

DROPLET-BASED MICROFLUIDICS FOR QUANTITATIVE CELL-BASED REPORTER GENE ASSAYS

J.-C. Baret^{1,*}, Y. Beck^{1,2}, I. Billas-Massobrio², D. Moras² and A.D. Griffiths^{1,**}

¹ISIS-UdS-CNRS, STRASBOURG, FRANCE and
²IGBMC-UdS-CNRS-INSERM, ILLKIRCH, FRANCE

ABSTRACT

We developed a two-color flow cytometer for the quantitative analysis of cells in droplets. Our system combines the advantages of standard flow cytometry (single cell analysis and high throughput) with the compartmentalization of the assays. This enables to screen for the activity of chemical compounds at the single cell level and to extract statistical informations on cell populations. We measure the dose-response of an hormone at the single cell level in an automated manner using droplets as microreactors controlled by microfluidic devices.

KEYWORDS: Droplet, Microreactor, Cell-based assay, Dose-response

INTRODUCTION

Despite the fact that it has been known for many years that the biological effects of chemical compounds can display complex concentration-dependent relationships [1], in pharmaceutical primary drug screening, just a single measurement at a single concentration (typically 10 μ M) is obtained for each compound [2]. It is, therefore, extremely important to develop fast, quantitative and reliable techniques for cell-based assays which allow the measurement of dose-response profiles using only small amounts of reagents and small numbers of cells.

In droplet-based microfluidic systems, aqueous microdroplets dispersed in an immiscible carrier oil act as the functional equivalent of microtiter-plate wells that are actuated on-demand (see [4] and ref. therein) with many advantages: (i) compartmentalization in droplets prevents diffusion and Taylor-Aris dispersion of reagents; (ii) highly monodisperse droplets can be created and on-the-fly analyzed at rates of up to $\sim 10^4$ droplets per second with the possibility to perform series of operations on the droplets; and (iii) human cells and even multicellular organisms encapsulated in droplets remain viable for several days, allowing assays for cellular enzymatic activity and viability to be performed [5,6,7].

EXPERIMENTAL

Here, we developed a two-color laser induced fluorescence set-up for droplet-based microfluidics, used as a flow cytometer, to perform a quantitative cell-based reporter gene assay for a nuclear receptor ligand and automatize the quantitative measurement of the Effective Concentration (EC_{50}) of an hormone on the cells.

Biological system - The assay is based on the response of transformed *Bombyx mori* cells in the genome of which a transgene encoding green fluorescent protein under *ecdysone response element* control has been inserted. The cells endogenously express the ecdysone receptor (EcR, NRH1) and Ultraspiracle (USP, NR2B4), two nuclear receptors. In the absence of the hormone 20-hydroxyecdysone (20E), EcR and USP cannot form the active complex able to induce reporter gene expression [8] (Figure 1A). In the presence of 20E, the EcR ligand, a complex constituted by EcR, 20E, and USP, specifically binds the *ecdysone response element* and induces GFP gene expression (Figure 1B).

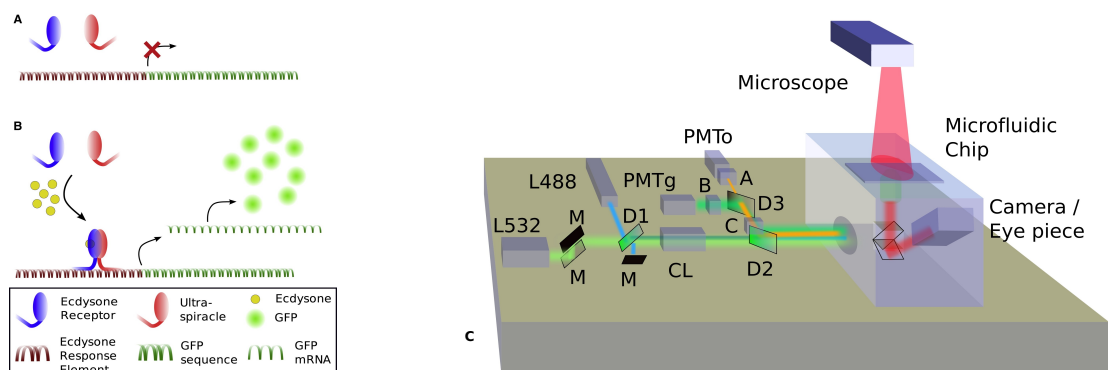


Figure 1: The cell-based reported gene assay performed in the droplets (see text and ref 10 for details) and the two-color laser induced fluorescence setup. Fluorescence is excited with two lasers (488 nm and 532 nm) and emission is recorded on photomultiplier tubes (green: PMTg and orange-red : PMTo)

Droplet-based microfluidic system - Microfluidic systems for cell encapsulation in droplets and droplet reinjection are manufactured by replica molding in PDMS of a mould of SU8 on silicon wafer [9]. The channel dimensions are tuned to produce droplets of ~ 1 nL which provide sufficient nutriments for the cells for an overnight incubation. Solutions are

pumped into the microfluidic chip using harvard apparatus syringe pumps. The concentration of the hormone is varied in the droplets by connecting 3 syringes with 100 μ M, 10 μ M and 0 μ M of hormone. By varying the relative flow rates of the respective streams – keeping the cell flow rate constant – a dilution series of the hormone is generated in the droplets over 3 decades required for an accurate determination of dose-response. We used here 10 different hormone concentrations in the droplets. Droplets are collected in a glass capillary for overnight incubation and reinjected in the production chip for fluorescence readout.

Oil, aqueous phase and surfactant – We used aqueous droplets in perfluorinated oil. The aqueous phase consist in the standard medium for *Bombyx Mori* culture. The oil phase is a HFE-7500 (3M). This perfluorinated oil dissolves very well respiratory gases which is important for an assay requiring an overnight incubation. The surfactant is a PEG-PFPE block-copolymer (Raindance Technologies, Inc) which is biocompatible and emulsion stabiliser at concentrations down to 0,5 % w/w: droplets are therefore stable against coalescence upon incubation and reinjection.

Optical Encoding – In order to know at the reinjection of the droplet the concentration of the hormone in the droplet, the concentration of the hormone is encoded by a red fluorescent molecule, Dextran Texas Red (DTR). This molecule has been chosen for 3 reasons: first, the fluorescence signal of DTR does not interfere with the GFP signal over a wide range of concentrations; second, the GFP fluorescence does not leak into the red fluorescence of the DTR. Third, the dye does not exchange between droplets. It is important to notice that this dye is not bound to the hormone but free in the droplet. This means that the response of the cell is indeed the response to the hormone and is not influenced by a fluorophore attached to the hormone.

Optical setup - The sketch of the optical setup is provided in Figure 1C. The first color (488 nm excitation; 514/30 nm emission) is used to measure GFP signals of the cells while the second color (532 nm excitation; 625/26 nm emission) is used to measure DTR signals. The simultaneous measurement of the GFP signal (response of the cells) and of the red dye (coding the dose of hormone) provides an automatic measurement of dose-response and subsequent determination of EC_{50} .

RESULTS AND DISCUSSION

For each hormone concentration, droplets are generated for about 1 min ($\sim 7500 \pm 2500$ cells in $\sim 17,000$ droplets). In a single run, the effect of the ten concentrations of hormone on the cells is assayed simultaneously and under the same conditions. The emulsion is then incubated at room temperature for 24 hr and reinjected for fluorescence measurement at ~ 60 Hz. The response of the cells is, as expected, a function of the hormone concentration and has a relatively wide distribution (over a decade). There is always a population of non-responding cells (consistent with other studies of reporter gene expression). To circumvent this and account for responding cells only a fluorescent threshold is defined below which the cells are not considered. Formally, the response R of the cells is then defined as the mean value of the fluorescent signals detected beyond this threshold (Figure 2A and 2B).

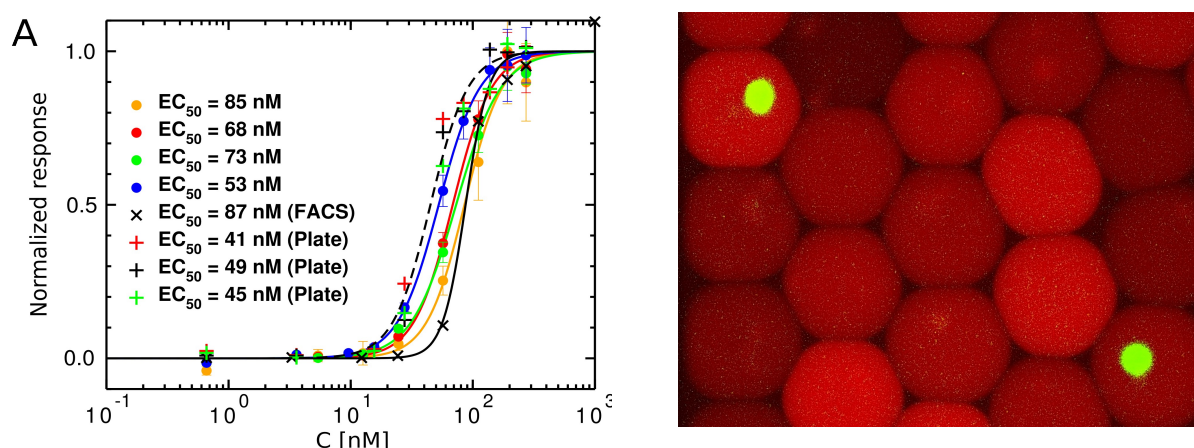


Figure 2: A - Quantitative results extracted from the analysis of the droplet fluorescence for the 10 concentrations of hormone and comparison with standard analytical techniques (reprinted from 10 with permission). B - Optical micrograph (fluorescent composed image) of cells in droplets after incubation (droplet diameter $\sim 100 \mu$ m). The red-encoded droplets contain different levels of DTR to encode the hormone concentration. Positive cells are GFP fluorescent.

Despite the large standard deviation of cell fluorescence (on the order of the R), the large number of data points results in a $\pm 8\%$ confidence limit for the mean ($\alpha = 0.05$) for all hormone concentrations tested. By fitting R versus the hormone concentration, C , with a four-parameter Hill function, EC_{50} values were determined and ranged from 53 to 85 nM in four independent experiments. The intra-experimental precision of the EC_{50} in each case is $\pm 8\%$ ($\alpha = 0.05$) and the

inter-experimental precision for the mean EC_{50} (70 nM) is $\pm 12\%$ ($\alpha = 0.05$). These EC_{50} values are in close agreement with EC_{50} values determined in microplates (40–49 nM), using flow cytometry (87 nM), and with the literature ($EC_{50} = 75$ –100 nM) [8]. The droplet-based experiments led to EC_{50} values intermediate between plates and flow cytometry, and the general shape of the dose-response curve (including the exponent) was similar for all methods and experiments.

CONCLUSIONS

Droplet-based microfluidic systems provide a quantitative and automated method to measure dose-response profiles using droplets as microreactors and to determine EC_{50} values using cell-based reporter gene assays in droplets. The dose-response profile and EC_{50} in microfluidics are consistent with microtiter plate and flow cytometry measurements as well as the value reported in the literature.

Assays such as the one demonstrated here, based on quantifying cellular transcriptional responses, require incubation times of ~24 hr, but the same system could easily be adapted, by the use of on-chip delay lines, to measure rapid cellular responses such as receptor-mediated calcium signals, as well as to *in vitro* assays. Indeed, the platform can be generalized to perform most homogeneous fluorescence-based assays commonly performed in microtiter plates. Finally, the flexibility of droplet manipulation also enables more complex operations to be performed. For example, cells can be preincubated in droplets with test compounds and then assay reagents can be added via drop fusion before readout [6], which would increase the range of assays that can be performed in droplet-based format.

We believe that miniaturization and automation of cell-based assays using droplet-based microfluidics opens the door to the quantitative screening of compounds in droplet-based formats and will enable chemical compound screening using cell types that are difficult or expensive to obtain in large quantities such as primary or stem cells and with small amount of reagents for a drastic reduction of the cost of assays.

ACKNOWLEDGEMENTS

J.-C.B. was supported by a European Molecular Biology Organization long-term fellowship. This work was also supported by the European Commission Framework 6 (EC FP6) MiFem Network, the Ministère de l'Enseignement Supérieur et de la Recherche, Centre National de la Recherche Scientifique (CNRS), and Agence National de la Recherche (ANR) (ANR-05-BLAN-0397).

We are grateful to C. Rick, T. Mangeat, A. El Harrak, C. Merten, V. Taly, M. Ryckelynck, O.J. Miller, P. Kessler, C. Ebel and the IGBMC cell culture common resource for their help and valuable discussions, to K. Iatrou and L. Swevers (National Center for Scientific Research “Demokritos,” Athens, Greece) for their generous gift of the BM5 cell line (Material Transfer Agreement) and to D. Link and RainDance Technologies for the kind gift of the surfactant used in this study.

Figure 1A, 1B and 2A, as well as some excerpts of this text have been reproduced with permission from ref 10.

REFERENCES

- [1] A.J. Hill, The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves, *J. Physiol (London)* **40**, 4-7 (1910)
- [2] N. Malo *et al.*, Statistical practice in high-throughput screening data analysis, *Nat Biotechnol.* **24**, 167-175 (2006).
- [3] M.H. Wu *et al.*, Microfluidic cell culture systems for drug research, *Lab Chip*, **10**(8) 939-956 (2010).
- [4] A.B. Theberge *et al.* Microdroplets in Microfluidics, *Angewandte Chemie, Intl Ed.* In press (2010)
- [5] J. Clausell-Tormos *et al.*, Droplet-based microfluidic platforms for the encapsulation and screening of Mammalian cells and multicellular organisms, *Chem. Biol.* **15**, 427-437 (2008).
- [6] E. Brouzes *et al.*, Droplet microfluidic technology for single-cell high-throughput screening, *Proc. Nat. Acad. Sci. USA* **106**, 14195-14200 (2009).
- [7] H.N. Joensuu *et al.*, Detection and Analysis of Low-Abundance Cell-Surface Biomarkers Using Enzymatic Amplification in Microfluidic Droplets”, *Angew. Chemie Int. Ed.* **48**, 2518-2521 (2009)
- [8] L. Swevers, *et al.*, A cell-based high-throughput screening system for detecting ecdysteroid agonists and antagonists in plant extracts and libraries of synthetic compounds. *FASEB J.* **18**, 134–136.
- [9] Y.N. Xia and G.M. Whitesides, *Soft-lithography Annu. Rev. Mater. Sci.* **28**, 153–184
- [10] J.-C. Baret, Y. Beck *et al.*, Quantitative cell-based reporter gene assays using droplet-based microfluidics, *Chemistry and Biology*, **17**(5), 528-536 (2010).

CONTACT

- * J.-C. Baret, Max-Planck Institute for Dynamics and Self-Organisation – Droplets, Membranes and Interfaces
Bunsenstrasse 10, 37073 Göttingen [D], jean-christophe.baret@ds.mpg.de
- ** A.D. Griffiths, griffiths@unistra.fr