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An Integrated Device for Monitoring Time-Dependent in vitro Expression From Single Genes in Picolitre Droplets

Fabienne Courtois,[a, b] Luis F. Olguin,[a, b] Graeme Whyte,[c] Daniel Bratton,[c] Wilhelm T. S. Huck,[c] Chris Abell,*[a] and Florian Hollfelder*[b]

Microdroplets have great potential for high-throughput biochemical screening. We report the design of an integrated microfluidic device for droplet formation, incubation and screening. Picolitre water-in-oil droplets can be stored in a reservoir that contains ~10^6 droplets. In this reservoir droplets are stable for at least 6 h, which gives an extended timescale for biochemical experiments. We demonstrate the utility of the system by following the in vitro expression of green fluorescent protein. The high efficiency allows protein expression from a single molecule of DNA template, creating "monoclonal droplets" in which genotype and phenotype are combined in one emulsion compartment.

Introduction

Compartmentalisation of chemical or biological reactions in droplets[1–3] is an emerging and powerful format for performing ultra-high-throughput assays on a very small scale. Individual droplets (typically fL to nL volume) act as discrete reaction vessels.[4–6] Such microdroplets can be generated in bulk,[1] but microfluidic devices offer a much higher level of control necessary for quantitative analysis of, for example, reaction kinetics.[4] Homogeneous droplets of defined size can readily be formed and manipulated: they can, for example, be split,[7,8] fused,[9–11] analysed[12] and sorted.[13] The contents, size and frequency of droplets can be precisely controlled by appropriate adjustment of flow rates. The rapid mixing of aqueous reagents in droplets, by chaotic advection,[14,15] leads to short dead times ($<$ 2 ms), allowing fast reactions—on the second to millisecond timescale—to be followed.[9,16] Many chemical and biochemical assays require longer reaction times. Although storage devices are available for plugs (e.g., capillaries),[17–21] the storage of microdroplets in microfluidic device is still a challenge.

In this paper we describe the design of an integrated device allowing precisely controlled formation of water-in-oil droplets, their storage for several hours in a reservoir, and individual analysis of droplets by laser detection.[12,22] We demonstrate the utility of the new device by measuring the kinetics of in vitro expression of a reporter protein within droplets over several hours through the use of a suitable emulsion formulation. The high efficiency of our system also allows us to express proteins in microreactors containing single copies of a gene. This paves the way for directed evolution experiments in such devices.

Results and Discussion

Device fabrication

Many high-throughput assays involve an incubation step. One approach that has been exploited in microfluidics is the use of plugs of fluids in capillaries to look at many discrete reaction compartments. These plugs have been shown to be stable for months, enabling a variety of chemical,[19] biochemical[20,21] and crystallographic experiments[18,21] to be performed. This approach, however, appears to have limited throughput[17] and a restricted range of applications as a consequence of the relatively large volumes (nL) of the plugs.

In an earlier study, Dittrich et al. demonstrated cell-free expression of a protein in droplets.[22] The reactions were carried out in small (5 μm, 65 fL) droplets that were stored at the "end reservoir" of the device. After incubation, the droplets were pumped back through the channel for analysis. However, it was reported that droplet fusion occurs on the assay timescales, leading to loss of compartmentalisation. Following the same basic principles as the work performed by Dittrich et al., this paper addresses droplet storage and stability further. Integrated devices that combine droplet formation, storage and analysis will be required in order to allow the use of microdroplets in microfluidics as single-droplet reaction chambers.

The device design used in this study is shown in Figure 1. The key components are a flow-focusing device for droplet for-

[a] *Dr. F. Courtois, Dr. L. F. Olguin, Prof. C. Abell*
Department of Chemistry, University of Cambridge
Lensfield Road, Cambridge CB2 1EW (UK)
Fax: (+44) 12223-336362
E-mail: ca26@cam.ac.uk

[b] *Dr. F. Courtois, Dr. L. F. Olguin, Dr. F. Hollfelder*
Department of Biochemistry, University of Cambridge
80 Tennis Court Road, Cambridge CB2 1GA (UK)
Fax: (+44) 1223-766002
E-mail: fh111@mole.bio.cam.ac.uk

[c] *Dr. G. Whyte, Dr. D. Bratton, Prof. W. T. S. Huck*
Melville Laboratory for Polymer Synthesis
Department of Chemistry, University of Cambridge
Lensfield Road, Cambridge CB2 1EW (UK)

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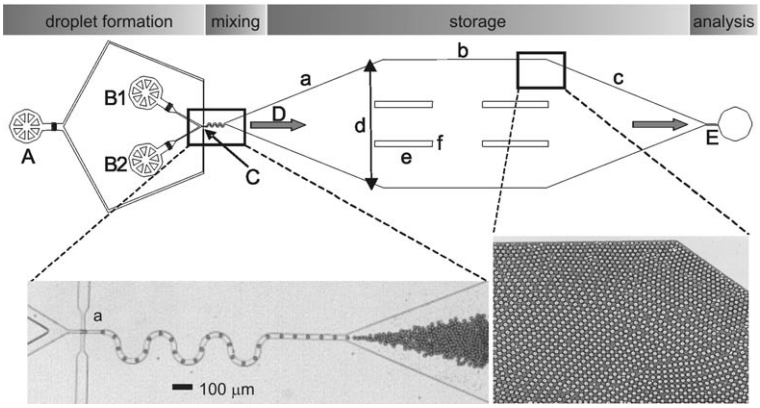

Figure 1. Design of the integrated device. The device contains an oil inlet (A) that joins two aqueous inlet channels (B1 and B2) at a narrow junction (C: 10 μm) to form droplets of ~15 μm size. A wiggle channel allows rapid mixing of droplet components. The droplets flow directly (D) into a reservoir (a: 7 mm, b: 7.3 mm, c: 7.2 mm, d: 5.8 mm) containing four pillars (e: 3 mm, f: 300 μm) and a narrow outlet channel (30 μm) for droplet exit and detection (E). The channels and reservoir are 25 μm deep.

mation, and a droplet storage reservoir (total volume ~2 μL). Droplets (typically 10–30 μm, 0.5–15 pL) are generated with a flow-focusing design in a PDMS/glass device [PDMS: poly(dimethylsiloxane)].[23] The different reaction components are introduced through two aqueous inlet channels (Figure 1 B1 and B2), joining each other in a narrow orifice. Droplets, created at the junction by the shear forces from two perpendicular oil flows, then pass through a short sinusoidal channel that promotes rapid mixing by chaotic advection and accumulate in a large reservoir. The geometry of the reservoir has been optimised iteratively. It possesses a V-shaped entrance that causes the droplets to slow down gradually as they enter the chamber, thereby avoiding droplet splitting upon collision. To store up to 10^6 droplets a large reservoir with a large aspect ratio (width/height) of ~230 was designed. Such a large ratio could lead to sagging or collapse of the device structure.[24] This potential problem was avoided by inserting four supporting pillars in the middle of the reservoir, effectively reducing the aspect ratio.

Once the reservoir has been filled with droplets, the entrance and exit are sealed. Droplets could be retrieved by opening the reservoir and introducing oil from the inlet to force droplets to flow through the narrow outlet channel (Figure 1 E). The exit is tapered in a similar way to the entrance to stop droplets getting trapped in deadspace in the corners.

Emulsion stability

The main mechanisms of breakdown of thermodynamically unstable emulsions are Ostwald ripening (growth of larger droplets at the expense of smaller ones) and coalescence with other droplets.[25] Smaller droplets are more stable: 2 μm droplets, for example, have been used in bulk emulsion containing different mixtures of surfactants for in vitro compartmentalisation.[2,26–28] However, in the microfluidic system described above, larger droplets (10–30 μm, 0.52 to 14.14 pL) are used. Such droplets are inherently less stable and need to be stabilised by a good surfactant formulation. A study was therefore carried out with different formulations of Abil EM 90, which has been used previously in bulk emulsions.[29,30]

Droplet stability was evaluated by image analysis to compare droplet size over time for at least 3000 droplets per reservoir. It was found that 1% Abil EM 90[31] was insufficient to form stable emulsions, whereas 2% Abil EM 90 stabilised droplets for up to four hours. In both cases, the droplets eventually coalesced.

Addition of a co-surfactant has been shown to stabilize water-in-oil emulsions against coalescence.[25] Studies of droplet size from pictures taken over time with the previously used 2% Abil EM 90 formulation together with 0.5% Triton X 100 showed stabilisation of droplets for a minimum of six hours, but ≈25% of droplets had coalesced after this time. In contrast, a formulation containing 3% Abil EM 90[28] suffered less coalescence (≈10%). This formulation of surfactant was used throughout the subsequent study. Unfortunately, the increase in surfactant concentration caused the droplets to aggregate,[25] as shown in Figure 2. The droplets were initially uniform and packed in ordered arrays in the reservoir (Figure 2 A). Over time the mineral oil was absorbed into the PDMS walls of

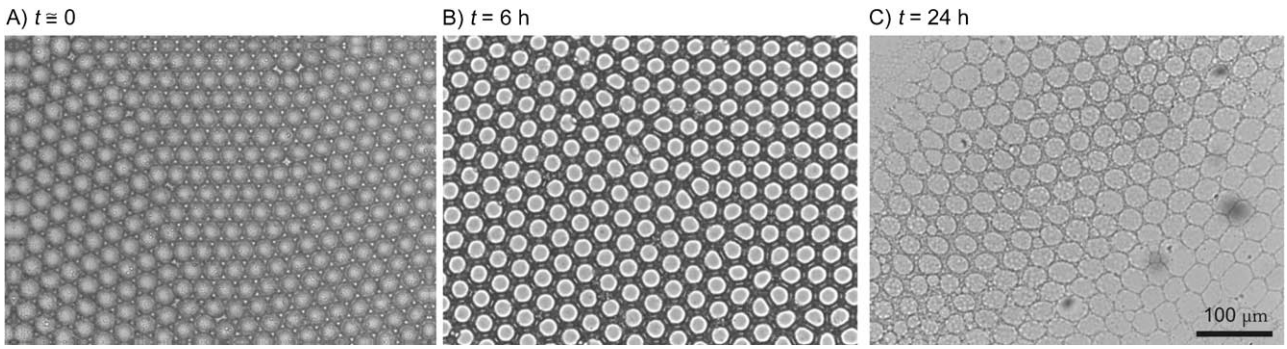

Figure 2. Microscopic observation of droplet behaviour in the reservoir. Droplets of water in mineral oil containing 3% Abil EM 90 stored in the reservoir imaged over time: A) droplets in the freshly filled reservoir, B) after 6 h droplets adhere to each other as the oil is absorbed into the device; these droplets can be recovered by addition of oil, C) after 24 h, droplets are aggregated and cannot be recovered.

the device,[32] decreasing the amount of oil separating the droplets. As a result, the droplets deformed and adhered to each other (Figure 2B). Up to this point, droplets could still be recovered by introducing more oil (through inlet A) into the system, which led to the reversal of the deformation and the regeneration of free-flowing droplets. After 24 h the droplets had eventually aggregated and stuck to the device (Figure 2C).

Retrieval and detection of droplets

The droplets were studied to quantify the extent of droplet fusion, Ostwald ripening and leakage of material from the droplets by using the successful droplet formulation described above. To do so, droplets containing green fluorescent protein (GFP) were prepared and stored in the reservoir shown in Figure 1. A green fluorescent mutant protein with increased fluorescence relative to the wild-type GFP was used throughout this study because of its ease of detection.[33] At selected time intervals, droplets were extracted from the reservoir and their laser-induced fluorescence was detected in the outlet channel. Figure 3A shows a typical trace of droplets containing GFP leaving the reservoir by the outlet channel. If droplets had shrunk, their fluorescence signals would be more intense. If droplets had swollen, the measured fluorescence intensities would fall. Droplet fusion would lead to larger droplets with no change in concentration. The detected signal would then show similar fluorescence intensity, but have a larger peak area. The extent of droplet fusion and changes in droplet size were quantified by peak intensity and peak area studies of droplets containing GFP stored in the reservoir. Measurements after 6 h showed that ~10% of droplets had shrunk and another ~10% of droplets had fused and thus increased in size. (Histograms of the distribution of peak area and peak height after 6.55 h are shown in the Supporting Information.) Studies of droplet size from pictures taken over time also confirmed these results. Figure 3B shows the average total fluorescence in 3000 droplets measured at different time points. It was found that the GFP fluorescence intensity changed by less than 10% over 6 h (Figure 3B). The relatively constant fluorescence suggests that the concentration of GFP had not changed, indicating that there is no leakage of GFP from the droplets.

In vitro expression of green fluorescent protein in microdroplets

The system described in this paper allowed us to store and analyse droplets containing GFP in a sensitive and high-throughput manner. The linearity of detection of GFP in droplets was assessed by making dilute droplets of GFP and recording their fluorescence (Figure 4A). The lowest concentration measured (20 nM) was readily resolved. We therefore used a calibration curve (Figure 4A) in all further experiments to estimate concentrations as low as 5 nM. As previously reported,[22] the fluorescence observed for GFP inside a droplet is about 30% less than that measured for the flow in the inlet channel prior to droplet formation.

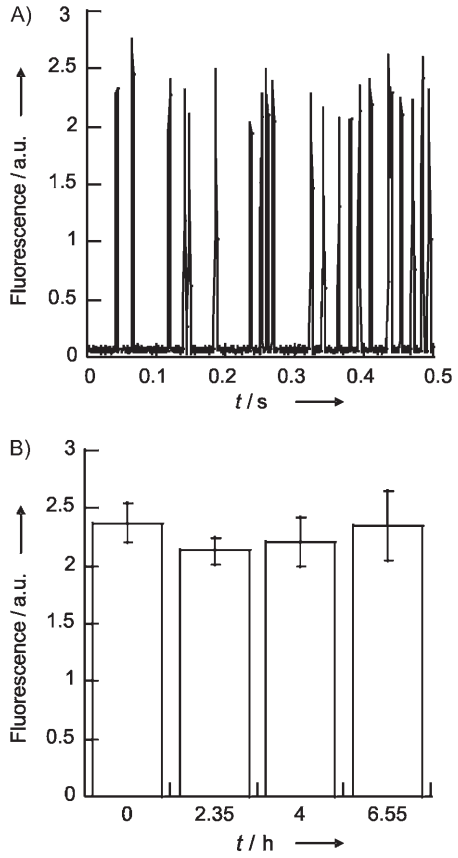

Figure 3. Detection of fluorescence emission from droplets in the outlet reservoir channel (E, Figure 1). A) Trace of droplets going out of reservoir after 4 h storage. The droplets contained GFP (375 nM). Each droplet passing through the laser beam (excitation 488 nm) in the detection channel gives rise to a peak of fluorescence intensity (emission above 516 nm) linearly related to the fluorophore concentration inside the droplet. B) Average of droplet fluorescence intensity stored in the reservoir for 2.35 h, 4 h and 6.55 h (~3000 droplets per time point). Droplets were formed with mineral oil containing 3% Abil EM 90, GFP (750 nM) and Tris/HCl (50 mM, pH 7.5) mixed on the chip at a 1:1 ratio. The flow rates were oil mixture/GFP/buffer: 100:5:5 $\mu L\,h^{-1}$; mean droplet diameter: 15 μm; droplet formation frequency: 440 Hz. Error bars represent the standard deviation of the measured values.

As a demonstration of the utility of this system, in vitro expression of GFP was carried out, and the resulting fluorescent protein was detected. A commercial in vitro transcription and translation kit (IVTT), including ribosomes, tRNA, translation initiation, elongation, termination factors, T7 polymerase, amino acids and ribonucleotides, was used (RTS100, *E. coli* HY kit, Roche Applied Sciences). As emulsions are more stable at lower temperatures, GFP expression in bulk solution was tested at 37 °C and 20 °C in a 96-well plate. In vitro expression was found to be only about 20% less efficient at 20 °C than at 37 °C over a 5 h incubation period (data not shown). Therefore experiments in droplets were performed at room temperature (20 °C). (Experiments at higher temperatures may require further optimisation with regard to droplet stability or possible evaporation, which is not addressed here).

The droplets were formed by mixing two solutions (in B1 and B2) at the same flow rate. One contained the *E. coli* lysate and the amino acids, whilst the second contained the "reaction

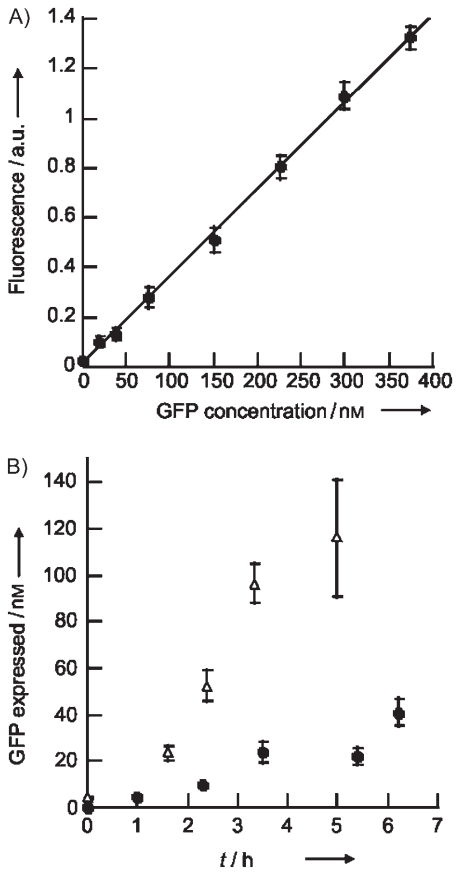

Figure 4. A) Detection of GFP fluorescence emission inside droplets. Droplets were formed with mineral oil/Abil EM 90 (3%)/GFP [750 nM] and Tris/HCl [50 mM], pH 7.5. The oil mixture flow rate was set up at 100 $\mu L\,h^{-1}$. The GFP concentration was decreased by dilution on chip with the buffer, the total aqueous flow rate being kept at 20 $\mu L\,h^{-1}$. The data are corrected for the background signal (~0.06). B) Time course of in vitro expression of GFP in the integrated microdroplet device. The GFP expression in microdroplets was monitored by the increase in fluorescence above 516 nm. Conditions: 5.2 pM (●) or 30 pM (△) plasmid inside aqueous droplets of 15 and 30 μm diameter, respectively, in mineral oil containing 3% Abil EM 90 at room temperature (~20 °C).

mix" and the DNA template (pIVEX-*gfp* containing a T7 promoter). The droplets were stored in the reservoir at room temperature. The presence of the IVTT mix did not destabilize the droplets, which appeared to be as stable as the droplets containing GFP.

At selected time intervals, the reservoir was opened and the fluorescence intensity of the droplets passing through the outlet channel was recorded. The high number of droplets provided large sample sizes (>1500 per time point) for analysis. Two time courses of GFP expression—for 30 μm (14 pL) droplets containing 30 pM plasmid template, and for 15 μm (1.8 pL) droplets containing 5 pM plasmid—are shown in Figure 4B. Similar expression profiles were observed in each case.

The larger droplets contained on average 250 molecules of plasmid and produced 10^6 molecules of GFP, corresponding to 4000 molecules of protein per molecule of template. The smaller droplets contained on average five molecules of plasmid and produced approximately 40000 molecules of GFP, corresponding to 8000 molecules of protein per molecule of template. A very similar yield has been reported with the same IVTT kit in vesicles (approximately <8000 copies per gene).[34] In the early experiments reported by Dittrich et al.[22] the corresponding figures were three molecules of GFP produced from each molecule of template. However a different IVTT system, a higher plasmid concentration (20 nM) and different conditions (50 min at 37 °C) were used, so a direct comparison cannot be made. The bulk emulsion protocol also results in lower protein copy number per gene, possibly due to partial protein deactivation at high homogeniser speed that is avoided by the milder droplet formation in microfluidics.[28]

In vitro expression of GFP from a single DNA template molecule

The high yield of GFP in the IVTT expression experiments in droplets made it possible to consider the expression of GFP from a single plasmid in a droplet. This is a pivotal experiment, allowing these devices to be used for directed evolution in droplets. To screen a library of different templates, it is desirable that each droplet should contain a single molecule of template to generate a unique protein, linking each genotype with its phenotype, ensuring the maximum enrichment.

To investigate the possibility of expressing and detecting GFP from a single copy of template in the droplet reservoir, the plasmid solution of pIVEX-*gfp* was diluted to final concentrations of 0.5, 0.3 and 0.1 pM. At these concentrations there is on average less than one molecule of template per droplet (Table 1). The percentage of droplets expected to contain a single copy of the template was calculated by Poisson statistics. For example, in droplets formed from a solution of 0.5 pM plasmid, there should be 80% empty droplets, 18% with only one plasmid and 2% with more than one copy of DNA. The calculated concentration of one molecule of plasmid in a drop-

Table 1. GFP expression in droplets containing a single DNA template molecule after incubation at room temperature (~20 °C) for 6 h 30 min. All droplets contained equal amounts of in vitro expression mixture (see Experimental Section).

[Plasmid] in loading solution [pM]	Droplet diameter [μm]	Droplet volume [pL]	[Plasmid] in droplet [pM]	% empty droplets	% droplets containing >1 plasmid (calcd)	% droplets containing a single plasmid (calcd)	% droplets containing GFP (obs)	GFP expressed [nM]	Molecules GFP per molecule plasmid[a]
0.5	11.1	0.7	2.3	80.3	2	17.7	26	45	20000
0.3	12.7	1.1	1.6	82.7	1.6	15.7	23	46	30000
0.1	10.5	0.6	2.7	96	0.2	3.8	4	32	12000

[a] In droplets containing one gene copy and expressing GFP.

let is 1.6–2.7 pM, depending on the droplet volume (varying between 0.6 to 1.1 pL).

The droplets were stored in the device reservoir for 6.5 h at room temperature (~20 °C) to ensure the completion of protein expression. The background fluorescence was measured for droplets containing the IVTT mixture but lacking plasmid (Figure 5A and C). The droplets in which GFP was expressed had a mean fluorescence signal more than three times background (Figure 5B and D).

It was found that the percentage of droplets in which GFP was expressed was in relatively close agreement with the calculated percentage of droplets expected to contain plasmid, coinciding exactly with 0.1 pM plasmid in the loading solution (third entry in Table 1). The majority of droplets that expressed GFP did so within a relatively narrow range (45 ± 20 nM), as would be expected if each contained a single molecule of plasmid genes. Typically, 65 to 95% of the droplets analyzed (~4×10^4) were empty in this type of experiment, in comparison with less than 20% empty droplets when higher plasmid concentrations were used (Figure 6).

The number of molecules of GFP produced per droplet (i.e., per molecule of plasmid), in droplets containing single genes, was between 12000 and 30000 (Table 1). These experiments give higher numbers of protein molecules per DNA template than in the experiments carried out with multiple plasmid molecules (4000–8000; see above). Two factors could explain the lower expression efficiency observed in the presence of a higher plasmid concentration. It might be that oxygen is limiting and so not all the GFP can fold properly and form the oxidised fluorophore.[35,36] An alternative explanation could be saturation of the translation machinery.[34,37,38]

Conclusions

By using microfluidic devices, it is possible to generate droplets (at rates of up to 10^3 s^{-1}) of uniform size, enabling quantitative studies in a high-throughput way. Until now, the timescale for transit of a droplet through a typical microfluidic device has usually been seconds, too fast for experiments that require extended incubation times.

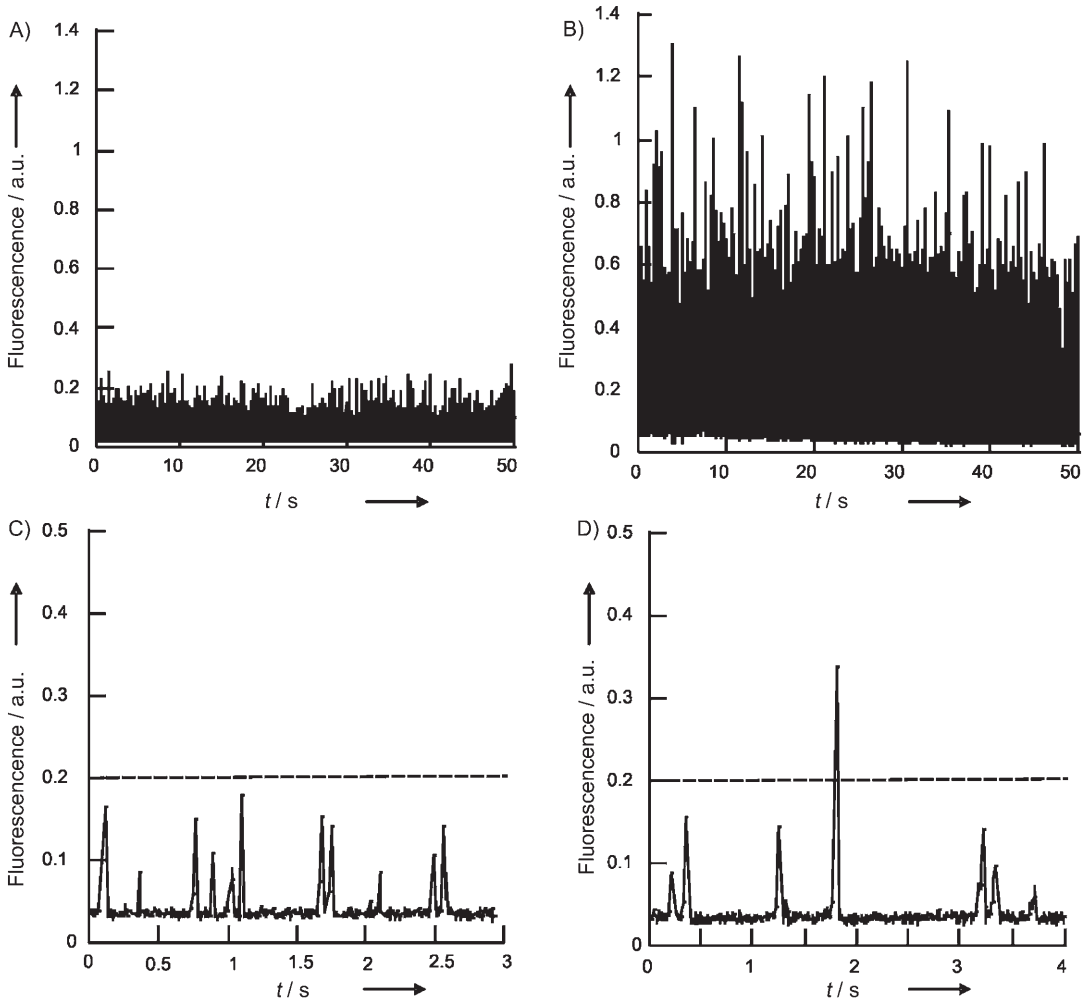

Figure 5. Analysis of droplet fluorescence. Fluorescence intensities of droplets measured after 6.5 h incubation at room temperature. Each peak corresponds to one droplet. Droplets contain the cell-free expression system either without (A, C) or with (B, D) pIVEX-gfp template (0.3 pM). In A, >98% of all peaks show less than 0.2 fluorescence units. This was taken as a conservative upper limit for the background in order not to miss any positives. In B, all peaks with a fluorescence value above 0.2 were considered as droplets expressing GFP. The distribution of peaks in a similar experiment is shown in Figure 6.

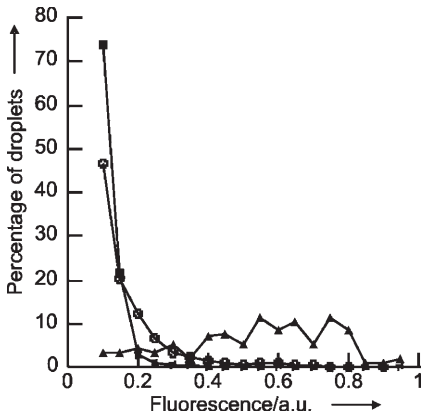

Figure 6. Histograms of peak values obtained when GFP was expressed in vitro in droplets with no plasmid template (■), with 0.26 DNA molecules per droplet (○; observed percentage of droplets containing one or more plasmids—Table 1, first row) or with 250 DNA molecules per droplet (▲). Larger amounts of DNA template led to smaller percentages of empty droplets.

This paper describes the design and development of an integrated microfluidic device for droplet generation, incubation and screening for experiments that need extended reaction or incubation times. Together with a surfactant formulation that keeps droplets stable for hours, this system can be used to make uniform droplets in a range of sizes (typically 10–30 μm diameter; 0.5–14 pL) and to store large numbers of droplets (~10^6) in a reservoir for several hours, during which time there is less than 10% erosion of the droplet quality. Longer residence times in droplets can be necessary in order to follow relatively slow enzymatic or nonenzymatic kinetics or in order to pre-incubate enzymes with inhibitors before a reaction assay is started. The utility of the system is demonstrated in a series of experiments on in vitro expression of GFP.

High yields of GFP were obtained, comparable with the best reported in IVTT-IVC systems,[34] and much higher than have previously been reported in droplets.[22] These high yields made it possible to perform protein expression from single copies of the DNA template, thereby generating monoclonal droplets. In vitro compartmentalisation experiments using droplets in bulk emulsion[39–42] and liposomes[43] with excesses of compartments over DNA molecules have been reported previously. However these systems suffered from a lack of uniformity of compartment size, rendering quantitative studies difficult and decreasing the stringency possible for any selection (e.g., for product formation). In contrast, microdroplets in microfluidics have low polydispersity, making them amenable to quantitative analysis. It is noteworthy that even though the concentration of DNA template is picomolar in the monoclonal droplets, efficient translation was obtained. The availability of monoclonal droplets is a major step towards future in vitro directed evolution experiments under stringent, quantitative selection pressure in droplet compartments in microfluidics.

Experimental Section

Materials: Poly(dimethylsiloxane) (PDMS, Sylgard 184) was obtained from Dow Corning (UK). Glass coverslips (microscope slides) were obtained from AGAR Scientific (UK). Mineral oil was supplied by Aldrich, and ABIL EM 90 by Golsdschmidt GmbH (Essen, Germany). The RTS 100 *Escherichia coli* HY kit was supplied by Roche Diagnostics GmbH (Mannheim, Germany). The laser was obtained from Picarro Cyan and the photomultiplier tube (H8249) from Hamamatsu Photonics. The CCD camera (Model A247) was from Pixelink and the microscope (IX71) from Olympus. LabView 8.2 software was supplied by National Instruments.

Cloning, expression and purification of GFP: The GFP used in this study contains three mutations (S65A, V68L and S72A) that increase its fluorescence 20-fold relative to the wild type.[33] The gene was amplified from the plasmid pDXA-GFP2[44] by PCR with oligonucleotides (5′-aca aag cgg ccg cca tca tca tca tca tca tga-3′ and 5′-ctt gag tcg aca ctt gaa ttg atc ctc tag-3′) that introduced *Not*I and *Sal*I restriction sites. The purified DNA was digested and ligated into the plasmid pIVEX2.6d (ROCHE) to give the plasmid pIVEX2.6d-*gfp*. The ligation mixture was used to transform *E. coli* DH5α. DNA sequencing (Department of Biochemistry, University of Cambridge) from minipreps (QIAprep Spin Miniprep Kit, QIAGEN) of the transformed cells confirmed the correct GFP sequence.

The pIVEX2.6d-*gfp* plasmid used for in vitro expression was further purified by phenol/chloroform extraction: phenol buffer equilibrated at pH 8.0 (USB, 200 μL) was mixed with a plasmid solution obtained from a miniprep (100 μL). The mixture was shaken extensively and centrifuged (13000 *g*) to recover the aqueous phase. Phenol/chloroform/isoamyl alcohol (25:24:1, Gibco BRL, 100 μL) was added, and after extensive shaking the aqueous phase was recovered by centrifugation (13000 *g*). Two extractions with ethyl ether (~300 μL) were used to clean the aqueous phase further. The DNA was concentrated by ethanol precipitation.

The S65A-V68L-S72A-GFP was cloned into the expression vector pET21d (Novagen) and transformed into *E. coli* BL21(DE3) cells, to provide an N-terminal *His6*-tagged protein. The protein was expressed at 37 °C and with IPTG (1.0 mM) for four hours and purified by use of a HisTrap FF (1 mL, GE Healthcare).

Device fabrication: The microfluidic PDMS/glass devices were fabricated by conventional soft lithographic methods.[24] A master consisting of a 25 μm thick layer of SU8–2025 patterned onto a 76 mm diameter silicon wafer was first constructed by conventional photolithography. A mixture of poly(dimethylsiloxane) (PDMS, Slygard 184) and cross linker (ratio 10:1 *w/w*) was poured over the master, and the system was degassed and then cured for 6 h at 70 °C. The cured device was cut and peeled from the master, and holes for tubing were cut with a biopsy punch. After treatment with air plasma for 30 s, the device was sealed against a glass slide and baked for 4 h at 70 °C. Dimensions of the device are given in Figure 1.

Microfluidic experiments: The flow in A, B1 and B2 (Figure 1) was driven with Harvard Apparatus 2000 syringe infusion pumps. Glass syringes (50 μL and 100 μL Hamilton Gastight syringes (1700, cemented needles, 22 s gauge) connected to polyethylene tubing (Beckman and Dickinson, US) were used for precise control of aqueous flows. The droplet size was controlled by the ratio of carrier flow rate (i.e., oil-containing surfactant) to total aqueous flow rate, and the channel dimensions. We used a 10 μm wide junction channel and an oil/aqueous flow rate ratio of 6. This enabled us to form monodisperse droplets ranging between 10 and 30 μm in

diameter in separate experiments (0.52 pL to 14.14 pL reaction volume) at a typical frequency of 440 droplets per second. The droplet diameter is smaller than the height of the reservoir, resulting in spherical drops.

The microfluidic channels were visualized with a CCD camera (Model A247, Pixelink) mounted on a microscope (IX71, Olympus).

The image analysis to determine the size of the droplets was performed with software written with LabView 8.2. To calculate the properties of the droplets in an image they were first isolated from their surroundings with the aid of the local contrast at the droplet–oil interface. This produces a map of areas inside and outside of the droplets. These objects were then filtered by size and roundness to exclude non-droplets and artefacts. The resulting objects could then be analysed in terms of their diameter and area.

Laser set-up and fluorescence analysis: Fluorescence detection was performed with a laser-induced fluorescence setup consisting of a 20 mW, 488 nm diode-pumped solid-state (DPSS) laser (Picarro Cyan) and a low-noise photomultiplier tube (PMT; H8249, Hamamatsu Photonics). The system was in an epifluorescence arrangement, in which a single objective lens (UPLSAPO 40×, Olympus) was used to focus the laser light and to collect the fluorescence signal. The laser was expanded before entering the objective to give a spot size of ~20 μm.

The voltage from the PMT was fed into a computer through a National Instruments data acquisition card (PCI 6251) and processed offline with LabView 8.2 software (National Instruments).

The fluorescent intensity recorded from the PMT was analysed by use of a threshold peak detection algorithm with the upper and lower bounds defined by hand for each set of results. The area of the fluorescent peak was then calculated by integrating the signal lying above the noise baseline.

Determination of fluorescence detection limits: To quantify the detection limit of our system (Figure 4), the fluorescence of purified GFP was measured with a device with two inlets containing a wiggle channel and a short outlet channel for droplet detection (similar to the left-hand side of Figure 1). As in the other experiments, a mineral oil/Abil EM 90 mixture [3% (*w/w*)] was used. One aqueous inlet (B1, Figure 1) contained GFP (750 nM), and the other aqueous inlet (B2, Figure 1) contained Tris/HCl buffer (pH 7.5, 50 mM). The GFP concentration was decreased by dilution on chip, the total aqueous flow rate being kept at 20 $\mu L\,h^{-1}$. Fluorescence intensity was measured and analyzed as described above. The signals for oil and buffer were ~0.075 and ~0.1, respectively.

Retrieval and detection of droplets: All experiments were run in the type of integrated device shown in Figure 1. The reservoir was first filled with the oil mixture for at least 30 min before the experiment to saturate the PDMS material, minimizing the absorption of the oil into it later. The carrier fluid was a mixture of mineral oil containing ABIL EM 90 (3% *w/w*) flowing at 60 $\mu L\,h^{-1}$. Two aqueous syringes were prepared, one containing Tris/HCl buffer (50 mM, pH 7.55), the other one GFP (750 nM). The aqueous flow rates were each 5 $\mu L\,h^{-1}$. Once the reservoir was filled with droplets, it was closed by cutting and burning the tubes with a cigarette lighter.

Emulsion stability was estimated by image analysis of droplet sizes on images recorded at different time intervals and by analysing the fluorescence intensities of droplets. At selected intervals, the reservoir was emptied by pumping oil from inlet A at 300 $\mu L\,h^{-1}$. The droplets were thus forced out of the reservoir one by one through a 30 μm outlet channel where the laser beam was focused. Their fluorescence intensities were measured and analysed as above. The percentage of fused droplets was calculated by comparing the peak area for each droplet. The amount of shrunken droplets was obtained from the number of droplets presenting higher fluorescence intensity than the one at time zero. Theses values were compared to the values obtained by droplet size analysis. Experiments were performed three times, and the total number of droplets analysed was approximately 9000.

Measurement of long GFP in vitro expression kinetics: All kinetic experiments were conducted as described above. For the in vitro expression of protein, the RTS 100 IVTT kit from Roche was used. Two aqueous syringes were prepared, the first one containing the *E. coli* lysate, the amino acids and the methionine, and the second one containing the kit buffer, the "reaction mixture" and the circular DNA template (10.4 or 60 pM) in the proportions recommended by the manufacturer. The two solutions were introduced at the same flow rate (5 $\mu L\,h^{-1}$) to facilitate the droplet formation and to obtain the correct final concentrations of all components. The droplets were stored, and their fluorescence intensities were measured and analysed as previously described. The concentration of IVTT kit in all experiments (including the single DNA experiments described below) was identical.

Single DNA molecule experiments: To investigate the in vitro expression of GFP on the integrated chip, experiments were performed at different plasmid concentrations. To determine the fluorescent background of the *IVTT* reaction, a control experiment was performed without plasmid. Droplets were formed, stored and analysed as described above. The fluorescence was recorded after 6.5 h. The average peak intensity was ~0.12, but 60% of the droplets had slightly higher intensities. For the analysis of droplet content (Table 1), all peaks showing fluorescence intensity below 0.2 were considered to be droplets in which no reaction had taken place. Less than 1.3% of the droplets show up as false positives (i.e., above an intensity of 0.2).

The plasmid stock solution (0.02 $ng\,mL^{-1}$) was used to form droplets of ~10 to 13 μm diameter (Table 1). The distribution of the fluorescence signals (Figure 6) is consistent with a Poisson distribution of plasmids if each plasmid expresses 45 nM ± 20 nM of GFP. The number of GFP molecules expressed per gene was calculated as follows: the measured fluorescence value (peak height) was reduced by the constant background fluorescence and then converted to a GFP concentration by use of the calibration curve in Figure 4A. The concentration was multiplied by the volume and by Avogadro's number ($c \times vol \times N_A$ = number of molecules). After 6.5 h the reservoir was emptied, and the fluorescence intensities of the droplets were recorded at the outlet channel. More than 20000 droplets were analysed. Only those peaks presenting fluorescence higher than the cut-off value (i.e., a value of 0.2) were considered to contain expressed GFP.

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Keywords: high-throughput screening · in vitro protein expression · microfluidic devices · microreactors · monoclonal droplets

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