A HOMOGENEOUS ASSAY FOR BIOMOLECULE INTERACTION ANALYSIS IN DROPLETS BY FLOURESCENCE POLARIZATION

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ABSTRACT

We present a novel homogeneous assay for detecting biomolecule interactions in microdroplets by fluorescence polarization (FP) for the first time. The FP assay allows the detection of target biomolecules directly after incubation without removing the detection reagent by separation or washing, making the assay amenable to automation. Using this assay we evaluate protein-protein and drug-DNA interactions. We detect these interactions at concentrations as low as 100nM and 69 pM respectively. This is a proof-of-concept homogeneous labeling assay in droplets for detecting biomacromolecules.

KEYWORDS: Microdroplets, Homogeneous assay, Fluorescence polarization, Biomolecule

INTRODUCTION

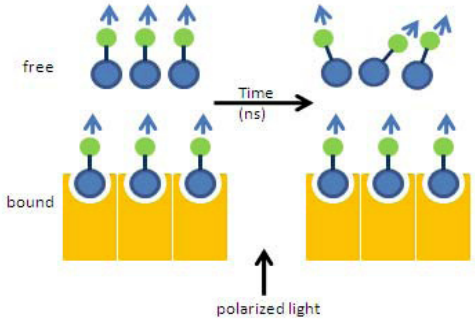
Droplets generated from microfluidic devices are efficient micro-reactors for various biological assays [1]. Thousands of monodispersed droplets compartmentalizing reagents can be generated per second and thus reactions can be run in parallel in a high-throughput manner [1]. A number of droplet applications involving biomolecule interactions such as high-throughput toxicity screening of drug candidates [2] and detection of cell surface marker on single cells, have been demonstrated [3].

A homogeous assay is a method which allows all the components to be present during measurement. No pre-labeling with detection reagents or subsequent washing in order to remove them is required. Homogenous assays are well-suited for droplet-based platforms since reactions are compartmentalized and droplets are not to be broken during measurement. The FP assay is a type of homogenous assay that does not require any separation of bound and free detection reagents [4]. The ratio of FP tracers bound to large molecules (*e.g.* proteins) to free FP tracers can be obtained from FP assays. They are readily adaptable to low volumes, facilitating the automation of droplet microfluidics [5].

THEORY

FP measurements are based on the assessment of molecular rotation [6]. The FP tracer is usually a rapidly rotating small molecule labeled with a fluorescent dye (Figure 1). Upon excitation by polarized light, the orientational distributions of free FP tracers are randomized during the excited-state life time (several ns) due to the rapid rotation, resulting in depolarized light emission and thus low FP. However, when the FP tracers bind to significantly larger molecules, its rotation is slowed down, emitted light is polarized and thus high FP can be detected. The degree of polarization can be determined by measuring the fluorescence intensity from the directions both parallel and perpendicular to the plane of the polarized excitation light. The polarization (P) and anisotropy (r) is calculated according to Equation 1 and 2.

FP indicates the binding of a tracer to a significantly larger ligand. It has been extensively employed in clinical diagnostics [7].



$$P = \frac{F_{II} - F_{\perp}}{F_{II} + F_{\perp}} \quad (1)$$

$$r = \frac{F_{II} - F_{\perp}}{F_{II} + 2F_{\perp}} \quad (2)$$

F_{II}= fluorescence intensity parallel to the excitation plane
$F_{\perp}$= fluorescence intensity perpendicular to the excitation plane

Figure 1: Illustration of the principles for FP

EXPERIMENTAL

Microfluidic devices were manufactured in Polydimethylsiloxane using standard soft lithography techniques. Two devices, described in Figure 2, were used. In the first one, monodisperse 14pL aqueous droplets were generated in a continuous fluorocarbon oil, containing a surfactant to stabilize the droplets, at a rate of ~1400 droplets/second by pressure driven flow. The device has two aqueous inlets that allow mixing of sample and detection reagent before reaching a droplet generating nozzle. The emulsion was collected in a syringe for incubation (>2 hrs) and re-injected into the second device where droplets were dispersed in an oil stream and analyzed by laser induced FP using a photomultiplier tube on either side of a polarizing beamsplitter cube.

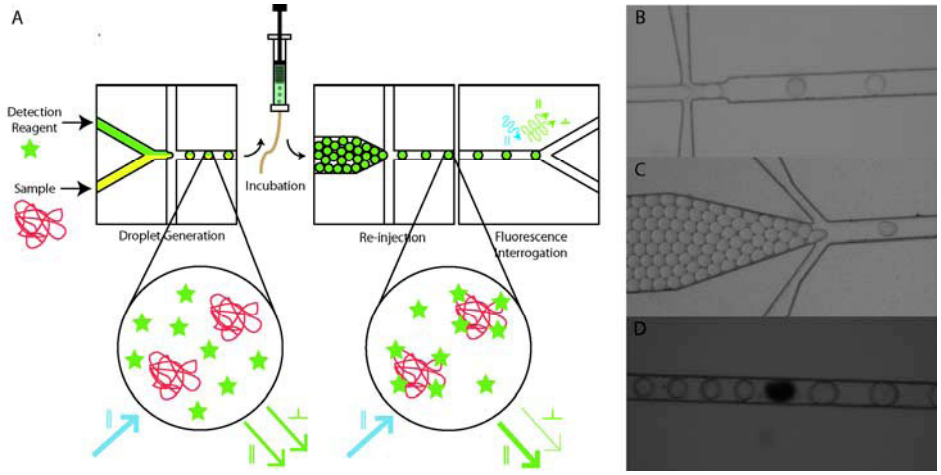


Figure 2: The concept of FP detection in droplets shown schematically. The degree of polarization of fluorescencent emission from a FP tracer excited by polarized light depends on the amount reorientation due to Brownian motion during its excited state lifetime. (A)The schematic depiction illustrates the experimental set-up. (B)Encapsulation of detection reagents and samples on the microfluidic device. (C)After incubation, droplets were re-injected for FP analysis. (D)Controlled pairwise droplet coalescence. Droplets from two populations are fused.

RESULTS AND DISCUSSION

To validate the experimental set-up, Streptavidin and Fluorescein-Biotin were mixed at varying concentrations, encapsulated and analyzed to determine the characteristics of FP in droplets (Figure 3), detecting Streptavidin with a limit of detection in the nM-range.

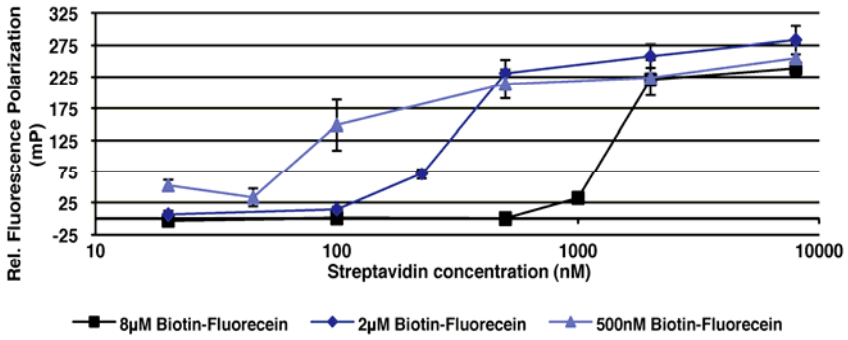


Figure 3: The interaction between Streptavidin and Biotin-fluorecein detected by FP in 14pL droplets. At a fixed concentration of Biotin-fluorescein, the amount of bound Streptavidin can be determined by measuring the FP.

Peptides are small in size and can be synthesized. They are widely used for the development of new vaccines and drugs [8]. It is possible to evaluate their binding to target proteins using the FP measurement. To demonstrate the application of our FP-based assay in droplets investigating peptide-protein interactions, we evaluate the binding of a synthetic peptide (Fc-III) to IgG (Figure 4). Fc-III consists of 13 amino acids (DCAWHLGELVWCT) as a set of contact residues for the binding site of IgG [9] and was coupled to fluorescein as an FP tracer. IgG and Fc-III were mixed at varying concentrations followed by a two-hour incubation. They were then encapsulated in droplets for FP detection (Figure 4A). Dose-response curves were obtained, suggesting that as a tracer, Fc-III concentration can be as low as ~100 nM. Correspondingly, the lowest detectable concentration of IgG is within the range between 150 nM and 1.5 μM. To investigate the limit of IgG detection as well as to demonstrate the possibility of combining FP assays and droplet fusion for potential automation, IgG and Fc-III were mixed in the microfluidic device and encapsulated in droplets. Following a two-hour incubation, the droplets were individually evaluated for FP. The results show that the limit of IgG detection is ~450 nM using our experimental set-up (Figure 4B) and that it is possible to automate the analysis.

We also evaluated the binding of Doxorubicin, a fluorescent anti-cancer drug, to DNA. Doxorubicin and DNA were mixed in the microfluidic device and encapsulated in droplets. Following overnight incubation, droplets were individually evaluated for FP. Droplets containing Doxorubicin and DNA showed a significantly higher FP compared to droplets containing only Doxorubicin (Figure 5).

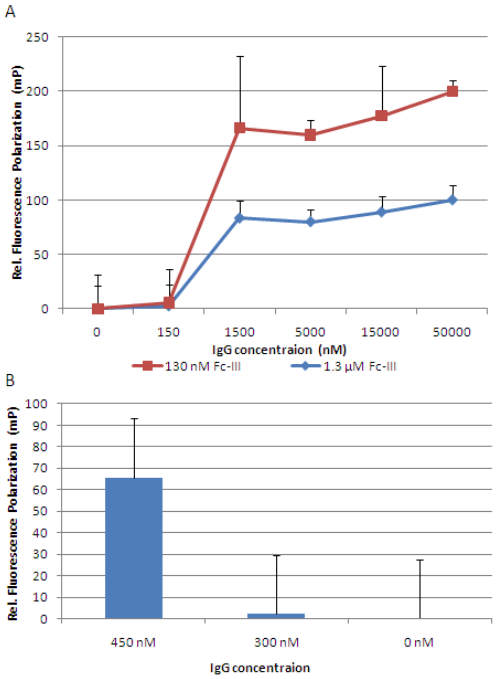


Figure 4: Detection of Fc-III binding to IgG. (A) Dose-response curve obtained for IgG and Fc-III mixed at varying concentrations. Fc-III concentration is as low as 130 nM. For detectable binding, the lowest concentration of IgG is within the range between 150 nM and 1.5 µM. (B) IgG and Fc-III were mixed and encapsulated in droplets for FP detection. At an IgG concentration of 450 nM, a significant FP signal can be detected.

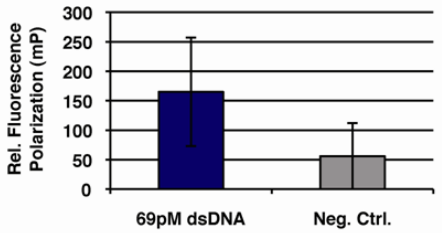


Figure 5: Detection of interaction between the cancer drug Doxorubicin and 48 kbp dsDNA. Concentrations of Doxorubicin and dsDNA were 3.45µM and 69 pM, respectively. Compared to the negative control where free Doxorubicin was presented in the droplets, the mixture of dsDNA and Doxorubicin in the droplets gave rise to significantly higher FP signals, indicating binding.

CONCLUSION

We demonstrate for the first time an FP-based homogenous assay in microdroplets. Using this assay we evaluate different biomolecular interactions which can be applied in different biological assays. The limit-of-detection is in the nM range. Combining the FP assay and droplet fusion in the microfluidic device, it will be possible to analyze biomolecular interactions in an automated, high-throughput and multiplex fashion.

ACKNOWLEDGEMENTS

This work was funded by the Swedish Research Council, the Knut and Alice Wallenberg Foundation and VINNOVA through the ProNova VINN Excellence Center. The surfactant used was graciously provided by RainDance Technologies, Boston, MA, USA.

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