# Microfluidic separation and capture of analytes for single-molecule spectroscopy†

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*Received 28th August 2007, Accepted 16th October 2007*
*First published as an Advance Article on the web 23rd October 2007*
**DOI: 10.1039/b713103h**

**A complex mixture of fluorescently labeled biological molecules is separated electrophoretically on a chip and the constituent molecules are confined in a sub-nanolitre microchamber, which allows analysis by various single-molecule techniques.**

Various single-molecule techniques have been developed in the last two decades to characterize different properties of diffusing fluorescent molecules in solution or even in living cells.[1] The distribution of these properties, including molecular brightness, diffusion coefficient, fluorescence resonance energy transfer (FRET) efficiency, and fluorescence lifetime, provides valuable insights into the mechanism of biological processes. As complexity of the sample under study increases, however, the resolving power of single-molecule analyses may not be sufficiently high to extract meaningful information. Additionally, the fluorescence labeling process itself, which is required to make "visible" most proteins and nucleic acids, often introduces heterogeneity into the sample under study because the presence of multiple labeling sites results in a mixture of species tagged in different permutations. It is critical to achieve highly specific labeling for quantitative data analysis. Therefore, a purification/separation procedure is usually involved prior to single-molecule measurements; otherwise, careful experimental design is needed. Usually, this problem is addressed by using chromatographic purification or sophisticated techniques in biochemistry and molecular biology.[2]

Here we present a general technique that uses a microfluidic platform to integrate a separation method with single-molecule spectroscopy. As a demonstration, we choose capillary electrophoresis (CE) as the separation method because of its high resolving power, short separation time, and small sample volume. For single-molecule analysis, we use the photon counting histogram (PCH) method,[3] which allows the determination of two parameters: the molecular brightness and the average molecule number in the observation volume. PCH has been successfully applied to study interactions between nucleic acids and polycationic polymers, pH-induced conformational change, and the size distribution of actin oligomers.[4] We show that by combining these two techniques, proteins labeled with different numbers of dye molecules can be separated and individually analyzed.

Previous use of microchannels for single-molecule fluorescence experiments such as photon burst detection, single-molecule FRET measurement, and fluorescence-lifetime imaging has been limited to continuous flow.[5] In the case of a microfluidic separation, the analyte passes across the detection point transiently, preventing the accumulation of sufficient data for statistical analysis. We overcome this problem by adding a pair of microvalves on the flow channel. When the sample of interest reaches the valves, they are closed so that the sample is captured between the two valves. Subsequent single-molecule experiment can be performed in the closed chamber for an extended period of time.

Fig. 1 presents a schematic representation of the experimental setup. The poly(dimethylsiloxane) (PDMS) chip with a microvalve was fabricated using a multilayer soft lithography technique.[6] The standard configuration of a double-T junction allows electrokinetic injection of the sample (more details are presented in the ESI†). When pressure (15–20 psi) is applied to the valve layer (valve closed), the thin layer of PDMS between the valve and the channel layers collapses to form a sub-nanolitre chamber (100–300 pL) in the channel layer due to the elastomeric property of the material. When the valve is open, the channel shape is restored in less than a second, which is measured by analyzing the video microscopy images, frame by frame. The restoration time depends on the aspect ratio of the structure and the thickness of the intermediate layer. In the CE mode, the valves are kept open, and the fluorescence signals from the migrating species are collected *via* laser-induced fluorescence (LIF) detection at the center of the

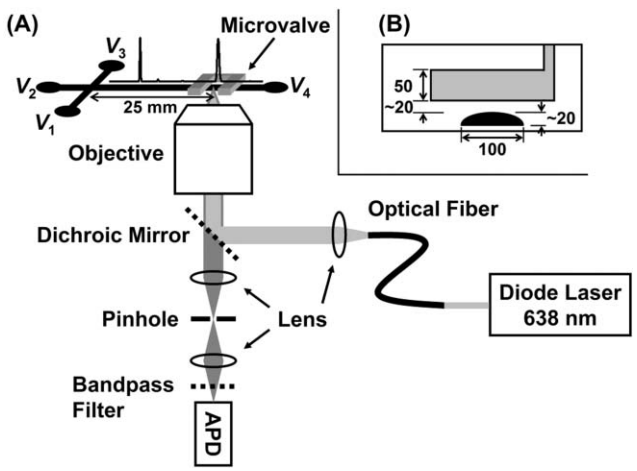


**Fig. 1** Experimental setup and the microchip. (A) Optical setup for LIF detection and single-molecule spectroscopy. APD = avalanche photodiode, $V_n$ = the voltage applied to each electrode. (B) Cross-sectional view of the double-T microchip at the capturing region. The valve layer is shown in gray and the channel layer in black. The drawing is not to scale, but the dimensions are in μm.

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† Electronic supplementary information (ESI) available: Experimental details, Table S1, Fig. S1–S3. See DOI: 10.1039/b713103h

capture region. In the CE-PCH mode, when the peak of interest migrates into the capture region, the high voltage across the separation channel is turned off, and the valves are closed. Then, the fluctuating fluorescence signal from the captured fraction is recorded and analyzed by the PCH method.

We tested the CE-PCH method by measuring the molecular brightness of a mixture of simple dyes with similar fluorescence characteristics: Cy5 and Alexa Fluor 647 (A647). Conventional PCH analysis was performed for each fluorophore in a glass-bottom chamber, which revealed the following molecular brightnesses: $\varepsilon_{Cy5}$ = 69 000 ± 3 000 (counts per second per molecule, cpsm), and $\varepsilon_{A647}$ = 82 000 ± 3 000 (cpsm). Because these two brightnesses are very close, it is extremely difficult for PCH analysis to resolve these two dyes in their mixture.[7] On the other hand, Cy5 and A647 can be separated by CE according to their different electric charges (−1 for Cy5 and −2 for A647). Fig. 2(A) shows a representative electropherogram where two dyes are baseline separated within 1 min. The calculated spatial widths at half maxima of Cy5 and A647 peaks at the detection point were 360 μm and 400 μm, respectively, assuming constant migration velocities. The distance between the capture valves was varied between 100 μm and 300 μm, ensuring complete capture of separated fractions. When one of the migrating peaks was captured by the pair of microvalves, the fluorescence signal became stabilized in about 10 s (Fig. 2(B)). This phenomenon can be explained by the diffusive mixing of the dye molecules within the confined volume before it reaches a homogeneous concentration. The fluctuation of fluorescence signal caused by the diffusion of A647 molecules at the laser focus was then recorded. PCH analysis of this fluctuation yielded brightness values of $\varepsilon_{Cy5}$ = 67 000 ± 3 000 (cpsm) and $\varepsilon_{A647}$ = 79 000 ± 3 000 (cpsm), which were statistically indistinguishable from conventional PCH analysis of individual dyes. Moreover, consistent with the 1 : 1 molar mixing ratio, similar values of average number of molecules ($N$) are observed for the two dyes (see Table S1 of the ESI†). Therefore, we concluded that the geometry of our microcapillary does not affect the PCH parameters significantly. It is noteworthy to compare our result with a recent application of PCH measurement in a microcapillary of cylindrical geometry, where the $z$-position of the laser focus was critical to the collection efficiency and the molecular brightness decreased by a factor of two in the capillary from the beam distortion.[8] In contrast, our microchannel has a flat bottom surface that allows an observation volume profile with almost no distortion.

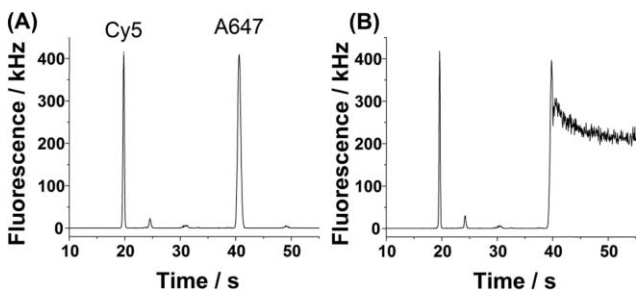


**Fig. 2** CE-PCH analysis of a dye mixture. (A) Electropherogram of the mixture of Cy5 and A647. (B) Fluorescence signal after capturing the A647 peak. Once captured, the intensity drops because of diffusive mixing. Fluctuations caused by molecular diffusion can be observed.

We then applied the CE-PCH method to analyze streptavidin that was labeled with A647 fluorophores. A truncated version of the streptavidin, which is used for most commercial products, has 125–127 amino acids per subunit including 3–5 lysine residues.[9] The streptavidin molecule exists as a tetramer, allowing 12–20 labeling sites for amine-reactive succinimidyl ester if the hindered locations of some residues are disregarded as shown in Fig. S2.† To monitor the conjugation reaction, samples with different dye : protein mixing ratios were prepared and analyzed by the CE mode. Fig. 3 shows the electropherograms of these samples. The protein species conjugated with A647 fluorophores (15–30 s) migrated faster than the free or hydrolyzed dye molecules (30–45 s), resulting in baseline separation and making unnecessary the use of size-exclusion chromatography for purification. Although streptavidin is negatively charged at pH 7.5, which is above its isoelectric point (pH 5–6), it has higher mobility than A647 because the larger hydrodynamic radius of the protein reduces significantly the electrophoretic mobility, which is in the opposite direction to the prevailing electroosmotic flow.

As the degree of labeling increased, more slowly migrating species emerged with increased absolute peak heights; this pattern, called a protein charge ladder,[10] strongly indicates that the resolved peaks result from different numbers of dye molecules that are introduced into the protein. Cy5-labeled streptavidin showed similar patterns but with less well-resolved electropherograms (Fig. S3†), which confirms the role of incorporated negative charges in the resolving power of the CE technique. This data also

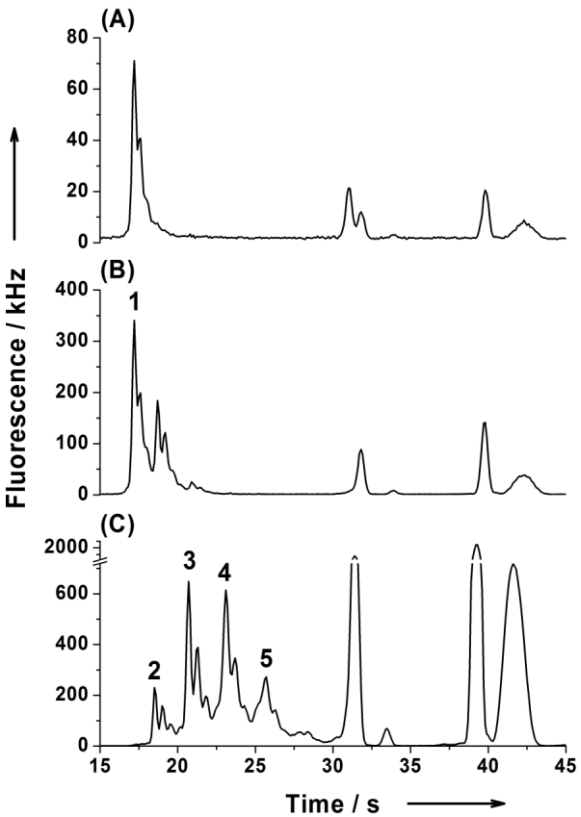


**Fig. 3** Electropherograms of A647-streptavidin reaction mixtures with protein to dye ratios of (A) 10 : 1, (B) 1 : 1, and (C) 1 : 10. The selected peaks for the CE-PCH analysis are numbered.

**Table 1** PCH parameters obtained for A647-labeled streptavidin

| Peak | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| $t_m$/s | 17.2 | 18.5 | 20.7 | 23.1 | 25.7 |
| $\varepsilon$/cpsm | 12 900 ($\pm$400) | 28 000 ($\pm$500) | 34 000 ($\pm$300) | 41 000 ($\pm$1,000) | 46 000[a] ($\pm$500) |
| $N$ | 7.7 ($\pm$0.2) | 1.90 ($\pm$0.01) | 4.59 ($\pm$0.1) | 4.72 ($\pm$0.3) | 3.86[a] ($\pm$0.03) |

[a] A reasonable fitting of peak 5 was obtained only with a 2-species PCH model. Here, the molecular brightness and the number of molecules for the dimmer species are used.

suggest that the system can be optimized by varying the charge state of protein (*i.e.*, pH) and choosing the right fluorophore.

To study each of the separated fractions further, we selected major peaks (1–5 in Fig. 3) and captured them with a microvalve as described earlier. The migration times ($t_m$) and the PCH parameters ($\varepsilon$ and $N$) obtained from these peaks are summarized in Table 1. The PCHs obtained from fluctuating fluorescence signals for all peaks except 5 were fitted well with a 1-species model, yielding a single brightness parameter for each. Peak 5 required at least 2 species with different molecular brightnesses to be fitted with a reasonable reduced $\chi^2$ value. It is clear from Fig. 3(C) that the resolution of separation around peak 5 becomes poor and consequently the captured fraction of the sample solution will be less homogeneous. Nonetheless, when the molecular brightness of the dimmer species for peak 5 was taken into account, a definite trend existed; a larger molecular brightness was observed for a protein species with a longer migration time. Although a brighter molecule is consistent with the increased number of incorporated dye molecules, the molecular brightness does not increase linearly with the number of fluorophores. It appears that, at a higher degree of labeling, A647 molecules are more likely to be conjugated at a more buried site, which may quench the fluorescence. Alternatively, dye molecules can quench each other when they are located in close proximity. It should be noted that a similar quenching effect has been observed for streptavidin when it is labeled with Alexa Fluor 546 succinimidyl ester.[11]

Because of the excessive number of different fluorescent species in the protein labeling mixture, the information we learned here is difficult to obtain using conventional PCH measurement unless the separated fractions are collected and analyzed off-chip. Although our scheme often lacks the ability to resolve the statistical mixture of protein species with the same number of dye molecules labeled at different locations, the fact that we can separate proteins by their charge states can significantly benefit single-molecule measurements in which the number of fluorophores on the target molecule plays an important role, such as quantifying the oligomerization of biomolecules using their molecular brightnesses.

In summary, this work demonstrates that complex biological samples can be separated and confined in a sub-nanolitre microchamber, which then can be used for further single-molecule analyses. Our method can be readily extended to other single-molecule fluorescence techniques such as fluorescence correlation spectroscopy (FCS) and single-pair FRET (spFRET) experiments because they share the same optical configuration as PCH. It should be noted, however, that the extremely small sample volumes of ultra-dilute samples (*e.g.*, spFRET) makes it more difficult to obtain high-quality single-molecule data because of the more pronounced photobleaching effect and the limited observation time. From the other point of view, this method also allows the use of the highly sensitive single-molecule spectroscopy as the detection modality for various separation techniques including CE and liquid chromatography. In this way, detailed information can be retrieved for each of the separated species. Therefore, we believe that our method of interfacing microfluidic sample manipulation and single-molecule detection will have wide applications.

## Acknowledgements

S.K. thanks the Stanford Bio-X Program for the Bio-X Graduate Student Fellowship. This work was supported by the National Science Foundation under BES-0508531.

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