Supporting information for the manuscript:

"Using droplet-based microfluidics to improve the catalytic properties of RNA under multiple turnover conditions"

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		Sequence																																									k_{cat}^{ss} (min^{-1})	Fold Improvement
		Substrate-binding Site 1								½ Stem 1				Loop 1				½ Stem 1						½ Stem 2			Loop 2					½ Stem 2					Substrate-binding Site 2									
						20					25					30					35					40					45					50					55					
	wt	G	G	A	G	U	A	G	C	C	A	A	U	G	U	G	A	A	U	U	G	A	G	A	G	C	C	U	U	A	A	G	C	U	G	U	A	U	C	A	G	G	A	C	0.010	1
Round 2	1					C							G									G						C						C											0.000	-
	2						G																				U																G		0.027	2.7
	3																																												0.046	4.6
	4	C																																											0.072	7.2
	5			G																																									0.082	8.2
	6						G																																						0.084	8.4
	7																																											U	0.087	8.7
	8			G																																			U						0.110	11.0
	9		A	G																																									0.110	11.0
	10		A																																										0.097	9.7
	11		A																																										0.113	11.3
	12			G			G																																						0.117	11.7
	13						G											G																									G		0.122	12.2
	14					C																																							0.124	12.4
	15	A																																									G		0.141	14.1
	16					C								A																										G			G		0.149	14.9
	17	C																G											C											G					0.152	15.2
	18						G											G																						G					0.152	15.2
	19						G																																	G					0.185	18.5
	20		A				G					G	C					G	C																										0.217	21.7

Table S1. Sequence analysis and activity screening of variants selected from round 2. Each variant was *in vitro* transcribed for 1 h before 2 μL of the mixture was transferred to 8 μL of activity assay mixture containing fluorogenic substrate (4 μM final concentration). Assuming that the yield of RNA was identical for the X-motif (wt) and each variant, it was possible to calculate k_{cat}^{ss} and the improvement in k_{cat}^{ss} of each molecule relative to the X-motif. The residues were numbered according to their position in in the extended X-motif.

		Sequence																																													k_{cat}^{ss} (min^{-1})	Fold Improvement
		Substrate-binding Site 1								½ Stem 1				Loop 1				½ Stem 1					½ Stem 2			Loop 2				½ Stem 2				Substrate-binding Site 2															
						20							25				30				35					40				45				50				55											
	Wt	G	G	A	G	U	A	G	C	C	A	A	U	G	U	G	A	A	U	U	G	A	G	A	G	C	C	U	U	A	A	G	C	U	G	U	A	U	C	A	G	G	A	C	0.010	1
Round 3	1						G								C											U																	G		0.001	0.1
	2								A																		C																U	0.003	0.3
	3	A																																	A					G			G		0.003	0.3
	4						G																									A							U	G					0.005	0.5
	5											G																												U					U	0.059	5.9
	6												G																																U	0.074	7.4
	7						G																																	G					0.123	12.3
	8		U											A																															0.146	14.6
	9			G																																				A				G		0.151	15.1
	10						G																				G															G				0.151	15.1
	11	U																																											U	0.153	15.3
	12	C																																										G		0.161	16.1
	13						G											G																									U				0.175	17.5
	14						G																																					A			0.179	17.9
	15	C																									C															U				0.192	19.2
	16	C																									C															U				0.196	19.6
	17						G																																	C			G				0.217	21.7
	18						G						C					G																									U				0.219	21.9
	19		A										C					G																											G		0.233	23.3
	20		A																																							U				0.245	24.5

Table S2. Sequence analysis and activity screening of variants selected from round 3. Each variant was *in vitro* transcribed for 1 h before 2 µL of the mixture was transferred to 8 µL of activity assay mixture containing fluorogenic substrate (4 µM final concentration). Assuming that the yield of RNA was identical for the X-motif (wt) and each variant, it was possible to calculate k_{cat}^{ss} and the improvement in k_{cat}^{ss} of each molecule relative to the X-motif. The residues were numbered according to their position in in the extended X-motif.

		Sequence																																									k_{cat}^{ss} (min^{-1})	Fold Improvement
		Substrate-binding Site 1								½ Stem 1				Loop 1				½ Stem 1						½ Stem 2			Loop 2					½ Stem 2					Substrate-binding Site 2									
						20					25					30					35					40					45					50					55					
	Wt	G	G	A	G	U	A	G	C	C	A	A	U	G	U	G	A	A	U	U	G	A	G	A	G	C	C	U	U	A	A	G	C	U	G	U	A	U	C	A	G	G	A	C	0.010	1
Round 6	1		A					C										G																					U						0.004	0.4
	2		A																C																								G	A	0.005	0.5
	3		A						U																														U					U	0.011	1.1
	4		A	U											C	A			A																				U						0.056	5.6
	5		A	G																																							G	U	0.073	7.3
	6		U																																				U						0.151	15.1
	7		A				G																																				G	U	0.174	17.4
	8						G									A																												A	0.195	19.5
	9		A												C																								U						0.218	21.8
	10		A				G									A		G																									G	A	0.176	17.6
	11		A				G									A		G																									G	A	0.220	22.0
	12		A																																								C		0.220	22.0
	13		A													A																											G	A	0.226	22.6
	14						G									A																											U	G	0.234	23.4
	15		A																																				U						0.188	18.8
	16		A																																				U						0.234	23.4
	17		A																																				U						0.240	24.0
	18		A																																				U						0.241	24.1
	19	A	A																																				U						0.256	25.6
	20		A										C					G											C										U						0.261	26.1

Table S3. Sequence analysis and activity screening of variants selected from round 6. Each variant was *in vitro* transcribed for 1 h before 2 µL of the mixture was transferred to 8 µL of activity assay mixture containing fluorogenic substrate (4 µM final concentration). Assuming that the yield of RNA was identical for the X-motif (wt) and each variant, it was possible to calculate k_{cat}^{ss} and the improvement in k_{cat}^{ss} of each molecule relative to the X-motif. The residues were numbered according to their position in in the extended X-motif.

		Sequence																																											k_{cat}^{ss} (min^{-1})	Fold Improvement
		Substrate-binding Site 1								½ Stem 1				Loop 1				½ Stem 1						½ Stem 2			Loop 2					½ Stem 2					Substrate-binding Site 2									
						20					25					30					35					40					45					50					55					
	Wt	G	G	A	G	U	A	G	C	C	A	A	U	G	U	G	A	A	U	U	G	A	G	A	G	C	C	U	U	A	A	G	C	U	G	U	A	U	C	A	G	G	A	C	0.010	1
Round 9	1		A													A		G										C	C						A				U						0.001	0.1
	2		A										C		C			G																					U					U	0.003	0.3
	3		A									U															A						A						U						0.003	0.3
	4		A	C					U																														U						0.009	0.9
	5		A	C								G			C	A			C										G															U	0.065	6.5
	6		A				G						C					G																									U		0.109	10.9
	7		A	U											C	A																											G	U	0.160	16.0
	8		A	U											C	A																												U	0.173	17.3
	9		A	U											C	A																												U	0.226	22.6
	10		A													A													C														U	G	0.192	19.2
	11		A										C					G																						G	A		G	A	0.192	19.2
	12		A																																								G	A	0.201	20.1
	13		A												C	A												C														U	G	A	0.215	21.5
	14		A													A																											G	A	0.243	24.3
	15		A													A																											G	A	0.248	24.8
	16		A													A																											G	A	0.294	29.4
	17		A													A																										U	G	A	0.254	25.4
	18		A													A													C														G	U	0.289	28.9
	19		A	C												A																											G	A	0.289	28.9
	20		A													A																									U		G	A	0.289	28.9

Table S4. Sequence analysis and activity screening of variants selected from round 9. Each variant was *in vitro* transcribed for 1 h before 2 μL of the mixture was transferred to 8 μL of activity assay mixture containing fluorogenic substrate (4 μM final concentration). Assuming that the yield of RNA was identical for the X-motif (wt) and each variant, it was possible to calculate k_{cat}^{ss} and the improvement in k_{cat}^{ss} of each molecule relative to the X-motif. The residues were numbered according to their position in in the extended X-motif.

Supporting Figures

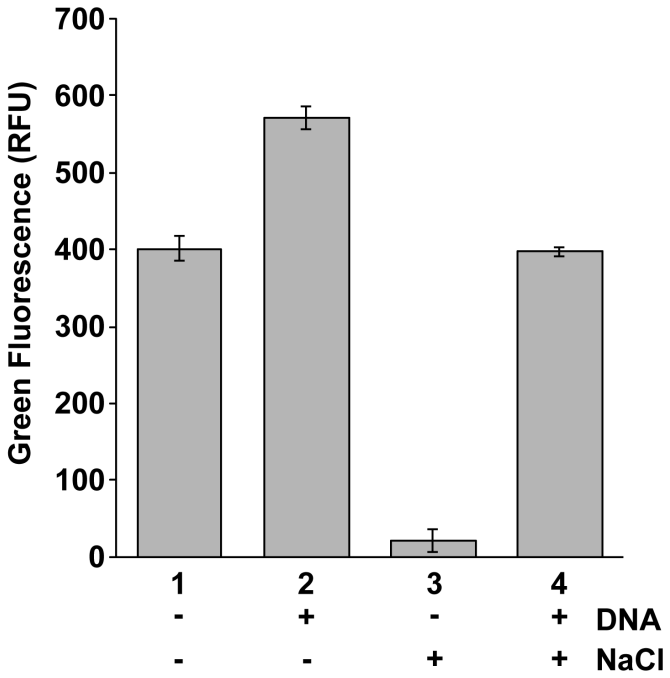

Figure S1. T7 RNA polymerase-generated background fluorescence and effect of NaCl. An in *vitro* transcription (IVT) mixture was prepared as for enrichment measurement and incubated in the presence or in the absence of template DNA for 30 minutes at 37°C. 17 µL aliquots of IVT mixtures were then mixed in a total volume of 20 µL with 100 pmol of fluorogenic substrate in the presence or in the absence of 250 mM NaCl. After incubating for 30 minutes at 37°C the green fluorescence of each mixture was measured. While the presence of template DNA in IVT led to a limited increase in fluorescence when the fluorogenic substrate was added alone (compare lanes 1 and 2), the signal was clearly detectable in the presence of 250 mM NaCl (compare lanes 3 and 4), mainly through a reduction of background fluorescence (compare lanes 1 and 3). The values are the mean of 2 independent measurements and error bars correspond to ±1 standard deviation.

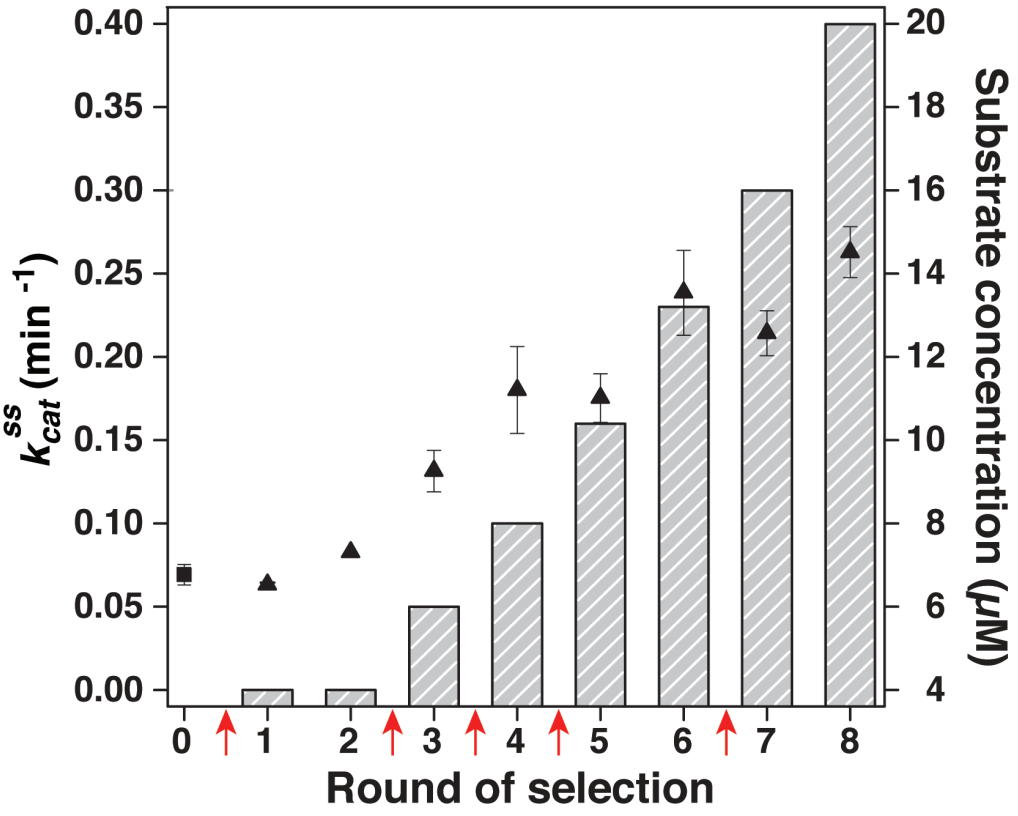

Figure S2. *In vitro* evolution of the extended X-motif at low mutation rate (0.8 mutations per gene). Multiple-turnover activity of the selected libraries at low mutation rate (0.8 mutations per gene) in different rounds of selection. The k_{cat}^{ss} was determined for the parental extended X-motif (square) and the selected library (triangle). Steps at which new genetic diversity was introduced by random mutagenesis are indicated by red arrows. The total concentration of substrate RNA (fluorogenic and non-fluorogenic) used in the selection is represented by the dashed bars. The concentration of fluorogenic substrate was constant in all rounds (4 μM). The values are the mean of at least 3 independent measurements and error bars correspond to ±1 standard deviation.

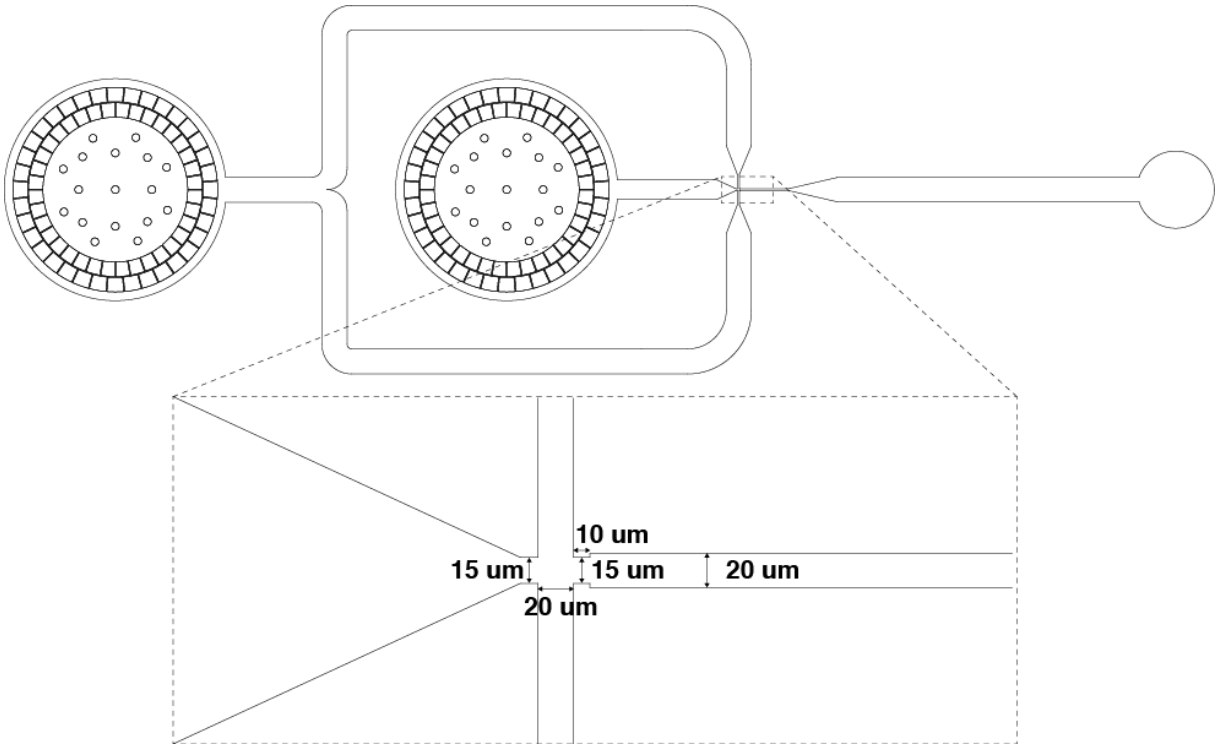

Figure S3. PCR droplet generation device. Key dimensions of the device are indicated in μm. 10 μm deep microfluidic devices were used.

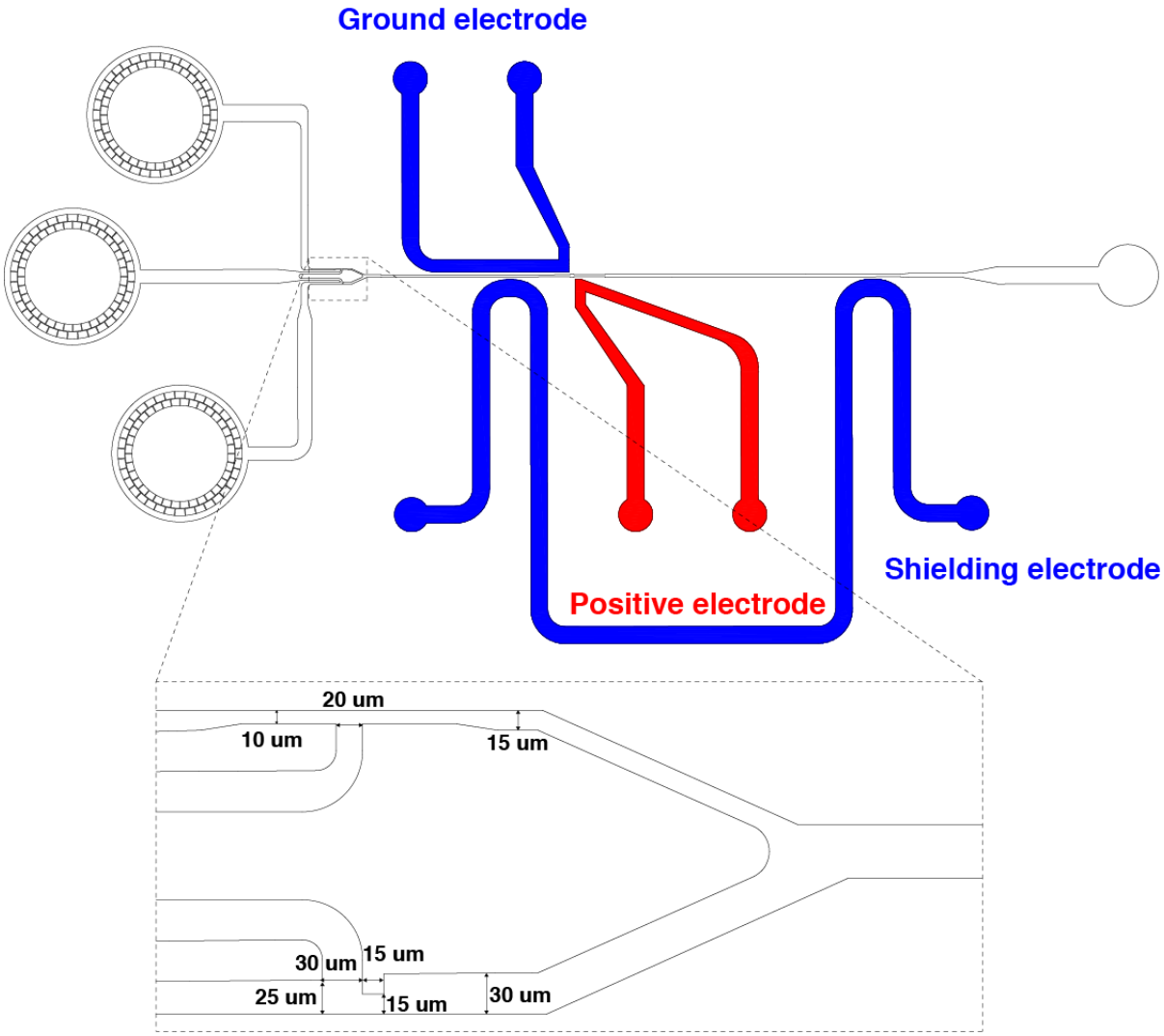

Figure S4. Droplet fusion device. Key dimensions of the device are indicated in μm. 15 μm deep microfluidic devices were used.

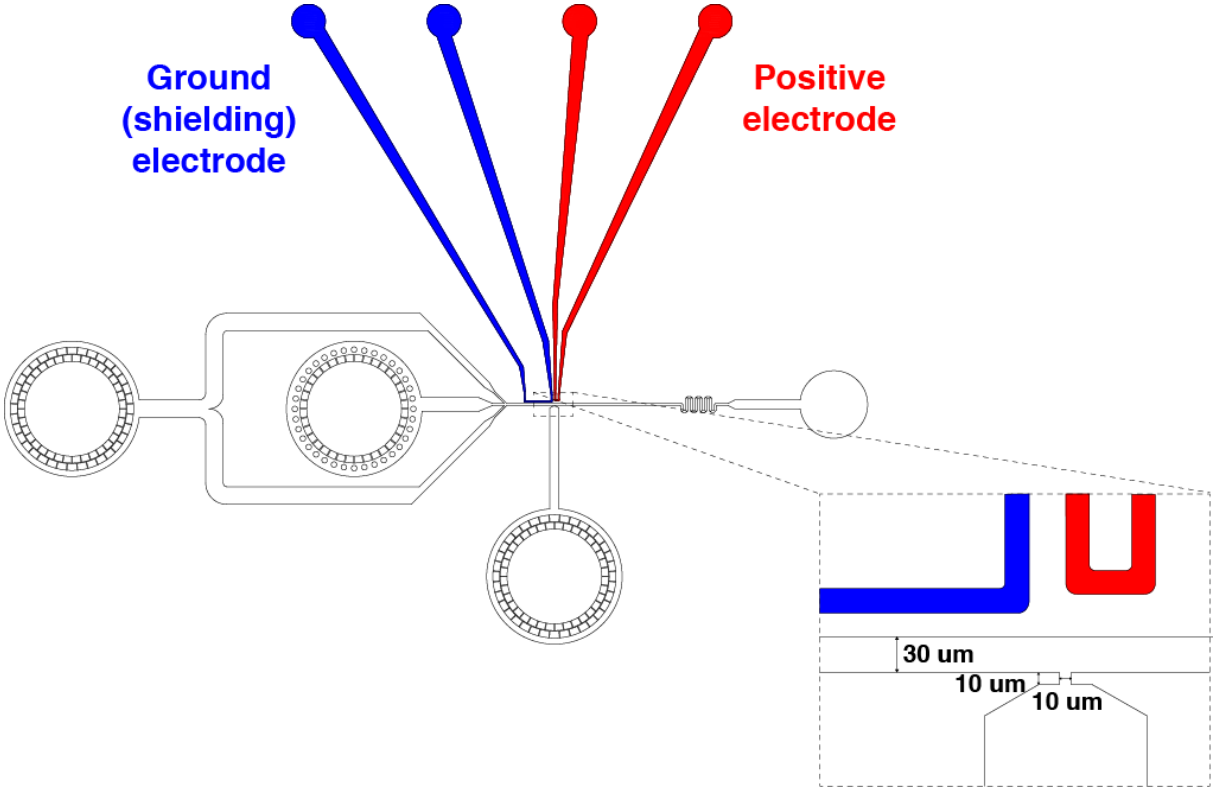

Figure S5. Pico-injection device. Key dimensions of the device are indicated in μm. 20 μm deep microfluidic devices were used.

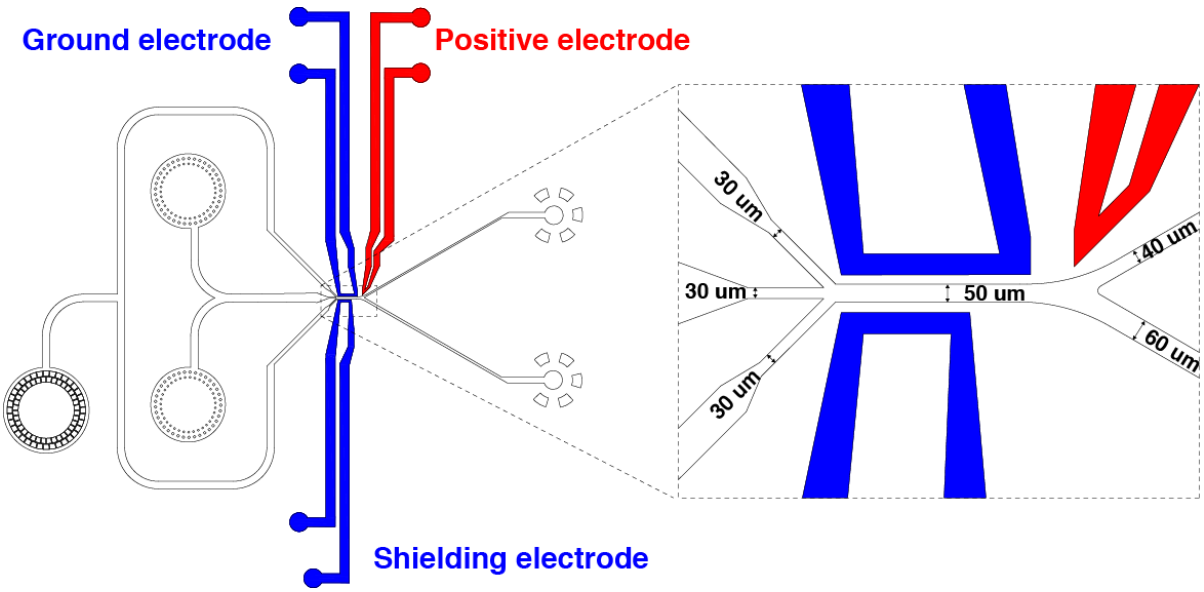

Figure S6. Fluorescence Activated Droplet Sorting device. Key dimensions of the device are indicated in µm. 25 µm deep microfluidic devices were used.