Anal. Chem. **2002,** *74,* 1171−1176

Two-Beam Fluorescence Cross-Correlation Spectroscopy in an Electrophoretic Mobility Shift Assay

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Two-beam fluorescence cross-correlation spectroscopy (FCCS) was used to resolve the bound and unbound fractions of fluorescently labeled single-stranded DNA (ssDNA) in a ssDNA-protein complex as the analyte solution flowed continuously through an electrophoresis capillary. Cross-correlation of the single molecule fluorescence from two spatially separate excitation laser beams resulted in cross-correlation functions that consisted of well-resolved peaks characteristic of the different electrophoretic flow velocities of the bound and unbound ssDNA. This decoupled the molecular parameters of the bound and unbound ssDNA used to model the cross-correlation function, which enabled the relative concentrations to be determined without prior knowledge of the pure-component cross-correlation functions, as would be required in an analogous autocorrelation analysis. The relative concentrations of the bound and unbound ssDNA were determined by two-beam FCCS within 2−6% precision, even for samples that contained as little as 5% unbound ssDNA, and were consistent with the results obtained by capillary electrophoresis (CE) separation of the same samples. Data sufficient to obtain these results was acquired in 10−15 s per sample. Fluorescently labeled poly(dT)$_{39}$ complexed with the single stranded DNA binding protein of *Escherichia coli* served as the model system. The measured dissociation constant of 2.5 ± 0.9 nM agreed with the literature value for this complex within experimental error. The CE/two-beam FCCS experiment described here is part of a family of techniques that use single molecule fluorescence detection to resolve different components in an electrophoresis system. Advantages of these methods relative to separations-based CE include enhanced sensitivity, the potential for higher speed analyses, elimination of the sample plug injection step, and the ability to carry out the analysis in shorter flow channels.

In 1995, Castro and Shera introduced the technique of single molecule electrophoresis (SME),[1] whereby the electrophoretic flow velocities of individual molecules flowing continuously through an electrophoresis capillary were measured from the temporal offset of their fluorescence signals detected at two spatially separate excitation laser beam foci oriented perpendicular to the flow axis. This method enabled discrimination of different molecules in a mixture on the basis of their characteristic electrophoretic flow velocities without the need to perform a chemical separation. As the authors reported, advantages of the SME technique relative to conventional separations-based capillary electrophoresis (CE) included enhanced sensitivity, the potential for higher speed analyses, and elimination of the sample plug injection step. The latter advantage overcomes the instrument complexity and poor reproducibility associated with introducing a small sample plug at the beginning of the CE separation and opens the way for continuous monitoring of time-dependent changes in analyte concentration. However, the relatively large, picoliter-sized illumination volumes that were used required a pulsed picosecond laser system for single molecule fluorescence excitation and time-correlated single photon counting electronics for time-gated suppression of Rayleigh and Raman scattered light. This, coupled with the relatively low light collection efficiency of the optical setup, has made SME, as originally described by Castro and Shera, difficult to implement in practice. Although detection of individual chromophores was accomplished, multicomponent analysis was only demonstrated for large DNA strands labeled with multiple chromophores and for multichromophoric proteins.

A related technique involving multicomponent analysis in a CE system by fluorescence correlation spectroscopy (FCS) was first demonstrated in 1998 by Van Orden and Keller.[2] In this technique, termed CE/FCS, autocorrelation analysis of the single molecule fluorescence from a near-diffraction limited excitation region was used to determine the relative concentrations of different analytes in a mixture on the basis of their different electrophoretic flow velocities. Here again, the analysis was carried out on analytes continuously flowing through an electrophoresis capillary and did not require sample plug injection or chemical separation. CE/FCS takes advantage of the fact that in modern FCS,[3−5] Rayleigh and Raman scattered light are suppressed by the use of confocal optics that limit the detection volume to the nearly diffraction limited focal region of a single excitation laser beam positioned inside the sample. Fluorescence emitted from this detection volume is collected through a high-efficiency

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10.1021/ac015647w CCC: $22.00 © 2002 American Chemical Society
Published on Web 01/26/2002

objective lens, which allows single molecule fluorescence detection with high signal-to-background ratio using an inexpensive cw laser as the excitation source and a more compact optical and electronics setup than that used by Castro and Shera in their SME study. Single-beam FCS with autocorrelation analysis has been used for a number of years to characterize the diffusion rates of individual molecules in static solutions[3−5] and has also been used to characterize the flow parameters of molecules undergoing unidirectional translation.[2,6−8] The CE/FCS report by Van Orden and Keller described the first application of FCS to the discrimination of analytes based on their different electrophoretic flow velocities. More recently, CE/FCS was used to determine the binding ratios in a DNA-protein binding assay.[8] The advantages of SME outlined by Castro and Shera relative to separations-based CE also apply to CE/FCS, but the ability to study single chromophoric molecules is enhanced. Additional advantages to CE/FCS in multicomponent analysis become apparent in comparison to conventional diffusional FCS, wherein different analytes are discriminated on the basis of their characteristic diffusion rates.[4] Resolution of the different diffusion rates requires at least a 2-fold difference in the diffusion coefficients (D) of the analytes, which corresponds to an ~8-fold difference in molecular mass due to the $\sim D^{-3}$ dependence of the molecular mass on D. In CE/FCS, discrimination of analytes is based on the difference in their electrophoretic flow velocities, which is proportional to the charge-to-mass ratio, allowing for greater sensitivity to mass differences.

Two-beam fluorescence cross-correlation spectroscopy (FCCS) is an alternative FCS-based technique for measuring the flow parameters of single molecules that uses cross-correlation analysis of the fluorescence from two spatially separate laser beams to determine the travel times of individual molecules between the two beams.[9−11] Two-beam FCCS differs from the analysis technique used in Castro and Shera's SME experiment in that both laser beams are focused to near diffraction limited spots by a single high-numerical-aperture objective. The focal regions are offset from each other by only a few micrometers, as compared to the hundreds-of-micrometer separation distance used in SME, which enables the fluorescence from both focal regions to be collected by the same high efficiency objective. As in single-beam FCS, two-beam FCCS can be carried out with an inexpensive cw excitation laser and a compact optical and electronics setup and is readily applied to the study of single chromophoric molecules. Two-beam FCCS has been used in the past to characterize the transport properties of single molecules in microstructured flow channels.[9−11] Previously demonstrated advantages of this technique relative to single-beam FCS for the measurement of flow parameters include the ability to measure slower flow velocities at higher precision and to characterize the direction of flow. To our knowledge, multicomponent analysis has not previously been demonstrated using two-beam FCCS.

We report the application of two-beam FCCS in a CE-based electrophoretic mobility shift assay (EMSA), wherein the bound and unbound fractions of fluorescently labeled DNA in a DNA−protein complex are resolved on the basis of their different electrophoretic flow velocities. This work introduces another important advantage of two-beam FCCS relative to single beam FCS in this type of multicomponent analysis. In two-beam FCCS, the measured cross-correlation function consists of well-resolved peaks corresponding to the different electrophoretic flow velocities of the bound and unbound analytes, which decouples the molecular parameters of the different analytes used to model the cross-correlation function. Thus, determination of the relative concentrations can be accomplished with a single measurement of the cross-correlation function. In autocorrelation analysis, the molecular parameters for the different components of a mixture are strongly coupled such that accurate determination of the relative concentrations requires independent measurement of the pure component autocorrelation function for each analyte. This limits the multicomponent analysis capabilities of single-beam FCS to two or, at most, three analytes under favorable circumstances, whereas two-beam FCCS has the potential for much higher peak capacity. As with SME and CE/FCS with autocorrelation analysis, the measurements are carried out on molecules continuously flowing through the capillary, which eliminates the need for sample plug injection and chemical separation.

Other techniques related to those described here include two-color FCCS[12−14] and parallel imaging of single molecules flowing through a CE column.[15] Two-color FCCS uses two excitation laser beams of different wavelength to probe the same detection volume within a static solution. Each laser beam excites a different chromophore attached to two different analyte molecules, and a cross-correlation signal is observed only when both chromophores are simultaneously present in the excitation region as a result of binding of the two molecules. This method does not require prior calibration of the diffusion parameters, and a large decrease in the diffusion rate of the bound complex is not required. However, two-color FCCS is unable to directly measure the relative concentrations of the bound and unbound analytes in a single measurement, is limited to systems in which both binding partners can be fluorescently labeled, and does not have the same potential for high peak capacity as CE/two-beam FCCS. In the single molecule imaging approach, the electrophoretic flow velocities are measured directly for individual molecules as they flow continuously through the capillary by time-resolved imaging of the fluorescence from many single molecules in parallel.[15] This technique enables extremely fast data acquisition relative to both separations-based CE and techniques involving one-at-a-time single molecule detection described here. However, to date, this method has only been used to study multichromophoric molecules because of the need for a relatively large illumination region relative to confocal microscopy, which increases the background level and diminishes the contrast between the single molecule fluorescence signals and the background radiation. Additionally,

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the image processing required slows the data analysis, whereas real-time data analysis is achieved in one-at-a-time single molecule detection.

It should be carefully noted that none of the techniques involving single molecule detection in CE, including the one reported here, can rival conventional separations-based CE in terms of peak capacity and separation efficiency. Hence, at present, single molecule techniques are only applicable to the analysis of samples for which extremely high separation efficiency is not required. Despite this fact, there are several key aspects of CE/FCS and other single molecule electrophoresis techniques that make continued development of these methods a worthwhile pursuit. Although high-speed separations, on the order of seconds, have been reported in the literature, this seems to be the limit for most analyses based on separations-based CE. In CE with single molecule detection, acquisition and analysis times on the order of seconds are the norm,[1,2,8] and much shorter times are possible.[15] As noted above, separations-based CE typically requires a sample plug injection step, which adds time to the analysis, complicates the instrumentation, and can suffer from poor reproducibility. Single molecule techniques characterize the flow velocities one molecule at a time for molecules continuously flowing through the capillary, so there is no sample plug injection step. In addition, there is growing interest in carrying out separations in microchip electrophoresis devices. Analysis speed and device miniaturization would be greatly enhanced if the additional electrophoresis channel used for sample injection could be eliminated and if the separation channel could be significantly shortened. Indeed, the length of the channel needed to achieve an efficient separation, typically on the order of centimeters, is often the limiting factor in device miniaturization. In CE/FCS and other single molecule electrophoresis techniques, there is no channel length dependence on the efficiency with which different molecules can be discriminated. For example, in principle, it would be possible to carry out the same analysis as the one described here in an electrophoresis channel only slightly longer than the ~5- to 10-μm distance between the two laser beam foci, although the need for sample reservoirs and electrical contacts would introduce some limitations on the ultimate degree of miniaturization. Finally, there is strong evidence, discussed in more detail below, that improvements in the resolving power of single-molecule-based electrophoresis will be forthcoming. Hence, we anticipate that single molecule detection methods will come to play a major role in various aspects of microchip electrophoresis device fabrication, particularly for those devices involving arrays of electrophoresis channels for high-throughput screening applications.

EXPERIMENTAL SECTION

Sample Preparation. The model DNA−protein complex that we have chosen to study is the 1:1 complex formed between poly-$(dT)_{39}$ single-stranded DNA, labeled at the 5′ end with carboxytetramethylrhodamine (TAMRA-ssDNA, Operon, Alameda, CA), and the single-stranded DNA binding protein from *Escherichia coli* (SSB, Promega, Madison, WI). Solutions were prepared in pH 8 buffer (10 mM Tris−HCl, 0.5-mM EDTA) containing 0.028-wt % poly(vinylpyrrolidone) ($M_W \sim 10^6$ g/mol, Sigma, St. Louis, MO), which served as a dynamic coating to suppress electro-

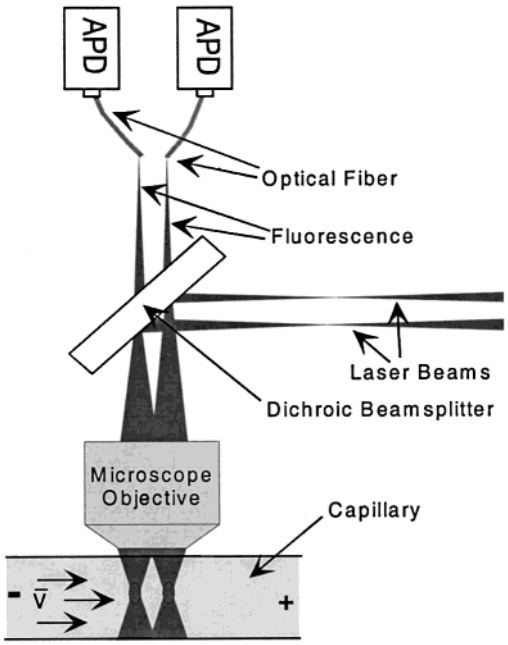

Figure 1. Schematic representation of the CE/two-beam FCCS experiment.

osmotic flow (EOF) and prevent adsorption of the biomolecules to the capillary walls.[16] Samples were introduced into a 40-cm-long, 40-μm-i.d. glass capillary (Polymicro, Phoenix, AZ) by applying 20 psig of N_2 to the capillary inlet for several seconds to completely fill the inner volume of the capillary with the analyte solution.

Instrumentation. Figure 1 displays a schematic representation of the CE/two-beam FCCS experiment. A 1 mW, 514.5-nm laser beam from an air-cooled cw Ar^+ laser (Melles-Griot, Carlesbad, CA) was split and then recombined into two nearly parallel beams by two 50/50 beam splitters (Newport, Irvine, CA). The two beams were reflected by a 530-nm long-pass dichroic beam splitter (CVI, Albuquerque, NM) and focused through a small window created in the capillary using a 100×, 1.25 NA oil immersion microscope objective (Edmund Industrial Optics, Barrington, NJ) to form two nearly identical focal regions ~6 μm from the inner glass surface. The distance between the focal regions was ~5 μm. A −30 kV potential was applied across the capillary by means of platinum electrodes connected to a high-voltage power supply (Spellman, model CZE1000R, Plainview, NY), resulting in an electric field of −750 V/cm. Fluorescence from each focal region, caused by excitation of the TAMRA-labeled ssDNA, was collected by the same objective, filtered (530 nm long pass, Chroma, Brattleboro, VT), and imaged onto the apertures of two 100-μm-i.d. multimode optical fibers (Thorlabs, Newton, NJ), which transmitted the fluorescence to two single photon counting avalanche photodiode detectors (APD, PerkinElmer Optoelectronics, model SPCM-AQR-14). The CE apparatus and optical setup were enclosed in a Plexiglas box interlocked to the high-voltage power supply, which prevented access to the high voltage electrodes during operation of the experiment.

The fluorescence signal from the two detectors was cross-correlated using an ALV5000E digital correlator card (ALV,

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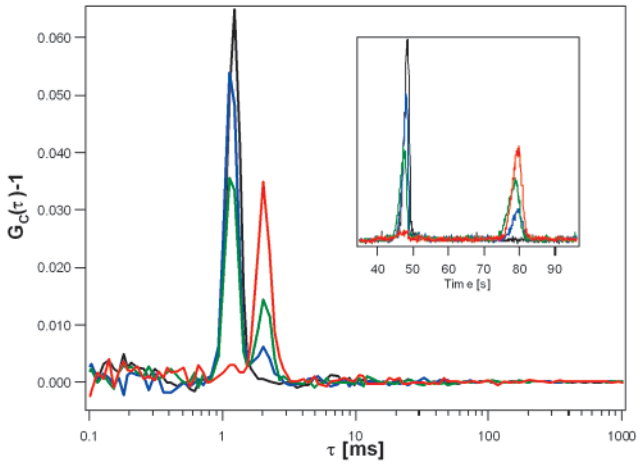

Figure 2. Cross-correlation functions obtained for 10-nM TAMRA-ssDNA and 0 (black), 9 (blue), 11 (green), and 23 nM SSB (red). Data acquisition times were 15 s per sample. The inset displays CE separations of the same samples. For the CE separations, the samples were injected by applying 4 psi N_2 to the capillary inlet for 1 s. The applied potential was −15 kV, and the detection point was 14 cm from the inlet.

Langen, Germany) mounted in a Pentium computer. Photocounts from each detector were accumulated into successive time bins, with multiple sampling times per bin varying from 200 ns to tens of seconds. The normalized cross-correlation functions were calculated in real time by the digital correlator using

$$G_C(\tau) = \frac{M\sum_{t=1}^{M} n_1(t)n_2(t+\tau) - \sum_{t=1}^{M} n_1(t)\sum_{t=1}^{M} n_2(t+\tau)}{\sum_{t=1}^{M} n_1(t)\sum_{t=1}^{M} n_2(t+\tau)} \quad (1)$$

where M is the total number of time bins for each detection channel, $n_1(t)$ is the number of photocounts accumulated into time bin t from detector 1, $n_2(t+\tau)$ is the number of photocounts in time bin $t+\tau$ from detector 2, and τ is the lagtime between detection channels 1 and 2.

RESULTS AND DISCUSSION

Figure 2 displays the two-beam cross-correlation functions measured for solutions containing 10 nM TAMRA-ssDNA and 0−23 nM SSB. Data acquisition times of 15 s per sample were used. Capillary electropherograms obtained for the same solutions are also shown for comparison. Note that both the cross-correlation functions and the capillary electropherograms consist of two peaks corresponding to free TAMRA-ssDNA at the earlier time and TAMRA-ssDNA−SSB at the later time. The cross-correlation function, $G_C(\tau)$ for the pure TAMRA-ssDNA solution was analyzed by nonlinear least-squares fitting to eq 2.[11] This equation assumes that the direction of flow is parallel to the axis between the two laser beam foci.

$$G_C(\tau) = 1 + \frac{1}{N}\left(1+\frac{\tau}{\tau_d}\right)^{-1}\exp\left[-\frac{|\bar{V}|^2(\tau^2+\tau_f^2-2\tau\tau_f)}{\omega_0^2(1+\tau/\tau_d)}\right] \quad (2)$$

Table 1. Cross-Correlation Function Parameters[a,b]

	[SSB] (nM)			
parameter	0[c]	9	11	23
		DNA peak[d]		
a		0.1264(15)	0.0863(13)	0.0056(16)
$\bar{V}$ (mm/s)	3.45(18)	3.66(19)	3.41(18)	3.45(24)
τ_f (ms)	1.221(15)	1.168(21)	1.175(21)	1.178(47)
		DNA−SSB peak[e]		
b		0.0166(19)	0.0416(16)	0.0956(23)
$\bar{V}$ (mm/s)		1.54(21)	1.98(13)	2.03(12)
τ_f (ms)		1.972(10)	2.076(10)	2.056(65)

[a] Numbers in parentheses are 1-s uncertainties in the last digits. [b] ω_0 was held constant at 0.40(2) μm for all the fits. This parameter was determined from autocorrelation analysis of a pure rhodamine 6G solution by using the known value of the diffusion coefficient D and the relationship $\tau_d = \omega_0^2/4D$. [c] $N = 6.53(6)$ for the pure ssDNA solution. [d] The parameter τ_d for ssDNA was fixed to the value of 0.89(5) ms, determined by autocorrelation analysis, in all of the fits. [e] The parameter τ_d for the bound complex was fixed to 1.1(1) ms in all of the fits.

Here, N is the average number of molecules occupying one of the focal volumes, τ_d is the transit time of TAMRA-ssDNA through the focal volume due to diffusion, $\bar{V}$ is the electrophoretic flow velocity (assuming no contribution from EOF), τ_f is the transit time between the two focal volumes as a result of unidirectional flow, and ω_0 is the $1/e^2$ radius of the focal volume in the lateral dimension. The parameters of the fit are presented in Table 1 under the "0 nM SSB" column. Analysis of the two-component mixtures was accomplished using a linear combination of the pure-component cross-correlation functions (eq 3).

$$G_C(\tau) = 1 + a[G_{C,DNA}(\tau) - 1] + b[G_{C,DNA-SSB}(\tau) - 1]$$

$$a = Q_{DNA}^2(1-R)/[(1-R)Q_{DNA}^2 + RQ_{DNA-SSB}^2] \quad (3)$$

$$b = Q_{DNA-SSB}^2 R/[(1-R)Q_{DNA}^2 + RQ_{DNA-SSB}^2]$$

$G_{C,DNA}(\tau)$ and $G_{C,DNA-SSB}(\tau)$ are the pure component cross-correlation functions (eq 1, absent the $1/N$ term) for TAMRA-ssDNA and TAMRA-ssDNA−SSB, respectively; Q_{DNA} and $Q_{DNA-SSB}$ are fluorescence yields of the TAMRA chromophore for free and bound TAMRA-ssDNA, respectively; and R is the ratio of bound TAMRA-ssDNA, given by

$$R = \frac{[\text{ssDNA} - \text{SSB}]}{[\text{ssDNA} - \text{SSB}] + [\text{ssDNA}]}. \quad (4)$$

Table 1 presents the parameters obtained by nonlinear least-squares analysis of the cross-correlation functions for the two-component mixtures, and Figure 3 displays a sample fit. R was determined for each solution from the parameters a and b and $Q_{DNA}/Q_{DNA-SSB} = 2.60 \pm 0.01$ measured by spectrofluorimetry. The parameters a and b could be determined without prior knowledge of the pure component cross-correlation functions, as would be required in an analogous autocorrelation analysis. Comparison of the R values obtained by cross-correlation analysis with those determined from the areas of the electropherogram peaks (Table 2) confirms the accuracy of the cross-correlation

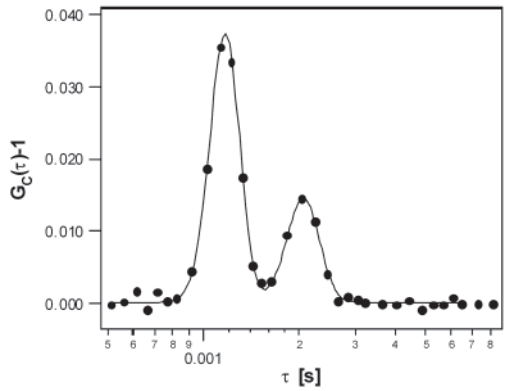

Figure 3. Experimental two-beam fluorescence cross-correlation function for a solution containing 10 nM TAMRA labeled poly(dT)$_{39}$ and 11 nM SSB protein (filled circles). The solid curve is the result of a nonlinear least-squares fit of the experimental data to eq 2.

Table 2. Binding Ratios (R) from Two-Beam FCCS and CE Separation[a]

	R	
[SSB], nM	FCCS	CE
0	0	0
9	0.470(29)	0.476(27)
11	0.7651(83)	0.731(25)
23	0.945(15)	0.964(31)

[a] Numbers in parentheses are 1-s uncertainties in the last digits.

measurements. The results obtained for the 23-nM SSB solution indicated that as little as 5% unbound TAMRA-ssDNA could be detected in the presence of 95% bound TAMRA-ssDNA−SSB. The dependence of R on the SSB concentration is consistent with a dissociation constant (K_d) for the complex of 2.5 ± 0.9 nM, determined from $R = \{0.5[\text{SSB}]\}/(K_d + 0.5[\text{SSB}])$,[17] which agrees with the literature value of 2 nM for 1:1 poly(dT)$_{30-40}$−SSB within experimental error.[18]

The efficiency with which different components in a mixture can be resolved is expressed as the coefficients of variance (CVs) of the peaks, defined as the standard deviation divided by the peak position. In our two-beam FCCS assay, the CVs of the TAMRA-ssDNA and TAMRA-ssDNA−SSB peaks, obtained by fitting the peaks to Gaussian functions, were 11 and 12%, respectively, which is adequate to achieve a value for the resolution of the two peaks of 1.4 (defined as the difference in the peak positions divided by the average baseline widths), corresponding to near-baseline separation. By comparison, Castro and Shera obtained CVs of ∼2−3% for the travel time histogram peaks in their SME study of 10- to 38-kilobase pair (kbp) DNA molecules,[1] and Shortreed et al. reported CVs of 1−3% for the electrophoretic flow velocities of 48- and 2-kbp DNA, respectively, in their single molecule imaging experiment.[15] In both cases, the analyte solutions contained a sieving medium. The larger CVs reported here are likely due to several factors, including the larger diffusion coefficients of the analytes, the lower solvent viscosity, and the smaller distance between the laser beam foci relative to the SME experiment. All of these factors cause an increase in the velocity distribution of molecules that transit both detection volumes and contribute to the cross-correlation signal. As noted above, none of these techniques attains the ∼0.2% CVs typically observed for capillary electropherogram peaks.

Clearly, the feasibility of applying this two-beam FCCS approach to a broader range of samples, particularly those that contain multiple components, would be enhanced if the CVs could be lowered. The following arguments suggest that by carrying out this same analysis in a planar microchip format, lower CVs will be obtained. First, in a planar microchip, the excitation laser beams will be focused through a glass coverslip rather than a curved capillary, which will result in focal radii that are closer to the diffraction limit of ∼250 nm, as compared to the ∼400-nm focal radii observed in this experiment. Reducing the size of the focal volumes would be equivalent to increasing the distance between the laser beam foci, which would narrow the velocity distribution of molecules contributing to the cross-correlation signal. Second, planar microchips are able to sustain higher electric fields than fused-silica capillaries without degrading the analysis as a result of Joule heating.[19] Electric fields as high as 2500 V/cm have been reported for microstructured electrophoresis devices,[19] which is higher than the 750 V/cm used here by a factor of 3.3. This will cause the analyte flow velocities to increase by the same factor. Using flow velocities of 11.4 and 6.6 mm/s for the unbound and bound TAMRA-ssDNA−SSB complex, respectively (obtained by multiplying the values in Table 1 for the 11 nM SSB solution by 3.3), ω_0 = 250 nm, and a 10-μm distance between the laser beam foci, we calculated an experimentally realizable cross-correlation function with very respectable peak CVs of 4% and a resolution between peaks of 3.6. Increasing the solvent viscosity would lower the CVs even further, resulting in significantly higher peak capacity.

CONCLUSIONS

In summary, we have demonstrated that two-beam FCCS can be used to resolve the bound and unbound ssDNA fragments in an EMSA-based ssDNA-protein binding assay. Because the cross-correlation function consists of separate peaks corresponding to the electrophoretic flow velocities of each analyte, the relative concentrations of the bound and unbound analyte could be determined without prior knowledge of the pure component cross-correlation functions. Since the analysis was carried out on solutions continuously flowing through the capillary, there was no need for separate sample plug injection and separation steps, which enables higher analysis speed and continuous monitoring of the analyte concentrations. In future work, the detection methods described here will be transferred to the microchannel format. Because the resolution in this case is not coupled to the channel length, it will be possible to carry out these analyses in electrophoresis channels that are significantly shorter than those used in separations-based microchip electrophoresis, and the need for a separate electrophoresis channel for sample plug injection will be eliminated. These factors will result in significant reductions in the size and complexity of microchip electrophoresis devices, particularly those that contain arrays of multiple electro-

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phoresis channels. Resolution enhancement of the cross-correlation peaks will be accomplished by taking advantage of the better optical quality and the higher electrophoretic flow velocities that can be obtained with microchip devices. Finally, we note that the methods described here are amenable to the study of other types of binding interactions in biomolecular systems, such as those involving antibody−antigen, enzyme−substrate, and drug−target binding, making these methods useful in such areas as medical diagnostics, environmental monitoring, and high-throughput drug discovery.

ACKNOWLEDGMENT

Funding was provided by Colorado State University, the Colorado Commission on Higher Education, the Petroleum Research Fund of the American Chemical Society, and 3M Corporation. We thank Jaemyeong Jung for technical assistance.

Received for review October 8, 2001. Accepted November 29, 2001.

AC015647W