Electronic Supplementary Material (ESI) for Lab on a Chip.

SeParate: Multiway fluorescence-activated droplet sorting based on integration of serial and parallel triaging concepts

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Section S1. Microfluidic chip designs

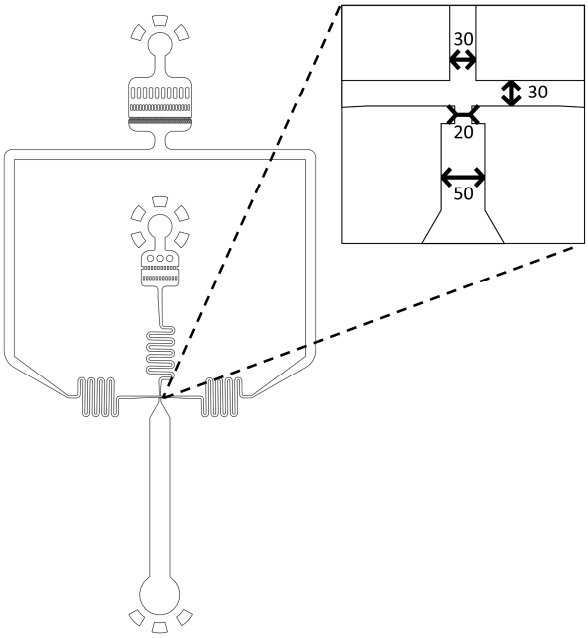

Figure S1. Microfluidic flow focusing design with dimensions depicted in µm.

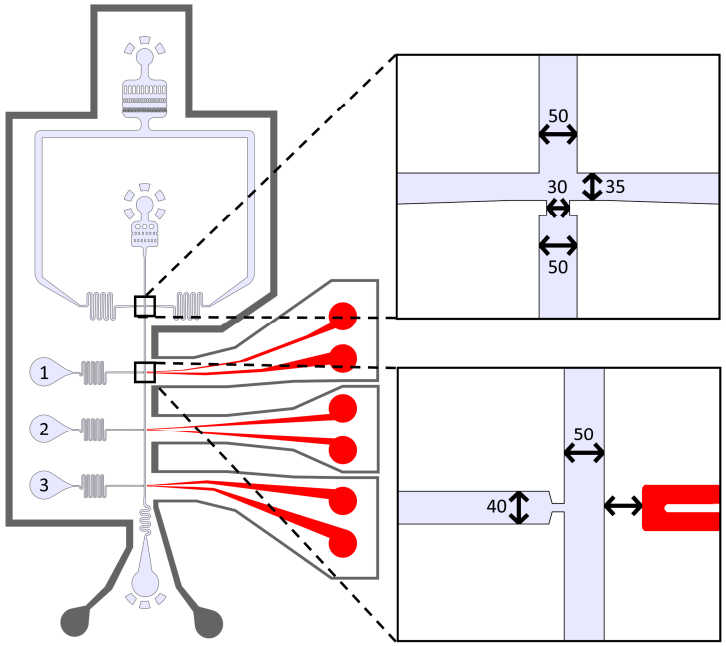

Figure S2. Picoinjection design with the most important dimensions in µm.

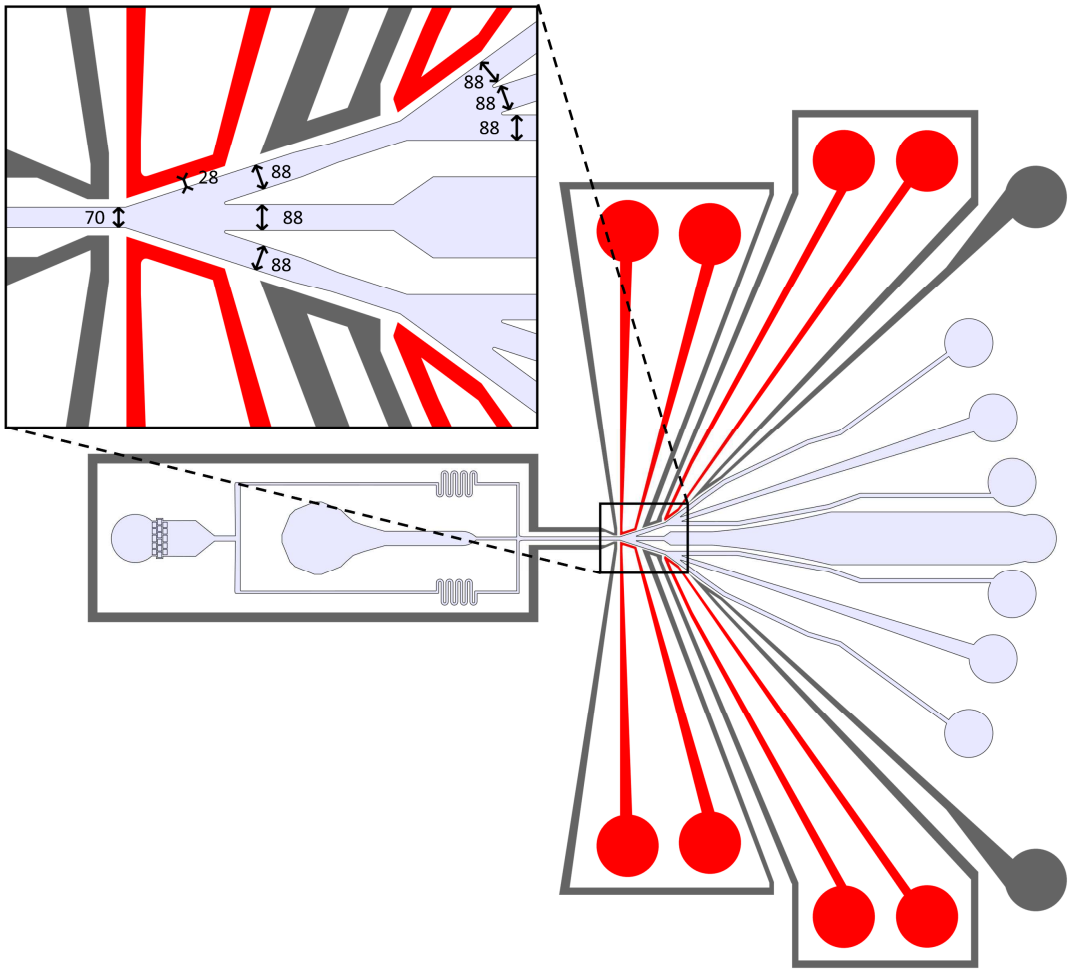

Figure S3. Microfluidic sorting design with the most important dimensions indicated in µm.

Section S2. Sorting of picoinjected droplets

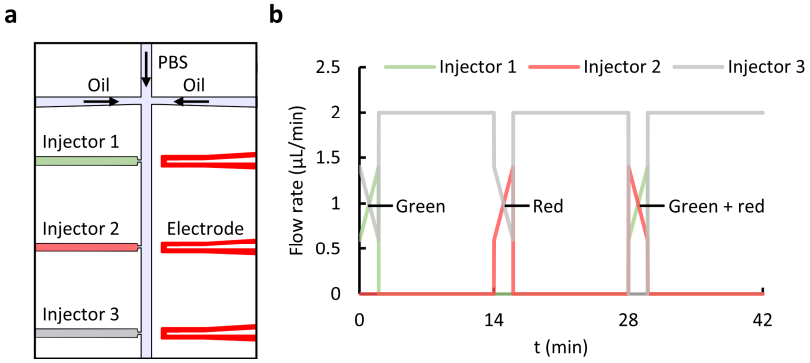

Figure S4. Schematic overview of the generation process of picoinjected droplets. (a) PBS droplets were created on-chip which subsequently passed the 3 injectors. Integrated electrodes were manipulated by means of a relay system. (b) Illustration of the action of the three injectors over time. Over the course of 42 minutes, 3 injector sweeps were performed by injecting with 2 out of 3 injectors in an inversely proportional manner for 2 minutes, creating a green fluorescent population, a red fluorescent population and a population showing both fluorescent signals. In between sweeps, only injector 3 was injecting for 12 minutes, resulting in a droplet population with no fluorescence.

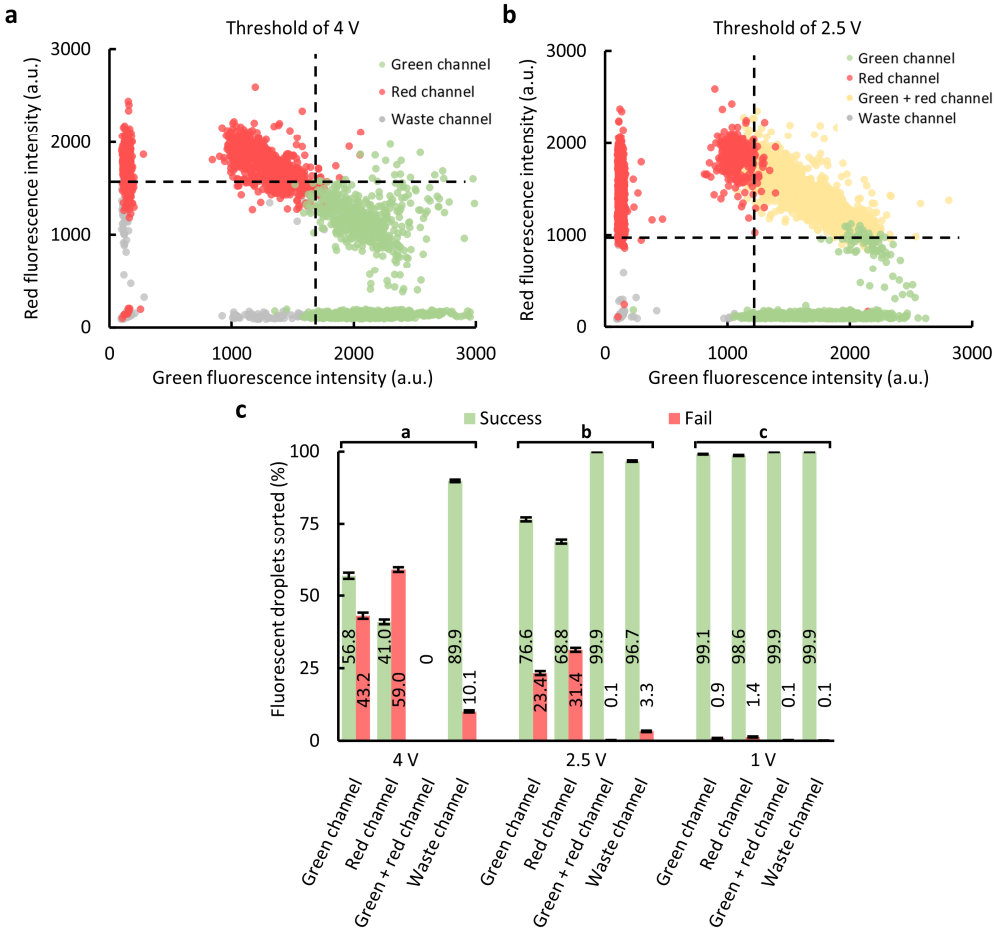

Figure S5. Sorting performance of the picoinjected droplet population for different thresholds. (a-b) The fluorescence intensities of every imaged droplet retrieved from their respective outlet. Dot colors represent the channel from which a droplet was retrieved at sorting threshold of 4 V (a) and 2.5 V (b). The dotted lines correspond to the fluorescence intensity value below which 99% of the droplets from the green and waste channel (for the horizontal line) or the red and waste channel (for the vertical line) are located. They indicate the effect the changing sorting threshold has on the sorting accuracy. (c) Sorting accuracy for all 4 channels for the 3 tested thresholds (the data for 1 V was already presented in Figure 5). Different letters above the bar indicate significant differences between the tested thresholds ($\alpha < 0.05$). For every channel and condition, at least 2185 droplets were analyzed (Table S2-3). Error bars represent one standard error of the mean (n = 3).

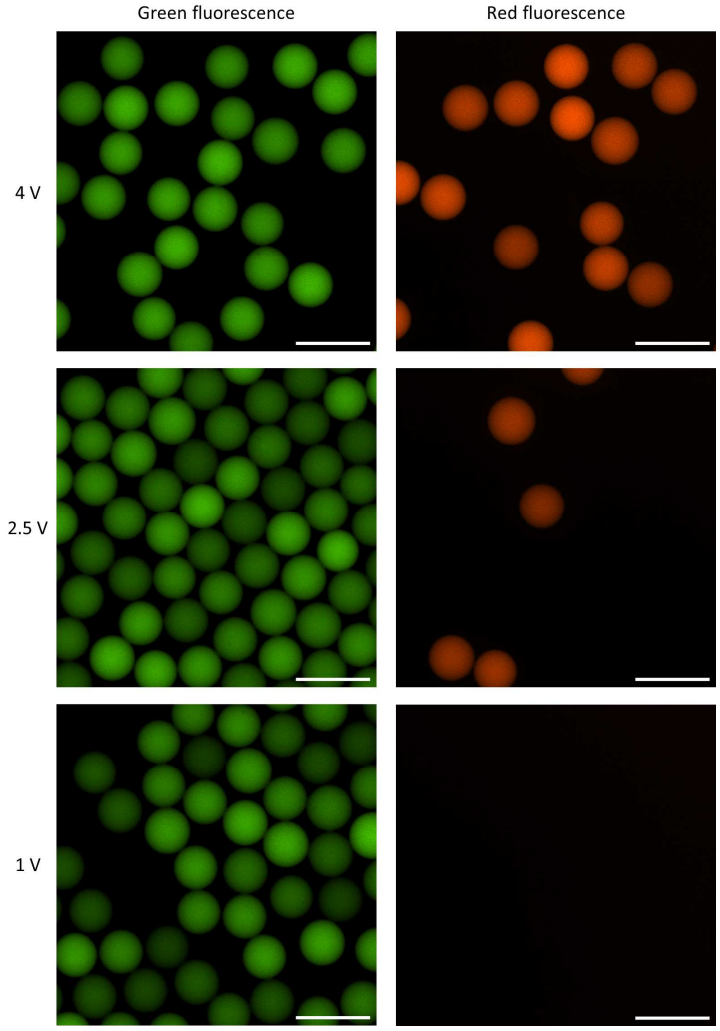

Figure S6. Green and red fluorescent widefield images of sorted picoinjected droplets for the three tested sorting thresholds (1, 2.5 and 4 V), retrieved from the green channel. Lowering the threshold to 1 V resulted in less green + red droplets in the green channel, increasing the sorting accuracy. Additionally, these images illustrate that an increase in threshold results in the lack of detection (and sorting) of low intense fluorescent droplets as for 4 V the droplets generally show a higher fluorescence intensity while for 1 V a mix between low and high fluorescence intensity droplets is observed. Scale bar = 100 µm.

Section S3. Sample variation in fluorescence intensity

Within one population (i.e. the whole library of droplets, existing of both fluorescent and non-fluorescent droplets), in the presented experiments, four subpopulations can be distinguished: (1) a green fluorescent subpopulation, (2) a red fluorescent subpopulation, (3) a green + red fluorescent subpopulation and an empty (non-fluorescent) subpopulation. In Figure S7a, a sample of the fluorescent bead population passing the interrogation zone over time is shown, illustrating that every bead type had its own, different, fluorescence fingerprint. However, within one subpopulation, beads showed a similar fingerprint, which simplified the determination of an optimal threshold, minimizing wrong sorting events. Figures S7b and c illustrate this for respectively picoinjected droplets and encapsulated cells. Compared to the low variation in fluorescence intensity between beads of the same type, picoinjected droplets and fluorescent cells showed higher intra-subpopulation variation in fluorescence intensity, potentially complicating accurate thresholding and sorting. These observations

are confirmed by Figure S8 as the average peak height and variation on this peak height are plotted, showing that the intra-subpopulation variation increases from beads to picoinjected droplets to cells.

Next to the cell sample showing the highest intra-subpopulation variation in fluorescence intensity, they pose an additional challenge as their fluorescence intensity is much lower compared to the other tested samples. This is reflected in the fact that a 40X short working distance was needed (instead of a more standard 20X) to capture fluorescent signals. While increasing the sensitivity, the background of the PMT measurements increased as well, which is clear from Figure S7c.

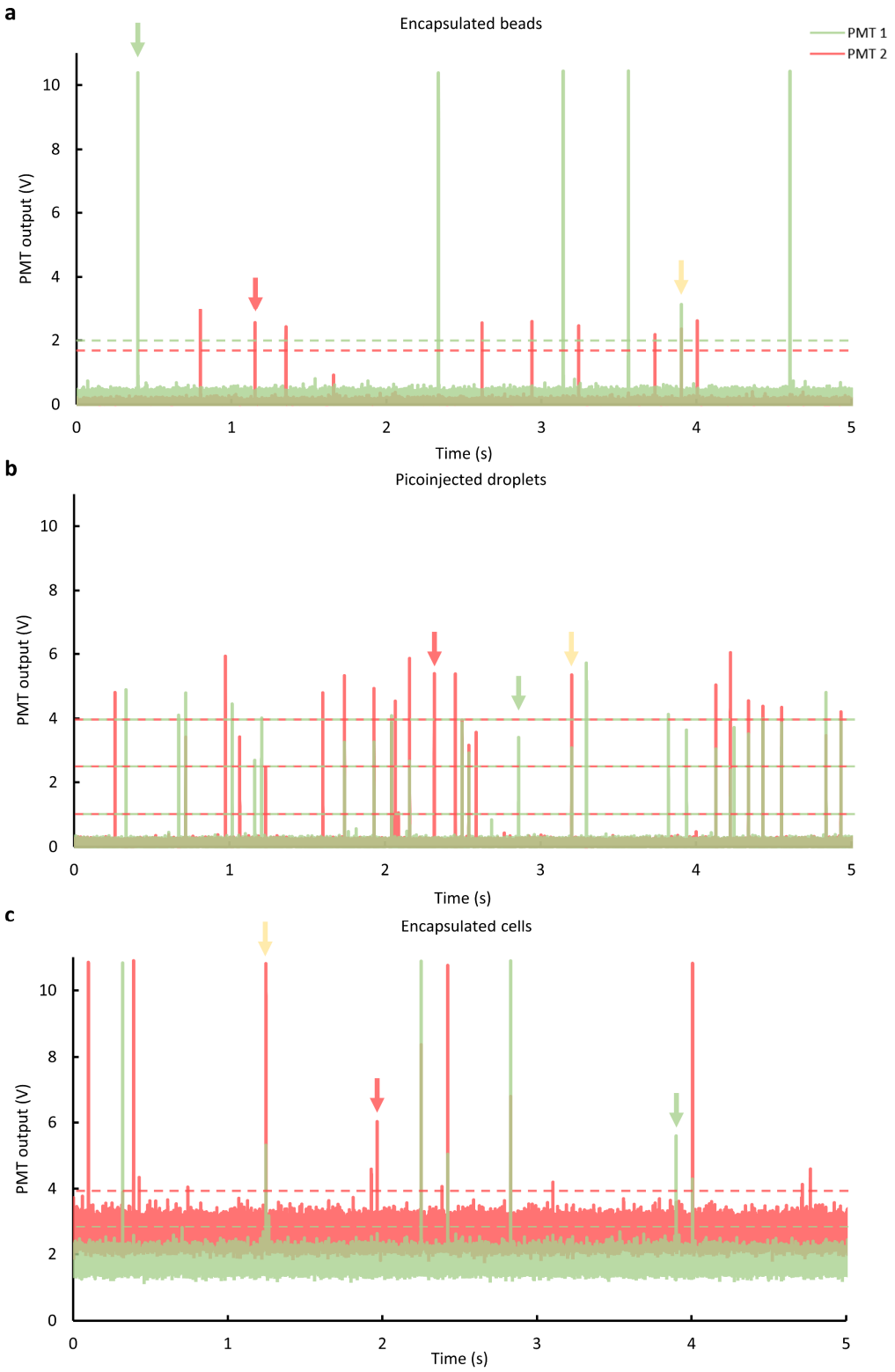

Figure S7. Recorded output signal from PMT 1 and PMT 2, depicted in green and red respectively, during the sorting process. Three peak types are visible: peaks in PMT 1 (i.e. green fluorescence), peaks in PMT 2 (i.e. red fluorescence) and in both PMTs (i.e. green + red fluorescence), indicated with respectively green, red and yellow arrows. Dotted lines represent the sorting threshold used for sorting. (a) The encapsulated mixed bead population, recorded using the 20X objective and 638 nm LP. (b) Picoinjected droplets, recorded with the 20X objective and 638 nm LP. Multiple thresholds are depicted as this population was sorted using 3 different thresholds. (c) The encapsulated cell population, recorded using the 40X objective and 590 nm LP.

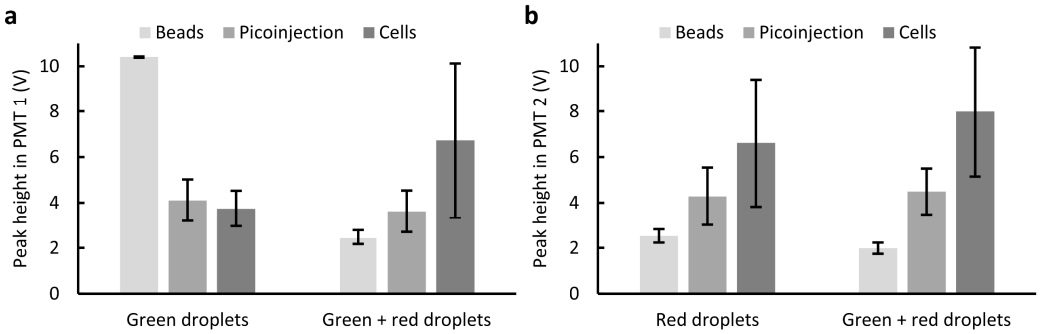

Figure S8. Average peak height as detected by the PMTs. (a) The peak heights for green and green + red droplets, as detected in PMT 1 (i.e. green fluorescent signal). For the green droplets, CV values were 0.3%, 22% and 21% for respectively beads, picoinjected droplets and cells, while the green + red droplets showed CV values of 12%, 25% and 50% for respectively beads, picoinjected droplets and cells. For green droplets, the signal in PMT 1 saturated. (b) Peak heights for red and green + red droplets detected by PMT 2 (i.e. red fluorescent signal). For the red droplets, CV values were 12%, 30% and 42% for respectively beads, picoinjected droplets and cells, while the green + red droplets showed CV values of 12%, 22% and 36% for respectively beads, picoinjected droplets and cells. Error bars indicate one standard deviation, n > 8.

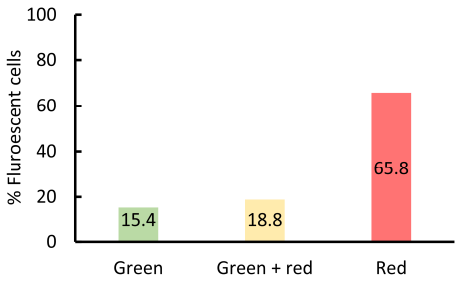

Figure S9. The relative frequency of green, green + red and red fluorescent cells, imaged prior to encapsulation.

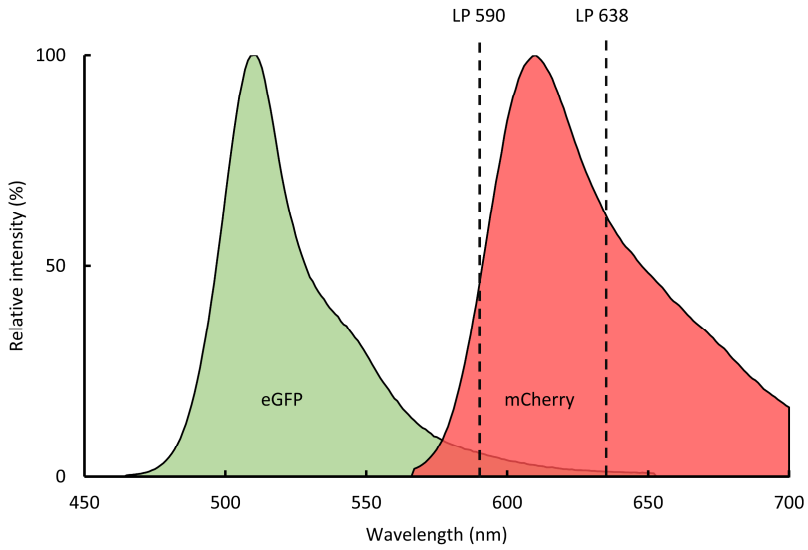

Figure S10. The emission spectra of eGFP and mCherry, the two fluorescent labels used in the cell sorting experiments. The dotted lines indicate the emission filter (for both optical setups used, 590 nm LP and 638 nm LP) positioned in front of PMT 2, letting all light above its value through to PMT 2. An eGFP tail is still observed above 590 nm, which leads to bleed-through when using the 590 nm LP, which is picked up when using a sorting threshold close to the background. Using the 638 nm LP configuration, together with the eGFP tail, the mCherry intensity peak is filtered out, leading to potential loss in sensitivity (i.e. low intense cells will not be detected).

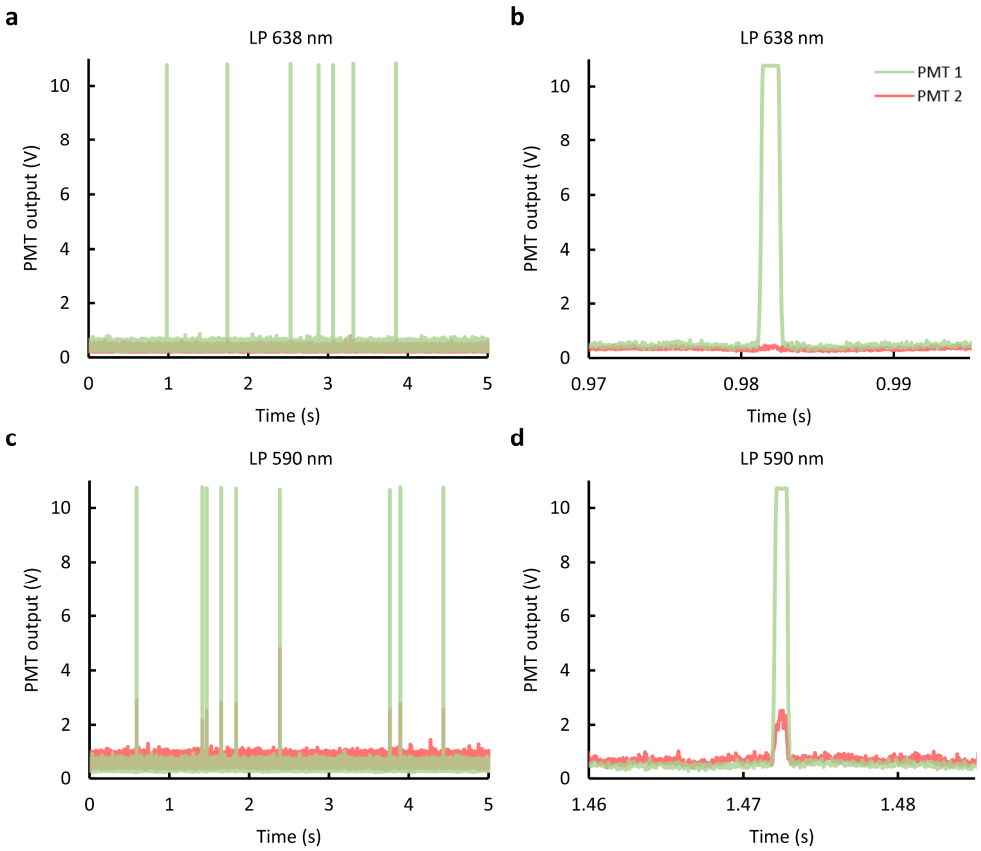

Figure S11. Recorded output of PMT 1 and 2 for green fluorescent beads in the two tested optical configurations. Every peak represents a green fluorescent bead passing by the interrogation zone. (a) Due to the more strict filtering by the 638 nm LP filter, green fluorescent beads only show in PMT 1 and not in PMT 2. (b) A zoom on one of the peaks, showing no bleed-through (i.e. no peak for PMT 2). (c) Using the 590 nm LP filter, green fluorescent beads also show small peaks in PMT 2, which could lead to wrongful identification during sorting. (d) A zoom on one of the peaks, clearly showing a peak in PMT 2 due to bleed-through.

Movie S1. An encapsulated bead gets sorted at junction 1 by electrode 1, leading to attraction to the red channel. Droplets that are not attracted flow straight into the waste channel.

Movie S2. Encapsulated bead sorting at junction 1 by electrode 3, leading to attraction to the green + red channel.

Movie S3. An encapsulated bead gets sorted at junction 2 by electrode 2, leading to attraction to the green channel.

Movie S4. A droplet arriving at junction 2 that is not attracted by an electrode, flowing to the red channel.

Table S1. The counts and sorting accuracy of sorted beads for three sorting repetitions on independent chips and the weighted average of these three repetitions.

Initial population									
	Green	**Red**	**Green + red**	**Empty**	**% Green**	**% Red**	**% Green + red**	**% Empty**	**Total**
	104	95	18	5112	2	1.8	0.3	95.9	5329

Chip 1									
	Green	**Red**	**Green + red**	**Empty**	**% Green**	**% Red**	**% Green + red**	**% Empty**	**Total**
Green channel	870	3	20	0	97.4	0.3	2.2	0	893
Red channel	18	801	3	6	2.2	96.7	0.4	0.7	828
Green + red channel	0	0	100	0	0	0	100	0	100
Waste	1	0	0	1712	0.1	0	0	99.9	1713

Chip 2									
	Green	**Red**	**Green + red**	**Empty**	**% Green**	**% Red**	**% Green + red**	**% Empty**	**Total**
Green channel	787	5	19	2	96.8	0.6	2.3	0.2	813
Red channel	19	938	0	0	2	98	0	0	957
Green + red channel	0	0	135	0	0	0	100	0	135
Waste	2	4	1	2737	0.1	0.1	0	99.7	2744

Chip 3									
	Green	**Red**	**Green + red**	**Empty**	**% Green**	**% Red**	**% Green + red**	**% Empty**	**Total**
Green channel	699	10	24	2	95.1	1.4	3.3	0.3	735
Red channel	43	820	0	7	4.9	94.3	0	0.8	870
Green + red channel	0	0	120	0	0	0	100	0	120
Waste	2	0	0	1394	0.1	0	0	99.9	1396

Weighted average											
	Green	**Red**	**Green + red**	**Empty**	**% Green**	**% Red**	**% Green + red**	**% Empty**	**Total**	**% Success**	**% Fail**
Green channel	2356	18	63	4	96.5	0.7	2.6	0.2	2441	96.5	3.5
Red channel	80	2559	3	13	3	96.4	0.1	0.5	2655	96.4	3.6
Green + red channel	0	0	355	0	0	0	100	0	355	100	0
Waste	5	4	1	5843	0.1	0.1	0	99.8	5853	99.8	0.2

Table S2. The counts and sorting accuracy of sorted picoinjected droplets for a threshold of 4 V and 2.5 V for three independent chips. Next to the counts, the weighted average of these three repetitions is shown.

Initial population									
	Green	Red	Green + Red	Non-fluorescent	% Green	% Red	% Green + Red	% Non-fluorescent	Total
	339	275	260	5420	5.4	4.4	4.1	86.1	6294

4 V threshold									
	Green	Red	Green + Red	Non-fluorescent	% Green	% Red	% Green + Red	% Non-fluorescent	Total
Chip 1									
Green channel	189	1	211	0	47.1	0.2	52.6	0.0	401
Red channel	3	378	747	16	0.3	33.0	65.3	1.4	1144
Green + red channel	0	0	0	0	0.0	0.0	0.0	0.0	0
Waste channel	48	42	30	903	4.7	4.1	2.9	88.3	1023
Chip 2									
Green channel	412	0	217	0	65.5	0.0	34.5	0.0	629
Red channel	2	531	586	4	0.2	47.3	52.2	0.4	1123
Green + red channel	0	0	0	0	0.0	0.0	0.0	0.0	0
Waste channel	121	110	65	2035	5.2	4.7	2.8	87.3	2331
Chip 3									
Green channel	641	0	514	0	55.5	0.0	44.5	0.0	1155
Red channel	0	490	645	9	0.0	42.8	56.4	0.8	1144
Green + red channel	0	0	0	0	0.0	0.0	0.0	0.0	0
Waste channel	79	42	13	1940	3.8	2.0	0.6	93.5	2074

Weighted average											
	Green	Red	Green + Red	Non-fluorescent	% Green	% Red	% Green + Red	% Non-fluorescent	Total	% Success	% Fail
Green channel	1242	1	942	0	56.8	0.0	43.1	0.0	2185	56.8	43.2
Red channel	5	1399	1978	29	0.1	41.0	58.0	0.9	3411	41.0	59.0
Green + red channel	0	0	0	0	0.0	0.0	0.0	0.0	0	0.0	0.0
Waste channel	248	194	108	4878	4.6	3.6	2.0	89.9	5428	89.9	10.1

2.5 V threshold									
	Green	Red	Green + Red	Non-fluorescent	% Green	% Red	% Green + Red	% Non-fluorescent	Total
Chip 1									
Green channel	694	6	493	0	58.2	0.5	41.3	0.0	1193
Red channel	11	1045	803	3	0.6	56.1	43.1	0.2	1862
Green + red channel	0	0	650	0	0.0	0.0	100.0	0.0	650
Waste channel	25	30	12	1649	1.5	1.7	0.7	96.1	1716
Chip 2									
Green channel	1228	2	303	2	80.0	0.1	19.7	0.1	1535
Red channel	3	572	215	4	0.4	72.0	27.1	0.5	794
Green + red channel	0	0	675	4	0.0	0.0	99.4	0.6	679
Waste channel	31	29	1	1429	2.1	1.9	0.1	95.9	1490
Chip 3									
Green channel	936	0	66	1	93.3	0.0	6.6	0.1	1003
Red channel	1	1249	268	2	0.1	82.2	17.6	0.1	1520
Green + red channel	0	0	1348	0	0.0	0.0	100.0	0.0	1348
Waste channel	11	8	0	1204	0.9	0.7	0.0	98.4	1223

Weighted average											
	Green	Red	Green + Red	Non-fluorescent	% Green	% Red	% Green + Red	% Non-fluorescent	Total	% Success	% Fail
Green channel	2858	8	862	3	76.6	0.2	23.1	0.1	3731	76.6	23.4
Red channel	15	2866	1286	9	0.4	68.6	30.8	0.2	4176	68.6	31.4
Green + red channel	0	0	2673	4	0.0	0.0	99.9	0.1	2677	99.9	0.1
Waste channel	67	67	13	4282	1.5	1.5	0.3	96.7	4429	96.7	3.3

Table S3. The counts and sorting accuracy of sorted picoinjected droplets for a threshold of 1 V for three independent chips. Next to the counts, the weighted average of these three repetitions is shown. The starting population was the same as depicted in Table S2.

	1 V threshold								
		Green	Red	Green + Red	Non-fluorescent	% Green	% Red	% Green + Red	% Non-fluorescent	Total
Chip 1	Green channel	952	1	8	0	99.1	0.1	0.8	0.0	961
	Red channel	27	1238	1	1	2.1	97.7	0.1	0.1	1267
	Green + red channel	1	0	399	0	0.3	0.0	99.8	0.0	400
	Waste channel	1	0	1	1533	0.1	0.0	0.1	99.9	1535
Chip 2	Green channel	1731	12	10	0	98.7	0.7	0.6	0.0	1753
	Red channel	11	1332	1	7	0.8	98.6	0.1	0.5	1351
	Green + red channel	0	0	1131	2	0.0	0.0	99.8	0.2	1133
	Waste channel	0	0	0	1846	0.0	0.0	0.0	100.0	1846
Chip 3	Green channel	800	0	1	0	99.9	0.0	0.1	0.0	801
	Red channel	1	1069	0	1	0.1	99.8	0.0	0.1	1071
	Green + red channel	1	0	1148	0	0.1	0.0	99.9	0.0	1149
	Waste channel	0	1	2	1357	0.0	0.1	0.1	99.8	1360

	Weighted average										
	Green	Red	Green + Red	Non-fluorescent	% Green	% Red	% Green + Red	% Non-fluorescent	Total	% Success	% Fail
Green channel	3483	13	19	0	99.1	0.4	0.5	0.0	3515	99.1	0.9
Red channel	39	3639	2	9	1.1	98.6	0.1	0.2	3689	98.6	1.4
Green + red channel	2	0	2678	2	0.1	0.0	99.9	0.1	2682	99.9	0.1
Waste channel	1	1	3	4736	0.0	0.0	0.1	99.9	4741	99.9	0.1

Table S4. The counts and sorting accuracy of sorted cells for three sorting repetitions on independent chips and the weighted average of these three repetitions for the optical setups using 638 nm LP.

	Initial population								
	Green	Red	Green + red	Empty	% Green	% Red	% Green + Red	% Empty	Total
	10	57	10	2656	0.4	2.1	0.4	97.2	2733

	LP 638								
	Green	Red	Green + red	Empty	% Green	% Red	% Green + Red	% Empty	Total
Chip 1	Green channel	307	0	90	17	74.2	0.0	21.7	4.1	414
	Red channel	3	674	2	3	0.4	98.8	0.3	0.4	682
	Green + red channel	0	2	218	0	0.0	0.9	99.1	0.0	220
	Waste channel	0	13	0	1112	0.0	1.2	0.0	98.8	1125
Chip 2	Green channel	360	18	155	14	65.8	3.3	28.3	2.6	547
	Red channel	1	1193	2	1	0.1	99.7	0.2	0.1	1197
	Green + red channel	6	12	519	5	1.1	2.2	95.8	0.9	542
	Waste channel	1	33	3	1437	0.1	2.2	0.2	97.5	1474
Chip 3	Green channel	428	4	138	36	70.6	0.7	22.8	5.9	606
	Red channel	0	1489	2	15	0.0	98.9	0.1	1.0	1506
	Green + red channel	5	6	667	6	0.7	0.9	97.5	0.9	684
	Waste channel	0	9	0	1006	0.0	0.9	0.0	99.1	1015

	Weighted average										
	Green	Red	Green + red	Empty	% Green	% Red	% Green + Red	% Empty	Total	% Success	% Fail
Green channel	1095	22	383	67	69.9	1.4	24.4	4.3	1567	69.9	30.1
Red channel	4	3356	6	19	0.1	99.1	0.2	0.6	3385	99.1	0.9
Green + red channel	11	20	1404	11	0.8	1.4	97.1	0.8	1446	97.1	2.9
Waste channel	1	55	3	3555	0.0	1.5	0.1	98.4	3614	98.4	1.6

Table S5. The counts and sorting accuracy of sorted cells for three sorting repetitions on independent chips and the weighted average of these three repetitions for the optical setups using 590 nm LP. The starting population was the same as depicted in Table S4.

	LP 590								
		Green	Red	Green + red	Empty	% Green	% Red	% Green + Red	% Empty	Total
Chip 1	Green channel	265	0	0	13	95.3	0.0	0.0	4.7	278
	Red channel	1	1080	0	9	0.1	99.1	0.0	0.8	1090
	Green + red channel	22	0	98	2	18.0	0.0	80.3	1.6	122
	Waste channel	0	3	3	1673	0.0	0.2	0.2	99.6	1679

		Green	Red	Green + red	Empty	% Green	% Red	% Green + Red	% Empty	Total
Chip 2	Green channel	566	15	1	29	92.6	2.5	0.2	4.7	611
	Red channel	0	1217	1	51	0.0	95.9	0.1	4.0	1269
	Green + red channel	146	14	991	13	12.5	1.2	85.1	1.1	1164
	Waste channel	0	9	2	1080	0.0	0.8	0.2	99.0	1091

		Green	Red	Green + red	Empty	% Green	% Red	% Green + Red	% Empty	Total
Chip 3	Green channel	390	4	8	25	91.3	0.9	1.9	5.9	427
	Red channel	1	1291	2	60	0.1	95.3	0.1	4.4	1354
	Green + red channel	101	14	791	24	10.9	1.5	85.1	2.6	930
	Waste channel	0	0	0	884	0.0	0.0	0.0	100.0	884

	Weighted average										
	Green	Red	Green + red	Empty	% Green	% Red	% Green + Red	% Empty	Total	% Success	% Fail
Green channel	1221	19	9	67	92.8	1.4	0.7	5.1	1316	92.8	7.2
Red channel	2	3588	3	120	0.1	96.6	0.1	3.2	3713	96.6	3.4
Green + red channel	269	28	1880	39	12.1	1.3	84.8	1.8	2216	84.8	15.2
Waste channel	0	12	5	3637	0.0	0.3	0.1	99.5	3654	99.5	0.5