An integrated platform for surface forces measurements and fluorescence correlation spectroscopy

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We describe an apparatus to measure the diffusion of dilute fluorophores in molecularly thin liquid films within a surface forces apparatus (SFA). The design is a significant modification of the traditional SFA in that it allows one to combine nanorheology with the single-molecule sensitive technique of fluorescence correlation spectroscopy. The primary enabling idea was to place a miniaturized SFA onto the stage of an optical microscope equipped with a long working distance objective and illuminated by a femtosecond laser. A secondary enabling idea was that the silver coating on the backside of mica, normally used in the traditional SFA design for interferometric measurements of the film thickness, was replaced by multilayer dielectric coatings that allowed simultaneous interferometry and fluorescence measurements in different regions of the optical spectrum. To illustrate the utility of this instrument, we contrast the translational diffusion of rhodamine dye molecules (in the solvent, 1,2-propane diol), in the unconfined bulk state and confined between mica sheets to the thickness 2.5 nm. The diffusion coefficient is found to decrease by 2 orders of magnitude under confinement. © *2003 American Institute of Physics.*
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I. INTRODUCTION

In recent years, there has been a significant increase of interest to study the structural and dynamical properties of nanometer to micrometer thick films confined between two solid surfaces. Whereas mechanical contact occurs at numerous asperities when dealing with most workaday surfaces, which are rough, the advent of tip-based microscopies and the surface forces apparatus (SFA) has enabled systematic investigation of interfacial problems in a single asperity contact. In particular, the surface forces apparatus, though it was originally designed to measure the van der Waals and Derjaguin, Landau, Verway, and Overbeek (DLVO) forces in simple liquids and in colloidal systems,[1] later was modified by several groups to study the frictional forces of molecularly thin films confined between rigid surfaces.[2] It has made a significant impact on understanding the origin of confinement induced solidification which is responsible for the ubiquitous phenomenon of static friction and ensuing stick–slip when motion begins. This in turn has consequences from tribology to geology, and possibly to biological issues such as the longevity of hip and knee joint replacements.

Though force measurement using the SFA is a productive technique, forces represent ensemble-averaged values and it is also desirable to have local, direct information at the molecular level. However, technical difficulties of combining spectroscopy with the SFA have until present precluded this. The semireflective silver layer at the backside of mica sheets, which is used to measure the film thickness by optical interferometry[1] does not allow sufficient excitation source intensity to illuminate the confined film. Though the power of spectroscopic techniques in the studies of confined fluid has been of speculative interest for a long time, only a very few successes have been reported so far. Safinya *et al.* investigated the structure of a thin smectic liquid crystal films under confinement using synchrotron x-ray scattering within the SFA.[3] But it was not used to study ultrathin (~nm) liquid films. Synchrotron waveguide methods were recently used to characterize the layering of molecularly thin films.[4] The microrheometer developed by Dhinojwala *et al.* can readily be combined with spectroscopy (Fourier transform infrared spectroscopy and dielectric spectroscopy) or scattering (x-ray and neutron) techniques.[5] It uses two parallel optically flat windows plates whose separation can be controlled from a few tens of nm to a tens of μm, but is more suited for thicker (0.1–10 μm) films. By replacing one of the plates with a prism, recently it was shown that this rheometer can be combined with the surface sensitive technique of infrared-visible sum frequency generation (SFG) in the total internal reflection geometry.[6] That combination can be used to probe the orientation, alignment and relaxation modes of organic molecules at the buried interface in a condition of flow or shear. Some years back it was shown that SFG can also be combined with the SFA to study 1-nm-thick films of one system of self-assembled monolayer confined between atomically smooth mica surfaces,[7] but implementation of this approach to other experimental situations has not been reported as yet.

In this article we present the experimental setup and principles of operation of an integrated platform in which to combine surface forces measurement and friction studies within the SFA, with measurements of translational diffusion using fluorescence correlation spectroscopy (FCS). In these fluorescence experiments, a dye molecule is doped into the sample, acting as a probe of its local environment. The sci-

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entific objective, in building this integrated platform, is to elucidate how forces and friction (ensemble-averaged numbers) are related to local, spatially-resolved rates of diffusion (translational and rotational). Elsewhere, we reported initial experimental findings obtained using this apparatus.[8] The known single-molecule sensitivity of the FCS technique in bulk experiments[9] is demonstrated to apply also to the present measurements.

II. PRINCIPLE OF FCS MEASUREMENT

FCS, an experimental method to extract information on dynamical processes from the fluctuation of fluorescence intensity, has enjoyed widespread application on chemical biology[10] but was not used previously to study the dynamics in ultrathin liquid films buried between two solids. The fluctuation of fluorescence, when dye molecules are dilute, can in principle result from diffusion, aggregation, or chemical reaction. Here we have chosen to use two-photon excitation such that fluorescence was induced in a very small volume ($\sim 10^{-15}$ l) in very dilute systems (from pM to nM).

Compared to other techniques for studying diffusion problems, such as quasielastic light scattering, fluorescence recovery after photobleaching, and laser induced transient grating spectroscopy, FCS presents the capability of measuring extremely dilute systems with high spatial resolution (down to the optical diffraction limit). On the average there can be as few as 1–5 dye molecules within the ~1 fl volume element of the focused laser beam (discussed later). However, they move in and out due to Brownian motion causing intensity fluctuations, which are observed as a low frequency noise on the mean fluorescence signal. By inspecting the autocorrelation function of this fluctuation, $G(\tau) \equiv \langle \delta I(t)\, \delta I(t+\tau)\rangle / \langle \delta I(t)\rangle^2$ (here I denotes fluorescence intensity and t is the time variable), and by choosing a suitable model to analyze the autocorrelation function, the rate of dynamic process is obtained.[11]

If the primary reason for fluctuation is translational diffusion, and assuming that fluorescence characteristics of the diffusing molecules do not change while traversing the laser volume, one can use Fick's second law to calculate the translational diffusion coefficient (D) from the autocorrelation function by using the relation, derived in Ref. 12, $G(\tau) = G(0)/(1+8D\tau/\omega_0^2)$. This result follows from the convolution of the concentration correlation with the spatial profile of the laser focus, which has been assumed to be two-dimensional Gaussian (of width ω_0). The magnitude of the autocorrelation function at time zero, $G(0)$, is related to the average number of fluorophores (N) in the observation volume by using the relation $G(0) = 1/(2\sqrt{2}N)$.[13] As will be seen below, measurements in this setup can be made with single-molecule sensitivity. This same setup with minor modifications can also be used to study the rotational diffusion, which also probes the local viscosity of a liquid medium, and could also be of potential interest.

III. THE EXPERIMENTAL SETUP

A schematic diagram of the experimental setup is shown in Fig. 1. The FCS portion of the setup consists of three parts: light source, microscope, and data acquisition.[14] Two-photon excitation was achieved by a femtosecond Ti:sapphire laser (Tsunami, Spectra Physics), which generated laser pulses with full width at half maximum of 50 fs at a repetition rate of 80 MHz. A Zeiss inverted microscope (Axiovert 135, Carl Zeiss) served as the operational platform for the whole experiment. The excitation light was focussed onto the sample with the objective lens [Achroplan, Zeiss 65X, numerical aperture (N.A.)=0.75] and the emitted light was collected through the same objective and was detected by a single photon counting module (Hamamatsu) at the bottom outport of the microscope. The photon counting output was recorded by an integrated FCS data acquisition board (ISS, Champaign, IL) and data analysis was conducted using the software provided. By introducing the laser beam through the objective lens, a small excitation volume (~1 fL) was generated within the sample.

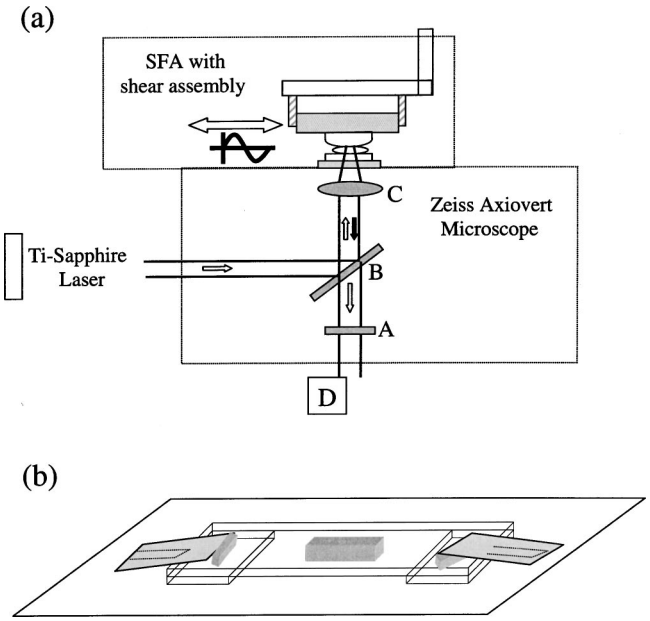

FIG. 1. (a) Schematic diagram of the assembly by which to perform fluorescence correlation spectroscopy within a surface forces apparatus equipped for shear experiments. A miniaturized surface forces apparatus sits on a commercial microscope stage. A mode-locked Ti–saphhire laser excites fluorescent dye molecules within a molecular thin liquid film contained between two opposed surfaces of muscovite mica. (A) Colored filter to remove the residual excitation light ($\lambda = 800$ nm) from the fluorescence light (400–550 nm) which is collected by the single photon counting module (D). (B) Dichroic mirror and (C) the objective lens used to focus and collect the light. An inchworm motor controls the separation of the surfaces from nanometers to up to several micrometers. (b) The small working distance of the microscope objective (~1.5 mm) requires significant modification of the box. The figure shows how the bottom lens (through which the excitation light enters and emitted light is collected) was attached with the SFA. It was clamped to the center of a stainless steel spring that itself was clamped onto the SFA box. The small gap (~0.2 mm) between the lens holder and the bottom surface of the sealed apparatus chamber box allowed the spring to deflect in the presence of surface forces or compressive forces applied by the motor.

The lateral dimension of the excitation spot was about ~0.35 μm, determined by a calibration experiment using a widely used dye, fluorescein (Aldrich), whose diffusion coefficient in water is known to be ~300 $\mu m^2/s$. The nonlinear process of two-photon excitation offers the advantage of in-

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herent spatial sectioning along the *z* direction, as only the focal plane receives sufficient intensity for significant absorption. The excitation power was at less than 1 mW to avoid photobleaching and heating effect of the sample. The typical concentration of the sample was 10 nM. Measurement time was limited to 45 min because photobleaching could not be avoided when the sample was illuminated for longer times.

The miniaturized SFA sat directly on the microscope stage. The traditional crossed-cylindrical geometry[2] produced a circular contact of parallel plates when the crossed cylinders were squeezed together such that they flattened at the apex. Using an inchworm motor (Burleigh IW-700), separation of the surfaces was controlled from nanometers to millimeters. To determine the separation between the surfaces we used multiple beam interferometry.[15] The operational details of the surface forces apparatus and the principle of surface separation measurement by multiple beam interferometry (MBI) have been described elsewhere.[1,15] A thin reflective layer on the backside of each mica sheet acted as an optical mirror, producing sharp interference fringes of equal chromatic order (FECO) in the focal plane of an imaging spectrograph (SP-500, Acton Research). Inspection of the FECO wavelengths enabled determination of the surface separation. But to integrate this technique with concurrent measurements using fluorescence spectroscopy required significant modification of the usual methods. In the following we discuss the challenges of combining SFA with spectroscopy.

IV. DESIGN REQUIREMENTS FOR FLUORESCENCE MEASUREMENT WITHIN A SURFACE FORCES APPARATUS

A. Challenges

In FCS experiments, the magnitude of the fluctuation autocorrelation function scales inversely with the number of fluorescent molecules in the observation volume, which requires that on the average there should be only a few dye molecules within the laser focus (which can be determined either by confocal optics or the two-photon excitation that we employed here).[10] The difficulty comes to detect and collect fluorescence efficiently and to separate it from background noise.

Background originates from many sources: Raman and Rayleigh scattering, fluorescence owing to impurities in the solvent, and—particularly for this experiment—the lens, glue, and mica (the glue attaches a cleaved mica sheet onto the supporting cylindrical lens). It is our experience that the typical counts from mica and glue can far exceed those from a dilute concentration of fluorophore molecules doped inside the sample of interest.

Another type of challenge comes from the limited photochemical lifetime of a fluorophore. A common fluorophore photobleaches irreversibly after emitting a finite number of photons (10^5–10^6). This problem becomes severe in the ultrathin films where the dynamics become extremely slow and a dye molecule resides for a long time within the laser focus.

A third difficulty, which is particular to experiments involving the SFA technique, is the necessity to perform spectroscopy at the same time as MBI to determine the film thickness. Traditionally a silver coating of thickness ~63 nm is used at the backside of mica for the purpose of MBI, but the high reflectivity of silver from the infrared to ultraviolet (UV) regime excludes this possibility here.

The final challenge is to incorporate the SFA and the needed optics. As the signal must be as large as possible, the maximum possible amount of fluorescence from the fluorophore of interest should be detected for a successful experiment. A high N.A. objective is desirable but such objectives have a very small working distance (~1–2 mm). This requires significant modification of the traditional SFA in order to make it possible to focus the laser beam on the sample.

B. Design solutions

We now discuss how we partially overcame all the problems mentioned in the earlier section, starting with the last problem mentioned above.

1. Surface forces apparatus

In our design, the SFA sits directly on the microscope stage. The working distance of the objective lens used is only ~1.5 mm. The cylindrical lenses we used (BK7) were the thinnest (center thickness ~1 mm) that we could obtain commercially (Edmund Industrial Optics). The large radius of curvature of the lens ($R \sim 2.6$ cm) minimized any significant distortion of the laser focus profile. The bottom lens was glued at the middle of a stainless steel spring (of thickness ~0.2 mm) which was supported on two thin stainless steel spring at both ends [Fig. 1(b)]. The whole assembly was then clamped at both ends and directly fixed with the SFA. The small (~0.2 mm) gap created this way between spring containing the lens and the bottom surface of the box allowed it to deflect vertically due to surface forces and with the application of the normal pressure as in the conventional surface forces apparatus.

The top cylindrical lens, which was oriented at right angle to the bottom cylindrical lens as in the traditional SFA, was suspended from the upper portion of the apparatus by two piezoelectric bimorphs, mounted symmetrically to two ends of the mount. Shear forces were generated by one of the piezoelectric bimorphs (the ''sender''), and the response of the device induced a voltage across the right-hand bimorph (the ''receiver''). The analysis of the response signal to determine the viscoelastic properties of the confined liquid film has been described previously.[16]

The traditional silver sheets for interferometric measurements of surface spacing in the SFA were replaced by multilayer dielectric coatings known in the field of optics but not previously applied to this purpose.[17] We produced these multilayers by successive evaporation of 13 layers of TiO_x and Al_2O_3 by electron-beam evaporation (inset, Fig. 2) at the Center of Microelectronics, University of Illinois. The optical thickness of each layer was approximately $\lambda/4$ ($\lambda \sim 650$ nm) as determined by the optical monitor within the coating chamber. The desired thickness of each coating was calculated using software (J. A. Woolam Co., Inc., Lincoln, NE) so that the windows of reflectivity and translucency

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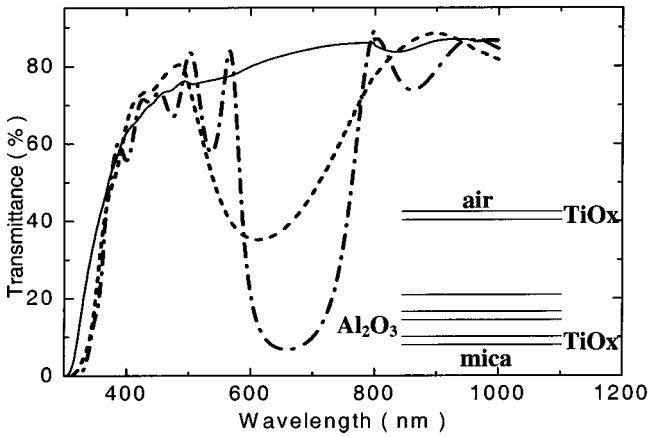

FIG. 2. Transmission spectra of mica with dielectric coating; bare mica (solid line), mica coated with seven layers of coating (dotted line) and with 13 layers of coating (dash–dot line). Thirteen layers were employed for the experiments shown in this paper. With successive layer of coating the reflectivity in the region 600–700 nm increased sharply, which allowed use of traditional multiple-beam interferometry of incident white light to measure the surface–surface separation. The large transmission in the region near 800 nm permitted fluorescence excitation and the large transmission in the region 400–550 nm enabled fluorescence detection. (Inset) The schematic diagram of the dielectric coating, composed of alternate layers of TiO_x (refractive index=2.6) and Al_2O_3 (refractive index=1.765).

were controlled by the deposition conditions. This approach produced high reflectivity in the region 600–700 nm, as well as translucent windows in the region ~800 nm (to allow fluorescence excitation) and 400–550 nm (to detect fluorescence). The reflectivity as a function of wavelength is shown in Fig. 2 for the bare mica surface and for surfaces with different number of multilayers.

The surface–surface separation was then measured by traditional multiple-beam interferometry of incident white light while at the same time affording translucency needed for the fluorescence studies. It allows one to excite fluorescence through one spectral window, detect the induced fluorescence through a second spectral window, and use multiple beam interferometry to determine the film thickness at a third spectral window.

2. Background and photobleaching

The problems associated with the background fluorescence and photobleaching were much more difficult to overcome. Every attempt was made to reduce the background fluorescence originating from the lens, glue, and mica. Prior to use, the lenses were kept in acid bath overnight, rinsed with de-ionized water, ultrasonicated in acetone, and finally rinsed with distilled ethanol before mounting. When not used they were stored in methylene chloride.

The adhesives that are traditionally used to attach mica sheets to a cylindrical lens (e.g., diphenylcarbazide or a mixture of galactose and fructose) could not be used for our application owing to the prohibitively large fluorescence that originated from multiphoton excitation in the presence of pulsed laser light. Instead we used NOA 63 (Norland Optics), an UV-cured optical glue (transparent above 350 nm), diluted with tetrahydrofuran at the ratio of 1:10 by volume for initial experiments. For later experiments, the glue was used as received.

An additional design consideration was to use two-photon excitation (TPE) to improve the signal-to-background ratio. Rather than rely on single-photon excitation using high-energy 400 nm photons to induce fluorescence, this strategy made it possible to excite the dye molecules using 800 nm photons of lower energy. As the most prominent scattering came from the incident light, well separated in wavelength from the induced fluorescence, this made it easy to separate the fluorescence emission from the excitation light and the scattered light. The TPE strategy also created a diffraction-limited small open volume element of ~1 fL by the focused laser beam, and this also reduced the background fluorescence. Unfortunately, the high power density at the laser focus reduced significantly the photochemical lifetime of the dye. Deoxygenating the sample before use and keeping the whole sample cell in a nitrogen atmosphere have been reported to reduce photobleaching significantly in the case of one-photon excitation, but consistent with other reports we found that it had little effect for (TPE).[18] To prolong the lifetime of the fluorophores we used the lowest possible power (<1 mW for most applications) and the most stable dyes available commercially.

V. EXPERIMENTAL RESULTS

As mentioned in Sec. I, the purpose of this experimental platform was to study the effect of confinement on the diffusion of small tracer molecules in ultrathin fluid films, and using this approach to contrast diffusion with friction. To demonstrate that our experimental setup is capable of measuring diffusion in 1-nm-thick fluid films, we now show experimental results in the bulk and in the confined fluid for one system.

Choice of a suitable experimental system for FCS applications requires some judgement. As the initial object of experiments using this platform, the liquid was chosen to be simple. The fluorescent tracer molecules should have low photobleaching rate and high quantum efficiency (~1). They should also dissolve readily in the solvent and should not adsorb at the surface. As a typical experiment can last for more than 12 h, the solvent should be nonvolatile, in order to avoid a time-dependent concentration change of the dye.

The typical signal-to-background ratio for the experiments described below was 1:5 before signal averaging and was improved by extensive signal averaging. The dark count from the detector was negligible (<20 counts/s). The typical thickness of the mica was between 2 and 4 μm. With our optical setup the background fluorescence from the mica (V2 quality, purchased from Lawrence and Co, MA) at the power of ~1 mW at the sample stage, was 200–2000 counts. This tenfold variability of background fluorescence probably stemmed from differing amounts of impurities at different spots within the mica sheets.

The effect of background on measurement of the autocorrelation function has been described extensively in the FCS literature.[19] The background does not alter the diffusion coefficient measurement, but it introduces a constant error

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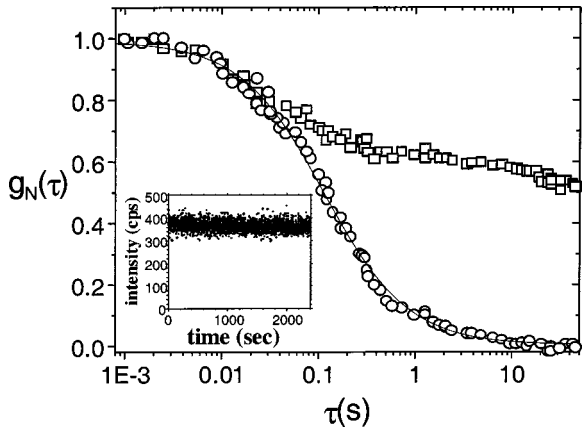

FIG. 3. Illustrations of autocorrelation functions (normalized to have the same value at the shortest times) for the system of 1,2-propane diol containing 40 nM rhodamine 123. The examples refer to a case of minimal photobleaching (circles) and significant photobleaching (squares). The effect of photobleaching is obvious; at long times, the autocorrelation function did not decay to zero. In the inset, fluorescent counts (counts/s) are plotted against time for a typical curve that showed minimal photobleaching (~2%–3% over the interval of averaging). No change in the autocorrelation function was observed with the change of laser power, indicating that the heating effect was negligible.

factor at all time scales in the autocorrelation curve $G(\tau)$ that leads to wrongly decreased apparent amplitude values $G(0)$, such that one overestimates the number of molecules within the laser focus.

One could account for this in principle if the photobleaching rate were known, but in practice it is difficult to separate quantitatively photobleaching and a diffusion process when a significant amount of photobleaching occurs during the measurement. From the definition of the autocorrelation function $G(\tau)=(\langle \delta I(t)\,\delta I(t+\tau)\rangle/\langle I\rangle^2)$, it is easy to verify that due to photobleaching the magnitude of the autocorrelation function will increase with time and that the value for $G(\tau\rightarrow\infty)$ will not be zero. Typically an autocorrelation curve with photobleaching of less than 10% should be analyzed. The effect of moderate photobleaching in the autocorrelation function is shown in Fig. 3, where it can be noted that the magnitude of the autocorrelation function $G(\tau\rightarrow\infty)$ does not decay to zero.

Note that the signal-to-noise ratio depends upon the square root on the total averaging time, so from this point of view, long averaging is desirable. But the longer the averaging, the larger the photobleaching. The problem can be alleviated by using fluorophores with high quantum efficiency and photo-stability, using samples with low background fluorescence, and low light intensity to excite fluorescence.

To illustrate a successful experiment, we now show the result of an experiment for 1,2-propane diol (Aldrich) containing 40 nM rhodamine 123 (Aldrich). Propane diol is a polar liquid (bulk viscosity ~40 CP at room temperature). The structure of the dye rhodamine is shown in the inset of Fig. 4. It has a quantum efficiency of ~0.9 and when mixed with this solvent, it does not adsorb to mica.[8] The autocorrelation curves in Fig. 4 were taken from data acquired with the same sampling frequency of 5 kHz and averaged over 45 mins. Note that we also observed similar findings for probe diffusion in a molecularly thin films of octamethylcyclotetrasiloxane, which has been extensively studied in surface forces measurements.[8]

The force–distance profile of propane diol is purely repulsive with the onset of repulsion starting at 2.8 nm. Control experiments showed that presence of small concentration of this dye did not change the rheological behavior of molecularly thin films of propane diol. To begin the experiment we first measured the dry thickness of mica by bringing the mica sheets into contact in air, and then inserted a small drop of liquid between the sheets after separating them.

The first measurement of the autocorrelation function, taken in the bulk, is shown in Fig. 4. The time at which the autocorrelation function decayed to half of its initial value provides a measure of the characteristic diffusion time (τ_D), the average residence time of the dye molecules within the calibrated laser focus. For bulk propane diol, we measured $\tau_D\sim 2$ ms, which gave the diffusion coefficient $D\sim 8\ \mu\mathrm{m}^2/\mathrm{s}$.[20]

Next we brought the two mica surfaces close together and found that the minimum spacing was ~2.5 nm; with the application of compressive force, the crossed mica cylinders flattened at their apex to produces a circular contact area of diameter ~20 μm. We equilibrated the compressed films for several hours and after that, the measurement of the autocorrelation function was performed around halfway between the center of the contact and the boundary.[8]

The comparison of bulk and thin-film response in Fig. 4 illustrates that the normalized autocorrelation function in the film (normalized to have equal value at short times) decayed on markedly slower times scales when the thin-film behavior was probed. If this is fit to a single Fickian diffusion process, the implied diffusion coefficient decreases by more than 2 orders of magnitude; this and other aspects of the physical interpretation are described elsewhere.[8] In this article we emphasize the instrumental aspects and note that the analysis of the magnitude of the autocorrelation function at $\tau\rightarrow 0$ implies that for the experiments performed here in the confined film, there was on the average $\leqslant 1$ molecule within the focal volume of the laser.

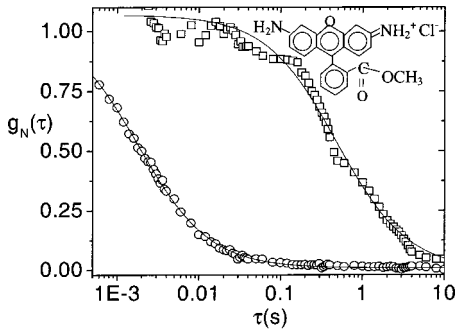

FIG. 4. Comparison of autocorrelation function in the bulk (circles), and at one spot within a film 2.5 nm thick, for 40 nM rhodamine 123 in the solvent 1,2-propane diol (squares). The magnitude of the autocorrelation function at the shortest times, $G(0)\sim 0.2$ for the thin-film experiment, implied that on average $\leqslant 1$ molecule resided within the focal volume of the laser. Analysis and interpretation of the differences between bulk and thin-film behavior has been presented elsewhere (Ref. 8). The inset shows the chemical structure of the rhodamine 123 dye. Coarse estimates of the length, width and thickness for the rhodamine 123 are $1.5\times 1.1\times 0.37$ nm.

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This implies that the FCS technique with TPE was capable of measuring diffusion in molecularly-thin films with single-molecule sensitivity. The combination of friction measurement and time-correlated fluorescence spectroscopy in the same apparatus has the potential to open up a class of experiments not previously possible regarding friction and lubrication. Although only the simplest systems have been studied to date, the same techniques can be applied to study aqueous systems, polymers, and systems of biological interest. Among the outstanding technological challenges are to employ these approaches to measure rotational diffusion times and dye lifetimes in similar confined geometries.

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