A New Embedded Process for Compartmentalized Cell-Free Protein Expression and On-line Detection in Microfluidic Devices

Petra S. Dittrich,*[a] Michael Jahnz,[b] and Petra Schwille*[b]

During the last century, an enormous number of protein functions were identified, many of which can be generally described as catalytic. Ongoing research currently focuses on two aspects, analysis of the native proteome to reveal new cellular functions, and adaptation of proteins with known functions to technical processes, with the additional aspect of how these functions could be modified or improved. To this end, directed evolution of proteins[1–4] has in many cases proved to be a successful strategy for designing new biocatalysts for chemical, pharmaceutical or even household use. It relies on the sensitive detection of mutants with new or improved properties, as well as their efficient singling out and amplification.

In this work, a combined approach based on ultrasensitive spectroscopy, microfluidic chips and artificial cells is provided, in order to contribute a new analytical tool for both proteome discovery and evolutionary biotechnology.

What is the artificial cell concept? Artificial cells[5–9] reduce the features of a complex microbiological organism down to the two basic properties that are needed for defined protein analysis: compartmentalization and in vitro protein expression. A promising approach for compartmentalization in miniature environments is the formation of a water-in-oil emulsion, in which micrometer-sized water droplets embedded in a hydrophobic layer serve as artificial biocontainers.[5] All essential compounds for transcription and translation, including the protein-encoding gene as well as a substrate to probe for catalysis, can be included within the same compartment, thus providing an essential requirement for in vitro evolution by linking genotype and phenotype. However, with classical methods for the generation of emulsions in batch (e.g. magnetic stir bar), individual droplets cannot be accurately addressed, and automated droplet handling, analysis and sorting is limited.

For in vitro protein evolution, tools for automated high-throughput formation, analysis and isolation of individual biocontainers are highly desirable.[5,10] Therefore downscaling and integration of all functional steps on a single microchip represents an exciting advance. In microfluidic channel networks, the formation of water-in-oil emulsions at high rates and with monodisperse droplets becomes feasible,[11–15] while small sample quantities can be controllably introduced, while automated droplet handling, sensitive detection of products and potential isolation of individual droplets can be performed.

In this work, we demonstrate the efficient implementation of microstructured devices to generate water-in-oil emulsions, and at the same time perform in vitro expression of proteins inside the water droplets (Figure 1). By using the red-shifted

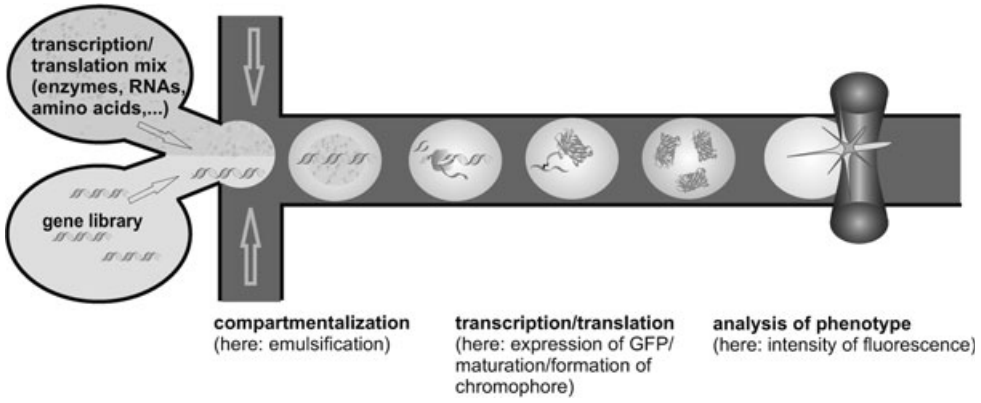

Figure 1. *Schematic of the in vitro evolution of proteins in microfluidic channels. During continuous formation of a water-in-oil-emulsion, the compounds for cell-free expression of proteins are mixed with templates from a gene library. Internal in vitro expression takes place in biomimicking artificial cells during transport through the channel. In our experiments, we used green fluorescent protein (GFP) as a model system. After the autocatalytic formation of the chromophoric group, GFP could be determined by fluorescence spectroscopy.*

[a] *Dr. P. S. Dittrich*
Department Miniaturization, Institute for Analytical Sciences
Bunsen-Kirchhoff-Straße 11, 44139 Dortmund (Germany)
Fax: (+49) 231-1392-120
E-mail: dittrich@ansci.de

[b] *Dr. M. Jahnz, Prof. Dr. P. Schwille*
Institute of Biophysics, Bio Tec, TU Dresden
Tatzberg 47–51, 01307 Dresden (Germany)
Fax: (+49) 351-46340-342
E-mail: pschwil@gwdg.de

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mutant of the green fluorescent protein (rsGFP) as a model system, the protein expression can directly be followed by extremely sensitive confocal fluorescence spectroscopy on a single microfluidic chip.

We used a PDMS (poly(dimethyl)siloxane) microstructure that is imprinted from a silicon master and comprises the positive relief of the microfluidic channels (experimental details in Supporting Information). The PDMS structure was sealed onto a glass plate.[16] Liquids were supplied by connecting the end reservoirs to syringes and syringe pumps. Droplets were

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formed in the microstructures by dispensing aqueous sample solution from a side channel into a stream of a hydrophobic liquid (mineral oil as carrier).

In vitro protein expression requires a number of complex compounds, such as tRNAs, amino acids, aminoacyl-tRNA-synthetases, ribosomes, initiation/elongation/release factors and, not least, the protein-encoding gene. To ensure the linkage of expressed protein with its genetic code, it is important that the compounds are mixed in the very moment of compartmentalization. Thus, we used a channel geometry in which the supplied liquids from two different input channels both merged into the main channel at the same position (Figure 2).

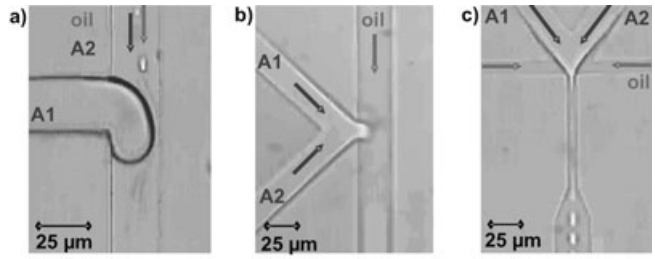

Figure 2. *Droplet formation with simultaneous mixing of two aqueous ingredients A1 and A2. a) A straightforward way to achieve the mixing of A1 and A2 in an emulsion droplet would be the fusion of droplets in a pre-existing emulsion with freshly formed droplets (filled with A2) in the microstructure. The pre-emulsion with droplets sizes of 1–2 µm was generated with a magnetic stirrer before introduction into the microchannels. We found that the mixing of both solutions in this case was rather random, irreproducible and hard to control. Most droplets of the pre-emulsion remained unmixed. b) A better strategy to achieve mixing of the two aqueous solutions A1 and A2 immediately before droplet formation is the merging of two channels into a main channel filled with the oily phase. The droplet diameter here was ~25 µm. c) Since channel geometry determines the diameter of the droplets under the experimental conditions, the narrowing section in the channel geometry enabled the formation of smaller droplets of ~5 µm in diameter.*

Under the experimental conditions, the crosswise diameter of the formed droplets is restricted by the channel dimensions, while the length is dependent on the applied flow rates of the aqueous liquids and the carrier (for a detailed description, see Tice et al.[14]). Generally, high flow rates of the carrier compared to the flow rate of the aqueous solution result in small aqueous droplets.

Droplets were continuously formed with a typical frequency 5–30 droplets per second, and with a length of 1–1.5× longer than the cross-section of the channel (movie in Supporting Information). As long as the flow rates of all liquids were constant, the dimensions of the formed droplets were extremely homogeneous. By introducing a short constriction into the channel network, the droplet diameter could be decreased to ~5 µm, that is, each droplet carried a volume of ~65 fL. To stabilize the emulsion, the mineral oil was mixed with a detergent (5% *v/v* SPAN 80). However, we could occasionally observe fusion of single emulsion droplets induced by precipitation on the rough channel surface, or inside the storage reservoirs.

High-sensitivity analysis of the emulsion was performed in an epifluorescence confocal setup,[16,17] in which laser-induced fluorescence of individual passing emulsion droplets was detected by aligning the centre of the microchannel with the focused light. The obtained periodic fluorescent time trace (Figure 3A) reflects the frequency of droplet formation (here 13 Hz) and is due to the alternating signals of oil and aqueous phases. For determination of the detection limit, we successively decreased the concentration of GFP (Figure 3B). At concentrations of approximately 5 nM, the fluorescence intensity of the water droplets equals the background signal obtained from the mineral oil. In principle, the detection limit can still be lower, as long as the fluorescence of the aqueous phase is distinguishable from the oil signal and higher than that of pure water (~10 kHz). In this optical setup, we determined a detection limit of 2 nM GFP. It should be mentioned that the fluorescence signal from the dye enclosed in the emulsion droplets is decreased to ~30% of that from the same solution before droplet formation. We assume that there is an additional thin hydrophobic layer surrounding the aqueous phase (also described by Tice et al.[14]), whose formation could be facilitated by the hydrophobic surface of the PDMS channels and the glass plate. The mismatch in refractive indices of water and oil is likely to contribute to the observed decrease in detection efficiency. The focal volume element is probably distorted by the oil phase, and stray light might increase. This assumption is in accord with the finding that the signal depends strongly on the position of the centre of the detection volume relative to the channel's height (*z*-axis). Therefore, this *z*-position requires careful alignment.

The flow velocity of the droplets was determined by calculating the autocorrelation function (acf; Figure 3C).[18] The obtained acf revealed two characteristic time scales, one corre-

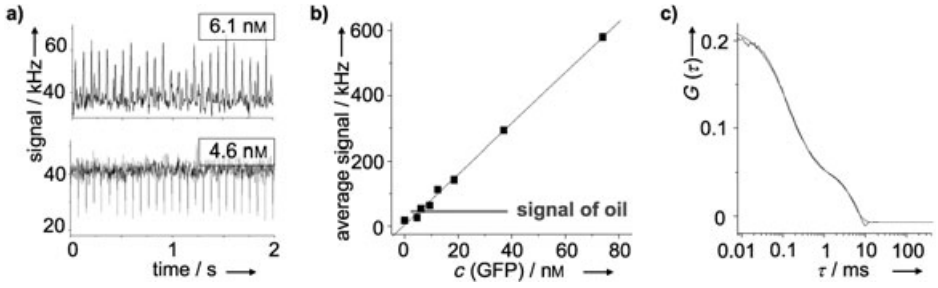

Figure 3. *Detection of fluorescence emission from emulsion droplets: a) Here, the droplets are filled with an aqueous solution of GFP and detected by confocal spectroscopy. The fluorescence bursts in the periodic fluorescence trace reflect the passing droplets. b) The fluorescence intensity increases directly with the concentration of the fluorescent protein, while the signal from the oil is constant at about ~40 kHz. For very low GFP concentrations, the fluorescence intensity of the water droplets can drop below that of the oil signal. c) The flow velocity was accessed by using fluorescence correlation spectroscopy (FCS). Here, the two-step autocorrelation curve reflects the residence times of GFP [τ_{fl} = 0.3 ms, Equation (1)] and the droplets with a diameter of ~7.3 µm (τ_{fl} = 5.3 ms) inside the detection volume. The flow velocity was 1.4 mm s^{-1} [Equation (2)].*

sponding to the mobility of the droplets themselves, and one reflecting the flow and diffusion of the dye molecules within the droplets (on a shorter time scale).

The following function was used to fit the autocorrelation curve $G(\tau)$ to the delay time τ:

$$G(\tau) = \textit{offset} + A_1\left(1+\frac{\tau}{\tau_{\text{diff}}}\right)^{-1}\exp\left[-\left(\frac{\tau}{\tau_{\text{f,dye}}}\right)^2\left(1+\frac{\tau}{\tau_{\text{diff}}}\right)^{-1}\right] + A_2\exp\left[-\left(\frac{\tau}{\tau_{\text{f,droplet}}}\right)^2\right] \quad (1)$$

Here A_1 and A_2 are amplitudes, and τ_{diff} is the characteristic diffusion time of the dye. The parameters $\tau_{\text{f,dye}}$ and $\tau_{\text{f,droplet}}$ reflect average residence times of dye and droplets within the detection volume (with the radius ω_0). The flow times of dye and droplets are related in the following relationship:

$$\frac{\omega_0}{\tau_{\text{dye}}} = \text{flow velocity} = \frac{\text{droplet size}}{\tau_{\text{droplet}}} \quad (2)$$

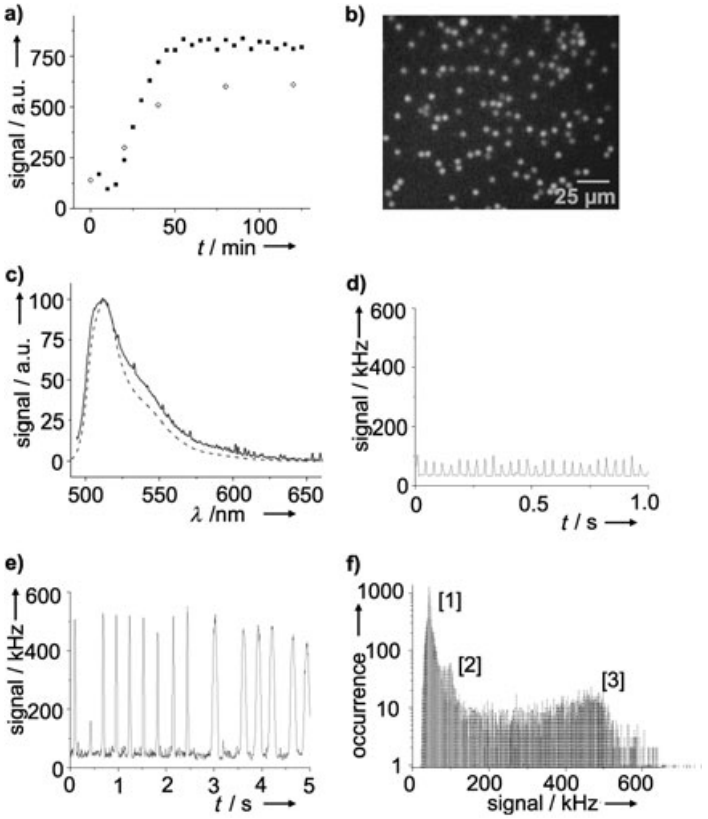

Figure 4. *Cell-free protein expression in emulsion droplets. a) Time course of GFP in in vitro translation: ■: bulk experiment under optimal conditions; ◇: same experiment in the microchannel (non emulsified). b) Fluorescence microscopy image taken from monodisperse water-in-oil emulsion after internal GFP in vitro expression. c) Fluorescence spectrum of GFP as measured from purchased protein in aqueous solution (-----) and from one individual emulsion droplet after in vitro expression (——). d) Fluorescence intensity time trace measured from emulsion droplets pumped through the channel directly after formation. e) The same time trace obtained from an emulsion after 50 min of incubation and internal GFP in vitro expression. Note that the emulsion is thereby pumped back from the storage reservoir in the reverse direction. This process, in general, is more inhomogeneous and shows lower flow rates than the forward flow directly after droplet formation. f) Histogram for 12500 data points of the fluorescence traces shown in (d) (light grey) and (e) (dark grey). The first section of each histogram [1] corresponds to the background signal from the oil. The shift of the maximum from [2] to [3] represents the increase of fluorescence intensity.*

For in vitro translation, all compounds were prepared according to Lesley's method.[8] In coupled transcription/translation reactions, a supercoiled DNA template with T7 promoter was used, and T7-RNA-polymerase was added. For expression of the rsGFP, we used the 6361 bp plasmid pQBI63 (Quantum Biotechnologies Inc., Montréal, Canada). Bulk fluorescent measurements showed that rsGFP expression, including chromophore maturation, started after a time lag of 10 minutes and that the reaction reached its saturation plateau after approximately 50 minutes (Figure 4A). This is a usual result and can be explained by gradual consumption of the added energy and amino acid mix.

To perform in vitro translation reactions in individual emulsion droplets, two batches of the transcription/translation mix are prepared with identical volumes. While one of these contained the GFP-encoding vector and RNA polymerase, the other provided the amino acids, nucleotides and energy equivalents. The solutions were filled in separate syringes, and identical flow rates of both liquids ensured the correct final concentration of all components within the droplets.

To make sure that there is no continual depletion of any component from the entire transcription and translation mix by for example, adsorption to channel surfaces, inside the microchannels or along the tubing system, we first performed the experiment in microchannels without emulsification. For this, we filled the channel system in the usual way, but terminated the flow of the mineral oil. Both reaction solutions were mixed continuously. After 5 minutes, the entire microstructured device was removed from the microscope stage and subjected to heating at 37 °C. During an incubation time of 2 h, the fluorescence intensity in the mixing zone was determined every 20 to 40 minutes (Figure 4A). It turned out that there is no significant difference in the temporal development of the fluorescence signal.

Next, we formed emulsion droplets in the microchannel and stored them at the end reservoir of the channel. We were able to observe green fluorescence from the microdroplets after they had been warmed at 37 °C for 50 min. (Figure 4B). The fluorescence spectrum measured from one of the emulsion droplets clearly confirmed the development of the green fluorescent protein (Figure 4C). We then repumped the emulsion through the microchannel, thereby recording the fluorescence intensity time trace. Comparing the signals obtained from GFP-expressing droplets before and after heating, we found a significant increase of a factor of about 6 in average. (Figure 4D and E). The brightness distribution of all emulsion droplets was

remarkably narrow; this reflected the homogeneous and precise mixing of the two separate aqueous liquids during droplet formation (Figure 4F). From concentration standard series (see Figure 3B), the concentration of newly in vitro-expressed GFP could be estimated to be about 60 nM on average.

As a conclusion, using this lab-on-a-chip approach, we have demonstrated for the first time the high-throughput formation of monodisperse artificial biocontainers for an in vitro expression of proteins that is compatible with extremely sensitive sequential detection. This allows us now to compartmentalize chemical or biochemical reactions in a huge number of separate containers of subpicolitre size, where different ingredients can be precisely and reproducibly added and mixed during formation of the droplets. Individual droplets are easily accessible to manipulation and can be directly analyzed and characterized on-line.

For this approach to be used in real molecular-evolution cycles, several features of our system will have to be improved. In particular, the insertion of a long, heatable channel section would be desirable so as to ensure a better temperature control for individual droplets, while droplet fusion would be prevented. Secondly, to achieve the extremely high required throughput rates for evolutionary approaches, a further reduction of the channel dimension would be necessary. This would generate even smaller emulsion droplets that each contained (statistically) just one DNA template. In further developments, supplementary functional units, for example, for droplet sorting, could easily be implemented. The whole system is readily suitable for parallelization and automation. Equipped with these features, our strategy might open the gate for a wide variety of new studies and applications in the fields of analytical proteomics and evolutionary biotechnology.

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