Supporting Information for

Uniform Amplification of Phage with Different Growth Characteristics in Individual Compartments Consisting of Monodisperse Droplets

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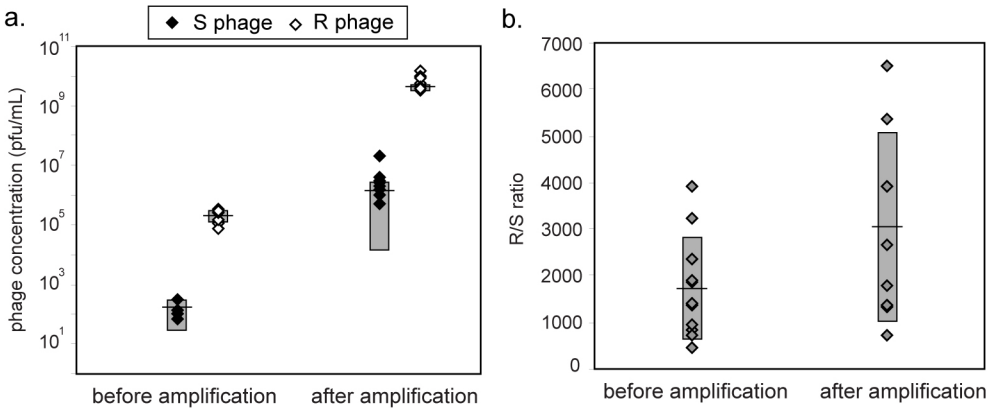


Supporting Information Figure 1. R/S ratio obtained by amplification of a ~2000:1 mixture of R and S phage in droplets. We used 2000:1 R/S ratio and we extrapolated that larger ratios will behave similarly. We formed 40 μm droplets from 1 mL of LB media containing a 1:10 dilution of the overnight K12 culture (10^8 cells/mL), $(2.0\pm0.75)\times10^5$ pfu/mL of R and $(1.5\pm1.2)\times10^2$ pfu/mL of S phage (R/S ratio $(1.7\pm1.1)\times10^3$). After seven hours of amplification produced $(4.2 \pm 0.83)\times10^9$ pfu/mL of R $(1.4 \pm 1.3)\times10^6$ pfu/mL of S, the R/S ratio $(3.0\pm2.0)\times10^3$. In amplification of this mixture, the R/S ratio increased only by a factor of 2.3 ($p=0.06$, calculated by the one-tail unequal variance Student t-test). All data are presented (three experiments); the overlaying grey bar is equal to 2x(standard deviation)).

The 1000:1 R/S ratio already presented a considerable challenge for characterization. In plaque-forming assay that we used to quantify the number of the phage clones, an average 14-cm agar plate contained >3000 white (R) and 1 to 5 blue (S) plaques per dish. Increasing the concentrations of plaques beyond 3000 per plate is impossible in practice, because it leads to incorrect analysis. When we attempted to quantify the 1,000,000:1 ratio that resulted from amplification of 2000:1 R/S ratio in the absence of compartmentalization, we could not detect any blue plaques in several 600-cm^2 trays containing >100,000 white plaques each.

Supporting Information Discussion

Dynamics of Growth of the Mixture of Rapid and Slow Phage in the Same Solution

Assume that phage B has an infection rate 25% lower than that of phage A. When the two phage are mixed with 10^9 bacteria, phage A goes through four cycles of infection and secretion ($1 \rightarrow 10^3 \rightarrow 10^6 \rightarrow 10^9 \rightarrow 10^{12}$), while phage B goes through only three cycles. The resulting population of phage B in the amplified mixture will be 1000 times less than that of phage A. Exponential amplification, and the large number of clones secreted at each cycle, makes the amplification process sensitive even to subtle differences in growth rate. Similarly, if phage B has an infection rate 50% lower than that of phage A, phage A can go through two rounds of infections (e.g. $10^5 \rightarrow 10^8 \rightarrow 10^{11}$) when phage B goes only through one (e.g. $10^5 \rightarrow 10^8$), that is, the A/B ratio is expected to increase by a factor of 1000.

Concentration of Phage in Droplets of Different Sizes.

When a solution of volume V containing N phage is broken into M droplets, the probability of finding at least one phage in a droplet is given by the Poisson statistics; the average number of infected droplets M_{inf}, is given by eq. 1:

$$M_{\inf} = M\left(1 - e^{-\left(\frac{N}{M}\right)}\right) \qquad (1)$$

In every infected droplet, phage is amplified to a plateau concentration of 10^{12} pfu/mL. Because the droplets are monodisperse, the volume of each droplet is V/M, and the number of phage in an infected droplet is $10^{12}V/M$. After combining all droplets in one solution, the final number of phage N_f in this solution is a product of M_{inf} and the number of phage per droplet (eq. 2):

$$N_f = 10^{12} V\left(1 - e^{-\left(\frac{N}{M}\right)}\right) \qquad (2)$$

Eq. (2) can be rewritten by expressing number of phage N using concentration of phage (C=N/V), and number of droplets M using volume of a droplet V_d (M=V/V_d) to yield eq. 3.

$$N_f = 10^{12} V\left(1 - e^{-CV_d}\right) \qquad (3)$$

For constant V and C, the final concentration of phage decreases with the droplet size.

In the above derivation, we assumed that in each droplet phage grows to a concentration of 10^{12} pfu/mL. This assumption is true if:

(a) Each droplet contains at least one bacterium, i.e., the probability of finding a droplet that has phage but no bacteria is < $1/M$ (eq 4, where N_b is number of bacteria in volume V):

$$e^{-\left(\frac{N_b}{M}\right)}\left(1 - e^{-\left(\frac{N}{M}\right)}\right) < \frac{1}{M} \qquad (4)$$

(b) Bacteria reach the same saturating concentration in each droplet. This assumption in general is not true, because maximum number of bacteria in droplets is also described by a probability distribution. We predict that for very small droplets that can support only ~10 bacteria, variations in the number of bacteria can lead to significant variations in the concentration of phage produced in each droplet. To minimize these variations for *E.coli* that reaches maximum concentration C_{max} ~10^9 cfu/mL; $C_{max}V_d$ should be >10, and V_d, thus, should be > 10^{-8} mL. This volume corresponds to spherical drops >26 micron in diameter.

Encapsulation in Droplets for Amplification of Large Libraries of Phage.

If a library contains N different phage clones (every clone is different), into how many

droplets (M) must it be split to minimize the probability of two different phage clones residing in the same droplet? To answer this question, the number of droplets (N_{2+}) containing two of more phages clones ("mixed droplets") can be estimated using a Poisson distribution (eq. 5):

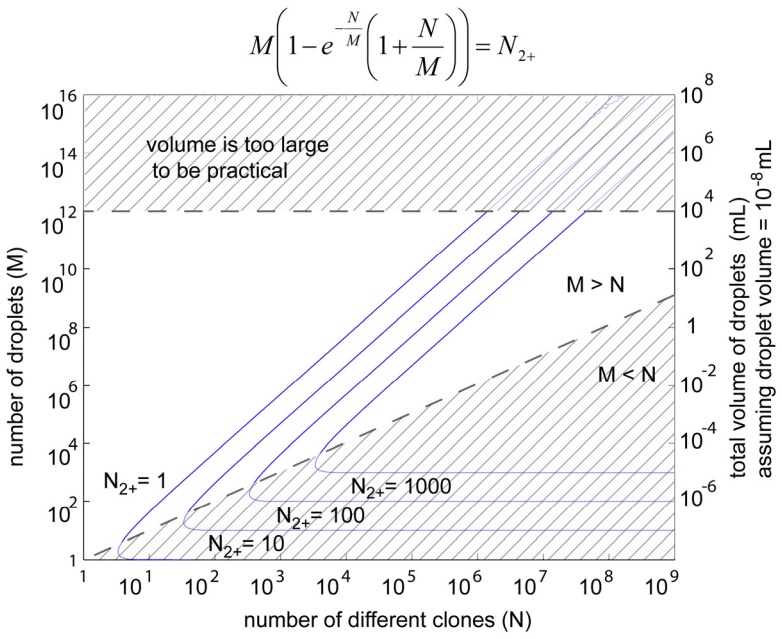
$$N_{2+} = M\left(1 - e^{-\left(\frac{N}{M}\right)}\left(1 + \frac{N}{M}\right)\right) \qquad (5)$$

To use this equation, one has to define an acceptable number of mixed droplets (N_{2+}). The total number of drops M, in turn, is found by solving equation (5) for given values of N and N_{2+}. **Supporting Information Figure S2** demonstrates (N,M) solutions for N_{2+}=1, 10, 100 and 1000. For example, to distribute a library that contains 10^5 different clones in a way that minimizes the number of mixed droplets to less than one (N_{2+}=1), one needs M=10^{10} droplets. This number requires several days of continuous droplet generation (at standard 2-3 kHz frequency for a single MFFD); more importantly, only 1 in 10^5 droplets is infected; thus, amplification generates impractically dilute libraries (<10^6 pfu/mL). Large volumes of liquid have to be processed to generate a number of phage sufficient for selection (~10^{11} pfu). If it is acceptable to lose 1000 clones in mixed droplets for the same library, however (N_{2+}=1000, 1% of the library), only 10^8 droplets are required. This number of droplets can be produced in 1-2 hours of operation of a standard one-channel generator, or in 30 min using four parallel generators; the expected concentration of phage after amplification is 10^9 pfu/mL.

These simple estimates demonstrate the practical limits of the system. Amplification of large libraries (>10^5 clones) must be done at a cost of losing a certain number of clones in mixed droplets. This limitation should not present a problem for the application of this method in a selection procedure. Although every selection starts from

a library of $>10^9$ clones, the first round of selection usually yields less than 10^5 clones. The vast majority of the clones do not bind the target, and hence are eliminated prior to any amplification. The remaining library of $<10^5$ clones can be easily distributed in separate droplets and amplified.

Supporting Information Figure S2.



Legend: A plot of solutions for the equation $M\left(1-e^{-\left(\frac{N}{M}\right)}\left(1+\frac{N}{M}\right)\right)=N_{2+}$ which describes how many droplets (M) are required to place N phage clones such that only N_{2+} of those end up in droplets with more than one phage type ("mixed droplets"). Four (M,N)-curves are presented for N_{2+}=1, 10, 100 and 1000. Practical solutions reside in between the two hatched areas*:* bottom hatched area designates solutions for $M<N$, which are not relevant; the top hatched area designates solutions that require an impractically high number of droplets (here 10^{12}). Solution for other values of N_{2+} can be easily extrapolated visually. Amplification of large libraries (>10^7) requires impractically large volumes and can be only be done at the cost of losing a certain fraction of the library to "mixed droplets" (assuming limited number of MFFD operating in parallel).

Experimental Details

Fabrication of the Microfluidic Channel

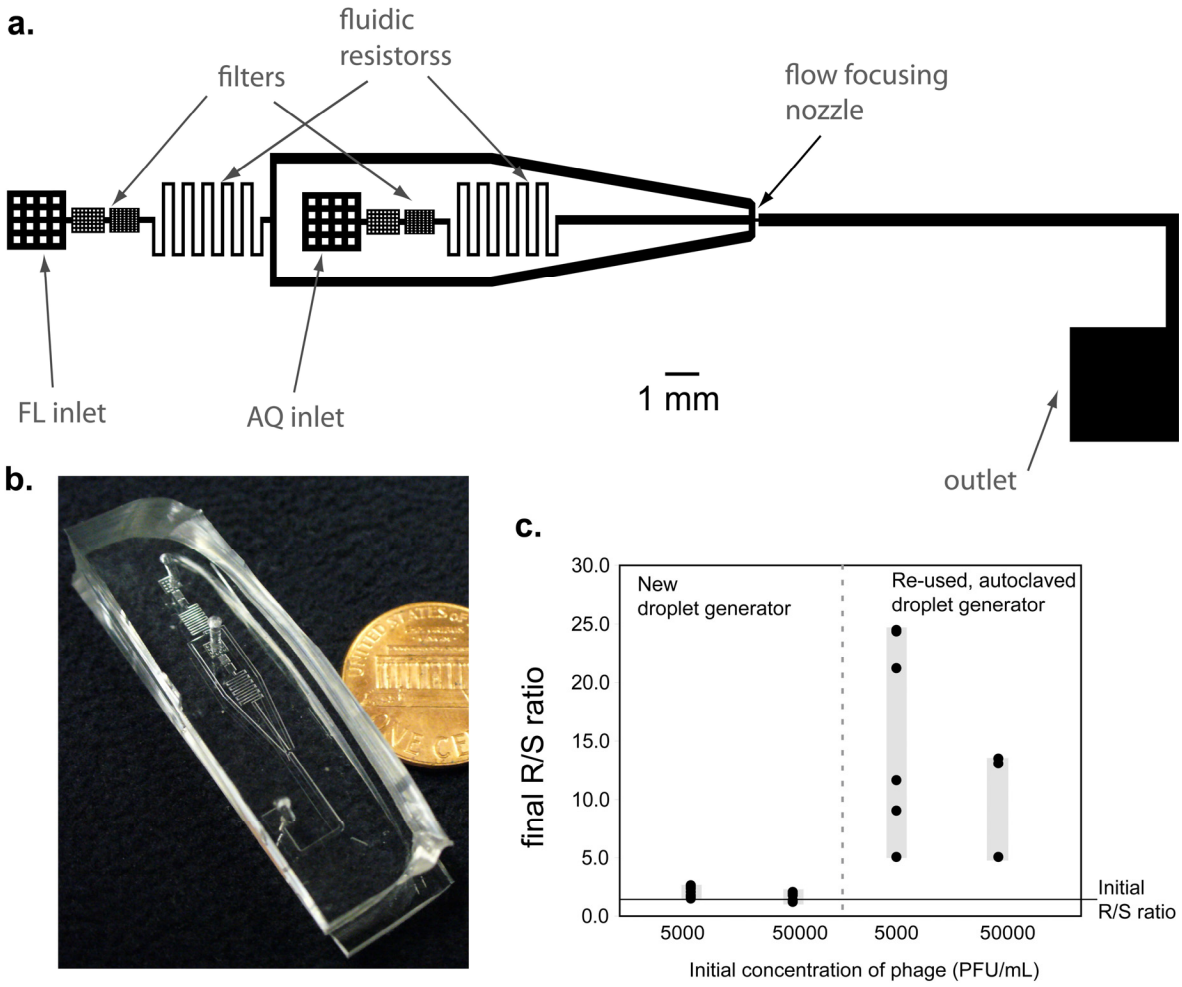
We fabricated the microfluidic channel using standard soft lithography.[1] We printed the design microchannel.dxf (attached as a separate supporting information file) at 5000 dpi resolution on a transparency (CAD/Art Services, Inc.). This transparency served as a photomask in contact photolithography to produce a master. We cleaned a 3 inch Si-wafer (Silicon Sense, Inc) using a plasma cleaner for 5 min, and spin-coated a 140 μm thick film of SU8-50 (Microchem) onto the wafer. Following instructions from Microchem, we soft-baked the wafer, exposed it to UV through the photomask at 500 mJ/cm^2 using AB-M mask aligner, and hard-baked it for 30 min. The master was then developed in SU-8 developer for 15 minutes, rinsed in isopropanol, dried, and silanized with trichloro(1H,1H,2H,2H-perfluorooctyl)silane.

To produce the top half of the microfluidic channel, we mixed PDMS elastomer base and curing agent (Dow Corning Sylgard 184) in 10:1 ratio, degassed the sample for 30 min, and poured the mixture onto the SU8 master. We also poured PDMS over a Petri dish to create the bottom flat half of the microfluidic channel. After incubation for >1 hour in a 60 ºC oven, we peeled both halves off their templating wafers, cut out ~1x4 cm areas, and punched holes at the inlets of the top half of the microfluidic channel using 1.2 mm biopsy puncher (Harris Uni-core). Both halves were oxidized in a plasma cleaner for 1 min and, no later than 2 minutes after oxidation, briefly pressed against one another to form a seal. We strengthened the seal by incubating the channel in an oven at 100 ºC for at least 30 minutes. We then coated the channel with Aquapel glass treatment (PPG Industries) to make the channel hydrophobic.

Sealed, treated channels can be stored for at least 6 months after their fabrication with no observable change in droplet generation. We observed an increase in R/S ratio when we re-used an old channel (Figure S3). We, thus, hypothesized that leftover phage

amplified and contaminated the microchannel, and even after autoclaving, a significant fraction of phage persisted. Fresh channel, thus, was used for every experiment.

Supporting Information Figure S3.



Legend: (a) Design of the microchannel (AQ and FL inlets are labeled, outlet is labeled). (b) Photograph of the channel. (c) Phage droplets generated in new vs. re-used channel

Generation of and Culture in Droplets; Harvesting Phage from Droplets.

We sterilized the channels in the autoclave. The continuous phase was a perfluorocarbon (HFE7500, 3M) with 1.8% EA surfactant (RainDance Technologies, Lexington, MA, USA), which is a PEG–PFPE amphiphilic block copolymer.[2] We used syringe pumps (Kent Scientific Corp.) to drive the flow of the liquids. The flow rates were 6 mL/h for the continuous phase, and 4 mL/h for the disperse phase containing bacteria and phage mixtures. Syringes were connected to the inlets of the channel using non-sterile polyethylene tubing PE60 (BD Intramedic™).

We started and stabilized the flow of the continuous phase (via FL inlet) before we introduced bacteria and phage. After the channel was filled with perfluoro phase, we mixed 10 μL of log culture of bacteria and 990 μL of phage solution in lysogeny broth (LB) medium. Within 1 min, we loaded the mixture in a 1-mL syringe and started the flow via AQ inlet. This sequence of steps minimized the time phage and bacteria were mixed together, and minimized the possibility that the first burst of phage progeny would occur prior to the separation of all phage into droplets.[1] We discarded droplets generated in first 20-40 sec until the flow stabilized and the drop size attained an equilibrium size. "We calculated the drop volume by dividing the volumetric flow rate by the number of drops generated per second. We counted the number of drops in movies recorded with a fast camera. Due to limited resolution of our imaging system, we could not measure the polydispersity of our drops. The reported polydispersity of drops generated in a similar flow-focusing nozzle is 1%."

We collected drops into a 3 cm petri dish filled with perfluorcarbon (FC40, Sigma Aldrich) to prevent coalescence of drops upon contact with the dish. The dish was then placed in a "humidity chamber" (a 14 cm-Petri dish containing a wet Kimwipe), and rocked for seven hours in the temperature-controlled shaker set to 40-60 rpm.

[1] If the experiment was conducted at 4 ºC instead of room temperature (about 20 ºC), phage production could potentially be delayed for many hours.

Once the culture was completed, we harvested the droplets floating on top of the perfluoro liquid using a P1000 pipeteman, and transferred them to 1.5 mL eppendorf tube. The suspension separated into layer of droplets (top) and excess of perfluoro (bottom) which we removed using a P200 pipeteman. To the remaining suspension of droplets, we added one-half of the volume of droplet-destabilizing solution (Raindance), vortexed for ~10 sec, and centrifuged for 2 min at 14,000 rpm. The top layer contained phage solution, the bottom layer contained the perfluorinated phase, and bacterial pellet was positioned on the interface of the two phases. The aqueous layer was immediately used for titering (see below) or stored in a -80 ºC freezer for later quantification.

Imaging of Droplets

The images of droplets in microfluidic channel (main text Figure 2b) were acquired using dissecting microscope with 20x objective. Images of bacteria in drops (main text Figure 2c) we aquired using inverted phase-contrast microscope with 20x and 100x oil-immersion objective.

Culture and Quantification of Phage and Bacteria.

A library of M13 phage engineered to express a library of 12-mer peptides and galactosidase reporter (slow phage) and F+ bacteria for amplification was purchased from New England Biosciences (Ph.D-12 kit). Wild type phage expressing neither peptide insert nor galactosidase was present at extremely low concentrations in the Ph.D-12 library; we isolated wild-type phage from this library after repetitive rounds of amplification and dilution.

Bacteria were cultured according to the manufacturer's instructions using tetracycline-containing (Tet) plates for streaking and maintenance of bacteria and antibiotic-free LB media to grow to late log-phase. To quantify the concentration of bacteria, 40 μL of solutions containing different dilutions of bacterial solutions were

spread over one-fourth of the agar plates; plates were inverted and incubated for 12-24 hours at 36 ºC. Plates that contain <50 colonies in the one-fourth plate were used for the estimate.

To quantify the concentration of phage, 180 μL of log culture of bacteria were mixed with 10 μL of phage solution in LB medium; the mixture was added to 3 mL of warm (50 ºC) 0.75% agar solution and poured onto 1.5% agar plates containing IPTG and X-gal. Plates were inverted and incubated for 12-24 hours at 36 ºC. For ratios 10:1 or less, we counted both blue- (S phage) and white-colored plaques (R phage) manually on the plates that contain <300 plaques per plate. To quantify ratios of R (white) and S (blue) plaques approaching 100:1, we selected the dilution of phage that yielded ~5-50 blue plaques per plate and up to 10,000 white plaques per plate. We counted blue plaques over the entire plate, counted white plaques in a small (1x1 cm) area and extrapolated to the area of the entire plate. For R/S ratios approaching 1000:1, we also used 12 cm-dishes instead of 7 cm to provide a better estimate of white plaques.

Titering Phage in Individual Droplets.

To confirm that distribution of phage in droplets followed Poisson distribution, we generated 400 μm droplets from a solution of 2.8×10^4 pfu/mL of S phage, 1.7×10^4 pfu/mL of R phage. One mL of solution was broken into 3.0×10^4 droplets; hence, expected numbers of phage per droplet were 1 for S and 0.5 for R phage (1 R phage in every other droplet). We picked individual droplets under a stereoscope using a P10 pipeteman and dispensed them into eppendorf tubes containing 20 μL of droplet destabilizing solution and 40 μL of LB media. After vortexing for ~10 sec, we added 200 μL of log culture of bacteria in LB, briefly vortexed, mixed with 1 mL of warm (50 ºC) agar solution and poured atop IPTG/X-Gal plates. The number of plaques resulting from titering indicated the number of phage in individual droplets.

Droplet #	1	2	3	4	5	6	7	8	Predicted average
R phage	1	0	3	1	2	0	2	2	0.93 pfu / droplet
S phage	0	0	1	1	0	1	0	2	0.56 pfu / droplet

Emergence of wt phage from phage libraries.

We used mixtures of wt and library phage to simplify monitoring R and S phage. The observation that wt does not amplify faster than the library, however, has immediate practical application. Faster amplification of wt phage poses serious problems for the selection process. For example, the product manual for commercial phage library states:

"In the absence of a corresponding strong *in vitro* binding selection during panning, even a vanishingly small levels of contamination can result in a majority of the phage pool being wild-type phage after 3 (of especially 4) rounds of panning" (*product manual for Ph.D.-12 phage display library by New England Biosciences*).

We observed that after as few as two rounds of 10^6 amplification without any selection, wt phage emerged from undetectable levels (estimate <0.001%) and constituted 6-60% of the library (see below). In contrast, during 10^6 amplification in droplets, the concentration of wt increased by no more than a factor of 3.

Experimental procedures:

We inoculated 3 mL of 1:100 dilution of overnight K12 culture (corresponding to ~ 10^6 cfu of bacteria) with 10^6 pfu of Ph.D.-12 library, amplified for seven hours, and purified the phage using the standard protocol (precipitation from PEG/NaCl). We then used 10^6 pfu of the purified phage to inoculate 3 mL of 1:100 dilution of K12 culture. After the second round of amplification, we observed that 6% - 60% of the plaques were clear (two independent experiments). LacZ-free wild type (environmental) phage indeed emerged rapidly when library was amplified in the absence of any other selection.

Complete author list for references 11 (Arap et al)[3] and 19 (Holtze et al)[2] and 24 (Clausell-Tormos et al)[4] of the main text are included below.

[1] J. C. McDonald, G. M. Whitesides, *Acc. Chem. Res.* **2002**, *35*, 491-499.

[2] C. Holtze, A. C. Rowat, J. J. Agresti, J. B. Hutchison, F. E. Angile, C. H. J. Schmitz, S. Koster, H. Duan, K. J. Humphry, R. A. Scanga, J. S. Johnson, D. Pisignano, D. A. Weitz, *Lab Chip* **2008**, *8*, 1632-1639.

[3] W. Arap, M. G. Kolonin, M. Trepel, J. Lahdenranta, M. Cardo-Vila, R. J. Giordano, P. J. Mintz, P. U. Ardelt, V. J. Yao, C. I. Vidal, L. Chen, A. Flamm, H. Valtanen, L. M. Weavind, M. E. Hicks, R. E. Pollock, G. H. Botz, C. D. Bucana, E. Koivunen, D. Cahill, P. Troncoso, K. A. Baggerly, R. D. Pentz, K. A. Do, C. J. Logothetis, R. Pasqualini, *Nat. Med.* **2002**, *8*, 121-127.

[4] J. Clausell-Tormos, D. Lieber, J. C. Baret, A. El-Harrak, O. J. Miller, L. Frenz, J. Blouwolff, K. J. Humphry, S. Koster, H. Duan, C. Holtze, D. A. Weitz, A. D. Griffiths, C. A. Merten, *Chem. Biol.* **2008**, *15*, 427-437.