Manipulating and probing the spatio-temporal dynamics of nanoparticles near surfaces

Minjoung Kyoung* and Erin D. Sheets*†‡

Department of Chemistry* and the Huck Institutes of the Life Sciences†
The Pennsylvania State University, University Park PA 16802 USA

ABSTRACT

In this report, we combine total internal reflection-fluorescence correlation spectroscopy (TIR-FCS) with a single optical trap to simultaneously manipulate and measure the dynamics of individual molecules near the substrate-solution interface. As a proof of principle, polystyrene particles (84 nm in diameter) are used as a model system to test our approach in studying their diffusion properties near surfaces, which are treated with polyethylene glycol 8000, bovine serum albumin or sodium hydroxide. The evanescent field of 543 nm excitation propagates ~100 nm into the solution, and the fluorescence detection is spatially confined by a 25 or 50 μm pinhole that is parfocal with the specimen plane. The optical trap is generated using a cw Ti:sapphire laser at 780 nm. Our results indicate that the particles’ diffusion is influenced by surface interactions, which might have further implications on biomembrane studies. Furthermore, the observed translational diffusion of individual particles can be manipulated using an optical trap. By combining the single molecule sensitivity of TIR-FCS with a noninvasive manipulation method, such as optical trapping, we will be able to probe molecular dynamics in biomimetic systems and living cells.

Keywords: total internal reflection, fluorescence correlation spectroscopy, optical trapping, evanescent depth, diffusion

1. INTRODUCTION

Molecular interactions at biological interfaces play important roles in many biological processes. Events based on receptor-ligand interactions in the cell membrane involve a variety of cellular communication and signaling processes, such as immunoreceptor [1, 2] and growth factor signaling [3], neurotransmitter signaling [4] and endo-[5] and exocytosis [6]. Desynchronized and stochastic properties of these biological events require single-molecule sensitivity without the population averaging inherent in conventional ensemble studies. As a result, single molecule studies allow us to understand the heterogeneous dynamics underpinning biological processes. Since the first reported detection of a single molecule in solution by Hirschfeld [7], extraordinary progress has been achieved to yield insight into the complexity of enzyme dynamics and kinetics [8, 9] and protein folding [10, 11]. In this paper, we demonstrate the feasibility of a new approach that combines TIR-FCS with optical trapping for measuring molecular interactions at solid/liquid interfaces with single molecule sensitivity.

TIR-FCS has enormous advantages over conventional fluorescence techniques for studying diffusion processes and chemical kinetics of equilibrated reactions at biological interfaces with single molecule sensitivity [12]. To obtain signals from fluorescently tagged individual molecules, it is crucial that the excitation volume is optically confined to minimize
background. When the excitation beam impinges at the interface between two media of different refractive indices at an
incident angle greater than the critical angle, a thin evanescent wave that propagates ~100 nm into the medium of lower
refractive index is generated, which can be used to excite fluorophores close to the substrate in TIR fluorescence
microscopy. FCS, whether in conventional confocal or TIR mode, can be used to measure the diffusion properties and
concentration of molecules in solution, on the membrane or inside the cell [12, 13]. Small fluctuations in fluorescence
occur spontaneously in an open system, which is in a state of thermodynamic equilibrium, due to stochastic processes
that cause changes in the fluorophores concentration (diffusion and chemical kinetics) or the fluorescence properties due
to conformational changes. These fluorescence fluctuations are correlated to obtain information about the average
number of fluorescent molecules in the detection volume, as well as the dynamic process leading to the fluctuations. The
latter is determined from the rate and shape of the temporal decay of the autocorrelation function. In confocal FCS, the
sample volume is defined by the diffraction limit of a focused laser beam, detection optics and a confocal pinhole.
However, the detection volume of TIR-FCS is defined by the depth of the evanescent field, which provides several times
higher resolution along the z-axis (~100 nm) as compared with the conventional approach with a pinhole (~1 μm).
Because of its advantage of greater discrimination along the z-axis, TIR-FCS has been used to measure diffusion of
proteins close to biomembranes and the kinetics of ligands binding to their receptors [14, 15].

A major challenge for studying single molecule interactions using TIR-FCS is the manipulation of individual molecules
in solution. Due to a fixed and confined detection volume and low concentration of the molecule of interest, it is not
efficient to collect the fluctuating signal from a single molecule. For single molecule studies, immobilization of the
molecule has allowed conformational changes of biomolecules to be studied [16], and various approaches of
manipulation have been used to segregate single molecules and sequentially transport them for analysis, such as isolating
the molecule using lipid vesicles [17] and trapping the molecules using electrophoretic [18], optical [19] and magnetic
[20] tweezers. To monitor the diffusional properties of molecules, optical trapping provides the most suitable
manipulation method for providing long residence times for TIR-FCS in a relatively simple way.

The development of our combined approach will help us understand molecular interactions at biological interfaces (e.g.,
biomembranes), which is key to explaining many biological processes, and the study of single molecules and single
molecular events offers unparalleled insight into the stochastic nature of individual biomolecule dynamics [17]. TIR-FCS
can measure molecular dynamics—diffusion and reaction kinetics—close to surfaces at the single molecule level [14];
however, due to the small (fL) detection volume, the observation of stochastic biological events is technically
demanding. The addition of an optical trap allows noninvasive manipulation of microscopic objects to the TIR-FCS
detection volume, which may facilitate the study of interfacial events at the single molecule level. As a proof-of-concept,
we measured the diffusion of trapped individual polystyrene particles (84 nm Φ) that are within ~100 nm of the glass-
water interface using TIR-FCS.

2. DESCRIPTION OF THE TIR-FCS/TRAP SET UP

A schematic description of our optical system is shown in Fig. 1. A HeNe laser (0.4 mW, 543 nm, Meredith Instruments)
was employed for prism-based TIR. The beam, which is steered using a periscope and focused with a focusing lens (f =
100 mm) located on a combined x, y and z-translation and rotation stage, passes through a fused silica cube that is
optically coupled, with glycerol, to a glass substrate to impinge on the solution/substrate interface at an angle greater
than the critical angle (Fig. 1). The evanescent wave propagates exponentially into the solution to selectively excite
fluorophores within ~100 nm of the substrate, and the depth $d$ of the evanescent field can be calculated as [21, 22]

$$d = \frac{\lambda_o}{4\pi} \left( n_2^2 \sin^2 \theta - n_1^2 \right)^{1/2}$$  \hspace{1cm} (1)

where $\lambda_o$ is the wavelength of the incident light in a vacuum; $\theta$ is the incident angle; and $n_1$, $n_2$ are the refractive indices
of the liquid and substrate, respectively. Different evanescent wave depths are achieved by adjusting the incident angle
using the upper periscope mirror.
The fluorescence fluctuation originating from the sample near the interface is collected through an objective (CFI Plan Apo IR 60×, 1.40 NA, Nikon). To confine the detection volume, a pinhole (25 or 50 μm in diameter) is placed immediately in front of a GaAsP PMT (H7421-40, Hamamatsu) in a plane conjugate to the sample (Fig. 1). The x-y position of the pinhole is adjusted using a custom mount to enhance detection efficiency. The fluctuations in fluorescence signal are counted in reciprocal counter mode and autocorrelated with the proper time interval. The autocorrelation of fluorescence fluctuations due to diffusion or chemical reactions is described by [13].

\[
G(\tau) = \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2}
\]

where \( F(t) \) is the fluorescence intensity at a given time \( t \); and \( \delta F(t) \) is the corresponding fluorescence fluctuation \( \langle \delta F(t) = F(t) - \langle F(t) \rangle \rangle \).

For optical trapping, an argon ion laser (Innova 90-6, Coherent [not shown]) is used to pump a continuous wave Ti:sapphire laser (899-01, Coherent) to produce an infrared output beam (700–830 nm range) whose radial intensity distribution is a Gaussian TEM\(_{00}\). A wavelength of 780 nm was chosen to minimize adsorption by water. To produce a clean, collimated Gaussian beam with a diameter that slightly overfilled the back aperture of the objective thus creating a tight focus at the sample plane, the beam is shaped by a spatial filter. The beam is then reflected to the back focal plane of the objective by a dichroic mirror located in a custom-designed infinity-space mount above the filter cube turret. The position of the detector pinhole is co-aligned with the trapping potential well to collect the fluctuating signal of a single trapped bead.

### 3. TIR-FCS OF PARTICLES EXCITED AT VARYING EVANESCENT DEPTHS

We needed to first characterize the lateral diffusion of fluorescent Nile red sulfate polystyrene (PS) nanospheres (84 nm \( \varnothing \); Invitrogen) that we used in subsequent TIR-FCS/trapping studies. The observation volume of our confocal FCS was
initially calibrated using 0.1–1 nM rhodamine green ($D = 2.8 \times 10^{-6}$ cm²/s, [23, and references therein]) using 488 nm excitation, which was then used to calculate the lateral diffusion of the PS in free solution. For subsequent FCS experiments using 543 nm excitation, the lateral diffusion of PS ($D = [3.4 \pm 0.9] \times 10^{-8}$ cm²/s, $n = 55$) was used to calibrate the system. To minimize nonspecific interactions between the PS particles and the substrate, we coated detergent-cleaned glass coverslips and slides with polyethylene glycol 8000 (PEG 8000) (10 mM in 50 mM Tris, pH 7.4, 100 mM NaCl; 1 h, room temperature, followed by extensive rinsing with water before use). PS particles were suspended in water for all FCS and trapping experiments described.

To characterize the TIR-FCS setup, we performed measurements at three different incident angles using 543 nm excitation (0.4 mW output power) and a 50 μm detector pinhole. To fit the TIR-FCS data, we used the following function [24]

$$G(\tau) = \frac{1}{2N} \left(1 + \frac{\tau}{\tau_{\omega}}\right)^{-1} \left[1 - \frac{\tau}{2\tau_{\omega}} \right] w \left[i\sqrt{\frac{\tau}{4\tau_{\omega}}} + \sqrt{\frac{\tau}{\pi\tau_{\omega}}}\right]$$

(3)

where $w = \exp(-x^2)erfc(-ix)$ and $x = i(\tau / 4\tau_{\omega})^{1/2}$; $N$ is the average number of particles in the detection volume; $\tau_{\omega}$ is the characteristic diffusion time along the $z$-axis; and $\tau_{\omega}$ is the characteristic diffusion time at the sample plane. The depth of the evanescent wave, $d$, and the diameter of detection volume, $\omega_{\omega}$, are calculated using $\tau_{\omega} = d^2 / 4D$ and $\tau_{\omega} = \omega_{\omega}^2 / 4D$, respectively. Increasing the incident angle of the excitation beam results in a corresponding decrease in the depth of the evanescent field (Eq. 1). Table 1 shows the theoretical and experimental penetration depths determined for three incident angles (Fig. 2). The observed changes in diffusion time with the angle of incidence can be attributed to

Table 1. Calculated and measured evanescent depths from three different angles of incidence.

<table>
<thead>
<tr>
<th>Incident angle</th>
<th>Theoretical depth of evanescent wave (nm)</th>
<th>Measured depth of evanescent wave (nm) b</th>
<th>Measured $D$ calculated from $\tau_{\omega}$ (×10⁻⁸ cm²/s) b</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>70.3°</td>
<td>82.6</td>
<td>111.0 ± 8.2</td>
<td>1.8 ± 0.5</td>
<td>35</td>
</tr>
<tr>
<td>71.9°</td>
<td>76.6</td>
<td>99.1 ± 5.5</td>
<td>2.1 ± 0.4</td>
<td>28</td>
</tr>
<tr>
<td>75.5°</td>
<td>68.6</td>
<td>84.7 ± 5.0</td>
<td>2.2 ± 0.5</td>
<td>25</td>
</tr>
</tbody>
</table>

a 10 nM PS in water, PEG 8000-coated substrates. b ± SD.
the expected increase of the observation volume. The diffusion time, $\tau$, was $0.90 \pm 0.13$ ms, $0.72 \pm 0.08$ ms and $0.53 \pm 0.06$ ms for $70.3^\circ$, $71.9^\circ$ and $75.5^\circ$, respectively. These values were used to estimate the diffusion coefficient in the $z$-dimension (Table 1). We found the diffusion of the PS nanospheres is reduced 35–46% when diffusing close to the substrate, as compared with PS particle diffusing freely in three dimensions (see above). These results agree with theoretical and experimental descriptions of a sphere diffusing within a few radii from the surface that show a reduced diffusion relative to the sphere in bulk liquid [25]. Furthermore, the measured depth represents an error of 23–34% with respect to the theoretical depth, which likely results from interactions between the PS particles and the PEG-coated glass surface. This deviation between measured and predicted evanescent depth agrees well with Harlepp et al. [26].

4. TIR-FCS OF PARTICLES DIFFUSING CLOSE TO MODIFIED SURFACES

To characterize particle-substrate surface interactions more fully, we performed TIR-FCS measurements of PS nanospheres using variously modified glass surfaces. The glass surfaces were coated with PEG 8000 (see above) and bovine serum albumin (BSA) (10 mg/mL in 50 mM Tris, pH 7.4, 100 mM NaCl; 1 h, room temperature, followed by extensive rinsing with water before use) by physisorption methods to reduce non-specific binding [27]. To increase the negative charge on the glass surface after detergent cleaning and thereby increase repulsion between the negatively charged PS nanospheres and substrate, we treated glass surfaces with NaOH solution (10 mM; 1 h, room temperature, and dried completely before use) [28]. The incident angle was 70.3° for all measurements, and the remaining experimental conditions were the same as for the previous section.

Figure 3. Representative TIR-FCS autocorrelation curves of freely diffusing particles (10 nM in water) close to modified surfaces. Circles, triangles and squares represent PEG 8000, NaOH and BSA modified glass surfaces, respectively. The incident angle was $70.3^\circ$.

Fig. 3 shows representative autocorrelation curves of particles diffusing close to the modified surfaces. Particles diffusing close to NaOH-treated and BSA-coated surfaces show similar diffusion properties along the $z$-axis, suggesting electrostatic repulsion between the PS particles and these surfaces due to the net negative charge of the NaOH- and BSA-coated surfaces. (Note that the pI of BSA is 4.7 [29], and the water used in these experiments is pH 5.6.) For diffusion in the $x$-$y$ plane, beads on BSA-coated surfaces exhibit a larger standard deviation as compared with the other surfaces, suggesting a non-uniform surface coating, which has been observed with AFM under different BSA incubation times (1 mg/mL, 10 min incubation) [30]. Unlike PS nanosphere diffusion close to NaOH- and BSA-treated surfaces, particles diffusing close to PEG-coated glass exhibit a slower diffusion due to the lack of a net surface charge and therefore weaker repulsive interaction forces [30, 31].
Table 2. Measured diffusion times of beads with different surface treatments.

<table>
<thead>
<tr>
<th>Surface treatment</th>
<th>Diffusion time along z-axis (ms)</th>
<th>Diffusion time in x-y plane (ms)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 8000</td>
<td>0.90 ± 0.13</td>
<td>44.94 ± 18.58</td>
<td>35</td>
</tr>
<tr>
<td>BSA</td>
<td>0.74 ± 0.06</td>
<td>68.94 ± 30.81</td>
<td>12</td>
</tr>
<tr>
<td>NaOH</td>
<td>0.79 ± 0.09</td>
<td>30.68 ± 5.91</td>
<td>24</td>
</tr>
</tbody>
</table>

*incident angle was 70.3°. b ± SD.

5. TRAPPING OF INDIVIDUAL NANOSPHERES CLOSE TO THE SURFACE

Individual PS beads (10 pM in water) were trapped in three dimensions near the glass surface using a 780 nm laser (~40 mW at sample plane). By imaging the particles using TIR excitation (543 nm, 0.4 mW; 0.5 s exposure, 0.7 s interval), we determined that the nanospheres could be successfully trapped as indicated by their stability at the trap position. Time-lapse CCD images were analyzed by line-scanning across each of the trapped particle images and integrating them over time. In this way, the fluorescence intensity of the trapped particles was calculated. Fig. 4 shows a representative trace of successive trapping of individual particles.

Figure 4. Successive trapping of individual polystyrene particles. The stepwise increases (Δ) in fluorescence intensity indicate when single particles diffuse into the potential well and were trapped.

6. SINGLE PARTICLE DIFFUSION IN A POTENTIAL WELL NEAR THE SURFACE

TIR-FCS was performed on single trapped particles; however due to the small number of particles, the noise level was higher than for TIR-FCS of freely diffusing particles. To reduce the possibility of collecting fluorescence signal from

Figure 5. Averaged TIR-FCS autocorrelation curve of single trapped particles (1–10 pM in water). 58 curves were averaged to present this unnormalized data. Black circles represent the averaged data and solid line indicates the fitted data. Experiments were performed on 1–10 pM bead solutions. Note from Eq. 3, $G(0) = 1/2N$; the average number of particles trapped is $0.42 ± 0.02$. 
outside the potential well, a 25 μm pinhole was used to confine the detection diameter to 450 nm in the x-y plane. By averaging the TIR-FCS curves of many individual trapped particles (Fig. 5) near the PEG-coated glass surface and fitting the resultant curves, we obtained diffusion times of 0.73 ± 0.11 ms and 45.1 ± 13.0 ms along the z-axis and in the x-y plane, respectively. The averaged number of beads in the detection volume was 0.42 ± 0.02, as determined fitting the averaged curve, which indicates a single particle is trapped in the potential well at a time. The less than unity number of particles trapped results from the larger spatial confinement of the optical trapping as compared to the detection confinement along the z-axis. The diffusion time was not significantly changed as compared to the untrapped beads on a PEG-coated surface (Table 2) along the z-axis, which was expected since the depth of evanescent wave was not changed. However, considering the effect of changing the pinhole size, the diffusion time in the x-y plane was increased. Using the relationship between diffusion time and diffusion coefficient ($\tau = \omega_0^2 / 4D$), the measured diffusion coefficient was ~24% less than the diffusion coefficient of freely diffusing particles not in a potential well (Table 2), which is likely due to the average position of the single bead being closer to the surface as a result of trapping.

7. CONCLUSION
We have reported the diffusion of single polystyrene nanospheres in potential wells near surfaces using a combination of TIR-FCS and optical trapping. After calibrating the relationship between evanescent wave depth and incident angle of excitation, we used TIR-FCS to measure the effect of different surface treatments on the diffusion of the PS particles. Individual particles could be trapped and their diffusion determined with TIR-FCS on a single trapped particle. In general we observe a reduction in diffusion when particles are close to the substrate, as compared with when they are in bulk solution. We attribute this retardation to electrostatic and other nonspecific interactions. TIR-FCS/trapping provides single molecule sensitivity and noninvasive manipulation to study single molecule events on surfaces, and this approach may be useful for biological investigations.

ACKNOWLEDGEMENTS
We thank Ahmed A. Heikal (Penn State University) for his invaluable comments and advice on all aspects of this work and Gabriel C. Spalding (Illinois Wesleyan) for helpful advice regarding optical trapping. Angel M. Davey (Penn State University) was a useful sounding board and provided excellent editorial advice for the preparation of this manuscript. This work was supported, in part, by the Pennsylvania State University, the Penn State Materials Research Institute and the Penn State MRSEC (under NSF grant DMR 0213623), and the Lehigh-Penn State Center for Optical Technologies, which is supported by the Commonwealth of Pennsylvania. Acknowledgment is also made to the Donors of the American Chemical Society Petroleum Research Fund, for partial support of this research. MK also thanks SPIE for their generous support, which allowed her to present this work at the 2006 Optics and Photonics Meeting in San Diego CA.

REFERENCES


