FINAL PUBLISHABLE SUMMARY REPORT

Project No: 221761

DevMicro - Directed Evolution in Femtolitre Microreactors – Exploring 'Promiscuity Space'

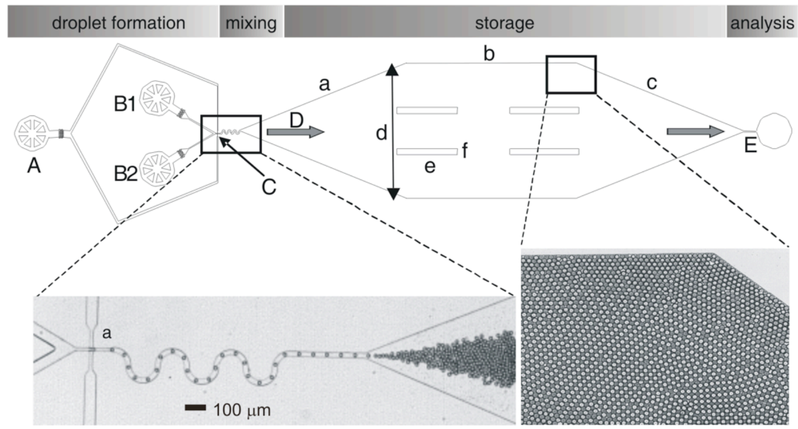

Figure 1. A typical microfluidic device used store droplets in which processes of exchange and catalysis were studied.

Research training. The use of water-in-oil droplets as microreactors to carry out chemical and biochemical experiments relies on the assumption that the contents of droplets are not leaking out into the oil phase and there is no communication between the reaction vessels. To assess the mechanism of possible leaking, the retention of various fluorescein derivatives from droplets formed in mineral oil and stored for hours in a reservoir on chip was studied. Leaking into the oil phase was observed and was shown to be dependent on the charge of the compounds and on the concentration of the surfactant used. We found that the addition of bovine serum albumin substantially reduced leaking. The improved retention of small fluorescent molecules inside droplets on chip using our modified formulation was demonstrated by single-cell experiments in which encapsulated *E. coli* cells producing an enzyme were distinguished clearly from those without cells, suggesting that cross contamination of product was minimized. These improvements in emulsion formulation enabled assays to be conducted for more than 18 h (Figure 2).

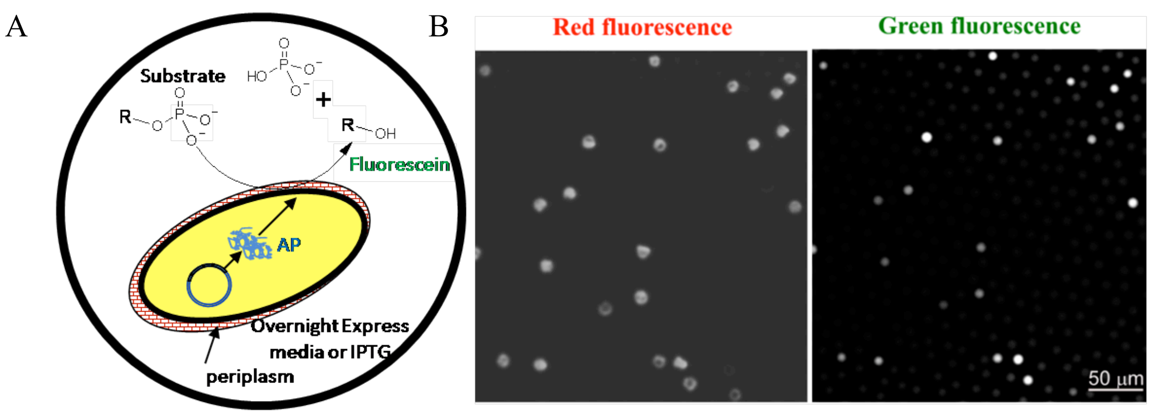

Figure 2. A- Schematic of periplasmic enzyme cell-based assay. B- Fluorescence microscopy image of droplets after in vivo co-expression of RFP and AP by encapsulated *E. coli* cells (accumulation of fluorescein, product of hydrolysis of FDP).

These results have been published in Analytical chemistry, **2009,** 81, 3008-3016 (*Controlling the Retention of Small Molecules in Emulsion Microdroplets for Use in Cell-Based Assays*, Fabienne Courtois, Luis F. Olguin, Graeme Whyte, Ashleigh B. Theberge, Wilhelm T. S. Huck, Florian Hollfelder, and Chris Abell).

Our objective was to develop an assay in microfluidics microdroplet for an enzyme presenting promiscuous activity which could be further enhanced by directed evolution. We chose to study the *Pseudomonas aeruginosa* arylsulfatase. In order to set up biochemical assays for this hydrolytic reaction in small emulsion droplets in microfluidic device, new fluorogenic substrates were needed. Fluorescein disulfate and fluorescein monosulfate were synthesised in our group and have been purified and characterised (Km and kcat determined) as substrates of our target enzyme, aryl sulfatase.

The access to monoclonal droplets, i.e. containing a single plasmid, a single linear DNA template directly purified from PCR or a single cell gives the ability to reliably compartmentalize genotype and phenotype, a requirement for directed evolution experiments.
We studied these different approaches by rather co-encapsulating single cells over-expressing the arylsulfatse and its substrate in microdroplets based microfluidic (Figure 2A) or by creating artificial cell containing a single gene, an *in vitro* expression system and the enzyme substrate (Figure 2B).

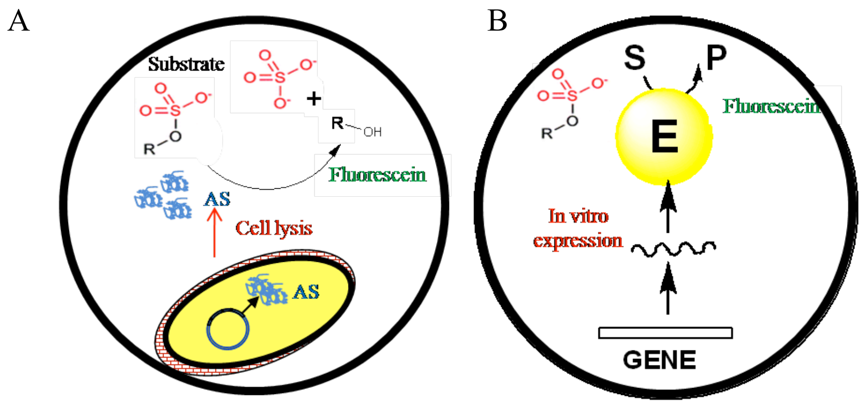

Figure 2. A- Schematic of cytoplasmic enzyme cell-based assay. B- Schematic of enzyme *in vitro* expressed assay.

We are able to follow the activity of aryl sulfatase from single cells encapsulated in droplets stored on chip. The activity of the wild-type enzyme expressed from single cells can be easily distinguished from the activity of mutants that are 8- and 36-times slower studied under the same conditions. These observations suggest that the technology is in principle viable for the screening of libraries.
We have also been able to detect production of fluorescein as a result of hydrolysis of fluorescein disulfate by aryl sulfatase. This assay was so sensitive that the catalysis by protein that was *in vitro* expressed in droplets could be detected, either from multiple copies of plasmids or from many copies of linear DNA templates. These results show that we are able to couple *in vitro* expression of an enzyme as well as detection of its activity in microdroplet on chip. Furthermore, detection of the wild-type activity of aryl sulfatase was detected for enzymes *in vitro* expressed from a *single* gene (may it be plasmid or a linear DNA template). This technology will be applied in the future for fast screening of libraries from PCR templates. This approach will allow the evaluation of libraries before sorting, determination of the selection parameters (time and threshold for sorting) to be applied. All these approaches can be based on kinetic characterisation of of an entire library, before, after and during rounds of directed evolution.

Additional leadership training. I had the opportunity to be trained and later to train PhD students and post-doctoral fellows on microfluidics, optical detection, enzyme assay development and biochemical *in vitro* experiments in bulk and in microdroplets. I also had the responsibility for supervision of a PhD student who conduct leaking studies and *in vitro* expression of enzymes coupled to their assays in droplets based on methods I had developed. A manuscript is currently in preparation based on the work produced joinly with a group at the Australian National University (N. Wu, F. Courtois, Y. Zhu *et al.*).
I also had the opportunity to disseminate my results by presenting my work at an international conference (TINE, Trends in Enzymology, July 2008) and also through discussion with scientists met during my collaborative research at Cornell University, NY, USA. During the collaboration with Cornell University, I also had the opportunity to learn techniques and expend my skills in a different but related field, where the work focused on identification of metabolites intermediates produced in mycobacteria.