

## MICRODROPLETS BASED DETECTION USING LASER INDUCED FLUORESCENCE FOR EVOLVING NEW ENZYME IN A MICROFLUIDIC DEVICE

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### KEY WORDS

Surfactants, Span 80, Abil EM90, Poly (methyl methacrylic) (PMMA), fluorescein sodium salt, dye,

### ABSTRACT

*In the development of microfluidic IVC platform for enzyme evolution, it is important to realize accurate droplet control and sensitive detection. For the application of droplets on a microchip in the field of in vitro compartmentalized protein expression, fluorescence induced by laser is usually used to acquire the corresponding information from each droplet. In this paper, droplet based fluorescence detection on a PMMA microchip was reported by comparison of two surfactants, Span 80 and Abil EM90 in mineral oil. It was found that 3% (w/w) Span 80 in mineral oil resulted in higher background than that of 3% (w/w) Abil EM90 at the same 488nm wavelength of laser excitation. The fluorescence of fluorescein sodium salt (dye) droplet below a certain concentration is smaller than the background of the mineral oil in the presence of Span 80. However, the fluorescence is higher than background even at very low concentration of dye droplet (50pM) when Abil EM90 is used. Changing the flow rates also changes the droplet sizes and rates of generation of droplets. At a given fluorescence concentration, with the increase of aqueous flow rate, the droplet size increased and the fluorescence intensity increased correspondingly. Further, the signals strongly depend on the position of detection area which mostly relates to z-axis in accordance with microchannel's height.*

### 1. INTRODUCTION

Creating and altering new functions of enzymes for specific purpose are demanding in the life science. Directed evolution [1] is a method used in protein engineering for developing new proteins such as enzymes. It can be carried out with or without living cells (in vivo or in vitro evolution). In vitro expression such as in vitro compartmentalisation (IVC) systems using water-in-oil emulsions has been gaining popularity recently since a combination of methods can be used to introduce variation into the selection pool [2]. IVC systems have been used for the selection of proteins, DNA and RNA enzymes, enzyme inhibitors and so on [3-8].

Recent advance in microfluids and microfabrication technologies has fueled the development of microfluidic IVC systems [9-14]. Such systems are capable of generating and manipulating microdrops in an efficient and controlled manner. For example, microdrops can be generated with desired size, fused for adding reagents or substrates, spilt for collection or analysis, or controlled to have temperature, pH and salt concentration variations [15-16].

In the microfluidic IVC systems, one of the tasks is to determine if proteins of interest are indeed synthesized in the drops. This task is very challenging since each microdrop contains only a limited number of gene molecules and therefore a limited number of protein molecules can be produced. Further, the microdrops are moving constantly along the microchannels and there is limited integration time for detecting the properties of the molecules in the microdrops. The aim of this study is to develop an optical detection method that can be sensitive enough for IVC applications and to examine the effect of operating and flow conditions on the detection sensitivity.

## **2. EXPERIMENTAL DETAILS**

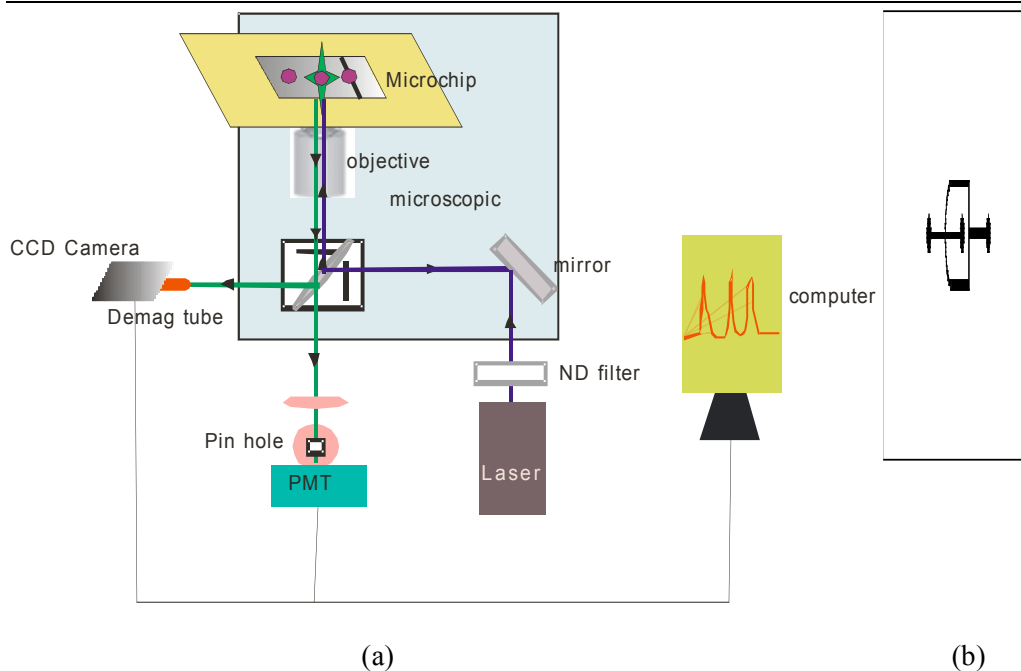
### **2.1 Methodology**

To develop an suitable detection system, a fluorescein sodium salt was used as a model dye. For the PMMA microchip experiment, the dye solutions with different concentrations were pumped through the droplet formation nozzle to generate monodisperse droplets by the shear force of the oil stream. The continuous phase was mineral oil doped with surfactant Span 80 and Abil EM90, respectively. After excitation with laser, the drops emit light which may have contributions from the microchip material, mineral oil, surfactants and the stray light. The signal of detected light would also have contributions of the electrical noise of equipments.

### **2.2 Experimental set-up**

The droplet experiments were carried out in the CSIRO Microfluidics Laboratory at Highett, Melbourne, Australia. The optical detection was carried out using an inverted epi-fluorescence microscope (NIKON TE2000U). The aqueous and oil streams are pumped by the two identical pumps (neMESYS) equipped with SGE (Supelco) syringes of 250 $\mu$ l capacity. Droplet formation at the junction point was monitored by the microscope and a video camera (Basler A 6021 C-2) equipped with a Nikon C-0.45 $\times$  demagnification lens. The camera is capable of capturing images at a frequency of 30 frames per second. A Sapphire laser (488nm) and a 460nm CDRH laser (Coherent) were used to induce fluorescence inside the aqueous droplets. The emitted light from the GFP molecules were collected by the objective lens of the microscope and detected by a photomultiplier tube (Hamamatsu). The whole experimental set-up is sketched in Figure 1(a).

The microchip was fabricated in a PMMA slide with dimensions of 75 mm $\times$ 25 mm $\times$ 2 mm. The microchip was fabricated at the CSIRO Microfabrication Laboratory, Clayton, VIC 3169, Australia. A flow focusing technique was used for droplet formation and only one nozzle was used in the chip. A sketch of the chip design is shown in Figure 1(b). The chip was fabricated using a standard photolithography technique in conjunction with electroplating and hot-embossing. The mask pattern was first exposed into a 70 $\mu$ m layer of laminar 5083 resist/stainless steel plate using a collimated UV exposure system. Then a nickel shim was made by electrodeposition onto the patterned surface. The nickel shim was then used as a mould to hot emboss the pattern of microchannels onto a PMMA plate at a temperature of around 110  $^{\circ}$ C. Finally the capping PMMA layer with a thickness of 125  $\mu$ m was thermally bonded onto the channel PMMA plate. Both the oil and water channel have a width of 315  $\mu$ m which is decreased to 115  $\mu$ m at the junction point. The production channel has a width of 207  $\mu$ m and all channels have the same depth of 70  $\mu$ m.



**Figure 1:** (a) Schematic graph of experimental set-up (b) flow focusing design in positive photo mask.

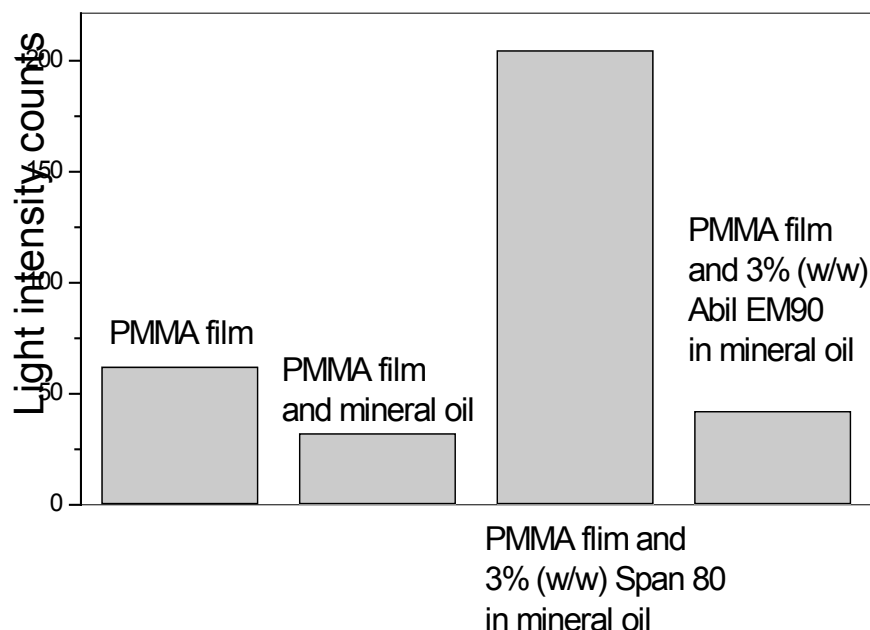
### 2.3 Materials

The fluorescence sodium salt (dye) (Ajax Finechem) was dissolved in dionised water and used as the aqueous phase while mineral oil was used as the oil phase. The mineral oil (molecular biology, Sigma) has a kinematic viscosity of 8.4 mPaS at 20°C and density of 840 kg/m<sup>3</sup>. The surfactants Span 80 (Fluka) and Abil EM90 (Goldschmidt GmbH, German) were added to oil at the same weight percentage of 3%. The pH of 40mM dye stock solution is 9.89. The dye solution was changed from 50pM to 200nM and all reagents used were filtered by 1.2 µm pore-sized syringe filters.

## 3. RESULTS AND DISCUSSION

### 3.1 Effects of surfactants on detection of droplets on a PMMA microchip

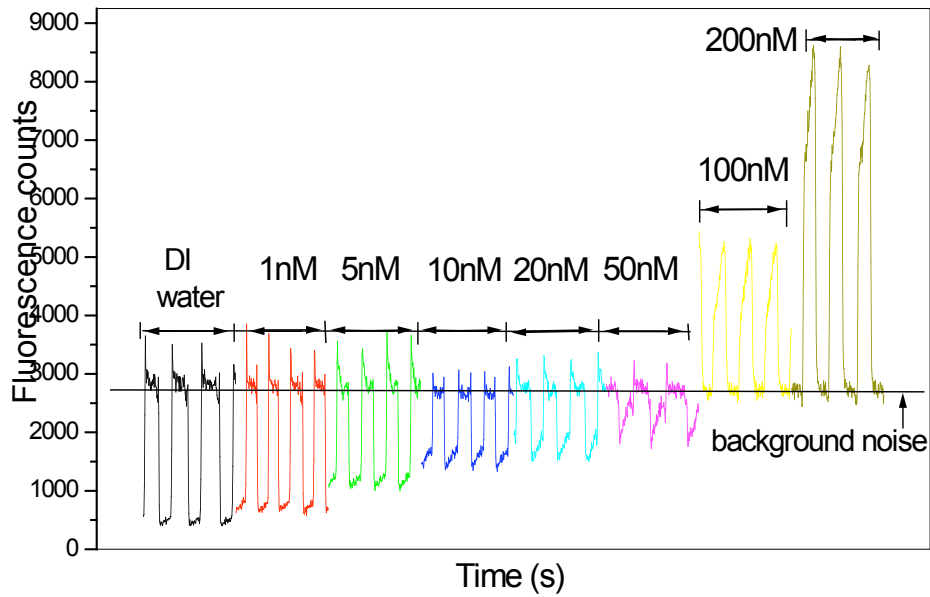
Before the microchip experiments, a preliminary test was carried out to understand the possible effect of surfactants materials on the detection efficiency. Several oil drops with different surfactant solutions were placed on a PMMA sheet with a thickness of 125µm. A solid state laser of 488nm wavelength was used to as the excitation source. The droplets were placed on the sheet using a pipeting device with precision control and the droplet volume was around 1µl. Some droplets contained 3% (w/w) Span80 while others contained 3% (w/w) Abil EM90 surfactant. A marker was used to fix the focal spot at a specific point inside of droplet throughout the measurement. The light intensity was measured using a Hamamatsu photomultiplier tube. Figure 2 shows the emitted light intensities for the four different samples. The mineral oil has the lowest light emission. It is even lower than that of the PMMA film. The light emission from the oil drops with 3% (w/w) Span 80 is the highest. When Abil EM90 surfactant was used, the light intensity was significantly reduced, only slightly higher than that of pure mineral oil. This indicates Abil EM90 provides much less contribution to background light emission than that of the Span 80 surfactant. Therefore, Abil EM90 is used throughout the whole study as the oil phase surfactant.



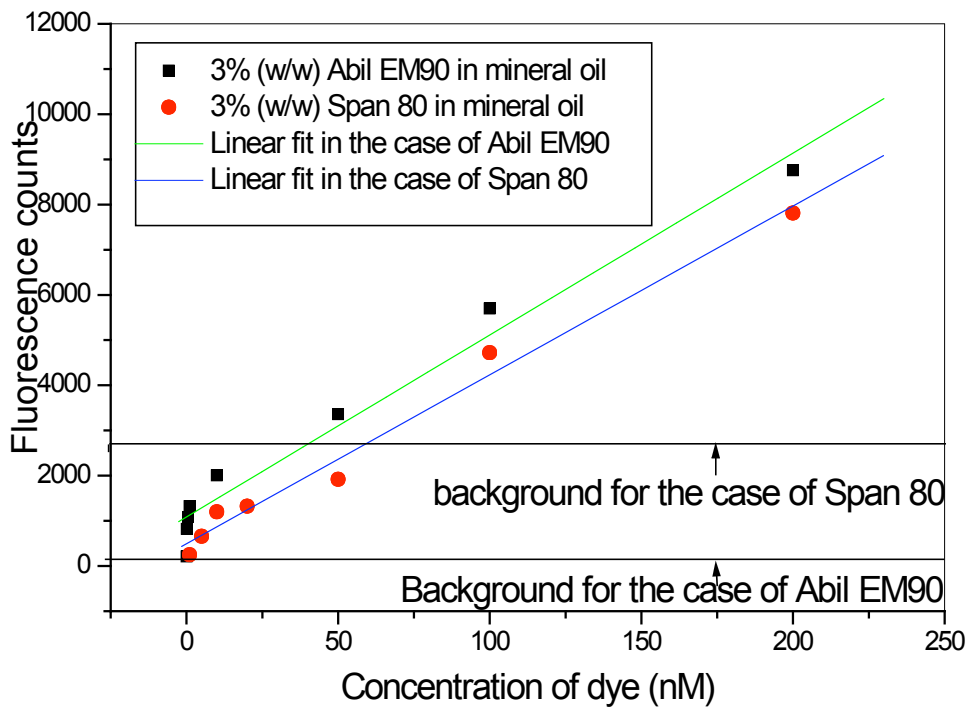
**Figure 2:** Light intensity of droplets with different composites on a PMMA film.

Figure 3 plots segments of signals detected from the droplets with different dye concentrations. The continuous phase is mineral oil with 3% (w/w) Span80. The black line indicated the level of background noise. It is revealed that when the dye concentration is below around 50nM, the light intensity of dye droplets is below the background noise and negative peaks occur. The negative peaks became larger with the decreasing concentration of dye in the droplets. When the dye concentration increases above about 50nM, negative peaks change to positive peaks. However, when Abil EM90 was used, the background noise level is much lower than that using Span 80, even though the concentration of dye droplets was lowered to 50pM, the signals appeared as positive peaks higher than background. This also proved that in the current detection system, the presence of Span 80 was the main source of the background value, and Abil EM90 contributed to the background much less than Span 80.

The laser induced fluorescence from the droplets is shown in Figure 4 as a function of dye concentration for the two oil phases, respectively. The two horizontal lines indicate the corresponding background values for the two cases. The fluorescence intensity increases approximately linearly with increasing dye concentration. However, at very low concentrations, the variation with dye concentration is more pronounced. The lowest dye concentration detected by the system is 50pM.



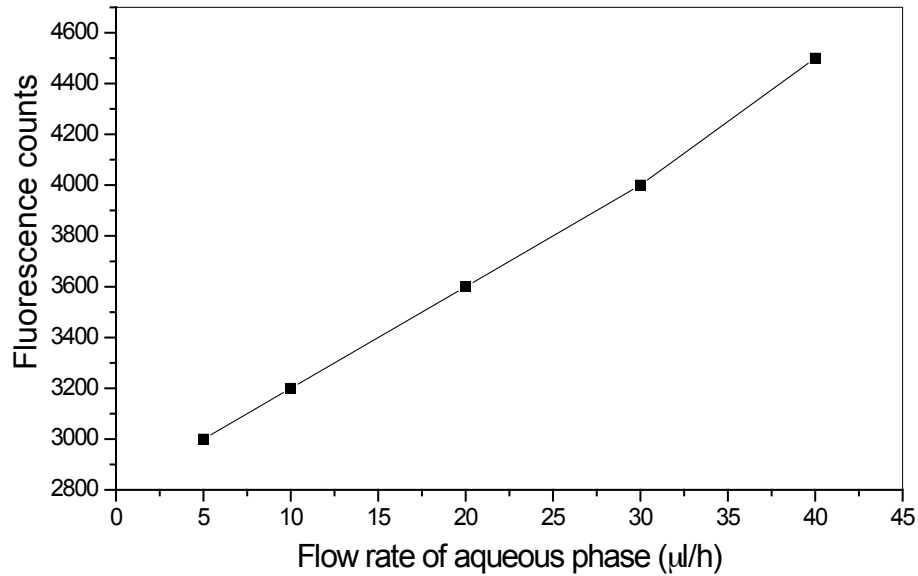
**Figure 3:** Segments of signals detected from droplets with different dye concentrations in the presence of 3% (w/w) Span 80 in mineral oil using 488nm laser on a PMMA microchip.



**Figure 4:** Fluorescence of dye droplets as a function of dye concentrations in the presence of 3% (w/w) Abil EM90 and 3% (w/w) Span 80 in mineral oil respectively.

### 3.2 Flow rates and fluorescence intensity

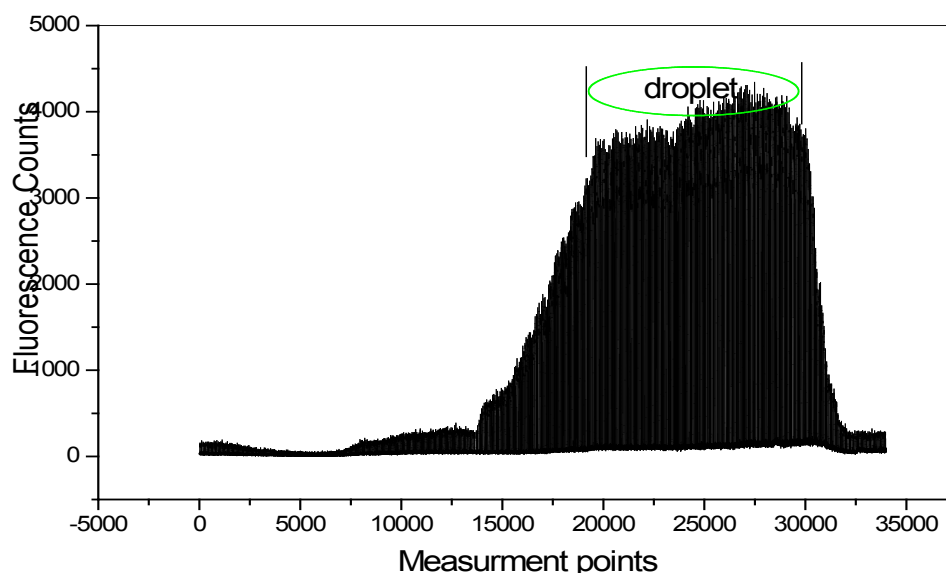
For a given dye concentration and oil flow rate, the flow rate of the aqueous phase was changed from 5  $\mu\text{l/h}$  to 40  $\mu\text{l/h}$  to examine the effect on detection. It can be seen from Figure 5 that the fluorescence intensity increased with the increase of aqueous flow rate. It has been mentioned [14] that the fluorescence intensity from dye enclosed in an emulsion droplet is about 30% less than that of its same solution before droplet formation. The droplet size increased when the aqueous flow rates increased. As a result, the drop volume increased and so was the detected fluorescence intensity. Further, larger droplets took longer time to pass the detection region and therefore the integration time of the detection is bigger. However, at a given flow rate of the dye solution on the same microchip, the fluorescence intensity had no distinguished change when the oil flow rates varied from 10  $\mu\text{l/h}$  to 60  $\mu\text{l/h}$ .



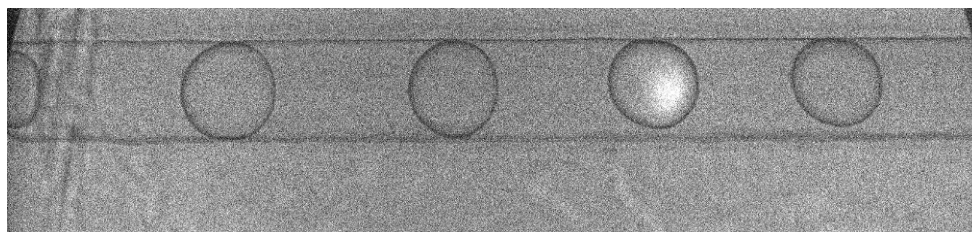
**Figure 5:** Fluorescence intensity of dye droplets as a function of the flow rates of aqueous phase from 5  $\mu\text{l/h}$  to 40  $\mu\text{l/h}$  at a given oil flow rate of 20  $\mu\text{l/h}$ .

### 3.3 Effect of detection position

In the current system, the dye droplets were produced on a flow focusing PMMA microchip with a PMMA bottom layer of 125  $\mu\text{m}$  thickness. The laser light was initially focused on the bottom layer by a 40 $\times$  objective lens. The focus was gently shifted up along the positive z-axis –perpendicular to the microchip surface, and the fluorescence intensity was recorded sequentially which is given by its numbers of measurements points as shown in Figure 6. The measurement point zero corresponded to the focal position at the bottom layer. In Figure 6, the labeled region showed the signals increased gradually where most of light was provided by the droplets as a consequence of the position of the detection volume entering into the droplets. Figure 7 showed and demonstrated that droplets passed by the detection spot and gave the fluorescence.



**Figure 6:** The position of detection volume shifting up along the positive z-axis from the bottom layer of PMMA microchip. Flow rate of oil phase is 30 $\mu$ l/h; aqueous flow rate is 10 $\mu$ l/h.



**Figure 7:** Droplet passing by the detection spot and giving fluorescence. The flow rate of oil phase is 30 $\mu$ l/h; aqueous flow rate is 20 $\mu$ l/h.

## 5. CONCLUSIONS

In this paper, the droplet detection on a PMMA microchip was investigated by using the surfactants of Abil EM90 and Span 80. No matter how the light signals appear, positive or negative peaks, as long as the fluorescence of dye is different from the background, the detection can still be carried out. However, the detection sensitivity in the case of Span 80 is far lower than that in Abil EM90 where 50pM dye droplets can be detected. The fluorescence intensity of dye droplets can also be affected by varying the flow rates of aqueous stream and changing the position of detection volume. By using the current microfluidic detection system, 16ng/ $\mu$ l GFP gene expression solution has been easily detected and expected to realize the detection of expression solution of single molecule of gene.

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## REFERENCES

- [1] Arnold, F. H., (1998). Design by directed evolution. *Acc. Chem. Res.*, **31**, 125–131.
- [2] Zhu, Y. & Power, B. E., (2008). Lab-on-a-chip in Vitro Compartmentalization Technologies for Protein Studies, *Adv Biochem Engin/Biotechnol*, DOI 10.1007/10\_2008\_098, Springer-Verlag Berlin Heidelberg
- [3] Sepp, A. & Choo, Y. (2005). Cell-free selection of zinc finger DNA binding proteins using in vitro compartmentalization. *J. Mol. Biol.*, **354**, 212–219.
- [4] Yonezawa, M., Doi, N., Kawahashi, Y., Higashinakagawa, T., & Yanagawa, H. (2003). DNA display for in vitro selection of diverse peptide libraries. *Nucleic Acids Res.*, **31**, e118.
- [5] Doi, N., Kumadaki, S., Oishi, Y., Matsumura, N., & Yanagawa, H. (2004). In vitro selection of restriction endonucleases by in vitro compartmentalization. *Nucleic Acids Res.*, **32**, e95.
- [6] Agresti, J. J., Kelly, B. T., Jäschke, A., & Griffiths, A. D. (2005). Selection of ribozymes that catalyse multiple-turnover Diels-Aldercycloadditions by using in vitro compartmentalization. *PNAS*, **102**, 16170–16175.
- [7] Griffiths, A. D. & Tawfik, D. S. (2003). Directed evolution of an extremely fast phosphotriesterase by in vitro compartmentalization. *EMBO J.*, **22**, 24–35.
- [8] Bernath, K., Magdassi, S., & Tawfik, D. S. (2005). Directed evolution of protein inhibitors of DNAnucleases by in vitro compartmentalization (IVC) and nano-droplet delivery. *J. Mol. Biol.*, **345**, 1015–1026.
- [9] Anna, S. L., Bontoux, N., & Stone, H. A. (2003). Formation of Dispersions Using “flow Focusing” in Microchannels. *Appl. Phys. Lett*, **82 (3)**, 364–366.
- [10] Nisisako, T., Torii, T., & Higuchi, T. (2002). Droplet Formation in a Microchannel Network. *Lab chip*, **2 (1)**, 24–26.
- [11] Link, D. R., Grasland-Mongrain E., Duri, A., Sarrazin, F., Cheng, Z. D., Cristobal, G., Marquez, M., & Weitz, D. A. (2006) Electric Control of Droplets in Microfluidic Devices. *Angew. Chem. Int. Ed*, **45**, 2556 –2560.
- [12] Tan, Y. C., Fisher, J. S., Lee, A. I., Cristini, V., & Lee, A. P. (2004). Design of Microfluidic Channel Geometries for the Control of Droplet Volume, Chemical Concentration, and Sorting. *Lab Chip*, **4**, 292–298.
- [13] Ahn, K., Kerbage, C., Hunt, T. P., Vestervelt, R. M., Link, D. R., & Waitz, D. A. (2006). Dielectrophoretic Manipulation of Drops for High-Speed Microfluidic Sorting Devices, *Applied Physics Letters*, **88**, 024104.
- [14] Dittrich, P. S., Jahnz, M., & Schwille, P. (2005). A New Embedded Process for Compartmentalized Cell-Free Protein Expression and On-line Detection in Microfluidic Devices. *ChemBioChem*, **6**, 811–814
- [15] Huebner, A., Srisa-Art, M., Abell, C., Hollfelder, F., DeMello, A. J., & Edel, J. B. (2007). Quantitative detection of protein expression in single cells using droplet microfluidics. *Chem. Commun.* 1218–1220.
- [16] Song, H., Tice, J. D., & Ismagilov, R. F. (2003). A Microfluidic System for Controlling Reaction Networks in Time. *Angew. Chem. Int. Ed.* **42(7)**, 767–772.