FINAL PUBLISHABLE SUMMARY REPORT

Project No: 220946

BioAssayDevice - A device for biological high-throughput assays based on *in vitro* compartmentalisation

Research training.

1. Studying gene expression of single cells in microdroplets.

We have developed a microfluidic system that can simultaneously monitor the time-dependence of co-induced protein expression of monomeric red fluorescent protein (mRFP1) and the enzymatic activity of alkaline phosphatase (AP) in compartmentalized *E. coli* cells. To demonstrate the utility of this methodology for applications in directed evolution, wild type AP (WT AP) was analyzed against its less active mutant (R166S AP). The device was designed to be able to store up to 4,000 droplets in storage wells. Also, the droplet shrinking due to water diffusion into the PDMS matrix that jeopardizes quantitative measurements of droplet contents was resolved by integrating a reservoir underneath the wells to continuously supply water to the stored droplets to keep the water content constant over long period.

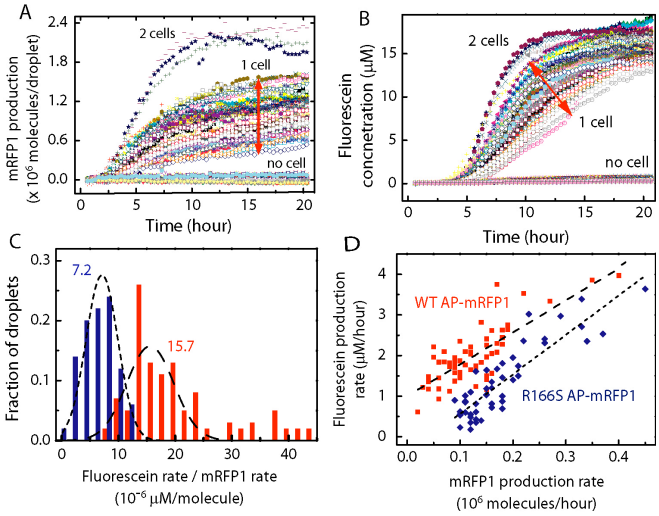

Figure.1 (A) Kinetics of mRFP1 production in cells bearing a plasmid co-expressing WT AP and mRFP1. Each time trace represents expression of mRFP1 in a different droplet. (B) Fluorescein concentration due to enzymatic activities of WT AP co-expressed with mRFP1 over time. Each series of symbols represents product formation in individual droplets starting with no cell (background hydrolysis), one or two cells. (C) Histograms displaying the number of droplets against fluorescein production rate normalized by mRFP1 expression rate. The bars in red and blue are distributions of droplets containing cells harboring plasmids producing wild-type and R166S AP, respectively. The values displayed next to each Gaussian are averages of the rate ratios. (D) The fluorescein production rate was plotted against the mRFP1 expression rate. Wild-type AP (squares in red) produces more product than R166S AP (diamonds in blue).

The significant differences in expression rates of mRFP1 between droplets are primarily due to different numbers of cells in droplets as a consequence of Poissonian encapsulation of cells (Figure 1A). This interpretation is backed up by the observation of distinctive groups that provide three regularly-spaced and well-defined peaks in the histogram of mRFP1 production, which correspond to the initial occupancy of zero, one or two cells per droplet. There is significant intra group variation (0.45 ~ 1.55 millions copies of mRFP1 per droplet) observed in the first group that started with one cell. The time courses for fluorescein formation in droplets are shown in Figure 1B. The rates observed for individual droplets fall into three distinct groups as observed for mRFP1 expression, again reflecting the number of cells initially compartmentalized. For directed evolution experiments it is crucial to be able to distinguish between different enzyme variants. The microfluidic platform was thus used to differentiate the activities of WT AP from its less-active mutant R166S AP having a smaller k_{cat}/K_m as shown in Figure 1C. The normalization correlates mRFP1 and AP expression levels, and removes the variance introduced by different copy numbers of plasmid DNA or different numbers of cells in droplets. Gaussian fits in (Fig. 3a) can be ascribed to fluctuations of the relative expression level of AP and mRFP1 despite being under the control of the same promoter. Mutant and wild-type enzyme can be distinguished, but there is a considerable overlap (~50% of the respective populations) despite a large difference in k_{cat}/K_M. By constructing a plot correlating mRFP1 and fluorescein production rate (Figure 1D), populations of WT AP and R166S become now clearly distinguishable. The linearity of each series implies that mRFP1 expression is positively correlated with fluorescein production and hence AP expression. By using the microfluidic system described in this work, it was possible to maintain thousands of droplets in a constant environment that allows quantitative measurements of each droplet and enabled the concurrent study of the kinetics of protein expression and enzymatic activity in individual cells. The ability to simultaneously monitor these properties provides an analytical tool for the assessment of members of a library in a directed evolution experiment or allows interrogation of the heterogeneity of cells generated from an identically prepared ensemble.

2. Studying single molecules in microdroplets.

We have shown that a PDMS microfluidic device can be used to perform single molecule fluorescence on molecules under fast flow up to 10 cm s^{-1}. This provides up to a two order of magnitude increase in the rate of data acquisition allowing us to detect femtomolar concentrations of target analyte in a few minutes. By encapsulating the molecules in water droplets in oil we can perform single molecule experiments on protein unfolding. This is a simple way to obtain significant improvements in single molecule fluorescence experiments and perform studies under non-equilibrium conditions. Figure 2A and 2B shows a schematic of the experimental set-up used which used a conventional single molecule fluorescence set-up based on an inverted microscope with single or dual laser excitation. We detected single molecules isolated in aqueous microdroplets in immiscible oil. In these

experiments we were using relatively lower flow rates of 1 cm s^{-1} but had the significant advantage that we could perform experiments under non-equilibrium conditions. We performed a protein unfolding experiment on ubiquitin labelled with a donor and acceptor fluorophore. The ubiquitin in 0.5 M guanidinium chloride was encapsulated in water droplets at 4.15 M guanidinium chloride, at which concentration it unfolds. The mixing time in this experiment is less than 100 ms. By monitoring the single molecule FRET signal at different times after mixing, we could follow the unfolding of the ubiquitin with time. It allows each conformation to be fitted with a Gaussian function and we observe that there is a reduction in the amplitude of the high FRET peak and simultaneous increase in the low FRET peak amplitude with time, as the protein unfolds (Figure 2C). These data can be analysed quantitatively and gives an unfolding rate in excellent agreement with ensemble measurements. The advantage of the use of microdroplets is that the protein is encapsulated in a compartment of denaturant at a fixed concentration and then we conformational changes can be monitored over time. This is a simple general configuration to perform single molecule experiments under non-equilibrium conditions and probe conformational changes and detect rare sub-populations. Since it is performed under fast flow the rate of data acquisition is much more rapid than the conventional method and hence the quality of the histograms we obtain significantly improved.

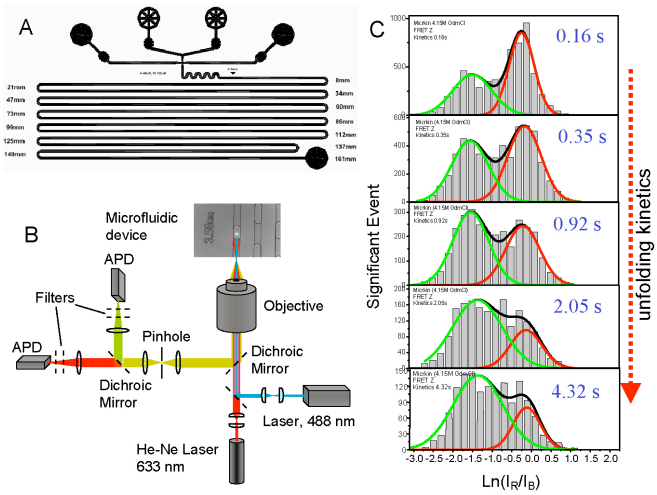

Figure.2 (A) The microfluidic device. The typical width and height of channels are 100μm and 25μm. (B) Optical setup. (C) Histograms showing kinetics of protein unfolding.

3. Controlling microenvironment in microdroplets.

In order to carry out multiple step reactions it is required to control the droplet condition after the formation. As aqueous droplets are emulsified in an immiscible carrier fluid, the droplet condition is not conveniently controlled on the down stream. The device is designed to be able to store droplets in storage wells and to have reservoir, which is separated from the wells by PDMS membrane. This multi-layered device enables transporting permeates of PDMS across the membrane from reservoir to stored droplets. We demonstrate when the solutes, macromolecules and cells encapsulated in droplets are impermeable to the carrier fluid and PDMS, the chemical condition can be controlled by delivering small hormone-like molecules to the droplets. The ability to control the droplet condition enables triggering the gene expression in cells by delivering auto-inducer. Many bacteria communicate with each other via a mechanism known as quorum sensing (QS) using small signaling molecules called auto-inducers (AI). To mimic the *P. Aeruginosa* QS in droplets we used *Escherichia coli* with Pmhlas plasmid. We report that a hormone-like small molecule (N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), Figure 3A) can be delivered through poly(dimethylsiloxane) (PDMS) membrane to microdroplets encapsulating cells. Figure 3B shows fluorescent protein production in encapsulated cells triggered by AI that was delivered from a reservoir through the PDMS barrier. The droplets encapsulating cells were stored in wells and various concentrations of OdDHL were introduced into reservoir. Also, the droplets enclosing both of cells and various concentrations of OdDHL were formed and stored in wells and the production were measured (Figure 3C). Cells rarely produced the protein with 0.01μM of auto inducer, the production jumped to its maximum production when concentration was above 0.03 μM (independent of how AI delivered to cells). Interestingly, the droplet fraction enclosing cells able to produce the protein differently behaved in either case (Figure 3D). When OdDHL was directly mixed with cells, the fraction of droplet started to increase at 0.01 μM and saturated around 1 μM. Meanwhile, if auto inducer was delivered through PDMS membrane, the droplet fraction slowly started to increase at 0.1 μM and saturated at 10 μM. This observation was ascribed to differences in actual amount OdDHL exposed to cells due to the slow diffusion process through the membrane. It was possible to deliver small molecules to control the gene expression in individual cells using a microfluidic device. The ability to change the condition in microdroplets encapsulating cells provides a tool to investigate responses of individual cells under controllable chemical environments.

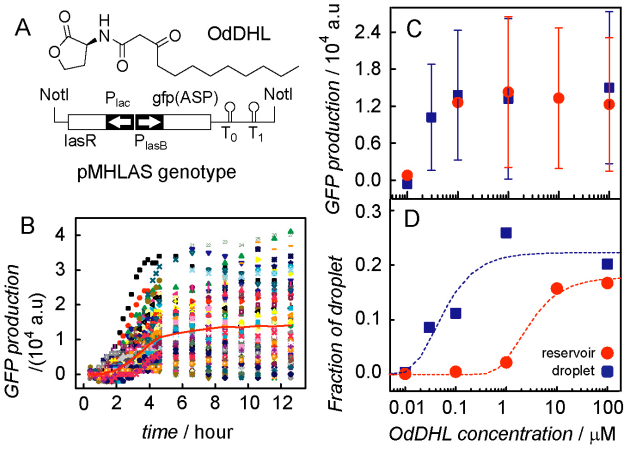

Figure 3 (A) Auto-inducer and plasmid DNA . (B) Protein production to time in droplets encapsulating cells. AI was delivered across PDMS. Each symbol represents droplets. GFP productions (C) and droplet fractions (D) to various AI concentrations. AI was mixed in droplet (red circles) or delivered from reservoir (blue squares).