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Characterization of the Surface Contribution to Fluorescence Correlation Spectroscopy Measurements

Salina A. Chowdhury and Manho Lim*

Department of Chemistry and Chemistry Institute of Functional Materials, Pusan National University, Busan 609-735, Korea
**E-mail: mhlim@pusan.ac.kr*
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Fluorescence correlation spectroscopy (FCS) is a sophisticated and an accurate analytical technique used to study the diffusion of molecules in a solution at the single-molecule level. FCS is strongly affected by many factors such as the stability of the excitation power, photochemical processes, mismatch between the refractive indices, and variations in the cover glass thickness. We have studied FCS near the surface of a cover glass by using rhodamine 123 as a fluorescent probe and have observed that the surface has a strong influence on the measurements. The temporal autocorrelation of FCS decays with two characteristic times when the confocal detection volume is positioned near the surface of the cover glass. As the position of the detection volume is moved away from the surface, the FCS autocorrelation becomes one-component decaying; the characteristic time of the decay is the same as the faster-decaying component in the FCS autocorrelation near the surface. This observation suggests that the faster component can be attributed to the free diffusion of the probe molecules in the solution, while the slow component has its origin from the interaction between the probe molecules and the surface. We have characterized the surface contribution to the FCS measurements near the surface by changing the position of the detection volume relative to the surface. The influence of the surface on the diffusion of the probe molecules was monitored by changing the chemical properties of the surface. The surface contribution to the temporal autocorrelation of the FCS strongly depends on the chemical nature of the surface. The hydrophobicity of the surface is a major factor determining the surface influence on the free diffusion of the probe molecules near the surface.

Key Words: Fluorescence correlation spectroscopy, Rhodamine 123, Free diffusion, Surface-molecule interaction, Surface hydrophobicity

Introduction

Fluorescence correlation spectroscopy (FCS) has become the method of choice for investigating various molecular properties such as diffusion,[1-7] the conformational dynamics of proteins,[8-10] and binding and reaction kinetics[10-13] at the single-molecule level due to the development of excellent laser sources with high beam quality and stability, sophisticated detectors with low noise and high sensitivity, development of objectives with long working distances and high numerical apertures (NAs), and high-quality optics.[14,15] In FCS, signals are recorded as fluctuations in the fluorescence signal from a molecule passing through a detection volume, which is on the order of femtoliters. The detection volume is restricted by laser beam focusing and a confocal aperture. The confocal aperture allows only fluorescence photons coming from molecules in the focal plane to approach the detector.[16,17] The fluctuation in the fluorescence signal arises from many possible sources that are responsible for changing the number of molecules in the detection volume; these include molecular diffusion,[18] chemical reaction,[17-19] intersystem crossing to a triplet state,[20,21] and conformational motion that alters the fluorescence property.[22,23]

For studying the free diffusion of molecules, FCS experiments have usually been performed far from the sample-solution interface to avoid the influence of the surface. Recently, FCS has been widely used to study the kinetics of biocells near the surface to understand the nature of membranes.[4,6,9,24,25] When investigating the dynamics of living cells or studying the properties of cell membranes, one cannot avoid taking into account the surface contribution; this is referred to as *surface artifacts*.[26-28] The surface contribution needs to be characterized to get an actual picture of the dynamical properties at the cell membrane.[29]

To characterize the surface contribution to FCS measurements, we have carried out experiments at the surface of the cover glass by using rhodamine 123 (Rh123) as a probe molecule; this is a standard dye used in FCS studies of mitochondria to determine potential membrane permeability.[30] In the present study, we have investigated how the surface influences the temporal autocorrelation function (ACF) of FCS by following a long time tail of the ACF, up to a few hundred milliseconds. The degree of surface influence on the autocorrelation decay strongly depends on the chemical property of the surface, suggesting that the interaction between the probe molecules and the surface dictates the surface contribution.

Materials and Methods

Fluorescence Correlation Spectroscopy. FCS is a statistical and analytical tool based on the temporal ACF of the measured fluorescence intensity $F(t)$ emitted by a probe dye molecule (fluorophore). Fluctuations in the fluorescence intensity are characterized by the normalized temporal ACF $G(\tau)$ at time lag τ, which is given by[4,24,29,31]

$$G(\tau) = \frac{\langle F(t)F(t+\tau)\rangle}{\langle F(t)\rangle^2} = 1 + \frac{\langle \delta F(t)\delta F(t+\tau)\rangle}{\langle F(t)\rangle^2}, \quad (1)$$

where $\delta F(t) = F(t) - \langle F(t) \rangle$ denotes the intensity fluctuation at time t and $\langle\ \rangle$ the time average.

Any physical or chemical processes that change the concentration of fluorophores in the detection volume can contribute to the fluctuation in the fluorescence intensity. Molecular motions such as free diffusion, singlet-triplet intersystem crossing, and chemical reactions can change the number of fluorophores in the detection volume and thus cause fluctuations in the fluorescence intensity. The dynamics of specific physicochemical processes is connected to the temporal profile of the ACF. The contribution of molecular diffusion to the ACF is mainly in the time range of tens to hundreds of microseconds. For a single chemical species diffusing in a dilute solution with a three-dimensional (3D) Gaussian intensity profile, the ACF is given by the following equation:[32,33]

$$G(\tau) = 1 + \frac{1}{N}\left(1 + \frac{\tau}{\tau_D}\right)^{-1}\left(1 + \frac{\tau}{\kappa^2 \tau_D}\right)^{-1/2}, \quad (2)$$

where N is the average number of fluorophores in the detection volume, $\kappa = z_0/r_0$, and τ_D is the diffusion time of fluorescent molecules, which is related to the diffusion coefficient of the probe molecule, D, as $\tau_D = r_0^2/4D$. Here, a focused Gaussian beam was assumed to take the form of a prolate ellipsoid with a beam radius of r_0 and a beam height of z_0.[4,32,33]

A beam radius of r_0 can be readily obtained by measuring the image of a small, dye-coated bead. When the detection volume contains a surface and a probe molecule interacts with the surface, the ACF exhibits a contribution from fluorophores interacting with the surface, as well as freely diffusing fluorophores. For example, for a fluorophore undergoing free diffusion in a solution and two-dimensional (2D) diffusion due to the interaction with a surface in the presence of a triplet dynamics, the ACF is given by:[20,29,34]

$$G(\tau) = 1 + \left(\frac{N_f}{\left(N_f + N_{2D}\right)^2}\left(1 + \frac{\tau}{\tau_D^{free}}\right)^{-1}\left(1 + \frac{\tau}{\kappa^2 \tau_D^{free}}\right)^{-1/2} + \frac{N_{2D}}{\left(N_f + N_{2D}\right)^2}\left(1 + \frac{\tau}{\tau_D^{2D}}\right)^{-1} \right)\left(1 + \frac{T_{eq}}{1 - T_{eq}} e^{-\frac{\tau}{\tau_T}}\right), \quad (3)$$

where N_f and τ_D^{free} (N_{2D} and τ_D^{2D}) are the mean number of molecules and the diffusion time of freely diffusing (surface-interacting) fluorophores, respectively. The factor T_{eq} represents the fraction of fluorophores in the triplet state and τ_T is the triplet lifetime. In this formula, the fluorescence quantum yield was assumed to be independent of the diffusing environments of the fluorophores.[32]

Experimental Setup and Sample Preparation. All experimentation was carried out using a homebuilt stage-scanning single molecule spectrometer, shown schematically in Figure 1. It consisted of an inverted microscope (Nikon, ECLIPSE TE2000U) equipped with an *x-y* piezo actuator with a digital piezo controller (Physik Instrument, E-710 & P-733.2CL), a picosecond diode laser (Becker & Huckl GmbH, BDL-473-SMC) coupled

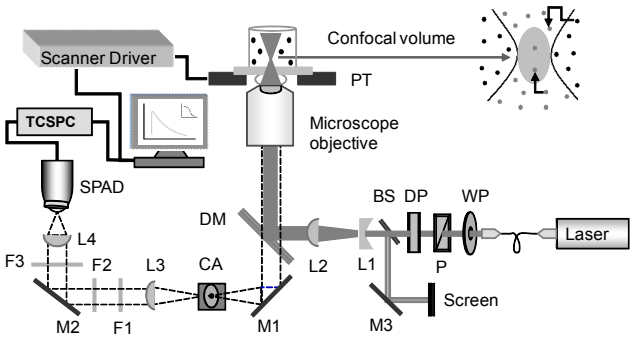

Figure 1. Schematic of our single molecule spectrometer. WP – wave plate; P – polarizer; DP – depolarizer; BS – beam sampler; DM – dichroic mirror; M1, M2, and M3 – mirrors; CA – confocal aperture; F1 – longpass filter; F2 and F3 – bandpass filters; L1, L2, L3, and L4 – lenses; SPAD – single photon avalanche diode; TCSPC – time-correlated single photon counter; PT – x-y piezo stage. The confocal volume is enlarged for better viewing.

with an optical fiber (Point-Source, kineFLEX-P-2-S-488-0.7-0.7-P2), a single photon avalanche diode (SPAD; Micro Photon Devices), a time-correlated single-photon counting (TCSPC) electronic (PicoQuant, TimeHarp 200), and a few optical components. The diode laser ran at 50 MHz, with a pulse width of *ca*. 60 ps at a wavelength of 470 nm. A single-mode optical fiber cleaned the laser pulse for a good Gaussian spatial profile. A combination of a half-wave plate (Thorlabs, λ/2 @ 400 - 850 nm) and a polarizer (Thorlabs, GT5) was used to continuously control the input intensity of the excitation light without changing the beam path. The laser pulse was then depolarized by a depolarizer (Thorlabs, WDPOL) to remove any influence from the polarization of the excitation source. A fraction of the light was reflected by a membrane beam sampler to characterize the input pulse. This membrane beam sampler also provided a reflection of the excitation light from the cover glass, which was used to monitor the microscope's objective position by checking the collimation of the reflected beam. Before entering into microscope, the laser beam was expanded to fill the aperture of the microscope objective. Finally, the laser beam was reflected by a dichroic mirror (Chroma, 505DCLP) and focused into the sample by a water immersion objective (60×, NA = 1.2; Nikon, plan-Apochromat). The fluorescence photons were collected through the same objective, transmitted through the dichroic mirror, and spatially filtered by a 50 μm confocal aperture (Thorlabs, P100S) that allows only fluorescence photons coming from the confocal detection volume. A pinhole with a 50 μm confocal aperture was enough to allow all the fluorescence originating from the focal spot and moving toward the detector. The spatially filtered fluorescence beam was then collimated by an achromatic doublet lens (Thorlabs, AC254-075-A1), passed through a long pass filter (Chroma, HQ 505LP) and two bandpass filters (Chroma, HQ 592/100m) to separate it from the scattered excitation light; it was then refocused onto the SPAD. The number of fluorescence photons was recorded by using a TCSPC electronic. This operated in the time-tagged time-resolved (TTTR) mode, which allowed us to record the arrival time of every detected photon with a temporal resolution in

the nanosecond range (macroscopic time), and its arrival time with respect to the last laser pulse with a picosecond timing resolution (TCSPC time).[35,36] The time delay between the excitation laser pulse and photon detection events gave direct information about the lifetime of the excited state of the molecules. All measurements and data analysis were performed using the SymPhoTime v.5.1.3 software (PicoQuant). The instrument was calibrated by measuring the diffusion coefficient of Rh123 in water.

The sample holder consisted of a cover glass (Corning, no.$1^1/_2$, 25 mm sq, thickness 0.17 mm) and a glass culture cylinder (Bioptechs, 14 mm × 5 mm).[15,35,37] Since background photons can easily outnumber photons from a single molecule, extreme care was taken to minimize any kind of contamination from solvent water, pipette tips, vials, cover slips, cylinders, *etc*., that could be a source of background photons. Sample holders were cleaned as follows. Firstly, they were immersed in a freshly prepared saturated NaOH solution and sonicated for 30 minutes. Then they were washed, soaked in concentrated H_2SO_4 solution, and sonicated at least for 30 minutes.[38] Finally, they were washed and sonicated in deionized water several times, dried with a flow of nitrogen gas, and kept in a UV oven overnight. If any exist, UV ray exposure decomposes large organic molecules that might emit background fluorescence. The deionized water (water filter system, Younglin, Aqua MAX-Basic 360 series and Ultra 370 series) used here had a nominal resistance of R ~18.3 M.

The surface of the cover glass was chemically functionalized to control the degree of hydrophobicity at the glass-water interface. The bare cover glass acted as a slightly hydrophilic surface because it carried the -OH group. By silanization with CH_3- and NH_2-terminated silanes, we introduced two types of surfaces (see Figure 2), namely hydrophobic and moderately hydrophilic surfaces. To modify the surface, the glass surfaces were immersed for 15 hours in a 2% solution of dodecyltriethoxy silane (DTES, 95%, Aldrich) or (3-aminopropyl) triethoxysilane (APTES, 99%, Aldrich) in methanol (MeOH)[34,39] to form CH_3- and NH_2-terminated surfaces, respectively. After washed with MeOH solvent, the substrates were washed with deionized water, dried with a flow of N_2 gas, and heated at 40 °C to obtain a stronger saline bond. A large chamber made of a cover glass and a large cylinder held 10 ml silane solution to modify only one side of the cover glass while keeping the other side clean. To prevent the evaporation of the silane MeOH solution, the surface modification process was carried out in an airtight box.

FCS measurements were carried out on three different types of surfaces by using a 400 μL aqueous solution of nanomolar Rh123 in the sample chamber and with a laser excitation power of 75 μW, measured in front of the microscope. To evaluate the influence of the surface hydrophobicity on FCS measurements, data were collected at various positions of confocal volume, specifically z = 0, 0.5, 1.0, 1.5, 2, 3, 5, and 10 μm (see Figure 3). Initially the confocal volume was positioned at the glass-water interface (z = 0 μm) and data were collected over five consecutive 60 s scans. Then, as the position of the objective moved upward along the z-axis to change the position of the confocal volume from the surface to the solution, data were collected

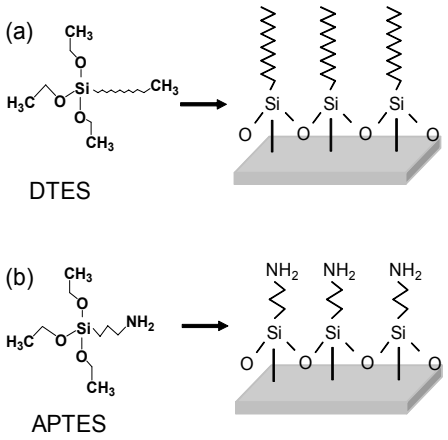

Figure 2. Schematic of the modified cover glass. (a) a dodecyltriethoxy silane (DTES)-terminated cover glass results in a strong hydrophobic surface and (b) a (3-aminopropyl) triethoxysilane (APTES)-terminated cover glass results in a moderately hydrophilic surface.

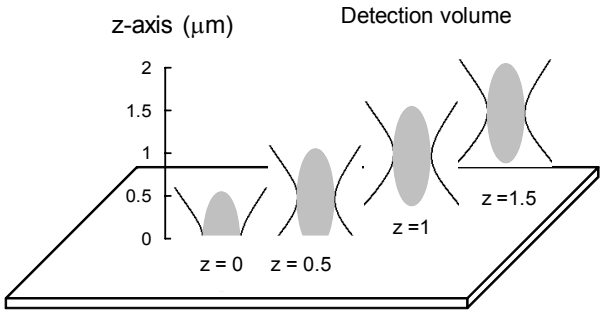

Figure 3. Schematic of the confocal detection volumes at various z positions. Note that the detection volume at z = 0 μm is half of that deep in the solution (z > 1 μm).

over three consecutive 600 s scans. For each z-position of the confocal volume, FCS was analyzed for an average of the temporal ACFs that were obtained from each scan.

Detection volume, estimated from the free diffusion of Rh123 in the solution (z = 10 μm, far enough from the surface-sample interface that the surface could not interfere with the free motion of Rh123 in the solution) with the observed diffusion time of 41 μs, was calculated to be ≈ 0.35 μm^3. This signifies that there was less than one molecule in the confocal volume for 1 nM of the solution. The focused beam size was confirmed by an independent measurement that scanned an image of a 45 nm dye-coated bead. The ACF of the single scan was calculated using SymPhoTime software; the averaging and global fitting were performed by MS Excel.

Results and Discussion

The temporal ACFs of the fluorescence fluctuation recorded on three different types of surface as a function of the position of the confocal volume are shown in Figure 4. When the confocal volume is deep in the solution (z = 10 μm), the ACF decays with a single decay characteristic and is well described by equation (2). When the confocal volume contains the water-surface interface, the ACF decay has a slow time contribution. The

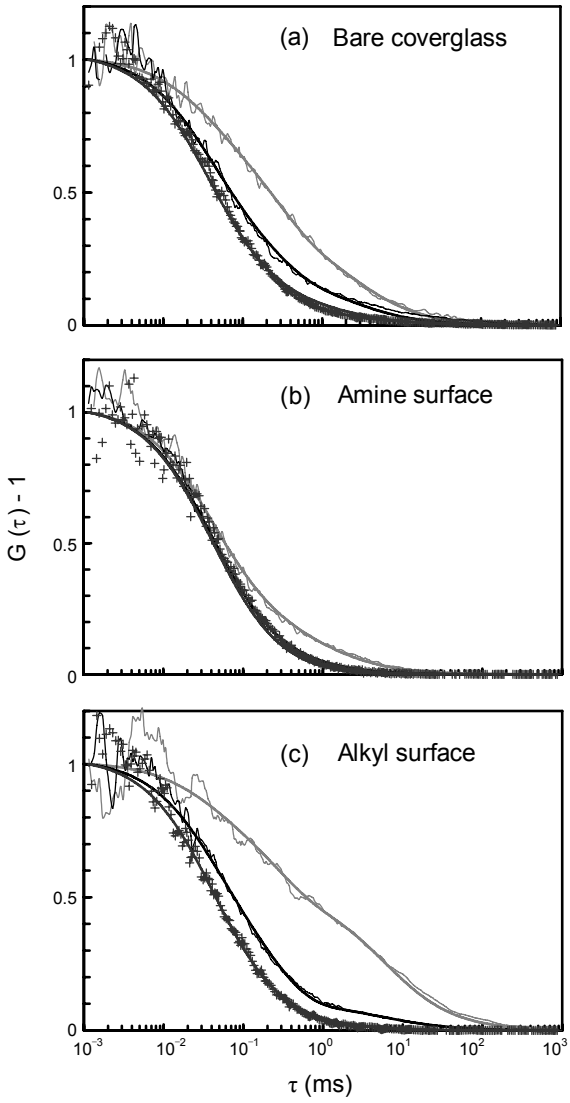

Figure 4. Representative normalized temporal ACF obtained for the confocal volume at z = 0 (thin gray line), 0.5 (thin black line), and 3 μm (crosses) recorded on (a) a bare cover glass, (b) amine-modified cover glass, and (c) an alkyl-modified cover glass. Data (thin solid lines and symbols) are well represented by equation (3) (thick solid lines).

slow decay time contribution is largest when z = 0 μm. As the position of the confocal volume moves up, the contribution of the slow component decreases and eventually the decay of ACF becomes the same as that deep in the solution. Clearly, the slow decay arises from the fluorophores interacting with the interface. The degree of surface contribution to ACF strongly depends on the chemical nature of the surface. For example, the ACF decay appears to have a slow time contribution when z < 1.5 μm with a bare cover glass, z < 1 μm with an amine-terminated cover glass, and z < 2 μm with an alkyl-terminated cover glass (see Figure 4).

Rh123 is a lipophilic, membrane-permeable molecule that contains a delocalized positive charge.[30] As a member of the xanthene dye family, it has three conjugated cycles that make it a hydrophobic molecule.[40,41] This means that its motion is influenced by the polarity of the surface when it is near the surface. The hydrophobicity of Rh123 is likely responsible for the difference observed in ACF calculated from data near the surface and a large depth in the solution. As shown in Figure 4, the temporal ACF measured at the surface is distinctly different from that for freely diffusing molecules in the solution. When a molecule interacts with the surface, it cannot move freely, and shows a long time tail on the temporal ACF. The temporal ACF can have contributions from molecules interacting with surface (slowly decaying component) as well as freely diffusing ones. Since the free diffusion of a molecule does not depend on the chemical nature of the surface, only the slowly decaying component of the temporal ACF varies with surface modification.

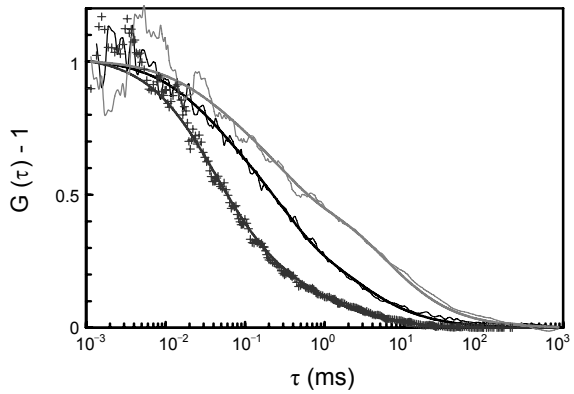

Figure 5. Normalized temporal ACF at z = 0 μm, the position of the confocal volume for bare cover glass (thin black line), amine-modified cover glass (crosses), and alkyl-modified cover glass (thin gray line). Thick lines are the fit.

The slowly decaying component can arise from interaction between the surface and molecules, such as that during a chemical reaction or an on/off reaction involving permanent or temporary adsorption of dye on the surface. A temporarily attached molecule stays in the focal volume for longer than the characteristic diffusion time of Rh123 in the solution, and has a 2D movement. Permanently adsorbed dyes will eventually be photobleached, or the chemical reaction leads to a removal of the fluorophore molecule; thus, the concentration of molecules slowly diminishes due to the photobleaching or chemical reaction. The count rate of fluorescence was found to be almost constant for hours of excitation, implying that there are no photobleaching or chemical reactions that change the fluorescence property as Rh123 interacts with the surface.

The influence of the chemical nature on the extent of the surface contribution to the ACF can be better viewed by comparing ACFs at the surfaces of various cover glasses. As shown in Figure 5, when the confocal volume was positioned at the surface of the cover glass, the contribution of the slow decay to ACF was the largest for the alkyl-terminated cover glass and the smallest for the amine-terminated cover glass. Clearly, the more hydrophobic the surface of the cover glass becomes, the slower the ACF decays. The slow decay component of the ACF was suggested to arise from the 2D diffusion of fluorophores due to their interaction with the surface.[29,34] To quantify the surface contribution, we made this assumption and modeled the measured ACF with equation (3). For a given cover glass,

all the ACFs for various z-positions were simultaneously fit to equation (3) with three local parameters (N_f, N_{2D}, and T_{eq}) and four global parameters (τ_D^{2D}, τ_T, τ_D^{free} and κ). Here, τ_D^{free} and κ are predetermined by globally fitting data collected in the solution ($z > 3$ μm) and then fixed in the final fitting. The recovered global parameters are $\tau_D^{free} = 41 \pm 4$ μs and $\kappa = 5.7 \pm 0.5$. The diffusion time of freely diffusing molecules with a calculated beam radius of $r_0 = 0.22$ μm results in a diffusion coefficient of Rh123 in water of $(3.0 \pm 0.3) \times 10^{-6}$ cm^2/s, which is consistent with the reported value in the literature, 3.0×10^{-6} cm^2/s.[42,43] The diffusion time of surface-interacting fluorophores was 2.3 ms for the bare cover glass, 1.3 ms for the amine-modified surface, and 5.9 ms for the alkyl-modified surface, suggesting that hydrophobicity is responsible for the interaction between the fluorophores and the surface and that the degree of retardation in molecular diffusion at the surface is proportional to the hydrophobicity of the surface. The diffusion time of surface-interacting molecules was 31 - 140 times slower than that of freely diffusing molecules. Clearly, the surface interaction dramatically slows down molecular diffusion near the surface. The recovered triplet state lifetime was 0.31 ± 0.03 ms, which is consistent with the literature reporting a few hundred μs.[30] Since the diffusion time of freely diffusing molecule is much smaller than the triplet state lifetime, transition to the triplet state (intersystem crossing) is negligible while the molecule is in detection volume when it is in solution. The recovered T_{eq} was 0 when all the molecules are in solution. The fitted T_{eq} increases up to 0.44 as the number of the molecule interacting with the surface increases. Clearly, when the molecule is exposed to the excitation for longer time due to the slow diffusion, more molecules go into the triplet state.

Figure 6 shows the evolution of the fraction of fluorescent molecules diffusing freely in the solution (f_f) and in 2D (f_{2D}) as a function of the position of the detection volume; here, $f_f = N_f / (N_f + N_{2D})$ and $f_{2D} = N_{2D} / (N_f + N_{2D})$. The fraction of molecules interacting with the surface strongly depends on the nature of the surface and rapidly decreases as the position of the detection volume moves further away from the surface. The influence of the surface weakens rapidly when the surface is an $-NH_2$-terminated hydrophilic surface, but a $-CH_3$-terminated hydrophobic surface retains its influence on molecular diffusion up to a few micrometers from the surface. Clearly, the hydrophobicity of the surface is the major factor determining the influence of the surface on molecular motion near it. Interestingly, a bare cover glass behaves as a hydrophobic surface up to 1.5 μm, suggesting that FCS measurements near the interface of the bare cover glass should consider the influence of the surface. Note that the influence of the interface disappears and the probe molecule undergoes a pure 3D diffusion in the solution when the position of the detection volume is at $z = 3$ μm and farther from the surface for all the surfaces we investigated. This suggests that the influence of the surface on the ACF can be avoided in FCS measurements if the detection volume is farther than 4 μm from the surface.

Figure 7 shows the normalized on- and off-time distribution of Rh123 for a bare cover glass; this distribution was calculated from fluorescence fluctuations recorded when the position of the detection volume was at the solution-glass interface ($z = 0$ μm) or deep in the solution ($z = 10$ μm). To calculate the on- and off-time distribution, we binned the fluorescence signal in a given time interval (binning time) and set a suitable threshold

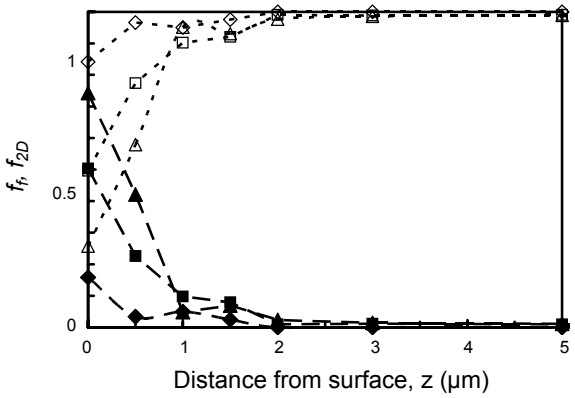

Figure 6. Evolution of the fraction of fluorescent molecules diffusing freely in the solution (f_f) and in 2D (f_{2D}) as a function of the position of the confocal volume for bare cover glass (squares), amine-modified cover glass (rhombuses), and alkyl-modified cover glass (triangles). Here, $f_f = N_f / (N_f + N_{2D})$ and $f_{2D} = N_{2D} / (N_f + N_{2D})$, where N_f and N_{2D} are the average numbers of freely diffusing and surface-interacting Rh123 molecules, respectively. Open symbols represent f_f and filled symbols f_{2D}. Dotted lines are to guide eyes for f_f and dashed lines for f_{2D}.

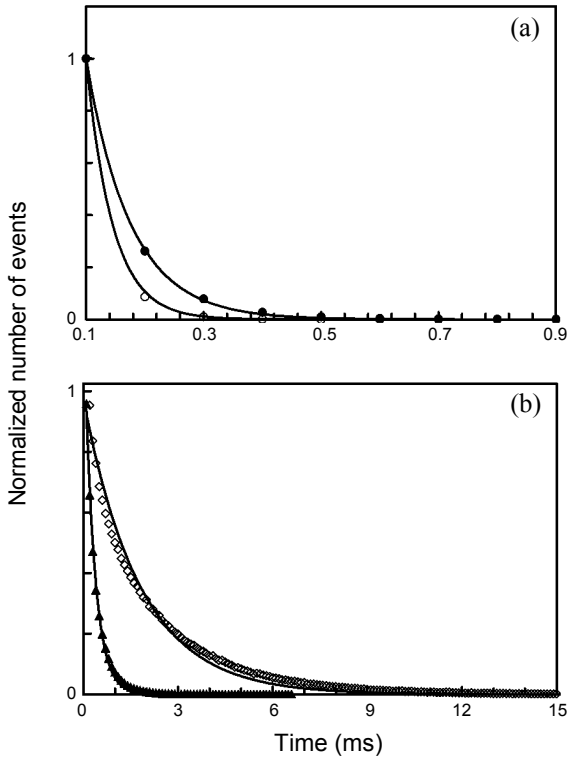

Figure 7. (a) On-time and (b) off-time distribution of fluorescence fluctuation with a 100 μs time bin for bare cover glass, the confocal volume of which was positioned at the surface-water interface ($z = 0$ μm, filled symbols) and deep in the solution ($z = 10$ μm, open symbols). The distribution is well described by an exponential function (solid lines), the characteristic time of which is the mean distribution time. The value at $t = 0$ was set to 1 for better comparison.

to differentiate the fluorescence signal from the background signal. A small binning time is ideal for a high time resolution, but the binning time has to be long enough to accumulate fluorescence photons that are distinguishable from background photons. A threshold should be set where the on- and off-time histogram does not change significantly with a slight change in the threshold.[25,44] For our data, 0.1 ms was the smallest possible binning time for an analyzable signal-to-noise ratio and a threshold of 1 was the suitable value. The on- and off-time distribution is well described by an exponential function, the characteristic time of which is the average distribution. The average on- and off-time for the freely diffusing fluorophores were 44 ± 4 μs and 1.8 ms, respectively. The mean on-time was almost identical to the diffusion time constant for the freely diffusing molecule, τ_D^{free}, which was found by fitting the data for the large depth in the solution to equation (3). The long off-time indicates that the sample used is very dilute and the fluorophore passes through the detection volume infrequently. The fluorescence signal for data collected at z = 0 μm contains fluorescence photons from molecules in the solution as well as from molecules interacting with the surface. Therefore, the on-time distribution of the fluorescence fluctuation data at z = 0 μm was modeled by a biexponential function, $p_1(0)\frac{1}{t_1^{ON}}\exp\left(-\frac{t}{t_1^{ON}}\right)+ p_2(0)\frac{1}{t_2^{ON}}\exp\left(-\frac{t}{t_2^{ON}}\right)$ where t_i^{ON} and $p_i(0)$ are the average times and the number of photons for the corresponding normalized exponential distribution, respectively. We also found that the average number of photons is about three times higher when z = 0 μm than when z = 10 μm. Since the focal volume at z = 0 μm is half of that in the solution (see Figure 2), only one-sixth of the photons originate from molecules in the solution; the rest come from molecules interacting with the surface. We fit the on-time distribution while fixing $p_1(0)/(p_1(0) + p_2(0)) = 1/6$ and found $t_1^{ON} = 44 \pm 4$ μs and $t_2^{ON} = 110 \pm 10$ μs. The on-time of molecules interacting with the surface is 2.5 times longer than that in the solution, which results in 2.5 times more fluorescent photons when there are equal numbers of fluorophores in the focal volume. The fact that six times more photons come from molecules interacting with the surface implies that there are more molecules at the surface than in the solution. This suggests that the Rh123 molecule has a tendency to stick to the bare cover glass, resulting in an increase in the dye concentration at the surface. The higher concentration of molecules near the surface can be also confirmed by the shorter average off-time for surface-interacting molecules. The average off-time of fluorescence fluctuation measured at z = 0 μm was found to be 0.32 ms, about six times shorter than that measured at z = 10 μm.

Since our data were collected in TCSPC, the fluorescence lifetime of Rh123 was obtained. When the dye molecule was deep in the solution (z = 10 μm), its fluorescence lifetime was independent of the modification to the cover glass; the lifetime was found to be 3.75 ± 0.05 ns, which is consistent with the reported fluorescence lifetime of Rh123 in water, 3.73 ns.[45] However, the fluorescence lifetime of Rh123 interacting with the surface strongly depends on the type of surface: 3.36 ns for the bare cover glass, 3.7 ns for an amine-modified surface, and 2.75 ns for an alkyl-modified surface. The lifetime decreases as the surface becomes more hydrophobic. A hydrophobic surface is likely to interact more strongly with hydrophobic molecules, resulting in a more dramatic change in the fluorescence lifetime of the molecules. Since there are two kinds of molecules when the detection volume is near surface, there are likely two fluorescence lifetimes. Our data do not allow a double exponential fit to the fluorescence decay with meaningful parameters. Therefore, the recovered lifetime with a single exponential fit is likely an average of two fluorescence lifetimes. Here, we view the fitted lifetime is the longer limit of the fluorescence lifetime of Rh123 interacting with the surface.

In conclusion, we have investigated the influence of the surface on FCS measurement by considering three types of surfaces and by using Rh123 dye as a fluorescent probe. The temporal ACF of the fluorescence fluctuation is strongly distorted by the influence of the surface when data are collected near the surface. Whereas the free diffusion time of Rh123 is 41 μs in our instrument, a slow diffusion time of a few milliseconds contributes to the ACF decay when data are collected near the surface of the cover glass. This slow component and the degree of contribution vary with the chemical nature of the surface and the position of the confocal volume. The contribution of the slow component is higher in the hydrophobic alkyl-terminated surface and is small in the hydrophilic amine-modified surface, suggesting that the interaction between the hydrophobic dye molecule and the hydrophobic surface is responsible for the slowly decaying component of the ACF. The surface contribution rapidly diminishes as the position of the confocal volume moves farther from the surface. For all the surfaces we investigated, the decay of ACF becomes identical to that in the solution when the position of the detection volume is farther than 4 μm from the surface. The bare cover glass behaves as a moderately hydrophobic surface and the slow component exists up to z = 1.5 μm from the surface. Our results suggest that the temporal ACF can be strongly affected by the chemical nature of the surface when FCS measurement is carried out near the interface at distances up to a few μm; thus, care has to be taken in the interpretation of FCS measurements near the interface, even when a bare cover glass is used.

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