Single-molecule detection with axial flow into a micrometer-sized capillary

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We characterize a new geometry for single-molecule detection with flow for use with a submilliliter drop of sample on an inverted confocal microscope. The solution is sucked into a glass capillary positioned above the ellipsoidal confocal volume so that molecules traverse the longest axis of the ellipsoid for greatest photon yield. Decreased spacing between the capillary tip and laser focus gives increased flow speed, as measured by fluorescence correlation spectroscopy, but also increased background from capillary autofluorescence. Flow can alleviate localized triplet and photobleaching effects and speed single-molecule sampling rates for fluorescence fluctuation spectroscopy determinations of slowly diffusing biomolecules in pharmaceutical drug discovery research. © 2007 Optical Society of America

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1. Introduction

The detection of individual molecules in solution has become increasingly prevalent in the examination of biological systems.1–4 Early work in this area included visual, as well as photoelectric observation of biological particles labeled with ~100 fluorescent molecules.5 The number of fluorophores necessary for detection was reduced by an increase of the light detection efficiency in a flow-cytometer configuration, in which a hydrodynamically focused sample stream forces molecules to pass through a focused laser beam.6,7 The detection limit was reduced to the single-molecule level by use of time gating to eliminate Raman scatter.8,9 The probe volume in all this early work was generally of the order of picoliters. In more recent studies, the use of confocal microscopy has reduced the probe volume to the order of femtoliters.10

Whereas these early studies observed the presence of individual fluorophores, fluorescence correlation spectroscopy (FCS) can be used to determine the ensemble averaged concentration and diffusion coefficient, once the probe volume dimensions are known, by the fitting of the normalized autocorrelation function (ACF) of the collected stream of photons. In FCS the temporal fluctuations of the fluorescence signal are generally caused by the variation in the number of molecules in the excitation volume. FCS was first developed in the 1970s as an extension of dynamic light scattering techniques to measure chemical reaction kinetics.11,12

Both single-molecule detection (SMD) and FCS have now been extensively applied to biological studies. For example, SMD has been used for the observation of molecular motors,13,14 and FCS has application to pharmaceutical drug discovery.15,16 The usefulness of FCS to pharmaceutical drug discovery arises from the ability to determine the relative concentrations of sample constituents with different diffusion coefficients. If a fluorescently labeled druglike molecule binds to an unlabeled target protein with high affinity, the mean molecular weight of the fluorescently labeled compound will be greater. This results in a slower mean diffusion coefficient that can be seen in the normalized autocorrelation. The addition of active transport allows a more rapid processing of these molecules, but other fluorescence fluctuation spectroscopy (FFS) techniques must be used, as differences in the diffusion coefficients become indistinguishable when transport is dominated by flow. The other techniques are based on the rotational diffusion, fluorescence brightness,17 or the coincidental detection of two colors.18–20

The use of flowing samples in SMD has been extensively investigated and applied to many areas. Early work concentrated on the use of flow to direct...
molecules through the probe volume in an effort to reduce the detection limit. In these studies, volumes of the order of picoliters were achieved. In Ref. 8, time gating was also used to discriminate background caused by Raman scatter. Another common feature of these early studies was that the excitation and collection paths were perpendicular.

SMD with flow in a microfluidic lab-on-a-chip device, with the use of electrophoretic separation of different molecule types, was first reported in 1998. In other work, the identification of different fluorescent species by a comparison of fluorescence lifetimes was demonstrated with the use of flow within a micrometer-sized capillary. SMD with flow has been extended for use in the counting and identification of labeled DNA molecules and amino acids, the binding of quantum dots to organic dyes, and the use of two-photon excitation. There has also been work done to improve the techniques of flow-cell fabrication, the instrumentation used for SMD with flow, and flow characterization by use of both FCS and photo-activated fluorophores. For a more comprehensive summary of the application of SMD for a flowing sample the reader is directed to Refs. 40 and 41.

The goal in this work is to develop a simple and inexpensive flow system for use with a total sample volume of ~100 ml in a confocal fluorescence microscope. In this flow system the molecules are transported along the focused laser beam, which differs from all previously reported investigations, which use flow transverse to the optical axis. Axial flow allows a longer interaction time compared with transverse flow at the same speed as the probe volume in a confocal microscope is well approximated by an ellipsoidal 3D Gaussian function in which the axial diameter is typically five to seven times that of the radial diameter. Also, in previously reported confocal experiments with flow, the probe volume is imaged through the walls of the capillary or microfluidic cell, which leads to a potential for aberration, whereas the imaging in the present system is only through the microscope coverslip. Here we describe the use of this axially aligned capillary flow system for SMD and its characterization using FCS. We show that the flow velocity increases quadratically with decreased distance between the tip of the capillary and the laser focus. However, this decreased spacing also results in greater background from capillary autofluorescence.

2. Materials and Methods

A. Experimental Setup

The custom-built microscope used in the experiments is shown schematically in Fig. 1. The laser beam passes through lenses that act as an adjustable beam expander and is then brought to the fluorescent solution by a fused-silica beam sampler (Newport Corp., Irvine, California, 10Q20NC.1) and focused into the solution with a water-immersion objective (Olympus UPlanApo, 60×, 1.2 N.A. UPLAPO60XW). The beam sampler allows the use of different laser excitation wavelengths without the need for realignment and gives ~10% Fresnel reflection, while the remaining 90% of the beam is dumped. Emitted fluorescence is then collected with the same objective lens, passed through the beam sampler, is focused through a spatial filter, and recollimated. After recollimation, the collected light passes through a bandpass filter (Omega Optical, Brattleboro, Vermont, 617DF45) to eliminate any Rayleigh scatter from the solvent molecules. The light that passes through the filter is then focused onto a single-photon avalanche diode (SPAD) detector (PerkinElmer SPCM-AQR15).

The excitation source most often used in our experiments is a synchronously pumped dye laser (Coherent 700), with a pulse repetition rate of 76 MHz running Rhodamine 6G and tuned to 585 nm. The dye laser beam was found to contain a small quantity of broadband fluorescence from the dye jet. To block this, the laser beam was reflected from a Raman notch filter (Kaiser Optical Systems, Inc., Ann Arbor, Michigan, HNPF-585-1.0) before it entered the mi-
microscope. The Raman notch filter was angle tuned to reflect the dye laser beam, while the broadband fluorescence was transmitted.

The detector pulses are passed to two data acquisition systems. For FCS and SMD, the detector output is passed to the gate input of a National Instruments PC1-6602 data acquisition card, which is configured to record the time of arrival (TOA) of each photon by using the internal clock with a resolution of 12.5 ns. The TOAs are then used to determine the instantaneous count rate and the photon amplitude of each burst as well as to numerically calculate the normalized ACF in LabVIEW (National Instruments). For fluorescence lifetime measurements, the detector output pulses are passed from the National Instruments card to the start-signal input for a TimeHarp 200 PCI card (PicoQuant GmbH). The stop signal is obtained from a small portion of the laser, which is diverted to a fast photodiode (Hewlett Packard, 5082-4203). The TimeHarp 200 includes software that displays a histogram of the time difference between the start pulse and the next laser pulse with 34 ps resolution.

The flow system consists of a small borosilicate glass capillary, drawn to a tip with a diameter of 5 or 10 μm (World Precision Instruments TIP5TW1LS01, Sarasota, Florida) aligned along the optical axis of the confocal microscope, as shown schematically in Fig. 1. The capillary is coupled to a manometer by a Luer connector with a three-way stopcock, which allows the flow to be turned on and off. The three-way stopcock also permits the attachment of a syringe to initially suck water up to the capillary from the manometer’s reservoir to initiate siphoning action. When the flow is turned on the water in the manometer tries to fall back to the reservoir, and a negative pressure difference is created, the result of which is that the sample is drawn vertically upward into the capillary tip. Control of the observed flow speed is achieved by either the adjustment of the water height in the manometer or by the alteration of the distance between the capillary opening and the laser focus.

The capillary is mounted on a precision xyz translation stage with submicrometer adjustability (Newport Corp., 462-XYZ-DM13). The alignment is done visually by the direction of collected light to either a microscope eyepiece or an intensified CCD camera (Princeton Instruments, I-Max). While the capillary is illuminated from above with white light, the eyepiece’s wide field of view is used to get the tip approximately into focus and to avoid the capillary tip hitting the coverslip. Then the optical collection path is switched to the CCD, and the white light is turned off. The sensitivity of the CCD, along with the lack of any optical filters in this path, allows the laser light that is reflected from the tip to be used to bring the capillary more precisely into focus and position it so that the focused laser beam is in the center. The capillary is finally translated vertically upward to the desired height above the laser focus, and the optical collection path is switched to the SPAD for data collection. An image of the capillary during the alignment procedure is shown in Fig. 2. In this image the focused laser is seen as the bright spot near the center of the circular 10 μm diameter capillary.

The fluorophores Alexa Fluor 594 carboxylic acid, succinimidyl ester (Invitrogen A-20004, molecular weight of 819.9) and Alexa Fluor 610 carboxylic acid, succinimidyl ester (Invitrogen A-30103, molecular weight of 1171.66) were used in the experiments reported here. These have good photostability and fluorescence quantum efficiency for SMD, and protein labeling kits are available. Alexa 594 and 610 can each be suboptimally excited at 585 nm, and they have emission peaks at ~620 and ~630 nm, respectively. The stock solutions were serially diluted in purified water (Barnstead Thermolyne Corp., NANO pure Infinity) to a concentration of 50 pM. The detergent Tween 20 (Cole-Parmer, EW-88248-24), was added to the dilution at a concentration of 0.1% by volume to reduce molecular adsorption to the glass capillary. Detergents such as Tween are commonly used in pharmaceutical screening studies to reduce nonspecific binding of reagents to surfaces that can change the concentration of the sample constituents.

B. Theory

The normalized ACF is defined as

$$G(\tau) = \frac{\langle F(t)F(t+\tau) \rangle}{\langle F(t) \rangle \langle F(t+\tau) \rangle}, \quad (1)$$

where $F(t)$ is the photon count rate at time $t$, and the angular brackets denote an average over all times, such that

$$\langle F(t) \rangle = \lim_{T \to \infty} \frac{1}{2T} \int_{-T}^{T} F(t') dt'.$$

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When diffusion is the dominant mode of transport through the sample volume, as is typically the case for FCS, then the normalized ACF, \(G_{\text{diff}}(\tau)\), is given by

\[
G_{\text{diff}}(\tau) = 1 + \frac{\alpha}{N} \left(1 + \frac{\tau}{\tau_D} \right)^{-1/2} \left(1 + \chi \frac{\tau}{\tau_D} \right)^{-1/2},
\]

(3)

where \(N\) is the average number of molecules in the excitation volume, \(\alpha\) is a factor taking any background count rate \(B\) into account:

\[
\alpha = \gamma \left(1 + \frac{B}{N\Phi} \right)^{-2},
\]

(4)

where \(\gamma = 2^{-3/2}\), which comes from the 3D Gaussian approximation of the probe volume, and \(\Phi\) is the fluorescence count rate per molecule. Also, in Eq. (3) \(\chi\) is the confocal parameter defined as

\[
\chi = \frac{(w_0^2)}{z_0^2},
\]

(5)

where \(w_0\) and \(z_0\) are the \(1/e^2\) radii of the ellipsoidal confocal volume in the \(x\)-\(y\) plane and the \(z\) direction, respectively. Also, \(\tau_D\) is the mean diffusional residence time, which is defined as

\[
\tau_D = \frac{w_0^2}{4D},
\]

(6)

where \(D\) is the translational diffusion coefficient. Calibration experiments with a fluorescent species that has a known diffusion coefficient \(D\) are typically performed to determine the confocal parameter \(\chi\), which can be held fixed in subsequent measurements. This calibration measurement can also be used to determine the confocal volume radius from Eq. (6).

If in addition to diffusion there is also an applied flow directed along the \(z\) direction, the fitting function is altered by an extra Gaussian term:

\[
G_{\text{flow}}(\tau) = 1 + \frac{\alpha}{N} \left[1 + \frac{\tau}{\tau_D} \right]^{-1} \left[1 + \chi \frac{\tau}{\tau_D} \right]^{-1/2}
\times \exp \left\{ -\frac{(\tau/\tau_D)^2}{1 + \chi^2/\tau_D^2} \right\},
\]

(7)

where \(\tau_F\) is the mean residence time due to flow, defined as

\[
\tau_F = z_0/u_F,
\]

(8)

with \(u_F\) as the flow velocity.\(^{45}\)

### 3. Results and Discussion

Initial experiments were conducted to characterize the background and reduce it as much as possible. The two major sources of background are the autofluorescence of the glass capillaries and scattered light, both of which cause a constant background and result in a lower amplitude of the normalized ACF, as seen from Eq. (4). To study the extent to which the spatial filter of the confocal microscope can discriminate background originating from the tip of the capillary, the count rate was observed as a function of distance between the tip and the center of the probe volume, with only water as a sample. The results for the total count rate with a 5 \(\mu\)m capillary and a 150 \(\mu\)m confocal pinhole are shown in Fig. 3(a), while the total count rate with a 10 \(\mu\)m capillary and a 75 \(\mu\)m confocal pinhole are shown in Fig. 3(b). This figure demonstrates that at distances \(\geq 50 \mu\)m between the capillary tip and the laser focus the background is dominated by the detector dark noise (\(\sim 100 \text{ s}^{-1}\)).

Time-resolved measurements were also performed with water and with the capillary tip located at various distances from the laser focus, as shown in Fig. 4, to determine the relative background contributions from the autofluorescence and the specular scatter from the tip that gets through the interference filter. Glass autofluorescence is identified in Fig. 4 as the slowly decaying component that diminishes with increasing distance between the tip and the laser focus, while the scattered light causes the peak at \(\sim 1.5\) ns and remains constant regardless of the separation between the tip and the laser focus. The major part of this promptly scattered light was found to be attrib-

![Fig. 3. Background count rate at various distances from the capillary: (a) 5 \(\mu\)m capillary with a 150 \(\mu\)m pinhole, laser power of 30 \(\mu\)W, \(\lambda = 585\) nm and (b) 10 \(\mu\)m capillary with a 75 \(\mu\)m pinhole, laser power of 30 \(\mu\)W, \(\lambda = 585\) nm.](image-url)
utable to broadband fluorescence light from the dye laser, and it was removed in all subsequent measurements by filtering the laser beam with a Raman notch filter, as described in the Subsection 2.A. With the specularly scattered light eliminated, almost all the autofluorescence contribution to the background can be eliminated by the spatial filter when the probe volume is 50 μm from the capillary tip, for both a 5 μm capillary with a 150 μm pinhole and a 10 μm capillary with a 75 μm pinhole.

Figure 5(a) shows the normalized ACF of Alexa Fluor 594 as a function of capillary distance from the laser focus, obtained at the center of the capillary. Also shown is the ACF obtained with only diffusional transport, which is used to find the confocal parameter, $\chi$ (0.021) and the mean diffusional residence time $\tau_D$ (2.1 ms) for the given configuration. By use of Eq. (7) to obtain a least-squares fit of the data, it is seen that the flow time increases with increasing distance from the tip. From Eq. (4) it can be seen that the lower amplitude of the ACF obtained near the capillary tip is caused by the higher background signal, which is attributed to glass autofluorescence.

To determine the flow velocity from the flow time, the probe volume must first be characterized by use of a standard fluorescent dye that has a known diffusion coefficient. The Alexa Fluor 594 used here has a measured diffusion coefficient of $6.5 \times 10^{-7}$ cm$^2$/s. This diffusion coefficient and the fitted parameters of the mean diffusional residence time, $\tau_D$, and confocal parameter, $\chi$, were used to calculate the radius of the confocal volume in the $x$-$y$ plane, $\omega_0 = 0.738 \pm 0.03 \mu$m, and the radius in the $z$ direction, $z_0 = 5.05 \pm 0.52 \mu$m for the 150 μm pinhole. The errors are estimated by adjustment of $\omega_0$ to fit the curve after the confocal parameter is forced to take on suboptimal values. The flow speed, $v_F$, can then be determined by the curve fit of the ACF with flow, and the use of Eq. (8). The leftmost ACF curve in Fig. 5(a) yields a maximum flow speed of $\sim 2$ m/s at the mouth of the 5 μm capillary tip, which corresponds to a volumetric flow rate of $\sim 4 \times 10^{-5}$ cm$^3$/s if the flow here is approximated to be pluglike. For points fur-

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ther from the tip, the plot of flow velocity versus distance from the tip is shown in Fig. 5(b). This is expected to decrease approximately quadratically with increasing distance, and in fact, the least-squares fit to the log-log plot has a slope of $-2.01$. With the 5 μm capillary tip 50 μm from the center of the confocal volume, the flow velocity is $\sim 7$ cm/s for a pressure difference induced by a 1 m column of water.

The main reason flow is desired is to increase the rate at which molecules pass through probe volume $M$, which can be calculated from the parameters extracted from the fitting of the ACF. For the case without flow this is given by $M_{\text{diffusion}} = N/2\tau_p$ and is measured to be 85 molecules/s. On the other hand, with flow, $M_{\text{flow}} = N/2\tau_p$, and with the capillary 50 μm from the laser focus, this works out to be 2630 molecules/s. Thus for the small Alexa 594 molecule, the sampling rate increases by a factor of $\sim 30$, while for large slowly diffusing molecules, the flow system yields an even greater increase.

The axial flow causes the molecules to traverse the major axis of the 3D Gaussian ellipsoid, which results in more collected photons per molecule than would occur if the flow were transverse to the optical axis. Also, the mean photon count rate was measured and divided by the mean number of molecules in the probe volume obtained from the ACF, which results in a time-averaged count rate per molecule of 18,274 and 18,184 photons s$^{-1}$ molecule$^{-1}$, for the cases with and without flow, respectively. Triplet kinetics and photobleaching are not evident in the ACF of Fig. 5(a), but when present, the time-averaged count rate per molecule is found to increase with flow, as molecules that have stopped fluorescing are more quickly replaced by new ones.

Although the rate of molecules passing through the probe volume is much greater with flow than without, the instantaneous count rate during each photon burst remains approximately the same, and the identification of individual bursts attributable to the transit of single molecules is readily achievable. A 1 s segment of the count rate versus time for a 50 pM solution of Alexa 594 is shown in Fig. 6 for the case where the capillary is 50 μm above the laser focus. While each burst corresponds to an individual chromophore, the burst amplitudes vary, as dye molecules travel through different parts of the radial laser profile.

Even though the apparent brightness of each detected molecule differs, the average of the amplitudes of a number of bursts can yield a precise measure of the mean brightness of that sample.$^{17}$ To this end, Fig. 7 shows a comparison of the probability density for the amplitudes of peaks in the Gaussian-weighted sliding sum of the photon data streams, with weights matching the linear transit of molecules across the axial confocal probe volume profile, obtained from a 50 pM solution of Alexa 594 and from a 50 pM solution of Alexa Fluor 610. In this case, Alexa 610 exhibits smaller photon bursts on average, due to the sub-optimal 585 nm excitation wavelength. This result demonstrates the possibility to determine sample components based on fluorescence brightness when flow is used, whereas molecules with different translational diffusion would no longer be distinguishable.

4. Conclusions

The use of gravity-driven flow in SMD experiments can be used in applications such as pharmaceutical drug discovery, where it is desired to more rapidly process large, slowly diffusing biomolecules. Fluorescence fluctuation spectroscopy techniques based on two colors, polarization-resolved detection, or fluorescence brightness could then be used to assay the binding affinities of labeled protein–ligand complexes.
Axial flow has the advantage of the molecules being transported along the major axis of the 3D Gaussian ellipsoid of the confocal volume, which results in more collected photons compared to transverse flow at the same speed. For the results shown here the count rate per molecule with flow was comparable to the case without flow; both measured to be ~18,000 s\(^{-1}\) molecule\(^{-1}\), while the rate of molecules passing through the probe volume increased by a factor of ~30.

In this work fluorophores are sucked through the probe volume by the application of a negative pressure difference to either a 5 or 10 µm capillary that is oriented along the optical axis. FCS was used to characterize this new geometry and to verify that the flow velocity decreases approximately quadratically with increasing distance from the capillary opening. There is a trade-off with this configuration between increased background autofluorescence of the glass capillary near the tip and decrease in flow velocity as the distance increases. However, when the tip is positioned 50 µm from the laser focus, the background can be spatially filtered, while the flow speed is adequate to dominate Brownian diffusion, even for small single-chromophore molecules. The potential for fluorophore adsorption to the capillary and other glass surfaces can be reduced by the use of detergents, which are commonly used in pharmaceutical screening assays. The axially aligned capillary provides an effective means for inducing flow within a small submilliliter drop of solution on the coverslip of an inverted confocal microscope.

References

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