High-throughput confinement and detection of single DNA molecules in aqueous microdroplets†

Monpichar Srisa-Art,[a] Andrew J. deMello*[a] and Joshua B. Edel*[ab]

Received (in Cambridge, UK) 27th August 2009, Accepted 21st September 2009
First published as an Advance Article on the web 8th October 2009
DOI: 10.1039/b917721c

A droplet-based microfluidic system combined with high-sensitivity optical detection is used as a tool for high-throughput confinement and detection of single DNA molecules.

Although droplet-based microfluidic systems that utilise flow instabilities between immiscible fluids have only been developed in recent years, this nascent technology has attracted huge interest and been exploited in a diverse range of applications.[1,2] As aqueous droplets within segmented microflows define confined volumes in the femto- or picolitre range, they have the potential to serve as isolated reaction compartments for both screening and synthetic purposes. For example, the compartmentalisation features associated with droplets are useful in isolating single cells and performing cell-based assays[3,4] and enzymatic reactions.[5] Importantly, this concept can in theory be extended to the encapsulation of other entities, such as nucleic acids or small molecules at the single molecule level.

Recently, there has been increased interest in studying chemical and biological events at the single molecule level to avoid the often misleading information obtained from averaging large populations.[6,7] Performing single molecule detection (SMD) using fluorescence spectroscopy is a challenging task involving the efficient reduction of background noise whilst maximising the (desired) fluorescence signal. Background noise typically originates from bulk solvents, impurities and scattered light, and varies with the detection volume. Accordingly, minimising sample volume is a direct way of reducing high background signals.[8] Indeed, microdroplets generated using piezoelectric pipettes have been used to confine single molecules within a picolitre volume for SMD experiments.[9–11] With such a minute volume, SMD using microdroplets has been successfully achieved with high signal-to-noise ratios. In addition, aqueous droplets were also used to perform sizing of single subcellular organelles and particles.[12] These droplets were prepared by vigorously mixing bead/synthetic vesicle aqueous solution with an oil solution and then transferred using a pipette onto a glass substrate for single molecule measurements.

[a] *Department of Chemistry, Imperial College London, South Kensington, London, UK SW7 2AZ. E-mail: a.demello@imperial.ac.uk; Fax: +44 (0)207 594 5801; Tel: +44 (0)207 594 5820*
[b] *Institute of Biomedical Engineering, Imperial College London, South Kensington, London, UK SW7 2AZ. E-mail: joshua.edel@imperial.ac.uk; Tel: +44 (0)207 594 0754*

† Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/b917721c

In a similar fashion, droplets generated from immiscible phases using microfluidic platforms also provide confined containers, which are advantageous to SMD. Whilst offering restricted reaction compartments, droplet-based microfluidic systems also hold great promise for the analysis of single molecules in a high-throughput manner (due to generation rates in excess of 1 kHz). To date, high-throughput SMD using droplet-based microfluidic systems has not been explored. In the current work, droplet-based microfluidics combined with a sensitive optical detection system have been used to demonstrate single molecule analysis. The ability to detect and characterise individual molecules within a discrete droplet enables the isolation and analysis of molecular events. Moreover, by means of droplet sorting,[13,14] an individual droplet containing a molecule of interest can be isolated for subsequent analysis at the single molecule level.

For the single molecule analyses presented herein, detection and isolation of individual molecules using droplets is the ultimate goal. Accordingly, the entire droplet must be monitored to ensure the detection of every molecule. Uniform illumination of the entire cross-section of a PDMS microfluidic channel is thus required to allow monitoring of all possible molecular events. In the current experiments, a lens is used to expand the optical detection volume to 4.4 pl (Fig. 1a), which is only 45 times smaller than droplet volume of 200 pl. This expansion allows probing of the entire microfluidic channel cross-section. Additionally, the 50 μm wide PDMS channel is restricted to 10 μm at the detection area and the entire channel network depth is reduced to 30 μm to ensure uniform illumination by the laser beam. Accordingly, when droplets travelled through the detection area, they squeezed through the constricted channel (Fig. 1b) and the entire droplet contents were probed. The ability to monitor the entire droplet allows for high-throughput isolation and detection at the single molecule level for use in applications such as molecular sorting and screening.

In all experiments double-stranded λ-DNA ~48 kbp in length was used as the target sample. The DNA was labelled with YOYO-1 at a ratio of five base pairs per one dye molecule.[16] The uniform illumination of the entire microfluidic channel cross-section ensures the detection of every encapsulated DNA molecule within a given droplet. DNA solutions between 10 fM and 10 pM were prepared in 1× Tris-acetate EDTA buffer at pH 8.0 (containing 40 mM Tris-acetate and 1 mM EDTA). Alexa Fluor 647 (AF647) at a concentration of 50 nM was added to the DNA solutions. This red dye was used to mark droplet boundaries.

All data were analysed using programmes written in MATLAB (Mathworks, UK). At the single molecule level,

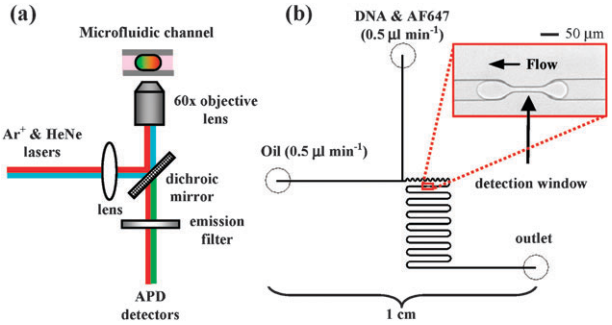

Fig. 1 (a) Schematic of the optical detection system for single molecule analysis. (b) Schematic of a microfluidic device fabricated using procedures described in previous work.[15] The main channel is 50 µm wide and restricted to 10 µm at the detection window as shown in the inset. As droplets 'squeeze' through the detection window, the contents are excited by the two lasers. All microfluidic channels are 30 µm deep. The inset shows a droplet squeezing through the detection window.

droplet boundaries cannot be observed and only spiked peaks representing DNA–YOYO-1 molecules transiting the detection probe volume are obtained in the green channel. Accordingly, the droplet signature in the red channel is used as a reference to visualise droplet location in the green channel. Fluorescence burst scans in the green channel are analysed to define DNA molecular events. Using our optical filter set, crosstalk between channels is negligible. Every peak above a background threshold is identified as a single DNA molecule event, as shown in Fig. 2a. To correlate signals between the green and red channels, droplets in the red channel (measuring emission from AF647) are initially identified and droplet location marked using a black-dashed line (Fig. 2b). The droplet trajectory from the red channel is then correlated with the fluorescence burst scan in the green channel to assign peaks to a particular droplet (Fig. 2c).

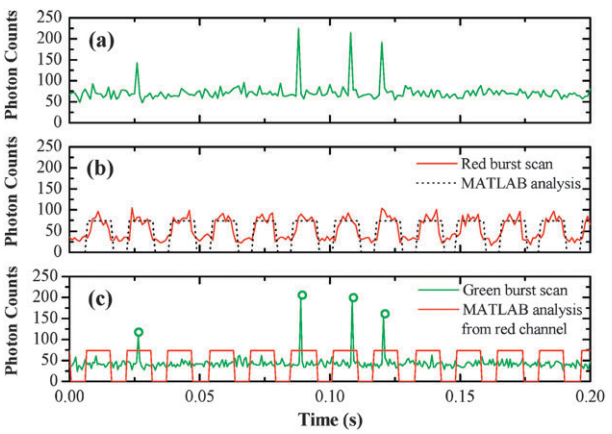

Fig. 2 Data analysis of single molecule experiments using droplets. (a) Data recorded from the green channel; spiked peaks correspond to single DNA events, but droplet boundaries cannot be observed. (b) Data recorded from the red channel; droplet signature can be visualised due to presence of AF647. The black-dashed line represents droplet localisation determined by MATLAB analysis. (c) Correlated fluorescence burst scans from the green and red channels. Droplet boundaries are symbolised as a red-rectangular sign.

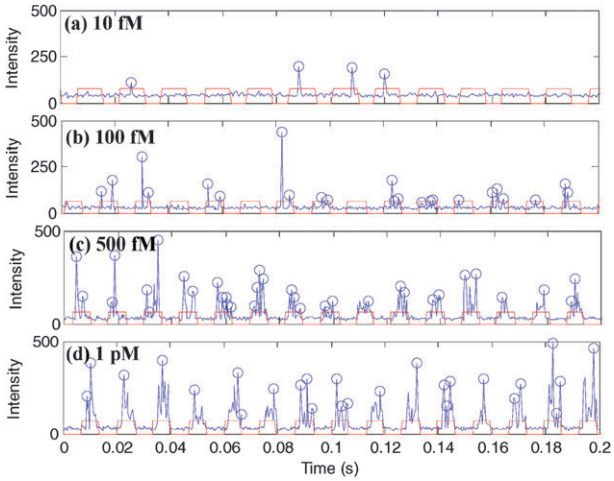

Fig. 3 Examples of fluorescence burst scans from samples of different DNA concentration: (a) 10 fM, (b) 100 fM, (c) 500 fM and (d) 1 pM. Each spiked peak above the threshold corresponds to a single DNA molecule event. The rectangular-shaped signal, which directly correlates with the droplet signature from the red channel, is used to localise droplet boundaries.

Fluorescence burst scans of a dilution series of DNA–YOYO-1 are shown in Fig. 3. As can be seen, the number of DNA molecules per unit time (and thus per droplet) increases as a function of DNA concentration. At the lowest DNA concentration studied (10 fM), single DNA molecule occupancy was achieved with most droplets being empty. Importantly, analytical throughput is not significantly impacted by the occurrence of empty droplets due to the fact that droplets can be produced at frequencies in excess of 1 kHz and only limited by the acquisition rate. At high DNA concentrations (1 pM and above), droplet boundaries were also observed in the green channel due to the large number of DNA strands per droplet.

It should be noted that the high-throughput SMD experiments in the current work were performed using a flow-based system. The droplets containing DNA molecules were monitored whilst they were moving through the detection area.

The open circles in Fig. 3 are used to highlight the exact location of a single DNA translocation event. It can be noticed that, at high concentrations (500 fM and above), some molecular events are not detected. This is because the MATLAB code only 'detects' peaks having their baselines level with the background. Since, in this communication we are only interested in establishing the feasibility of single molecule occupancy and detection, the analysis routine was not optimised further to handle high concentrations.

Statistical analysis of the data presented in Fig. 3 can be used to generate occupancy distributions. Examples of these are shown in Fig. 4. It should be noted that distributions are generated using approximately 2500 droplets. It was found that all distributions exhibit Poisson characteristics. Accordingly, DNA occupancy can be controlled by varying the concentration of DNA or changing the volumetric flow rate. For example, at 10 fM, the instantaneous occupation probabilities are 81.0% for an empty droplet, 17.0% for single occupancy and 2.0% for double occupancy. The percentage of single occupancy

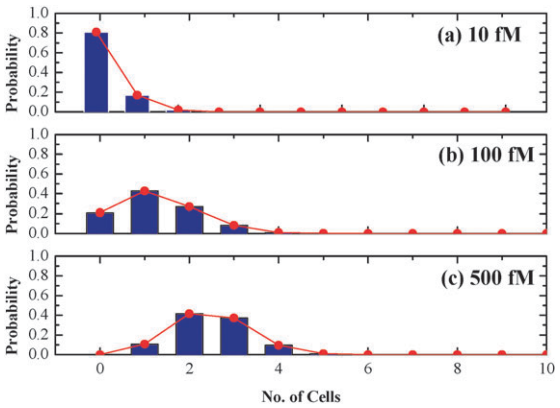

Fig. 4 DNA molecule probability distributions obtained from the experiments at DNA concentrations of (a) 10 fM, (b) 100 fM and (c) 500 fM. Data were obtained from approximately 2500 droplets.

increases to 43.0% with only 21.0% for empty droplets when increasing DNA concentration to 100 fM. However, multiple occupancy is also obtained when using high DNA concentrations (100 fM and above). For the current proof-of-principle experiments, single occupancy is required, thus using sample concentrations between 10 fM and 100 fM is preferable to obtain single molecule occupancy and to minimise the number of empty droplets. In addition, optimising flow rates to control droplet size is an alternative route to achieving single molecule occupancy.

In conclusion, the results obtained from these studies have raised the hope of exploiting droplet-based microfluidics for high-throughput analysis at the single molecule level. Encapsulation and detection of single DNA molecules has been successfully achieved. However, to subsequently facilitate DNA fragment sizing and purification in a high-throughput manner, some experimental aspects must be refined. These include uniform illumination of the entire cross-section of a microfluidic channel and reduction of background noise. In current studies, we are implementing a cylindrical lens arrangement to replace the plano-convex lens. This lens will create a uniform sheet-like observation probe volume to enable the detection of every molecular trajectory.[17] Such uniform illumination will improve the uniformity of height and area distributions of the fluorescence bursts of the DNA molecules, which is crucial for DNA fragment sizing applications. In addition, high background signals are a significant issue due to the absence of a pinhole to block out-of-focus light. With the implementation of a cylindrical lens, a confocal slit could be installed to reduce background noise and improve sensitivity. This rectangular-shape slit is compatible with the sheet-like probe volume created by a cylindrical lens. Considering these aspects, droplet-based microfluidic approaches open new possibilities for SMD analysis. Although the work presented herein is proof-of-principle, it represents a significant step towards high-throughput applications of droplet-based microfluidic technology at the single molecule level.

This work was supported by the RCUK Basic Technology Programme. M.S.-A. acknowledges the Royal Thai Government for provision of a research scholarship.

Notes and references

1 A. Huebner, S. Sharma, M. Srisa-Art, F. Hollfelder, J. B. Edel and A. J. deMello, *Lab Chip*, 2008, **8**, 1244–1254.
2 S. Y. Teh, R. Lin, L. H. Hung and A. P. Lee, *Lab Chip*, 2008, **8**, 198–220.
3 A. Huebner, L. F. Olguin, D. Bratton, G. Whyte, W. T. S Huck, A. J. deMello, J. B. Edel, C. Abell and F. Hollfelder, *Anal. Chem.*, 2008, **80**, 3890–3896.
4 A. Huebner, D. Bratton, G. Whyte, M. Yang, A. J. deMello, C. Abell and F. Hollfelder, *Lab Chip*, 2009, **9**, 692–698.
5 A. Aharoni, G. Amitai, K. Bernath, S. Magdassi and D. S. Tawfik, *Chem. Biol.*, 2005, **12**, 1281–1289.
6 M. E. Lidstrom and D. R. Meldrum, *Nat. Rev. Microbiol.*, 2003, **1**, 158–164.
7 P. S. Dittrich and A. Manz, *Anal. Bioanal. Chem.*, 2005, **382**, 1771–1782.
8 A. J. de Mello, *Lab Chip*, 2003, **3**, 29N–34N.
9 W. B. Whitten, J. M. Ramsey, S. Arnold and B. V. Bronk, *Anal. Chem.*, 1991, **63**, 1027–1031.
10 M. D. Barnes, K. C. Ng, W. B. Whitten and J. M. Ramsey, *Anal. Chem.*, 1993, **65**, 2360–2365.
11 J. Tang, A. M. Jofre, G. M. Lowman, R. B. Kishore, J. E. Reiner, K. Helmerson, L. S. Goldner and M. E. Greene, *Langmuir*, 2008, **24**, 4975–4978.
12 J. C. Gadd, C. L. Kuyper, B. S. Fujimoto, R. W. Allen and D. T. Chiu, *Anal. Chem.*, 2008, **80**, 3450–3457.
13 D. R. Link, E. Grasland-Mongrain, A. Duri, F. Sarrazin, Z. D. Cheng, G. Cristobal, M. Marquez and D. A. Weitz, *Angew. Chem., Int. Ed.*, 2006, **45**, 2556–2560.
14 Y. C. Tan, Y. L. Ho and A. P. Lee, *Microfluid. Nanofluid.*, 2008, **4**, 343–348.
15 M. Srisa-Art, A. J. deMello and J. B. Edel, *Anal. Chem.*, 2007, **79**, 6682–6689.
16 G. A. T. Chansin, R. Mulero, J. Hong, M. J. Kim, A. J. Demello and J. B. Edel, *Nano Lett.*, 2007, **7**, 2901–2906.
17 K. J. Liu and T. H. Wang, *Biophys. J.*, 2008, **95**, 2964–2975.

