Supporting Information for

One in a million: flow cytometric sorting of single cell-lysate assays in monodisperse picolitre double emulsion droplets for directed evolution

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Contents

(C) Supplementary Movies

(A) Experimental details

1.1 Chemicals and Materials. The materials used were purchased from Sigma Aldrich unless otherwise noted. Fluorinated oils HFE-7500 and FC-40 were purchased from 3M Novec, and Krytox FSH for surfactant synthesis was from DuPont. The substrate fluorescein disulfate was synthesized as previously described.[1]

1.2 Device fabrication. All microfluidic devices for monodisperse emulsion formation were produced using soft lithography as previously detailed.[2] Chip designs are presented in the SI (Fig S-1), and are freely available to download in CAD-compatible formats from DropBase, a repository of microfluidic droplet device designs (http://www2.bio.cam.ac.uk/~fhlab/dropbase/dropgen.php). The master was fabricated by applying a layer of SU-2025 (15 or 20 μm) on a 3'' silicon wafer using conventional lithography. The master was coated with a mixture of poly(dimethyl)siloxane (PDMS, Slygard 184) and curing agent (Slygard 184) in the ratio 10:1 (w/w). After degassing and curing at 70 °C for 6 hours the PDMS device was removed from the master and holes for tubing connections were created using a biopsy punch. The device was then attached to a glass slide by treating with oxygen plasma (Diener Femto plasma asher) for 30 seconds.

For the construction of hydrophobic chips, the PDMS device was baked for 20 min at 70 °C and coated with a hydrophobic surface agent trichloro(1H,1H,2H,2H-perfluorooctyl)silane (Sigma) in fluorinated oil HFE-7500 (1%, w/w; 3M).

To produce hydrophilic chips, PDMS devices were coated with polyelectrolytes directly after oxygen plasma treatment.[3] First, the activated chip was flushed with a polycation solution (poly(diallyldimethylammonium chloride), 2 mg/mL in 0.5 M NaCl) and left for 10 min. After washing with NaCl (0.1 M), a polyanion solution (poly(styrene sulfonate), 2 mg/mL in 0.5 M NaCl) was applied. Ten

minutes later the microfluidic device was washed with ddH_2O and sealed with scotch tape to prevent drying, which would result in salt crystal deposition in the channels.

1.3 Device operation. For the generation of monodisperse microdroplets a microfluidic device with a single or double aqueous inlet flow-focusing junction was used. The device was connected *via* polythene tubing (0.38 mm ID, 1.09 mm OD, Smiths Medical) to syringes (100 µL, 500 µL SGE glass syringes, 2500 µL Hamilton Gastight syringe or 1 mL plastic syringe (Becton-Dickinson, Madrid, Spain)), which were driven by syringe infusion pumps (Harvard Apparatus 2000). Droplet formation was monitored using a Navitar 12× microscope with a 'pike' camera (ALLIED Vision Technologies).

Water-in-oil single emulsions were formed in a hydrophobic flow-focusing device with channel dimensions of 15 µm height/16 µm width or 20 µm height/20 µm width. The carrier oil phase was HFE 7500 (3M) with 1% (w/w) surfactant (RainDance EA surfactant or AZ2C, synthesized as described in the supplementary information, SI). Flow rates of 40 µL/h total for the aqueous phase(s) and 500 µL/h for the carrier oil phase were used to generate droplets with an average diameter of 13 µm (1.15 pL volume) in a 15 µm height/16 µm width device.

The formation of water-in-oil-in-water double emulsions was performed in hydrophilic microfluidic devices with a double aqueous inlet flow-focusing junction with dimensions of 15 µm height/16 µm width or 20 µm height/20 µm width. The aqueous carrier phase consisted of Tween 80 (1%, w/w) in 150 mM NaCl solution. The water-in-oil emulsion was diluted with FC-40 (3M), containing surfactant (1%, w/w; RainDance EA or AZ2C). Before injecting single emulsion into the device the carrier oil (HFE-7500) was exchanged for FC-40 (3M) containing 1% surfactant (RainDance EA or AZ2C). The emulsion was drawn into a 100 µl SGE glass syringe below a layer of mineral oil (Sigma) and mounted into the infusion pump in a vertical position. Flow rates were adjusted

based on visual inspection. For instance, for double emulsion formation using a 15 μm height/16 μm width chip the flow rates used were 20 μL/h for water-in-oil emulsion, 25 μL/h for spacing oil and 365 μL/h for the aqueous carrier phase.

1.4 Protein expression. *E. coli* TOP10 cells were transformed with pASK-IBA63b-plus plasmid containing the PAS gene and plated on LB agar supplemented with ampicillin (100 μg mL^{-1}). LB broth (5 mL) supplemented with ampicillin (100 μg mL^{-1}) was inoculated with a single colony and grown overnight at 37 °C with shaking at 225 rpm. The overnight culture (1 mL) was diluted in 19 mL LB medium containing ampicillin (100 μg mL^{-1}) and incubated at 225 rpm at 37 °C until an A_{600nm}= 0.5 was measured. The cells were induced by addition of anhydrotetracycline (200 ng mL^{-1}) and protein was expressed for three hours at 37 °C with shaking at 225 rpm.

1.5 Cell compartmentalization in droplets. Two cultures for protein production were grown: the first expressing active PAS and the second expressing the PAS variant H211A. An aliquot of each culture (2 mL) was centrifuged for 5 min (2,000 rcf) and the supernatant discarded. To wash cells, the bacterial pellet was re-suspended by gentle vortexing in buffer (500 μL; 100 mM MOPS, 100 mM NaCl, complete EDTA-free protease inhibitor (1 tablet per 50 mL, Roche), pH 7.5) and centrifuged for 2 min at 2,000 rcf. This step was repeated three times. The bacterial pellet was taken up in 500 μL buffer and the A_{600nm} was determined. The active and inactive variants were mixed to give the ratios outlined in Table 1, and diluted to enable encapsulation at occupancies of 1 or 0.1 cells per droplet (Table 1) according to the assumption that 1 mL *E. coli* suspension at A_{600nm}=1 contain 5 x 10^8 cells. The cell suspension was mixed with the density-matching agent Percoll (25% v/v final concentration) in 100 mM MOPS, 100 mM NaCl, pH 7.5 and taken up into a syringe (inlet 3, Fig. 2). A second syringe (inlet 2, Fig. 2) contained a mixture of substrate (20 μM fluorescein disulfate) and cell lysis agents (rLysozyme, 0.4%, v/v; Novagen (30 KU/μl) and

BugBuster, 3% v/v of 10X stock; Merck). When compartmentalized on-chip, a flow rate of 20 μL/h for each of the aqueous solutions was used. The sample was collected under a layer of mineral oil in a microtube. Subsequently the water-in-oil emulsion was transformed into double emulsion as described under device operation.

1.6 Flow cytometric sorting. Before FACS sorting, the surfactant-bearing carrier phase (1 % w/w Tween 80 in 150 mM sodium chloride) was exchanged for 150 mM sodium chloride to remove the surfactant, which lowers surface tension and so prevents stable (water-in-air) droplet formation in the FACS instrument. Prior to sorting, the sample collection tubes were filled with 100 μL double-distilled water containing 200 ng of carrier DNA (pKNOM; a pUC18 derivative that codes kanamycin resistance in place of the parental ampicillin resistance and carried no insert). Addition of carrier DNA was found to decrease DNA loss during the subsequent purification process and increase the number of transformants by 5-fold.

Samples were sorted in a DakoCytomation (now BeckmanCoulter) MoFlo MLS high-speed cell sorter using PBS as sheath fluid. A set-up with a 70 μm nozzle was chosen to give an average sort rate of 10,000 – 15,000 events per second. The threshold trigger was set on side scatter. The sample was excited with a 488 nm argon laser and the emission was detected using a 530±15 nm band-pass filter. The double emulsion population was gated from other populations in the sample on logFSC/logSSC (see Fig. S-2).

1.7 DNA recovery and transformation. Sorted samples were de-emulsified by addition of 200 μL 1H,1H,2H,2H-perfluorooctanol (PFO), vortexing and centrifugation (5 sec, 2,000 rcf) to achieve phase separation. After the removal of the top layer (aqueous layer containing DNA) the PFO bottom layer was again extracted with 200 μL double-distilled water to recover remaining DNA. The harvested plasmid DNA was recovered from the combined aqueous layers using

a spin column (DNA Clean & Concentrator-5, Zymo Research), eluting with double-distilled water (10 µL).

Electrocompetent *E. coli* cells (25 µL, *E. cloni* 10G ELITE, Lucigen) were transformed with 5 µL of purified DNA by applying one electric pulse of 1.80 kV (using an *E. coli* Pulser Cuvette, 0.1 cm electrode; Bio-Rad and MicroPulser). Sterile S.O.C Medium (500 µL, Invitrogen) was added and the sample was shaken for 20 min at 37 °C before being plated on LB agar containing ampicillin (100 µg mL^{-1}) and incubated overnight at 37 °C.

1.8 Determination of the transformation efficiency and enrichment. The transformation efficiency was determined by comparison of the number of colonies obtained after transformation to the number of sorted droplets. To determine the enrichment of active PAS after sorting, transformant colonies were lifted with a nitrocellulose membrane (BioTrace™ NT, PALL) and transferred to a fresh agar-ampicillin plate containing 200 ng mL^{-1} anhydrotetracycline. The membrane was placed with colonies facing up on the plate. After incubation for 2 hours at 37 °C to allow protein expression, the nitrocellulose membrane (colonies facing up) was transferred into an empty petri dish. The cells were lysed by three freeze-thaw cycles (-80 °C for 5 min, 37 °C for 10 min) and then covered with a solution of 1.5% agar in 100 mM MOPS, 100 mM NaCl (pH 7.5) containing the substrate, 5-bromo-4-chloro-3-indolyl sulfate (0.02%, w/v; Sigma) (Fig. S-3). After 15 min incubation at room temperature colonies containing active PAS were colored blue (reflecting turnover of the colorless substrate to blue product). The enrichment was calculated as follows:

$$Enrichment = \frac{\%\ positives\ after\ sorting}{\%\ positives\ before\ sorting}$$

1.9 Control of incubation time. To demonstrate that control of incubation time in droplets is possible, two water-in-oil emulsions, one containing wild-type PAS, and another lacking enzyme were produced. The enzyme-free emulsion served as a negative control and contained just the substrate 10 µM fluorescein disulfate

(in 100 mM MOPS, 100 mM NaCl, 1.25% BSA, pH 7.5) whereas the enzyme-containing emulsion was produced by combining lysate of cells expressing wild-type PAS and substrate-buffer-mixture at the flow-focusing junction immediately prior to droplet formation. Aliquots of cell lysate emulsions were taken every 15 minutes and collected into tubes already containing the same amount of negative control droplets. To minimize leakage from droplets, the carrier oil (HFE7500 with 1% AZ2C surfactant) of the droplet mixture was exchanged to a 3:1 mixture of fluorinated oils FC40 and HFE7500 with 0.25% AZ2C (Fig. S-4). Heat inactivation at 95 °C for 5 min was performed after following time points: t = 0, 15 min, 30 min, 60 min, 2 h, 4 h and 24 h. Once all of the heat-inactivated single emulsions were prepared, they were independently transformed into double emulsions as described under device operation then subjected to FACS analysis as described below in method 1.12. In parallel, a reaction progress curve with the same reaction mixture was recorded in a plate reader (SpectraMax M5, Molecular Devices).

1.10 Adjustment of double emulsion droplet size by osmosis. Shrinking of double emulsion droplets was achieved by 10- to 100-fold dilution of double emulsion into a solution with a higher osmolarity than the buffer inside the droplets. For example, droplets with a diameter of 23 µm, containing 150 mM NaCl solution, were reduced to 17 µm by dilution of 10 µL of double emulsion into 500 µL of 2 M NaCl solution. For droplet expansion, a solution with a lower osmolarity was used. The samples were mixed thoroughly and incubated at room temperature overnight. Expansion and shrinkage was performed with SDS or Tween 80 stabilized double emulsions.

1.11 Freezing droplets for storage in a discontinuous workflow. Double emulsions stabilized by Tween 80 were transferred into an aqueous glycerol solution (20% w/w in double-distilled water) and shock-frozen in liquid nitrogen. Thereafter, emulsion storage at -20 °C or -80 °C is possible. The

freezing process reduced the size of double emulsions due to the high glycerol content in the freezing solution inducing osmosis. After thawing, droplet rehydration was performed by exchanging the outer aqueous solution against a solution that was isomolar to the content of the inner droplet.

1.12 Flow cytometric analysis of double emulsion droplets. Water-in-oil–in-water double emulsion droplets were diluted into 150 mM NaCl and subjected to flow cytometric analysis (FACScan, Becton Dickinson). The sample was excited with a 488 nm argon laser and the emission was detected using a 530±15 nm band-pass filter. Double emulsion populations were gated on logFSC/logSSC (see Fig. S-2). Fluorescent readout was obtained from more than 15,000 droplets for each measurement. Flowing Software (version 2.5.0, Cell Imaging Core, Turku Centre for Biotechnology) was used for data analysis.

2. Notes on double emulsion formation, handling and stability.

2.1. Choice of the oil phase

We found that stable water-in-oil droplets are formed in the fluorinated oil HFE7500 (Table S-3). However, this oil is not suitable for stopping the enzymatic assay through heat deactivation due to the transfer of small molecules (Fig. S-4). Oil exchange of the water-in-oil emulsion surrounding oil to the fluorinated FC40 solves this problem. Additionally, stable double emulsion droplets could be formed with FC40 as the oil layer. Simple droplet stability tests can be performed by monitoring the behavior of double emulsion droplets using a microscope. When droplets are insufficiently stable decomposition of double emulsion by merging of the inner aqueous double emulsion droplet with the surrounding aqueous phase takes place leaving a small aqueous residue inside a plain oil droplet (Fig. S-9). This happens when the fluorinated oil Galden HT110 is used for double emulsion formation. Also, the surfactant type has an influence on double emulsion droplet stability, especially when droplets are heated, cooled, frozen or osmotically shocked (Table S-4).

2.2 Heating of water-in-oil emulsions

To stop reaction progress by heat shock, water-in-oil emulsions need to be stabilized by a surfactant to prevent merging at elevated temperature (95 °C). Usually, a surfactant concentration of 1% was used to obtain stable droplets during droplet formation. However, when droplets are heated small molecule transfer is facilitated by the presence of surfactant. For this reason, to minimize leakage during heating the surfactant concentration should be decreased, which compromises the droplet stability. In addition, as mentioned above, fluorinated oil HFE7500, which is well suited to production of stable droplets, supports small molecule transfer and has to be exchanged for FC40. FC40 was observed to have a contrary effect on the leakage and droplet stability behavior. To be able to heat droplets without merging and minimize small molecule transfer at the same time a compromise between the discussed parameters (oil types and surfactant concentration) is required. We observed good results for droplet stability and minimal leakage using a 3:1 mixture of fluorinated oils FC40 and HFE7500 with 0.25% surfactant (AZ2C or EA Raindance). A systematic exploration of the influence of different oils and surfactant concentrations on small molecule transfer behavior is in progress (unpublished data).

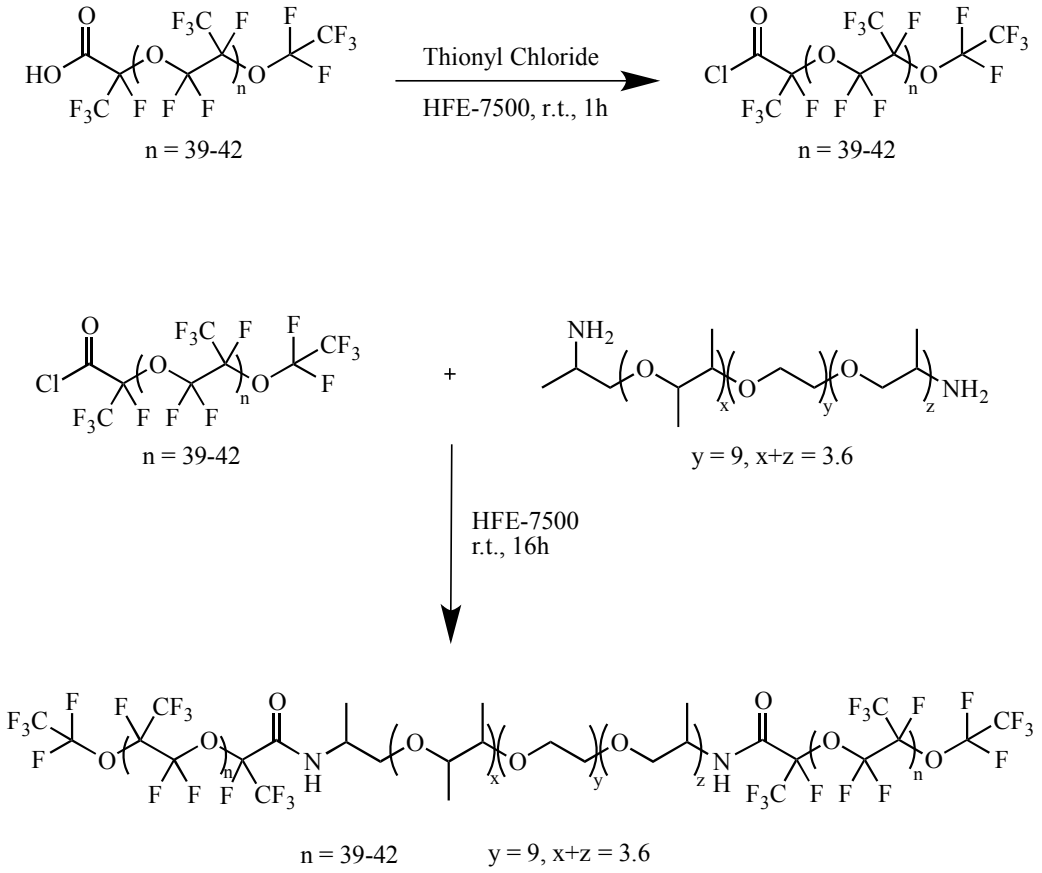
Before heating, water-in-oil emulsion samples were diluted at least 10-fold into the desired oil or oil mixture, respectively. A homogeneous sample mixture was achieved by shaking. Emulsions were covered with mineral oil to prevent both water evaporation and accumulation of emulsion on the side walls of the microtube during heating. After the sample separated into 3 layers (top: mineral oil, middle: emulsion, bottom: fluorinated oil) the majority of the mineral oil and fluorinated oil were discarded. In our experience, the interaction of water-in-oil emulsion with mineral oil may destabilize droplets during heating and foster merging. Removal of mineral oil to leave just a thin layer reduces this problem. The samples were heated at 95°C for 5 min (sufficient for inactivation of most enzymes). After cooling to room temperature, the samples were used for

downstream experiments (measuring fluorescence in a microscope or transformation into double emulsion for subsequent flow cytometry analysis).

2.3 Examination of aqueous surfactants for second emulsion

Expansion and shrinkage were addressed with several surfactant combinations. SDS had been used before in on-chip double emulsion formulation,[3] but proved incompatible with storage of samples in the fridge due to crystal formation at the surface of droplets, presumably caused by the limited solubility of SDS. Therefore, Tween 80 and Triton X-100 (which had been used previously[4,5]) were explored as alternatives. The stability of Triton X-100 formulation (1% w/v) was insufficient for expansion and shrinkage by osmosis as well as for heating and freezing. Tween 80 (1% w/v) was stable under all conditions and is therefore our reagent of choice (Table S-4).

3. Synthesis of surfactant AZ2C



Scheme 1. Reaction scheme for the synthesis of AZ2C through activation of Krytox FSH as an acyl chloride and subsequent coupling with Jeffamine ED-600.

Krytox 157 FSH (8.55 g, 1.5 mmol) was stirred vigorously under an argon atmosphere at room temperature. HFE7500 (1 mL) was added to reduce the solution viscosity followed by addition of thionyl chloride (217 µL, 3.0 mmol). The mixture was stirred at room temperature for 1 hour. Volatile compounds were removed *in vacuo* and the solution was cooled to 4 °C. Jeffamine ED600 (450 mg, 0.75 mmol) and HFE7500 (3 mL) were added. The reaction mixture was stirred at room temperature overnight. The solvent was removed *in vacuo.* The resulting material was used as a surfactant without further purification. Repeated syntheses of this material gave products that were identical in all functional respects.

(B) Supplementary Figures & Tables

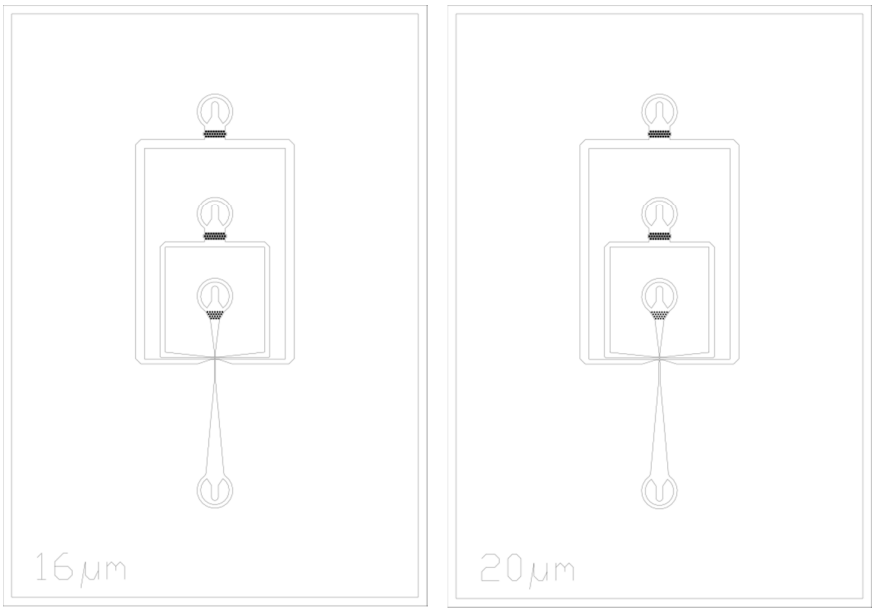


Figure S-1. Design of the microfluidic chips used in this research.

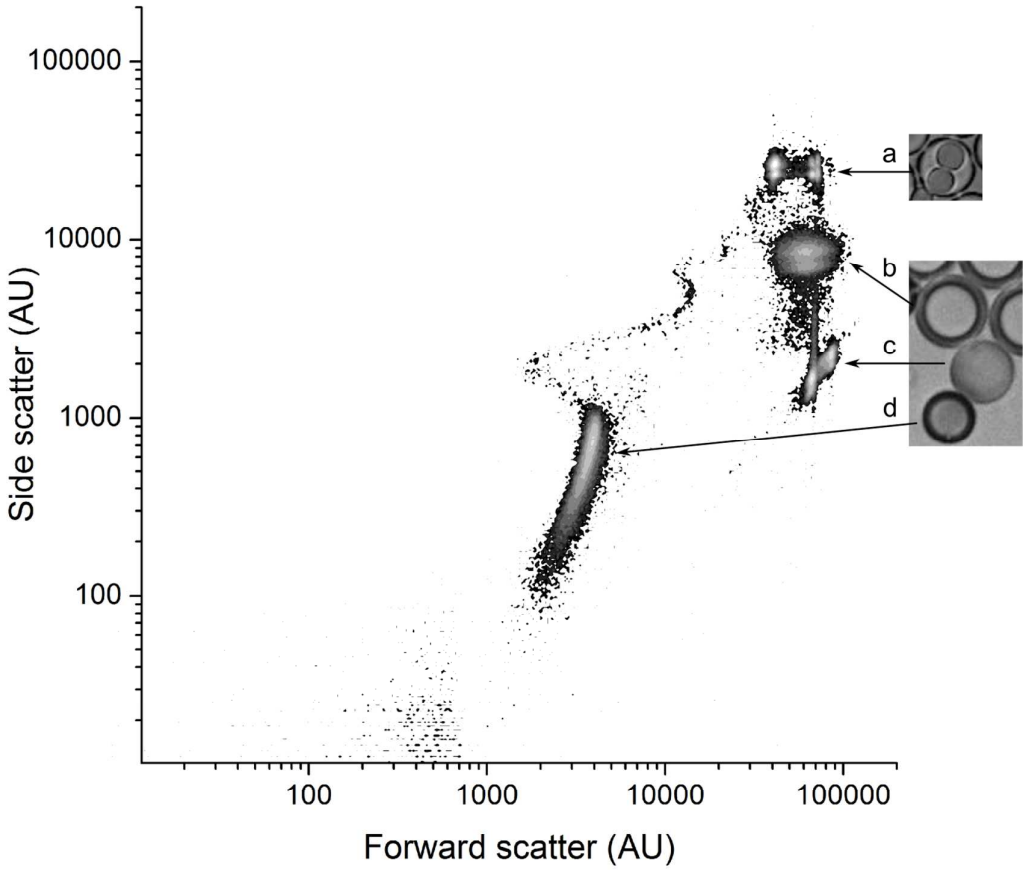


Figure S-2. Different droplet species and their positions in a forward scatter *vs* side scatter plot. The population with the highest side scatter (a) represents doublets, which contain two inner aqueous droplets. They are formed when the channel width of the double emulsion-forming chip is large (>1.3 times water-in-oil droplet diameter) and/or the flow rates for water-in-oil emulsion entering the chip are too high (in relation to the other flow rates). The population labeled with the arrow (b) consists of the desired monodisperse double emulsion droplets. This population should be gated in a FACS experiment. Empty oil droplets, formed when the flow rate for the 'spacing oil' (Fig. 2F) is too high, appear as population (c). Population (d), shifted towards the plot center, is made up of 'mineral oil-in-fluorinated oil-in-water' double emulsion. These droplets can be formed when the mineral oil that overlays the single emulsion in the syringe (Fig. 2D) enters the chip.

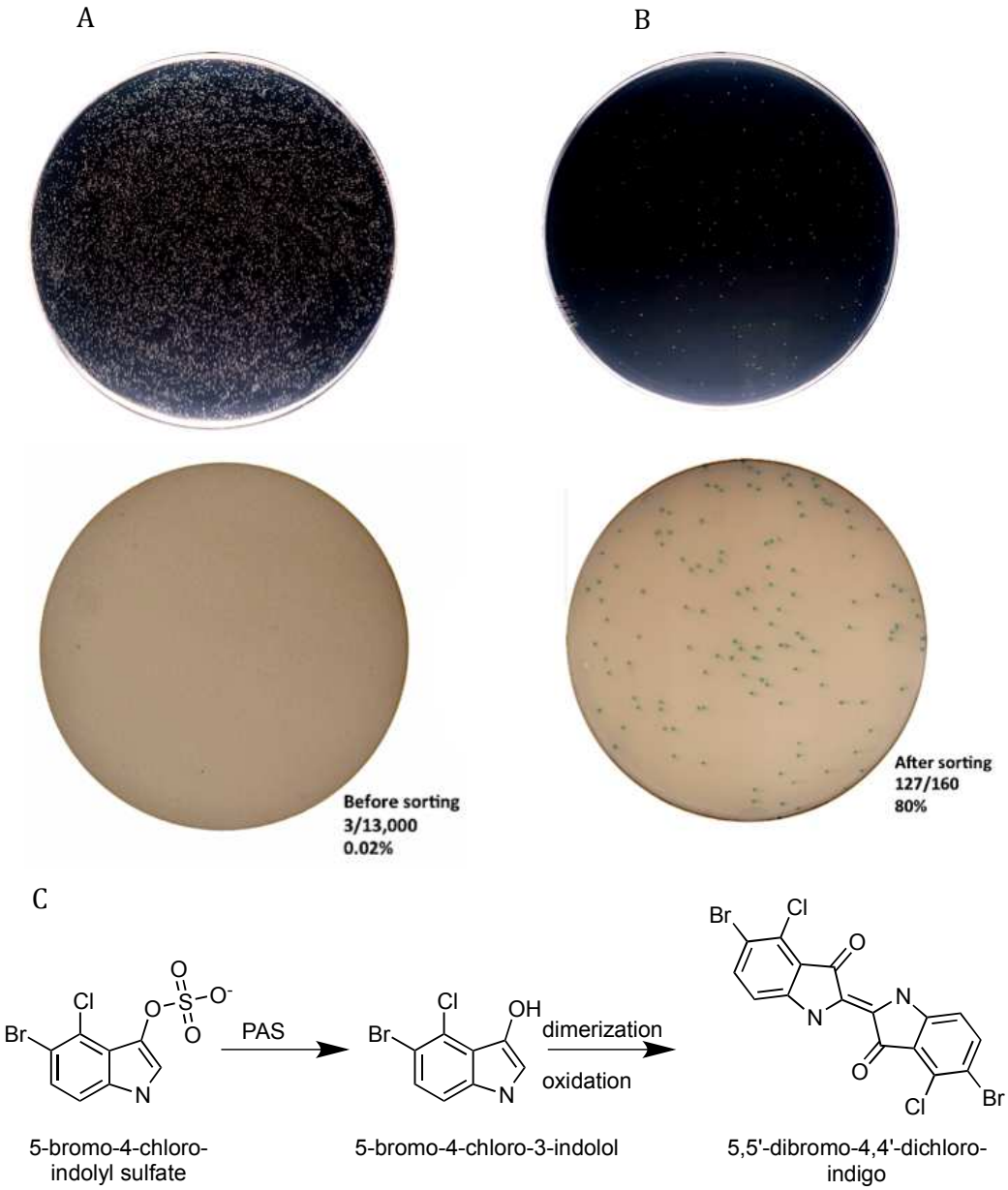


Figure S-3. Demonstration of the enrichment power of the double emulsion screening system by determination of active variants in the mixture before and after sorting. Images show transformed *E. coli* cells with DNA recovered from droplets before and after sorting (calculated 1:1,000 active:inactive ratio). Bacterial plates on black background – before enzymatic assay, on white background – after enzymatic assay. **(A)** Before sorting: 3 of 13,000 colonies show activity on indonyl sulfate substrate giving a ratio of 0.02% active cells in the mixture. **(B)** After sorting: 80% of colonies (127 of 160 colonies) show enzymatic activity. **(C)** Reaction mechanism of the substrate 5-bromo-4-chloro-3-indolyl sulfate to yield an insoluble blue product after sulfate group cleavage by PAS.

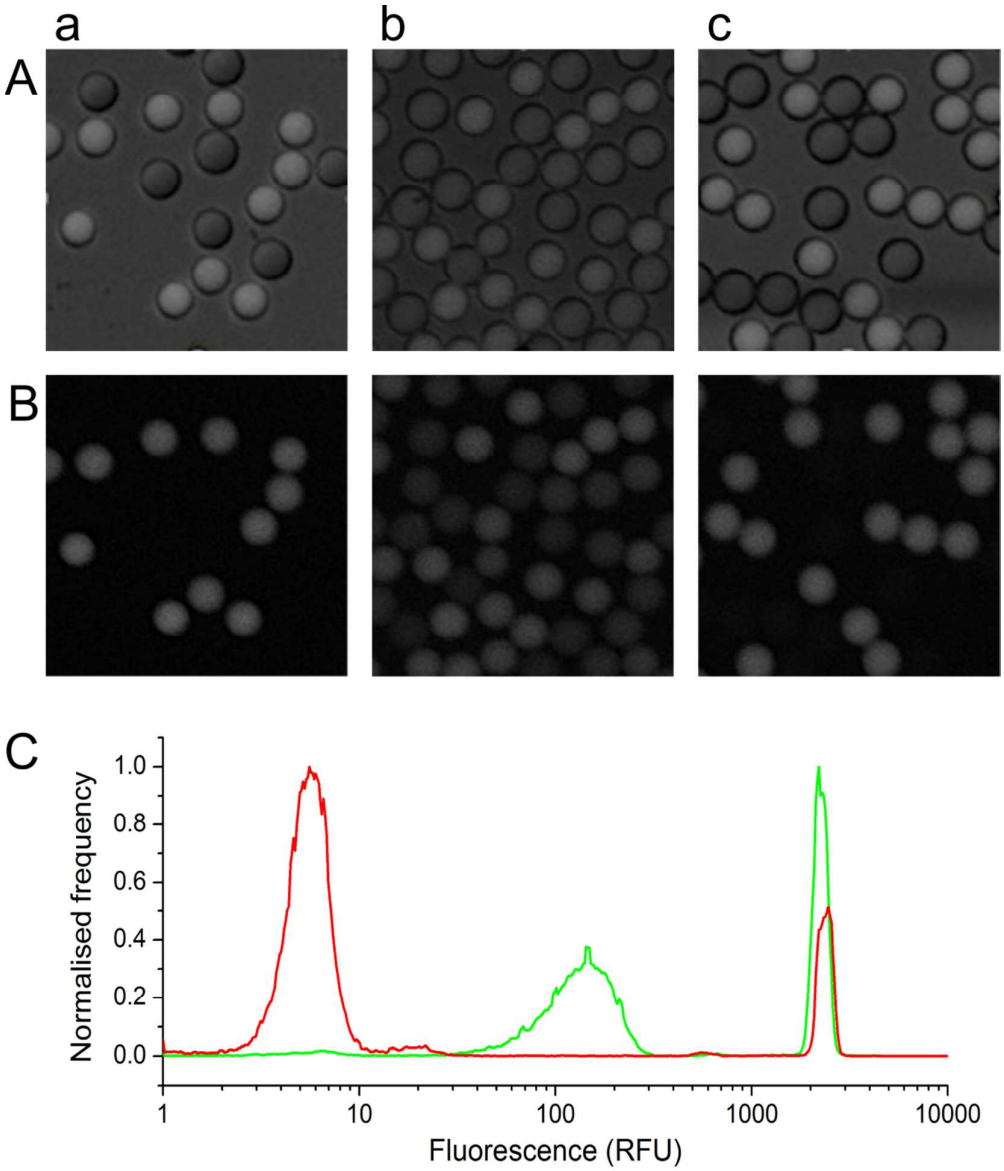


Figure S-4. Fluorescein transfer between droplets when a mixture of fluorescent and non-fluorescent droplet is heated to 95 °C for 5 min. The upper row **(A)** shows an overlay of fluorescent and visual microscope images. The bottom row **(B)** displays the corresponding fluorescent images for the sample before heating (a), the sample heated in HFE7500 with 0.25% AZ2C surfactant (b) and the sample heated in a FC40-HFE7500 (3:1) mixture with 0.25% AZ2C surfactant (c). A FACS fluorescence histogram **(C)** of a mixture of droplets with and without fluorescein before heating (red) and after heating in FC40-HFE7500 (3:1) mixture with 0.25% AZ2C surfactant (green). No transfer of DNA or protein was ever observed in droplet emulsion systems at relevant experimental timescales and we assume that this holds here, too.

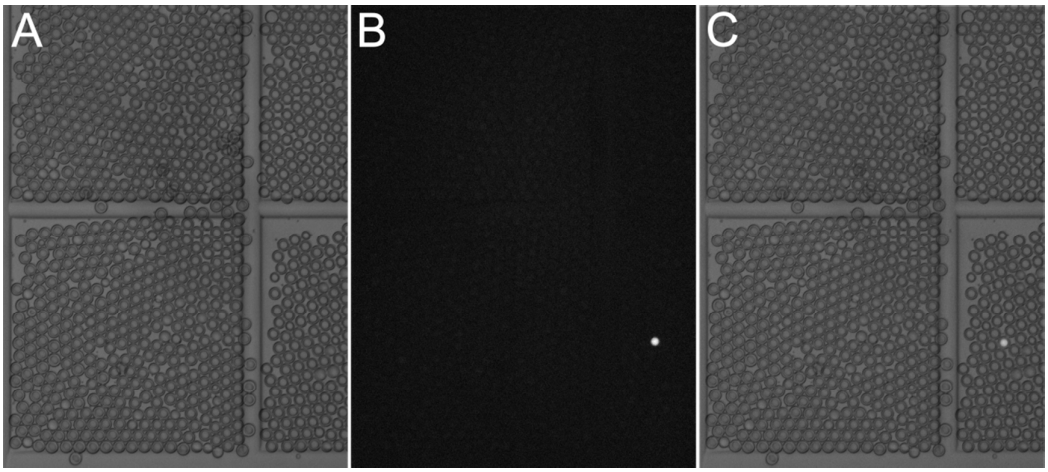


Figure S-5. Full-scale images used to generate Fig. 3A. **(A)** Bright-field image. **(B)** Fluorescence image. **(C)** Overlay of bright-field and fluorescence images.

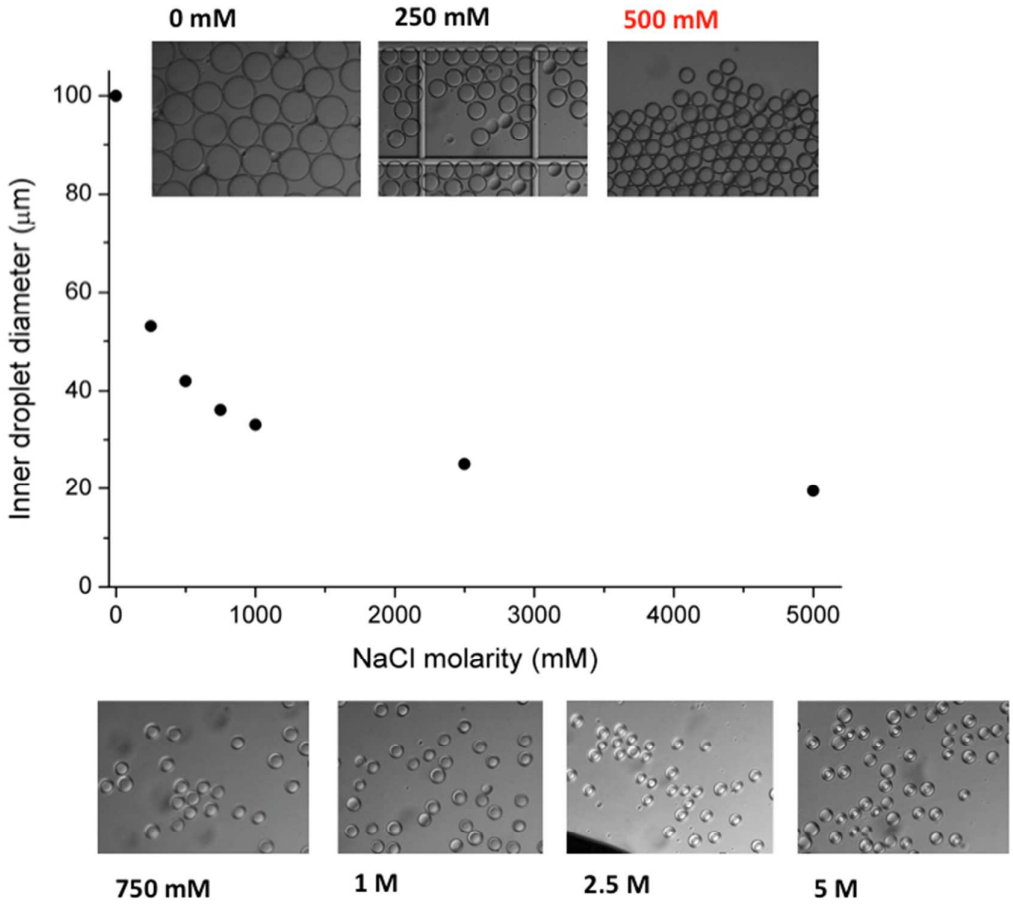


Figure S-6. Expansion and contraction of double emulsion droplets by osmosis. Double emulsion droplets with an avarage diameter 52 μm containing 500 mM sodium chloride solution were exposed to bulk solutions with a range of sodium chloride concentrations (shown on the x-axis). In bulk solution of higher salt concentration ([NaCl] > 500 mM), the droplets contracted, while in lower salt concentration ([NaCl]< 500 mM) expansion was observed. For each data point an image of corresponding droplets is shown. Double emulsion droplets with 500 mM sodium chloride in the inner solution were produced in a 500 mM sodium chloride outer solution containing 1% Tween 80.

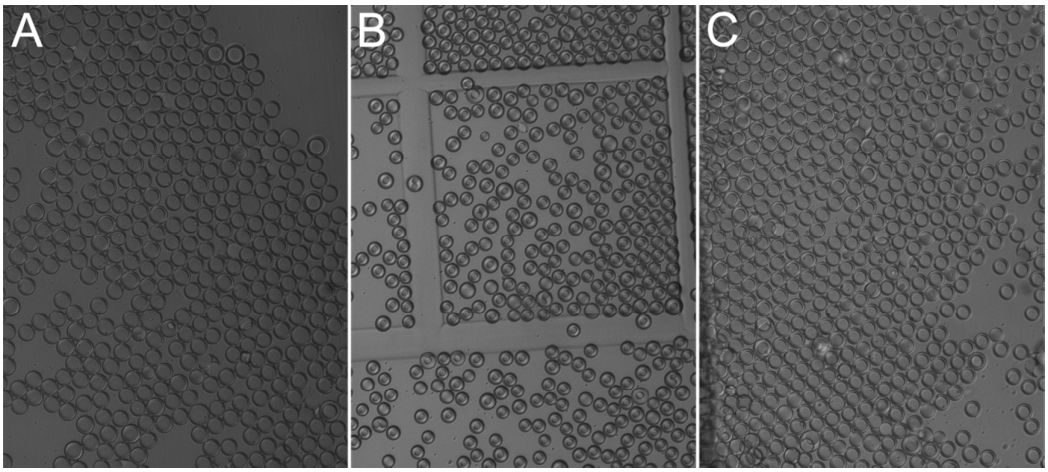


Figure S-7. Full-scale images of droplet freezing and rehydration (used to generate Fig. 5.) All images are of the same sample **(A)** before treatment, **(B)** after addition of glycerol (leading to droplet shrinkage) and **(C)** after thawing and rehydration.

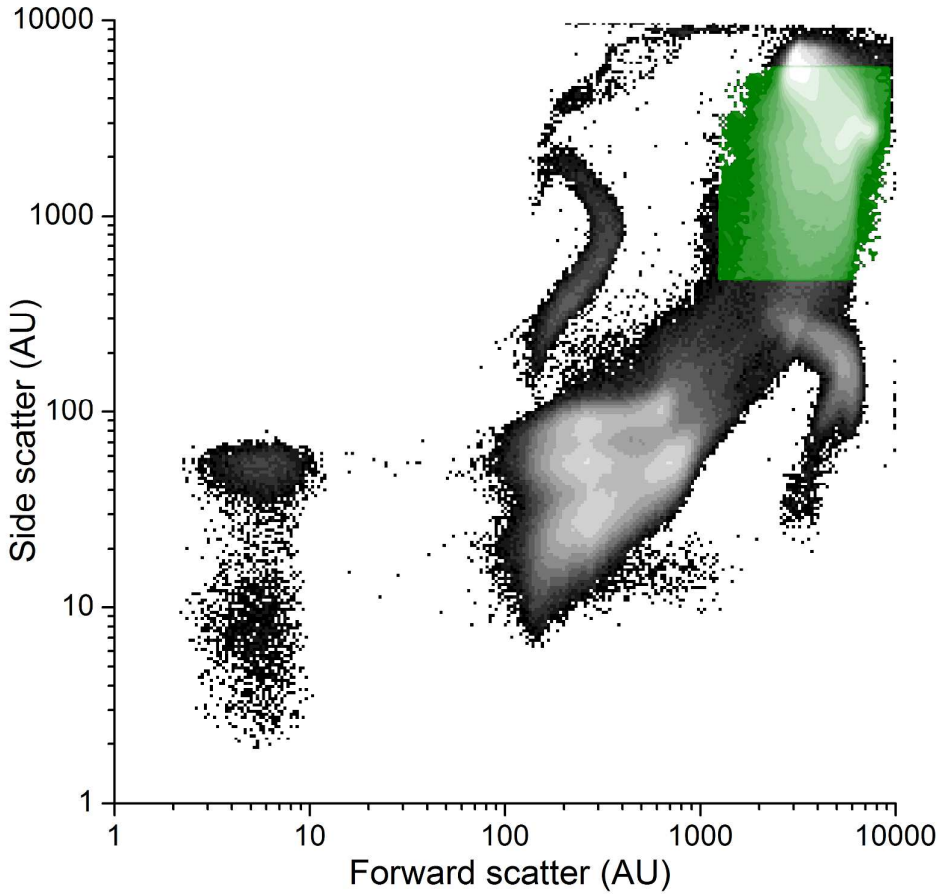


Figure S-8. A plot of forward scatter vs side scatter for Fig. 3. The gate was set on the green population to obtain forward scatter *vs* fluorescence plot shown in Fig. 3.

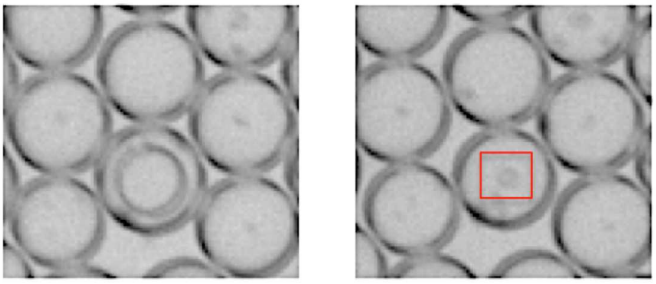


Figure S-9. A test system for assessing droplet stability. To identify suitable surfactant oil combinations the following test was carried out: When the type of oil surrounding aqueous droplets destabilizes double emulsions, then their formation is difficult and inner aqueous droplets combine with outer aqueous phase. As a consequence only a very small aqueous residual droplet inside an oil droplet remains behind (red square) and the format of double emulsion is lost.

Table S-1. Kinetic parameters of PAS variants used in this study. The substrate used to determine these kinetic parameters was *p*-nitrophenyl sulfate.

Variant	k_{cat}/K_M
Wild-type[a]	$(4.9 \pm 0.8) * 10^7$
H211A	$(8.2 \pm 0.8) * 10^2$
Mutant AZ0 (M72L, E74G, M110I, K193Q, A203S, K275M, G322D)	No detectable activity

[a] data taken from Babtie *et al.*[6]

Table S-2. Influence of molarity of the outer solution on droplet size and volume. Experimentally measured droplet diameters and calculated volumes after overnight incubation in buffers with different molarities. The initial solution concentration in inner droplets was 500 mM.

Molarity [mM] outer solution	Size [μ m] inner droplet	Size [μ m] whole droplet	Volume [pL] inner droplet	Volume [pL] whole droplet
0	100	110	524	697
250	53	63	78	131
500	42	52	39	74
750	36	50	24	65
1000	33	47	19	54
2500	25	45	8.2	48
5000	19.5	40	3.9	34

Table S-3. Overview of oil/surfactant combinations that were explored in this work.

oil	surfactant	range of surfactant tested	Optimal surfactant concentration	droplet formation	Flow rates aq./oil	Single emulsion stability
Galden HT110	Krytox 157 FSL	0.05–0.5%	0.25%	Ok but some deformation, satellites	50/500	~95% ok
Galden HT135	AZ2C		1.50%	low flow rates	30/500	low
Galden HT170	AZ2C		1%	low flow rates	20/500	low
HFE 7500	Raindance EA	0.5–1%	1%	good	50/500	high
HFE 7500	AZ2C	0.5–1%	1%	good	50/500	high
FC40	Krytox 157FSH	0.05–0.8%	0.10%	low flow rates	30/500	unstable
FC40	AZ2C		1%	low flow rates	25/500	unstable

Table S-4. Stability to different treatments of double emulsions stabilized by selected surfactants. All surfactants in this table are suitable for FACS sorting experiments.

	Treatment				**Limitations**
Surfactant	size change by osmosis	heating (95 °C)	freezing (- gly)	shock freezing (+ glyc)	
SDS	+	–			crystallization of surfactant
Triton X-100	–	–	–	+	no re-hydration after freezing
Tween 80	+	+	+	+	–

(C) Supplementary Movies

Two movies document the formation of single and double emulsion.

Movie S-1 Water-in-oil emulsion droplets were produced using a hydrophobically coated flow-focusing device (15 μm height / 16 μm width). The formation of these single emulsion droplets was monitored with a microscope (Olympus IX 71; 20X objective) and a fast camera (Phantom V7.2, Vision Research).

Movie S-2 Water-in-oil-in-water double emulsions were generated using a hydrophilically-coated flow-focusing device (15 μm height / 16 μm width). The formation of these double emulsion droplets was monitored with a microscope (Olympus IX 71; 20X objective) and a fast camera (Phantom V7.2, Vision Research).

The movies are available from the available free of charge via the Internet at http://pubs.acs.org from the entry for this article.

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