Supporting Information

Cell-free Directed Evolution of a Protease in Microdroplets at Ultrahigh Throughput

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Contents

S1. General Information and Procedures S2
S2. Supplementary Figures and Tables S6
S3. References S16
S1. General Information and Procedures

Chemicals and Materials

The materials and oligonucleotides used were purchased from Sigma Aldrich (St. Louis, Missouri, USA) unless otherwise noted. Fluorinated oil HFE-7500 was purchased from 3M Novec and 008-Fluorosurfactant from Ran Biotechnologies. The fluorogenic EnzChek casein substrate was obtained from ThermoFisher Scientific.

Library construction using Slonomics® for performing directed evolution in droplets

Six different Savinase® libraries have been generated via a ligation-based strategy for chemical gene synthesis based on the Sloning building block technology (Slonomics®).1 Six loops of the Savinase® gene consisting of 9-11 amino acids have been randomized. The following amino acid positions have been randomized for each library: Lib-1 = 53-63, Lib-2 = 94-104, Lib-3 = 124-134, Lib-4 = 152-163, Lib-5 = 181-191 and Lib-6 = 209-219. 73% wildtype are maintained in each position. The numbering of amino acid positions corresponds to that of the subtilase BPN' sequence.2 All six linear libraries were equipped with a T7 promoter and a ribosome binding-site (RBS) in front of the Savinase® gene. Each library was amplified using Sav-pUC19t primer pair (for: gttgtaaaacgacggcca, rev: ctggtgtaattgttatccgc), 5’-phosphorylated using T4 polynucleotide kinase (NEB) followed by circularization using T4 DNA ligase (NEB). Circularised genes were gel-purified and the concentration determined using a Nanodrop 2000 (Thermo Fisher Scientific, USA).

Chip design and microfluidic device fabrication

The designs for the poly(dimethyl)siloxane (PDMS) chip devices were prepared with CAD software (DraftSight, Dassault Systems). The corresponding CAD files can be downloaded from http://openwetware.org/wiki/DropBase. The devices were fabricated by standard soft lithography procedures using a high-resolution acetate mask (Microlithography Services Ltd.) and SU-8–2025 photoresist patterning.3,4 PDMS monomer and curing agent were mixed at a ratio 10:1 and then poured onto the lithographic plate before degassing. After PDMS solidification (65 °C, 4 h), PDMS was activated by exposure to an oxygen plasma and devices were sealed onto a microscope glass slide (or cover slip (thickness: 0.13mm) for the sorting chip). Hydrophobic modification of the channels surface was achieved by injecting a solution of 1% (v/v) trichloro(1H,1H,2H,2H-tetrafluoroethane) silane (Sigma) in HFE-7500 oil into the channels.

Flow-focusing generation of water-in-oil picolitre droplets to perform rolling circle amplification

Water-in-oil droplets were generated at high rates (~4 kHz) using a microfluidic double flow-focusing device (height: 30 μm; width: 30 μm at the flow-focusing junction; see SI Figure S15a) bearing three inlets. The microfluidic device was operated with syringe pumps (Nemesys or Cetoni) and gas-tight glass syringes (SGE) which were connected to the chip via fine pore PTFE tubing (ID 0.38 mm, OD 1.09 mm, Smith Medical). Two aqueous streams were mixed at the first junction and dispersed into the fluorinated oil HFE-7500 (3M) containing 2% w/w fluorosurfactant-008 at the second junction. The two aqueous phases used to generate the droplets contained reagents to perform RCA in droplets. RCA amplifications were performed using the commercial Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Saclay, France). Briefly, the DNA is heat-denatured then cooled in sample buffer containing random hexamers that non-specifically bind to the DNA. In a separate tube the second solution is prepared containing DNA polymerase (1/5 diluted with ddH2O), additional random hexamers, nucleotides, salts and buffers. The flow rates for oil, and the two aqueous solutions were 28 μL/min, 2 μL/min, and 2 μL/min, respectively, resulting in droplet volumes of about 14 pL. To obtain the required plasmid/droplet ratio after compartmentalization it was calculated that, by using 714,286 plasmids, 10% of the generated droplets contain a single plasmid, assuming a Poisson distribution for encapsulation. The droplet generation process was monitored on an inverted microscope (SP981, Brunell Microscopes) equipped with a high-speed camera (Miro cX4, Phantom Research). The generated droplets were collected into an inverted 500 μL microcentrifuge tube which was pre-filled with fluorinated oil containing 2% surfactant. The tube was modified by inserting tubing through access holes at the top and bottom of the microcentrifuge tube. This incubation chamber was sealed with...
adhesive glue (Scotch-Weld PR1500, 3M). The generated droplets were incubated at 30 °C for 6 hours to perform an isothermal amplification of DNA. After amplification the enzyme is heat inactivated for 15 minutes at 65 °C.

**Picoinjection of IVTT reagent and casein substrate**

The picoinjector devices were fabricated in PDMS, using the techniques described above, and consist of a droplet spacer and picoinjector (Supplementary Figure S15b). The devices have channel heights between 30 and 35 μm. Electrodes were made by filling channels with salt solution (5 M NaCl). The picoinjector injects fluid by merging the droplets with a pressurized channel containing the reagent. Picoinjection is triggered by an electric field, applied by electrodes connected to a high-voltage amplifier with a voltage output of 1.5 V at a frequency of 10 kHz. This voltage is applied to the electrodes to trigger injection. General flow rates for oil, the droplets and the solution to be injected were 3 μL/min, 2 μL/min, and 1 μL/min, respectively, resulting in injected volumes of 15 to 30 pL. In vitro transcription/translation (IVTT) in droplets was performed using the PURExpress In Vitro Protein Synthesis Kit (New England Biolabs, Hitchin, United Kingdom), according to the manual provided by the supplier. As a fluorogenic protease substrate the EnzChek Protease Assay Kit (Invitrogen, Carlsbad, United States) was used. For picoinjection in droplets the substrate was diluted 25-fold with ddH2O, to give an approximate concentration of 40 μg/mL. After injection of the fluorogenic casein EnzChek substrate the droplets were incubated in darkness for up to three days.

**Fluorescence-activated droplet sorting**

Water-in-oil droplets were re-injected into the sorting device (SI Figure S15c) at 0.5 μL/min and spaced out with oil (0.5% w/v 008-FluoroSurfactant in HFE7500) at 8 μL/min. As a result, droplets were sorted at a frequency of 200 Hz. A 488-nm laser was focused 180 mm upstream of the sorting junction through a 40x microscope objective (UPlanFLN, Olympus) for fluorophore excitation and the emitted fluorescent light was collected and amplified using photomultiplier tubes (H8249, Hamamatsu Photonics). The amplified fluorescence signal was processed by a data acquisition card operating at 38 kHz (National Instruments, USB-6009) that was linked to a peak detection algorithm, which recorded fluorescence distributions (LabView 8.2, National Instrument). Hardware triggering was implemented via a voltage comparator (LM339N, Texas Instruments), which compared the voltage readout by the photomultiplier tube with a user-defined arbitrary voltage generated via the acquisition card and doubled using an operational amplifier (LM358N, STMicroelectronics), to generate voltages between 0 and 10 V. A pull-up resistor (1 kΩ) was used to force the logical high state of the comparator to 5V and send the trigger signal to the pulse generator. Whenever the fluorescence peak reached a user-defined voltage threshold, a pulse generator triggered a single square pulse of 50 ms length and an amplitude of 0.7 Vp. This pulse was amplified 1,000-fold by a high voltage amplifier (610E, Trek) and applied to the electrodes on the sorting device. The sorting events were recorded with a fast camera (Phantom V7.2) that was triggered by the voltage comparator, to allow analysis of whether the desired droplets with increased fluorescence were indeed selected after the sort was carried out. Optical inspection of the movies thus recorded provided confirmation that only single droplets were selected for each pulse.

**DNA recovery**

Sorted droplets were collected into a 1.5 mL low DNA retention reaction tube (DNA LoBind, Eppendorf) and de-emulsified by adding 200 μL 1H,1H,2H,2H-perfluoroctanol (97%, Alfa Aesar). 25 μL of a 2 ng/μL salmon sperm DNA solution (Invitrogen) was added to the collected droplets. The tube was vortexed briefly and centrifuged for 1 min at 1,000 xg. The aqueous layer was transferred to a fresh reaction tube and the oil extraction was repeated twice. 5 μL of the aqueous phase were used for PCR amplification (30 s 98 °C, 25 x (10 s 98 °C, 30 s 55 °C, 30 s 72 °C), 300 s 72 °C, final hold 4 °C) using the sav-wt-Gibson primer pair

- for: gattacaaaaatcagccatgggtGCTGAAAGGCAAAGAAAATATTTAATGGG;
- rev: gcattcgctctcatcagAGCGCTTGCCGCTTCTGC).

The PCR product was purified by agarose gel electrophoresis. Isolated DNA was subeloned into the pCRII8a plasmid (Addgene) via Gibson-Assembly.
Quantification of enrichment of wildtype by quantitative PCR

To quantify the sorting efficiency, pUC19-sav-wt plasmid was mixed 1:250 with either pHAT-EtsB or pUC-EstB construct expressing the esterase EstB. Droplet generation, picoinjections and FADS were performed as described above. The amount of DNA was determined before and after sorting via quantitative PCR (qPCR). The following two primer pairs were designed that amplify a sequence of 100 nt of each gene and share the same melting temperature (T_m = 55 °C) and GC content (55%): Sav-qPCR-for gcaatcggtacctggaag; Sav-qPCR-rev tctctgatggagacca; EstB-qPCR-for gtatcggtactggaag; EstB-qPCR-rev ccaatcggtactggaag. 0.5 μM of each primer were mixed with 1x SensiMix SYBR green (Bioline) in a total volume of 25 μL. Quantitative PCR experiments were performed in duplicate in a CFX Connect (Bio-Rad) real-time PCR cycler. The cycling parameters were: initial DNA denaturation at 95 °C for 10 min, 40 cycles (95 °C for 15 s; 55 °C for 15 s; 72 °C for 20 s) followed by a temperature gradient enabling determination of the DNA melting temperature (between 79 °C and 85 °C). Reference curves using concentrations 1 ng/μL to 0.01 pg/μL plasmid DNA with both sets of primers were obtained with correlation coefficients R>0.99.

Re-screening of hits in B. subtilis as a host organism

Individual colonies of Savinase® variants transformed into B. subtilis were picked up and inoculated in 96-deep well plates containing 1 mL of 2YT medium supplemented with 5 μg/mL chloramphenicol. The cells were grown at 37 °C and 600 rpm overnight. 50 μL of these overnight cultures were used to inoculate 1200 μL of 2YT medium and grown for three hours at 37 °C. These cultures were induced with 500 μM IPTG and further incubated at 20 °C for 18 h. After recombinant production and secretion of proteins, cultures were centrifuged at 4000 xg for 30 minutes at 4 °C. 100 μL of the supernatant was assayed for activity by adding 0.2 μL EnzChek substrate (1 mg/mL in PBS) and 10 mM calcium chloride in reaction buffer (100 mM Tris-HCl, 2 mM calcium chloride, 2.5% Triton (v/v), pH 9). Fluorescence (λ_ex = 485 nm; λ_em = 515 nm) was measured over time using a plate reader. Variants showing fluorescence three times higher than standard deviation compared to wildtype were selected for sequencing and further testing.

Protein production and purification

For recombinant production of Savinase® and identified hits from the re-screening, the genes were amplified by PCR with Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific, F530L) using the sav-wt-Gibson primer pair. The amplified fragments were inserted into pCr18-a plasmid by Gibson-Assembly. The recombinant protein will be equipped with signal peptide for secretion and a C-terminal 6xHis tag for subsequent purification. 168 Marburg B. subtilis strain was transformed with pCr18a derivatives. The recombinant B. subtilis strains were grown in 2YT medium supplemented with 5 μg/mL chloramphenicol at 37 °C until an OD600 of 1 was reached. After induction by adding 0.5 mM IPTG, the culture was further incubated at 20 °C for 18 h. The culture was centrifuged (13,000 x g, 10 min, 4 °C) and supernatant applied to a Ni-NTA column equilibrated with buffer A containing 50 mM Tris-HCl, 150 mM NaCl and 50% glycerol (pH 8). After washing with 20 column volumes buffer A containing 20 mM imidazole, the bound proteins were eluted with 500 mM imidazole in buffer A. The enzyme in the elution buffer was further changed to buffer B by an Amicon ultra-4 centrifugal filter device (Millipore, USA) using the appropriate cut-off based on their molecular weight and then stored at -20 °C. Protein production was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and the concentration was determined by active site titration. Activity was tested by incubating 1 μM enzyme with 0.2 μL EnzChek substrate (1 mg/mL) and 10 mM calcium chloride in reaction buffer buffer (100 mM Tris-HCl, 2 mM calcium chloride, 2.5% Triton (v/v), pH 9). Fluorescence (λ_ex=485 nm; λ_em=515 nm) was measured over time using a plate reader. Initial velocities and end-point fluorescence was determined and compared to wildtype activity.
Active site titration

For the active site titration, a 10 mM solution of phenylmethylsulfonyl fluoride (PMSF) in 2-propanol was freshly prepared. 0.25 µL enzyme was mixed with 0-2 mM PMSF in reaction buffer (100 mM Tris-HCl, 2 mM calcium chloride, 2.5% Triton (v/v), pH 9). After a short incubation time the total content of enzyme was assayed for activity by adding 125 µM N-Succinyl-Ala-Ala-Pro-Phe \( p \)-nitroanilide (s-AAPF-pNA) substrate. The released \( p \)-nitroanilide can be measured by spectro-photometrical quantification at 410 nm and initial steady-state velocities determined. The residual activity was plotted versus the amount of PMSF and a line was fitted through the data points. The amount that is required for complete inactivation corresponds to the number of active sites.

Generation of a shuffled library using staggered extension process (StEP)

A shuffled library of identified hits was generated via StEPs \textit{in vitro} recombination following a procedure described in the literature. Briefly, 20 ng of mixed plasmid DNA were used as templates and mixed in a final volume of 50 µL with 0.15 μM of the sav-wt-Gibson primer pair, 1x PCR buffer, 200 µM dNTP mix, 1.5 mM MgCl\(_2\) and 2.5 U Taq polymerase. The PCR-cycler was programmed for 100 cycles using the following parameters: 94 °C for 30 s (denaturation) and 55°C for 17 s (annealing/extension). The PCR product was purified by agarose gel electrophoresis. Isolated DNA was further amplified by using the Sav-Sh-Lib-pHAT primer pair (for: tcacacatcattactcaacaacagactctca TTA ACG CGT TGC CGC TTC TGC GTT) and subcloned into the pHAT plasmid providing a T7 promotor and RBS for IVTT performance in picolitre droplets.
S2. Supplementary Figures and Tables

**Figure S1.** *In vitro* expression of green fluorescent protein (GFP) using IVTT after DNA amplification with different concentrations of phi29 DNA polymerase. pUC19-GFP plasmid DNA (6 pg/µL) with different concentrations of phi29 DNA polymerase (500 – 25 nM) and remaining RCA reagents to perform RCA for 3 hours at 30 °C. After addition of IVTT components, GFP expression was monitored over time in a plate reader measuring fluorescence (λ<sub>ex</sub> = 480 nm; λ<sub>em</sub> = 530 nm). 50 ng/µL pUC19-GFP was used as a positive control (‘positive Ctrl.’).

**Figure S2.** RCA is inhibited by IVTT reagents. RCA was performed by incubating pUC19-GFP plasmid DNA (6 pg/µL) with Φ29 DNA polymerase (100 nM) in presence of IVTT reagents for 2 hours at 30 °C or 37 °C (RCA + IVTT), respectively. As control experiments the IVTT reagents were added after performance of RCA (1. RCA/2. IVTT) and a mixture of plasmid DNA without Φ29 DNA polymerase was incubated with IVTT (-Phi29 + IVTT). *In vitro* expression of GFP was monitored over time in the plate reader measuring fluorescence (λ<sub>ex</sub> = 480 nm; λ<sub>em</sub> = 530 nm) for 4 hours at 37 °C.
Figure S3. Addition of EnzChek substrate after expression of sav-wt using IVTT results in higher fluorescent signal. pUC19-sav (10 ng/µL) was incubated with IVTT reagents (PURE) either in presence of the fluorogenic EnzChek substrate (2 µg/mL) or without. In latter case the substrate was added after expression of sav-wt performed at 37 °C for 4 hours. In both cases the fluorescent signal was measured after 12 hours reaction time ($\lambda_{ex} = 480$ nm; $\lambda_{em} = 530$ nm).

![Figure S3](image)

Figure S4. Enzymatic reaction in droplets. Single plasmids of a pUC19-sav-wt were compartmentalized in picolitre water-in-oil droplets ($\lambda = 0.1$) together with RCA reagents. After injecting IVTT components and the fluorogenic casein substrate, microscope images of the droplets were taken. Green fluorescent droplets can be detected showing savinase activity that are surrounded by droplets that lack enzymatic activity, because they are unoccupied. Scale bar: 400 µm.

Table S1. Enrichment of sav-wt by FADS based on proteolytic activity. For two separate enrichment experiments two sortings each were performed and fluorescent droplets similar to sav-wt activity were collected (see SI Figure S3). Starting ratio of active (sav-wt) to inactive (neg Ctrl.) plasmids were determined by qPCR. The enrichment values $\eta$ were determined after two different equations described in the literature.$^9,10$ According to Baret et al. the enrichment $\eta$ after one round of sorting was developed as a function of $\lambda$ (the occupancy based on a Poisson distribution) where $\varepsilon0$ is the initial ratio of active to inactive genes and $\lambda$ is the initial mean number of plasmids per droplet. The enrichment $\eta$ is defined as the ratio of $\varepsilon1$ after sorting to $\varepsilon0$ before sorting. Following Zinchenko et al. the enrichment $\eta'$ was determined by dividing the percentage of positives after sorting ($\varepsilon1'$) by that before sorting ($\varepsilon0$).
Figure S5. Histogram of 1:250 mixture of droplets containing sav-wt and a negative control gene screened via FADS for proteolytic activity. Droplets were incubated at room temperature in darkness for three days before sorting. The occupancy was $\lambda = 0.1$. The sorting gate was set up such that droplets showing increased fluorescence over the population average of a wild-type sample (sav-wt) were selected.

Figure S6. Standard calibration curve for sav-wt and EstB determined via qPCR. Dilution series of pUC-sav-wt and pUC-EstB plasmids were prepared ranging from 1 ng/µL to 0.001 ng/µL. After mixing with 1x SensiMix reagent and 0.5 µM gene specific primers, qPCRs were performed in duplicate. Calibration curves were generated from the measured Ct values and standard solution concentrations. Calculated linear regression and correlation coefficients are shown.
Figure S7. Nucleic and amino acid sequence of Savinase® (pdb: 1SVN). The signal peptide is indicated in purple and the pro-peptide in blue. Amino acids representing the catalytic triad are highlighted in yellow. Amino acids randomized for generating six libraries are highlighted in gray.
Figure S8. Calculated probability of mutations per gene. The mutational rate per gene generating a library with 73% chance of wildtype per randomized position is shown. The probability of the number of mutations was calculated using the binominal distribution with $k$ the number of successes in a sequence of $n = 11$ independent experiments having a probability $p = 0.27$ in a non-cumulative distribution function.\textsuperscript{11}

Figure S9. Screening of different Savinase\textsuperscript{®} libraries using fluorescence-activated droplet sorting (FADS). A)-D) Histograms of the fluorescence signal distribution of droplets screened for Savinase\textsuperscript{®} activity after three days of incubation at room temperature in darkness. The sorting gate was set up such that droplets showing increased fluorescence over the population average of sav-wt were selected.
Table S2. Overview of the ultrahigh-throughput screening in microdroplets and re-screening in microtiter plates. Hits were selected for sequencing after showing higher activity compared to sav-wt plus 3x standard deviation in a re-screening performed in 96-well plates using casein substrate and *B. subtilis* for secretion.

| Library | Droplets screened | Droplets sorted | Sequenced hits | WT hits | Unique hits |
|---------|------------------|----------------|----------------|---------|-------------|
| Lib-1   | 500,000          | 104            | 18             | 2       | 12          |
| Lib-2   | 1,300,000        | 130            | 22             | 0       | 7           |
| Lib-3   | 1,100,000        | 205            | 1              | 0       | 1           |
| Lib-4   | 2,600,000        | 34             | 1              | 0       | 1           |
| Lib-5   | 4,000,000        | 218            | 48             | 3       | 28          |
| Lib-6   | 2,600,000        | 100            | 34             | 2       | 9           |
| Sh-Lib  | 100,000          | 80             | 22             | 0       | 5           |

**Figure S10.** Enrichment of positive hits for each library after FADS. A-F) 88 variants that were randomly picked and secreted by *B. subtilis* from the corresponding original library (before sorting) were assayed against a fluorogenic casein derivative substrate (EnzChek). The data were normalized to the endpoint fluorescence of sav-wt. Activities are separated in higher activity than WT (>WT), 80-100%, 50-80% and 10-50% wildtype activity as well as variants showing less than 10% WT activity (<10%).
Figure S11. Validation of unique Savinase® hits selected for each library after re-screening in 96-well plates. Purified variants were tested against the fluorogenic casein substrate. A), C), E), G) and I) Time-courses of selected hits in comparison sav-wt. B), D), F), G), and J) The activity for each shuffled variant is plotted relative to that of sav-wt. The names of the variants correspond to the position of a 96-well-plate where it has been identified, the library it originated from and the plate number screened (e.g. D11-1-1 has been identified at position D11 in lib-1 in the first 96-well-plate screened).
**Figure S12.** Directed evolution of wt-sav for increased activity against a fluorogenic casein substrate. Activities of selected best variants from each of the six libraries are shown. Variants are plotted relative to that of sav-wt. Data are averages of duplicate values from three independent experiments and two protein purifications.

**Table S3.** Overview of selected best variants from each library and their mutations compared to the wild type sequence. Mutations are indicated in red. The numbering of amino acid positions corresponds to that of the subtilisin BPN’ sequence (Peptidase_S8 (PF00082)).

| Enzyme | Lib-1       | Lib-2       | Lib-3       | Lib-4       | Lib-5       | Lib-6       |
|--------|-------------|-------------|-------------|-------------|-------------|-------------|
| WT     | 53-GEFSTQQNG-63  |             |             |             |             |             |
| A8-1-3 | GEFRTQQNG     |             |             |             |             |             |
| D1-1-1 | WDPSTQQNG     |             |             |             |             |             |
| E1-1-1 | GKFRTQQNG     |             |             |             |             |             |
| H3-1-1 | GEFRTQQNG     |             |             |             |             |             |
| WT     | 94-KVLGASGSSSV-104 |             |             |             |             |             |
| A10-2-1| KVLGASGRGSSV  |             |             |             |             |             |
| C8-2-1 | KVLGASGSSSV   |             |             |             |             |             |
| G10-2-3| KVLGSGGTV     |             |             |             |             |             |
| WT     | 124-LSLGSPFSAT-134 |             |             |             |             |             |
| G2-3-6 | LSLSFRFSGT    |             |             |             |             |             |
| WT     | 152-AGGNSQGGS-163 |             |             |             |             |             |
| C4-4-1 | AGGNSQGGS     |             |             |             |             |             |
| WT     | 181-DQQNNRASFQ-191 |             |             |             |             |             |
| B4-5-3 | DQNNRASFST    |             |             |             |             |             |
| C2-5-6 | QNNRASFST     |             |             |             |             |             |
| E2-5-1 | QNNRFRASFQ    |             |             |             |             |             |
| WT     | 209-YPGSTYASLNG-219 |             |             |             |             |             |
| A1-6-1 | WPGGYAVLNG    |             |             |             |             |             |
| D5-6-3 | WPGGYAVLNG    |             |             |             |             |             |
| E8-6-1 | WPGGYAVLNG    |             |             |             |             |             |
| E8-6-2 | WPGGYAVLNG    |             |             |             |             |             |
Figure S13. Time-courses of shuffled Savinase® variants identified after re-screening in 96-well plates. Purified variants were tested against the fluorogenic casein substrate.

Figure S14. Positions of amino acid substitutions in shuffled variant G10+E2 projected into the structure of mature subtilisin Savinase® (pdb 1svn). Substitutions are indicated by red spheres and residue numbers. Residues D32, H64, and S221 form the catalytic triad in the active site of the protein.
Figure S15. Chip designs. (a) Flow-focusing device for the generation of monodisperse water-in-oil droplets. The two aqueous solutions, containing the plasmids and the RCA reagents were injected from inlet 2 and 3, respectively. Inlet 1 was used for the oil–surfactant mixture. Droplets were collected from outlet 4. The depth of the droplet generation device is 30 µm. The channel at the flow-focusing junction is 30 µm wide. (b) Microfluidic device for picoinjection. Number 2 indicates the inlet for reinjection of the droplet emulsion and number 1 for the spacing oil. The picoinjector marked as number 5 injects fluid by merging the drops with a pressurized channel containing the reagent. Picoinjection is triggered by an electric field, which is applied by the two electrodes marked as 3 and 4, respectively. Two different picoinjector devices with channel depths and widths of 30 µm and 35 µm were used, respectively. The width of the picoinjector nozzle is 10 µm. (c) Sorting device for the fluorescence-activated screening of droplets. Number 2 indicates the inlet for the droplet emulsion and number 1 denotes the inlet for the spacing oils. Inlets for the 5 M NaCl solution for generating a high voltage signal are marked by 3 and 4 for ground [GND(-)] and signal [signal (+)] electrodes, respectively. The outlets of the positive and negative channels are marked by numbers 5 and 6, respectively. The depth and width of the sorting channel is 50 µm. The corresponding CAD files can be downloaded from http://openwetware.org/wiki/DropBase.
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