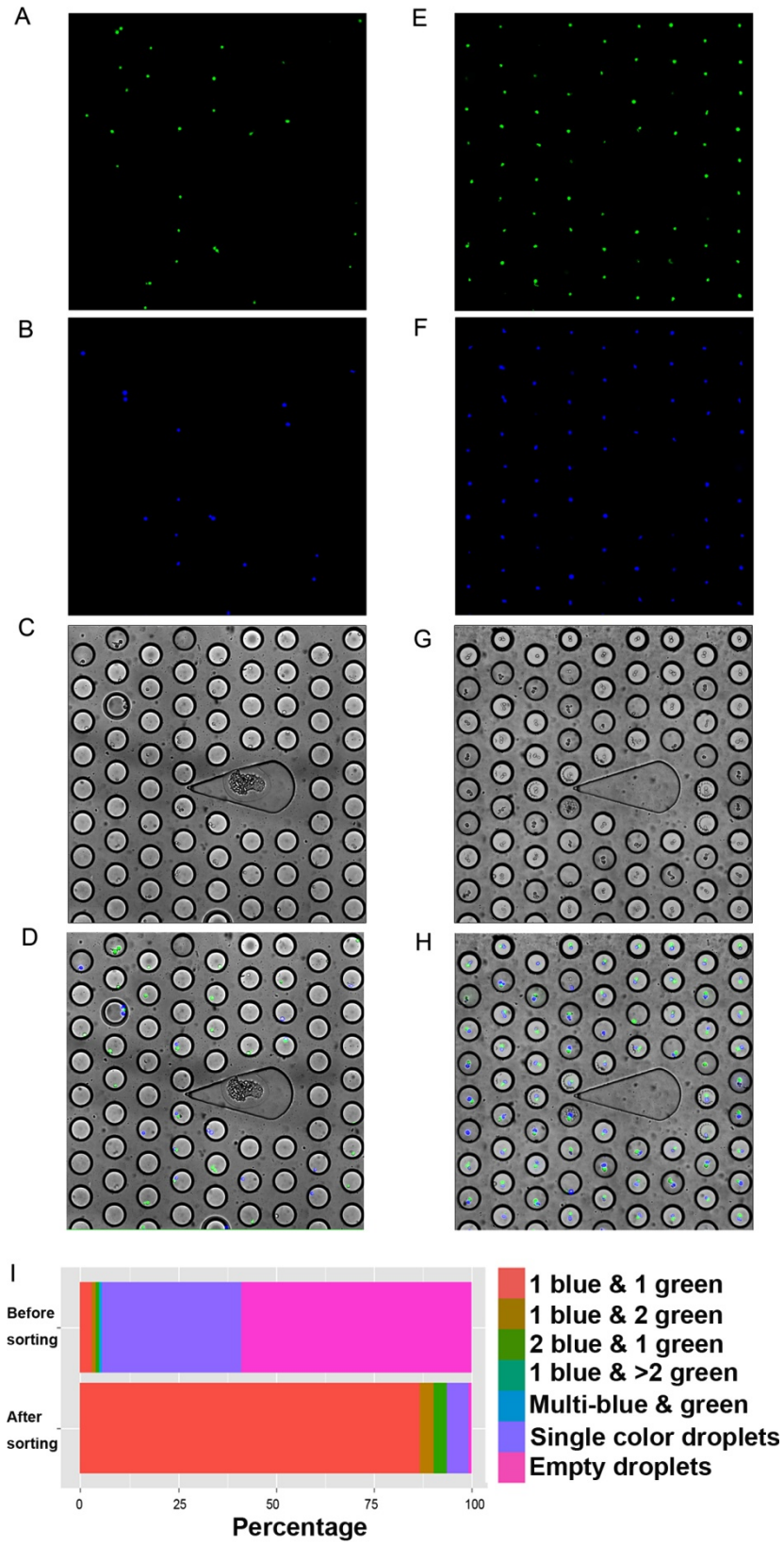

Fig. S9. Efficiency of the sorting process for droplets hosting differently stained Jurkat cells. Blue fluorescence of droplets captured in the collection chip

before **(A)** and after **(E)** sorting.Green fluorescence of droplets captured in the collection chip before **(B)** and after **(F)** sorting. Bright field images of droplets captured in the collection chip before **(C)** and after **(G)** sorting. Merged blue, green and bright field images before **(D)** and after **(H)** sorting. **(I)** Droplet occupancies in the collection chip before (top) and after (bottom) sorting.

Supplementary movie S1. Spacing of reinjected droplets upstream of the sorting junction (visualized using a 2-fold objective).

Supplementary movie S2. Droplet sorting (visualized using a 40-fold objective). The fluorescence signals of each droplet are measured in real time at 100 kHz and processed. Droplets hosting exactly one green and one violet cell are actively pulled into to the collection channel by switching on the electrodes. For all droplets with undesired occupancies, the electrodes remain switched off and the droplets follow the main flow into the waste channel.

Supplementary movie S3. Leakage of Calcein Violet from cells cultivated in glass bottom wells. Time lapse imaging of cells stained with Calcein Violet over a time period of 9.5 hours with 30 minute intervals.