Published in final edited form as:
J Phys Chem B. 2006 November 16; 110(45): 22557–22562. doi:10.1021/jp060402e.

Enhanced Fluorescence of Cy5-Labeled Oligonucleotides Near Silver Island Films: A Distance Effect Study Using Single Molecule Spectroscopy

Yi Fu and **Joseph R. Lakowicz***
Center for Fluorescence Spectroscopy, University of Maryland School of Medicine, Department of Biochemistry and Molecular Biology, 725 West Lombard Street, Baltimore, Maryland 21201

Abstract

We investigated fluorescence enhancements and lifetime reductions of Cy5 probe molecules at various distances from the deposited silver island film surface using single molecule spectroscopic methods. The proximity of fluorophore molecules to the surface was controlled by alternating layers of biotinylated bovine serum albumin (BSA-biotin) and avidin, followed by binding of Cy5-labeled oligonucleotides to the top of a BSA-biotin layer structure. We observed dramatically varied brightness of fluorophores with distances from metal structures as well with reduced blinking in the presence of silver island films. In addition, distributions of fluorescence lifetimes and apparent emission intensities from individual molecules indicate an inhomogeneous nature of local matrix surface near metallic nanostructures. These studies illustrate the exclusive information that is otherwise hidden in ensemble measurements.

Introduction

DNA micoarray techniques based on hybridization of complementary nucleic acids have been widely used in biotechnology and gene expressions.[1–3] In most such assays, the target sequence is detected by binding to fluorescently labeled oligonucleotides, which are specific for a sequence in the target DNA.[4] Since the dye-labeled DNA is only present on the surface, detection sensitivities are limited by the spectra properties and the photostability of the fluorophores. In addition, background noise from sample matrixes or instrumentation can result in a failure to detect weak signals. Among the different approaches being developed in attempting to overcome these issues, surface-enhanced Raman scattering (SERS)[5,6] and surface-enhanced fluorescence (SEF)[7–11] have been proved to be two potential detection methods for analytical applications. In contrast to SERS, surface-enhanced fluorescence is observed from molecules at a certain distance from the metallic surface.

The interactions of fluorophores with metallic particles have been studied theoretically[12–14] and empirically[15–20] since 1980s. In these reports, the major concern was the dependence of the emission rate on the distance between the dye layer and the metallic surface. The emission can be quenched due to radiation energy transfer to the metal as molecules adsorbed directly on the surface. Another consideration is the enhanced fluorescence due to increased electromagnetic field of surface plasmon and/or enhanced quantum yield.[19] The enhancement

*Corresponding author. lakowicz@cfs.umbi.umd.edu. Tel: (410)706-8409. Fax: (410)706-8408.

Supporting Information Available: The time traces presented in Figure 2 are analyzed by preparing histograms of the emission count rates. Dependence of emission intensity on the incident laser power. Brightness distributions from various proteins layers on glass and on SIFs. The material is available free of charge via the Internet at http://pubs.acs.org.

of fluorescence intensity generally depends on the surface features and the optimal distance of fluorophores from the surface.[9,16–18] Shifting a fluorophore only a few nanometers away from the metallic surface may lead to an enormous emission change by orders of magnitude. In addition, the strong energy transfer from the excited molecules to the metal particles or an increase in the radiative decay rate of the fluorophore can dramatically shorten the lifetime of the excited state.[16,21,22] This effect increases the number of excitation cycles a molecule can survive until photobleaching. As a result, a dramatic increase in the number of photons can be observed from a single fluorophore.[23]

In our opinion, the use of metallic particles to enhance fluorescence has great potential for advances in medical diagnostics and biosensor fields. In this work, we examined the effects of fluorophore-to-metal distance on enhanced fluorescence using the single molecule spectroscopy (SMS) technique. The main advantage of SMS method is its ability to observe phenomena hidden in ensemble measurements.[10,24–27] A single molecule can be a reporter of its local environment. The reporter properties of the fluorophores can be exploited to reveal inherent differences in local environments or to monitor time-dependent changes in the host. SMS completely removes the ensemble averaging and allows construction of actual distributions of the spectral parameters. Such details of the underlying distribution become crucially important when the system under study is heterogeneous. The technique also shows enormous potential for exploring the underlying nanoenviroments in biological systems. For biomolecules, heterogeneity can easily arise if various individual copies of a protein or oligonucleotides are in different folded shapes, different configurations. With the advent of single-molecule fluorescence technology, it becomes possible to study the emission of a single fluorophores and its interaction with interface.

One of the most commonly used approaches to investigate the distance dependence is the self-assembly and self-organization of protein layers.[7,11] In this experiment, we employed alternating monolayers of biotinylated bovine serum albumin (BSA) and avidin (Scheme 1). It is well-known that BSA adsorbs as a monolayer onto glass or silver surfaces, and the subsequent exposure to avidin and then biotinylated BSA results in additional protein monolayer.[7] The use of silver nanostructure increases the sensitivity of DNA detection, especially at single molecule levels. Time-dependent intensity data were extracted to characterize dynamic emission properties of fluorophores at various distances from silver nanostrutures. The experiment results suggest potential applications of the use as single molecule imaging in biotechnology applications.

Experiment Section

Sample Preparation

The oligonucleotides labeled with biotin, Cy5 (Biotin-3′-AGG-TGT-ATG-ACC-GGT-AGA-AG-5′-Cy5) were obtained from the Biopolymer Shared Service at the University of Maryland, School of Medicine. Nanopure water (>18.0 MΩ), purified using the Millipore Milli-Q gradient system, was used for all experiments. All buffer components were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. The coverslips (18 × 18 μm, Corning) used in the experiments were first soaked in a 10:1 (v:v) mixture of concentrated H_2SO_4 and 30% H_2O_2 overnight, extensively rinsed with water, sonicated in absolute ethanol for 2 min, and dried with air stream. The purity was checked by fluorescence measurements at single molecule levels.

Silver island films (SIFs) were deposited on cleaned glass coverslips by reduction of silver nitrate as reported previously.[21] The formed silver island films are greenish and nonreflective. Only one side of each slide was coated with SIF. The particles are typically 100–500 nm across and 70 nm high covering about 20% of the surface.[28]

Preparation of BSA-Biotin Modified Slides

Buffer components were purchased from Sigma-Aldrich. A solution of albumin, bovine-biotin labeled (Sigma, 1 mg/mL, 100 μL) was applied onto the surface and the slide was incubated in a humid chamber overnight at 5 °C and then extensively washed with distilled water and dried. A 0.1 × PBS solution of avidin (egg white, Molecular Probes) (1 mg/mL, 100 μL) was deposited and the slide was incubated for 1 h and washed extensively with 0.1 × PBS buffer. For multilayer formation, the above procedures were repeated the necessary number of times using 40–50-minute exposure times.

Immobilization of Cy5-Oligonucleotides

To investigate the fluorescence enhancement, both SIF coated coverslips and bare glass coverslips with identical BSA-biotin/avidin layers were incubated in a solution of biotin-Cy5-labeled oligonucleotides in 10 mM HEPS buffer (pH 7) for 1 h. The dye concentration was maintained at nanomolar levels (0.2–0.5 nM). After incubation, the dye-immobilized glass coverslips were thoroughly rinsed with HEPS buffer solution to remove loosely bound dye molecules.

Single Molecule Experiments

All single molecule studies were performed using a time-resolved confocal microscopy (MicroTime 200, PicoQuant). A single mode pulsed laser diode (635 nm, 100 ps, 40 MHz) (PDL800, PicoQuant) was used as the excitation light. The collimated laser beam was spectrally filtered by an excitation filter (D637/10, Chroma) before directing into an inverted microscope (Olympus, IX 71). An oil immersion objective (Olympus, 100×, 1.3NA) was used both for focusing laser light onto sample and collecting fluorescence emission from the sample. The fluorescence that passed a dichroic mirror (Q655LP, Chroma) was focused onto a 75 μm pinhole for spatial filtering to reject out-of-focus signals and then reached the single photon avalanche diode (SPAD) (SPCM-AQR-14, Perkin-Elmer Inc). Images were recorded by raster scanning (in a bidirectional fashion) the sample over the focused spot of the incident laser with a pixel integration of 0.6 ms. The excitation power into the microscope was maintained less than 1 μW. Time-dependent fluorescence data were collected with a dwell time of 50 ms. The data were stored in a time-tagged-time-resolved (TTTR) mode, which allows recording every detected photon with its individual timing information. Instrument response function (IRF) widths of about 300 ps fwhm can be obtained in combination with a pulsed diode laser, which permits the recording of sub-nanosecond fluorescence lifetimes extendable to less than 100 ps with reconvolution. Lifetimes were estimated by fitting to a χ^2 value of less than 1.2 and with a residuals trace that was fully symmetrical about the zero axis. All measurements were performed in a dark compartment at room temperature.

Results and Discussion

Imaging Experiments

Typical fluorescence images of 10× 10 μm regions recorded for films with a single BSA-biotin/avidin layer are illustrated in Figure 1. Images shown were recorded primarily for locating single molecules for further time dependent analysis. Each pixel has a 0.6 ms dwell time and the total number of photons counted in that time was displayed in a colorized gray scale, ranging from dark (fewer integrated photons) to light. Typical background signals in these images are less 1 count per pixel. These images provide initial, qualitative information on local sample properties. Figure 1A shows a representative fluorescence image of a dye-immobilized BSA-biotin/avidin layer on a bare glass slide, while Figure 1, parts B, C, and D, depict such images for single BSA-biotin/avidin layer on SIF with varied immobilized dye concentrations. These images were recorded under the same experimental conditions except Figure 1, parts B, C, and

D, were excited under lower incident intensities. As extremely high emission rates were observed occasionally on samples in the presence of SIFs, the laser power was greatly reduced to avoid saturation of the detector during the experiment. A number of well-separated bright round spots with diffraction-limited sizes of ~300 nm were observed in individual images. Silvered substrates yield images that are clearly distinguishable by eye from the unsilvered substrate as shown in Figure 1. The noticeable strong emission intensities of fluorescent spots indicate preliminary information of enhanced fluorescence on silver island films. The density of fluorescent spots on the film increases as incubated dye-labeled oligonucleotide concentrations increase, providing strong proof that the bright spots are due to single dye-labeled oligonucleotides bound to BSA-biotin/avidin surface. SIF film itself has low absorption at the excitation wavelength at 640 nm and typically generates relatively weak fluorescence emission[28–30] which were invisible in such images under similar circumstance. In addition, the peak signal of emission intensity of bright spots observed from bare glass substrates showed somewhat uniform emission intensity distributions with a typical value of about 20 per pixel. On the contrary, emission intensities on silvered surface varied dramatically from 80 to 500 per pixel. As shown in Figure 1, parts B, C, and D, some spots are very bright while others are relatively dim. The intensity variations shown first indicate that dye molecules were located in heterogeneous environments in the presence of SIF.

Fluorescence Time Traces

Some representative fluorescence intensity time traces (50 ms resolution) from individual dye molecules immobilized on various numbers of BSA-biotin/avidin layers excited at 635 nm are presented in Figure 2. Individual molecules suffer photobleaching as evidence by the discrete drop in fluorescence intensity to the background level, providing strong evidence that single dye molecules had been observed,[31] which obviously distinguishes them from the clear nondestructive “blinking” pattern from single silver particles.[30] During the study, we have selected the molecules in as unbiased a fashion as possible. To be ensuring that each bright spot corresponds to a single molecule and not to a small cluster or to an impurity, all single spots were checked for single-step photo-bleaching to make certain that the collected emission was from a single molecule. The general appearance of the time trace is representative of more than half of those observed in the experiment. A particularly high emission count rate is observed in Figure 2A from a single layer of BSA-biotin/avidin on silvered substrate with the relatively low excitation intensity (~20 au), which is more than 10-fold from that observed in the absence of SIF with the comparatively high excitation intensity (~500 au). As the fluorescence emission intensity and excitation intensity are nearly proportional in the present study (Supporting Information Figure S2), the enhancement shown in Figure 2A is estimated to be around 250-fold. An examination of the statistical distribution of fluorescence intensity dependence of distance was carried out over a sample population of around 100 individual molecules. Normalized brightness histograms for a collection of single molecule time traces over a single BSA-biotin layer are illustrated in Figure 3. The derived mean brightness values from various BSA-biotin layers are summarized in Table 1. There is no evidence that emission intensity on the bare glass substrates is distance dependent. However, higher emission intensities were always observed on the SIF surface relative to those on bare glass substrate and the intensity levels drops sharply as the number of protein layers increased to 3. As noted previously, only low laser intensity was used for exciting a single dye-immobilized BSA-biotin layer on SIF, it is clear that there is a manyfold enhancement in the presence of SIF. The effect is due to an enhanced local electromagnetic field in this region caused by the free electrons in the metal and the incident light,[9] and dye molecules immobilized within should experience the strongest fluorescent enhancement. The magnitude of the intensity levels is dramatically variable as shown in Figure 3 and is most likely due to the spatial heterogeneity as the distances range of the molecules from the silver islands varies. As statistically depicted in Figure 3, around 35% molecules collected show more than 100-fold enhancement in emission.

With one BSA-biotin/avidin monolayer (Figure 2A), the emission intensity is fairly constant on the time scale of the binned integration time. Close examination of the time trace shown in top insert (Figure 2A) indicates slight deviations of average values occurring on a slow time scale in the order of 100 ms. The intensity time traces of most molecules on a single BSA layer does not exhibit a tendency for blinking, an "on" count rate was maintained which was relatively constant within the millisecond temporal resolution, up to the moment when a photobleaching process would take place, whereas molecules on double or triple layers exhibit long "off" durations of up to seconds (Figure 2B,C). As mentioned in the experiment part, dye-labeled BSA-biotin/avidin monolayer on silvered surface was investigated under relatively low excitation intensity. One argument is that less frequent blinking could be the result of lower excitation intensities. However, this hypothesis is contradicted to previous reports, which revealed shortened off-states due to the high pumping power.[32–34] Additionally, the examination of time traces from the dye immobilized monolayer on SIF with higher excitation power completely eliminates this hypothesis, as the time profiles provided no evidence that the blinking is dependent on the excitation intensity on silvered surfaces (data not shown).

Further evidence of reduced blinking behaviors on metal surfaces can be confirmed from samples with multiple protein layers, which were excited at similar intensity levels. As shown in Figure 2, dye immobilized on multiple protein layers were frequently switched "on" and "off" in a similar manner with duration of a few hundred milliseconds up to tens to seconds, from which the fluorophores return to their fluorescent state spontaneously and an increase in nonfluorescence periods is observed as protein layers increase. Processes such as molecular rotation, spectra shift due to local interactions, or conformational changes that modify the absorption and/or emission properties of the molecule can cause blinking behavior.[31,35] Other experimental artifacts could induce blinking as well. To analyze these time traces, we determined the off-times by applying a threshold to the data.[25,36–38] Histograms of off times for multilayers on glass and silvered surfaces are present in Figure 4, respectively. The distribution of off states and also significant differences in the time scale of this off state from molecule to molecule suggest that the neighboring environment of the fluorophore could play an important role. Blinking could be the results of interactions with the surface of the protein layer. Although the probe molecules were attached to DNA with a few nanometers away from the surface, the momentary interaction of fluorophores with nearby immobilized oligonucleotides and the protein layers cannot be expelled. It is known that BSA consists of 60 lysines;[35] these macromolecules can serve as potential binding sites for dye molecules. We assume that frequent blinking observed were induced by fluctuations in the interaction geometry of the collapsed fluorophore moiety and amino acid residues located in close distance as shown in Scheme 1. As the number of the protein layer increases, the layers could become partially misaligned at some spots. Consequently, blinking behaviors of Cy5 are becoming more frequent with additional BSA-biotin layers. These dyes interact with neighboring residue sites, and the blinking probably occurs via a reverse electron transfer reaction from the ground state residue to the first excited singlet state of the dye. The discrete jumps in the intensity suggest that the electron migration is sufficiently rapid. The "off" state with duration of tens to seconds could be associated with a stabilized nonfluorescent Cy5 adsorbed to neighboring biomolecules.[39] The duration of "dim" or "off" state would be most likely to be associated with the time of sticking event. As an additional effect with silvered surfaces, the metallic nanostructure introduces new electromagnetic decay channels to the system, rendering excitons incapable of accepting electrons from the residue and creating a relatively reductive environment. Accordingly, we observed reduced frequency of blinking and relatively constant photon emission proximity to silvered surfaces as demonstrated in Figure 2.

Fluorescence Lifetime

A fluorophore usually emits photons within nanoseconds after absorption of the incident photon. The decay law is often monoexponential and characterized by a natural lifetime

$$\tau=(\Gamma+k_{nr})^{-1} \quad (1)$$

where Γ and k_{nr} are the radiative and nonradiative decay rate, respectively. The changes in k_{nr} are typically due to changes in a fluorophore's environment, quenching or FRET.[21] The radiative decay rate Γ is essentially constant, and any changes are primarily due to changes in refractive index. The quantum yield (Φ_F) is the fraction of the excited fluorophores which decay by emission (Γ) relative to the total decay (Γ + k_{nr}). Proximity of fluorophores to metals can result in an increase in the total radiative decay rate by addition of a new rate Γ_m. The lifetime is then given by[40]

$$\tau_m=(\Gamma+\Gamma_m+k_{nr})^{-1} \quad (2)$$

Since Γ_m is a rate process returning the fluorophore to the ground state, the change in Γ_m results in remarkable effects such as the increase in quantum yield and decrease in lifetime as Γ_m becomes comparable larger than Γ.[21] We implemented the time-correlated single photon counting (TCSPC) measurement on a single fluorescent spot. Once a single molecule is microscopically selected, the standard technique of TCSPC is applied to measure delay times between excitation by a pulsed laser source and emission from a single molecule. The excited-state lifetimes of dye molecules can be determined.

Figure 5 shows a pair of histograms of distribution of single-molecule lifetimes obtained from a single BSA-biotin/avidin layer on the bare glass surface as well as in the presence of SIF. Each distribution contains data from approximately 100 molecules and is fit to a Gaussian function. The mean of the lifetime distribution on silvered surface is 0.32 ns, which is much shorter than the mean on glass, which is about 2.43 ns. This result shows that the radiative decay rate of the Cy5 molecules is much larger on the SIF than on glass. We also measured lifetimes of single dye molecules on multiple BSA-biotin layers on glass substrates and on silvered surfaces. The approximate mean values and widths for the fit distributions are presented in Table 2. As depicted from these data, the lifetime distribution changes dramatically with increased BSA-biotin layers on silvered surface. As the number of layers increases, the lifetime distribution shifts to longer values on average. The distribution obtained from one single BSA-biotin/avidin layer on silvered film most clearly illustrates changes in fluorescence lifetime induced by the strength of metallic electromagnetic field. The lifetime is about 1 order below that of electromagnetic inert surface, which is consistent with bulk experiments.[41] When depositing three BSA-biotin layers on SIF surface, the obtained lifetime distribution appears to be relatively symmetric, showing similar widths and lifetimes to those found on unsilvered surface, indicative of a strongly weakened and fairly similar electromagnetic field around these probes. The mean values of lifetime and widths of the histogram distributions from various protein layers on bare glass surfaces are generally comparable. This implies that local nanoenvironments of immobilized dyes on such protein layers are relatively homogeneous in the absence of silver metallic nanostructure. With a single protein layer on SIF, lifetimes are found especially scattered in the range of 0.2 ns to 1 ns. The skewed Gaussian distribution shown in Figure 5B indicates the presence of a mixture of nanoscale environments in the sample. The possible origins could arise from the existence of environments differing in the strength of electromagnetic interactions that occur between the silver islands and the dye molecules.

Conclusion

In summary, the single-molecule spectroscopic method has been employed to study the distance effect of Metal-enhanced fluorescence (MEF) near silver nanostructures. Dye-labeled oligomers were attached to an avidin layer and were inferred from the surface properties of the protein monolayer. The controlled pattern of ordered macromolecules has the potential application in the field of biotechnology. SMS methods ensure to provide detailed new information on the emission of a single fluorophore and its interaction with interfaces. Intensity and lifetime distributions reveal a mixture of spectral properties of dye-labeled oligonucleotides near silver nanostructures. We believe that these are caused by the sample heterogeneity. The immobilized dye probes are located randomly and distantly near metallic surface which could lead to such variations. By the use of protein layers, we found that the maximum increase in intensity and maximum decrease in lifetime from a single BSA-avidin layer which positions the fluorophore about 10 nm[7] from the surface. Hence the optimal distance for MEF can be readily obtained using a protein monolayer. More uniform intensity and lifetime distributions are observed with multiple protein layers on silvered surfaces. The nanometer changes in distance can be used to adjust emission properties of the fluorophore.

Another advantage of the single-molecule approach is that one can study fluctuations of the molecular behavior which are usually buried when performing bulk experiments. The most striking example studied is the so-call “blinking” behaviors of single fluorophores, when the molecule switches repeatedly between a fluorescent state and a nonfluorescent state. We suggest that frequent “off” durations from multiple protein layers can be explained by the coupling of dye molecules to the protein layer residues. It is evident that the presence of SIF influences the photophysics of single Cy5 molecules based on the observation of reduced blinking behaviors.

For Cy5-labeled oligonucleotides, we found that the intensity increases greatly and the lifetime decreases dramatically in proximity to the SIF surface. It is well-known that many factors can result in increased intensities from fluorophores.[42] The absolute and relative brightness of a fluorophore are dependent on the values of the radiative and nonradiative rates. Like a radiating antenna, a fluorophore acts as an oscillating dipole, and nearby metal surfaces can respond to the oscillating dipole and modify the rate of emission and the spatial distribution of the radiated energy. The simultaneously decreased lifetimes and increased intensities observed proximal to silvered surfaces indicate that the radiative decay rate increases to a comparable larger value near the silver nanostructure. Decreased lifetimes should result in higher photostability because there is less time for chemical reactions to occur in the excited state. In addition, reduced blinking likely occurs because there will be less time for the fluorophores to go to the triplet state. One might speculate the work presented has the potential of increasing detectability of single molecules bound to surfaces which contain metallic structures, for either biophysical studies or high sensitive assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Center for Research Resources, RR-08119 and NIH, EB-00682.

References and Notes

1. Schena M, Heller RA, Theriault TP, Konrad K, Lachenmerier E, Davis RW. Trends Biotechnol 1998;16:301. [PubMed: 9675914]

2. Brown PO, Botstein D. Nat Genet Suppl 1999;21:33.
3. Deyholos MK, Galbraith DW. Cytometry 2001;43:229. [PubMed: 11260590]
4. Lipschutz RJ, Fodor SPA, Gineras TR, Lockhart DJ. Nat Genet Suppl 1999;1:20.
5. Nie S, Emory SR. Science 1997;275:1102. [PubMed: 9027306]
6. Kneipp K, Wang Y, Kneipp HT, Perelman LT, Itzkan I. Phys Rev Lett 1997;78:1667.
7. Sokolov K, Chumanov G, Cotton TM. Anal Chem 1998;70:3898. [PubMed: 9751028]
8. Tarcha PJ, DeSaja-Gonzalez J, Rodriguez-LIorente S, Aroca R. Appl Spectrosc 1999;53:43.
9. Kummerlen J, Leitner A, Brunner H, Aussenegg FR, Wokaun A. Mol Phys 1993;80:1031.
10. Yokota HSK, Yanagida T. Phys Rev Lett 1998;80:4606.
11. Lakowicz JR, Malicka J, Gryczynski I. BioTechniques 2003:34. [PubMed: 12866402]
12. Enderlein J. Biophys J 2000;78:2151. [PubMed: 10733992]
13. Enderlein J. Chem Phys 1999;247:1.
14. Bouhelier A, Renger J, Beversluis MR, Novotny L. J Microsc 2003;210:220. [PubMed: 12787088]
15. Leitner A, Lippitch ME, Draxler S, Riegler M, Aussenegg FR. Appl Phys B: Laser Opt 1985;36:105.
16. Aussenegg FR, Leitner A, Lippotch ME, Reinisch H, Reigler M. Surf Sci 1987;139:935.
17. Kühn S, Håkanson U, Rogobete L, Sandoghdar V. Phys Rev Lett 2006;97:017402. [PubMed: 16907406]
18. Kramer A, Trabesinger W, Hecht B, Wild UP. Appl Phys Lett 2002;80:1652.
19. Stefani FD, Vasilev K, Bocchio N, Stoyanova N, Kreiter M. Phys Rev Lett 2005;94:023005. [PubMed: 15698172]
20. Anger P, Bharadwaj P, Novotny L. Phys Rev Lett 2006;96:11302.
21. Lakowicz JR, Malika J, Gryczynski I, Gryczynski Z, Geddes C. J Phys D: Appl Phys 2003;36:R240.
22. Enderlein J. Biophys J 2000;78:2151. [PubMed: 10733992]
23. Lakowicz JR, Gryczynski I, Shen Y, Malicka J, Gryczynski Z. Photon Spectra 2001:96.
24. Moerner WE, Fromm DP. Rev Sci Instrum 2003;74:3597.
25. Garcia-Parajo MF, Veerman J, Bouwhuis R, Vallee R, van Hulst NF. ChemPhysChem 2001;2:347.
26. Tinnefeld P, Sauer M. Angew Chem, Int Ed 2005;44:2642.
27. Weiss S. Science 1999;283:1676. [PubMed: 10073925]
28. Lakowicz JR, Shen Y, Gryczynski Z, D'Auria S, Gryczynski I. Biochem Biophys Res Commun 2001;286:875. [PubMed: 11527380]
29. Lakowicz JR, Kusba J, Shen Y, Malicka J, D'Auria S, Gryczynski Z, Gryczynski I. J Fluorescence 2003;13:69.
30. Peyser LA, Vinson AE, Bartko AP, Dickson RM. Science 2001;291:103. [PubMed: 11141556]
31. Panzer O, Gohde W, Fischer UC, Fuchs H, Mullen K. Adv Mater 1998;17:1469.
32. Haase M, Hubner CG, Reuther E, Herrmann A, Mullen K, Basche T. J Phys Chem B 2004:108.
33. Fleury L, Segura JM, Zumofen G, Hecht B, Wild UP. Phys Rev Lett 2000;84:1148. [PubMed: 11017465]
34. Vosch T, Cotlet M, Hofkens J, Van der Biest K, Lor M, Weston K, Tinnefeld P, Sauer M, Latterini L, Mullen K, De Schryver FC. J Phys Chem A 2003;107:6920.
35. Fureder-Kitzmuller E, Hesse J, Ebner A, Gruber HJ, Schutz GJ. Chem Phys Lett 2005;404:13.
36. Weston KD, Carson PJ, Metiu H, Buratto SK. J Chem Phys 1998;109:7474.
37. Kohn F, Hofkens J, Gronheid R, Van der Auweraer M, De Schryver FC. J Phys Chem A 2002;106:4808.
38. Yip W, Hu D, Yu J, Vanden Bout DA, Barbara PF. J Phys Chem A 1998;102:7564.
39. Wirth MJ, Swinton DJ. J Phys Chem B 2001;105:1472.
40. Lakowicz JR. Anal Biochem 2001;298:1. [PubMed: 11673890]
41. Malicka J, Gryczynski I, Gryczynski Z, Lakowicz JR. Anal Biochem 2003;315:57. [PubMed: 12672412]
42. Lakowicz JR, Shen Y, D'Auria S, Malicka J, Fang J, Gryczynski Z, Gryczynski I. Anal Biochem 2002:301. [PubMed: 12413464]

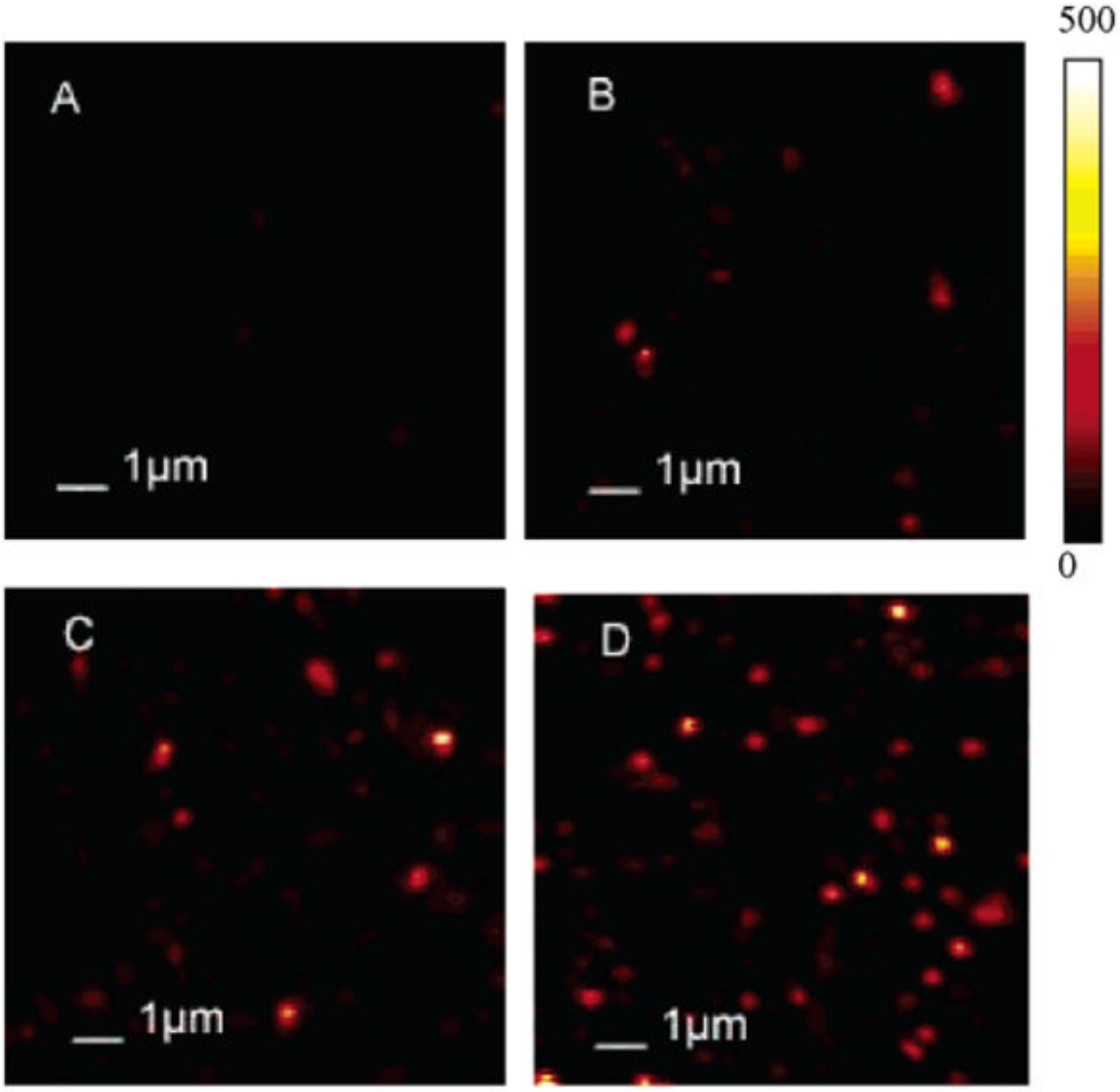


Figure 1.
Typical fluorescence images obtained from a single BSA-biotin/avidin layer. These two-dimensional 10× 10 μm images are 150 × 150 pixels. Each pixel has a 0.6 ms dwell time, and the fluorescence intensity is displayed in a colorized scale, ranging from dark to light. (a) Cy5-labeled oligomer on glass (laser intensity: 500 au, ~700 nW) with an incubated dye concentration of 0.5 nM; (b) Cy5-labeled oligomer on SIF (laser intensity: 20 au, 30 nW) with an incubated dye concentration of 0.5 nM; (c) Cy5-labeled oligomer on SIF (laser intensity: 20 au, 30 nW) with an incubated dye concentration of 1 nM; (d) Cy5-labeled oligomer on SIF (laser intensity: 20 au, 30 nW) with an incubated dye concentration of 1.5 nM.

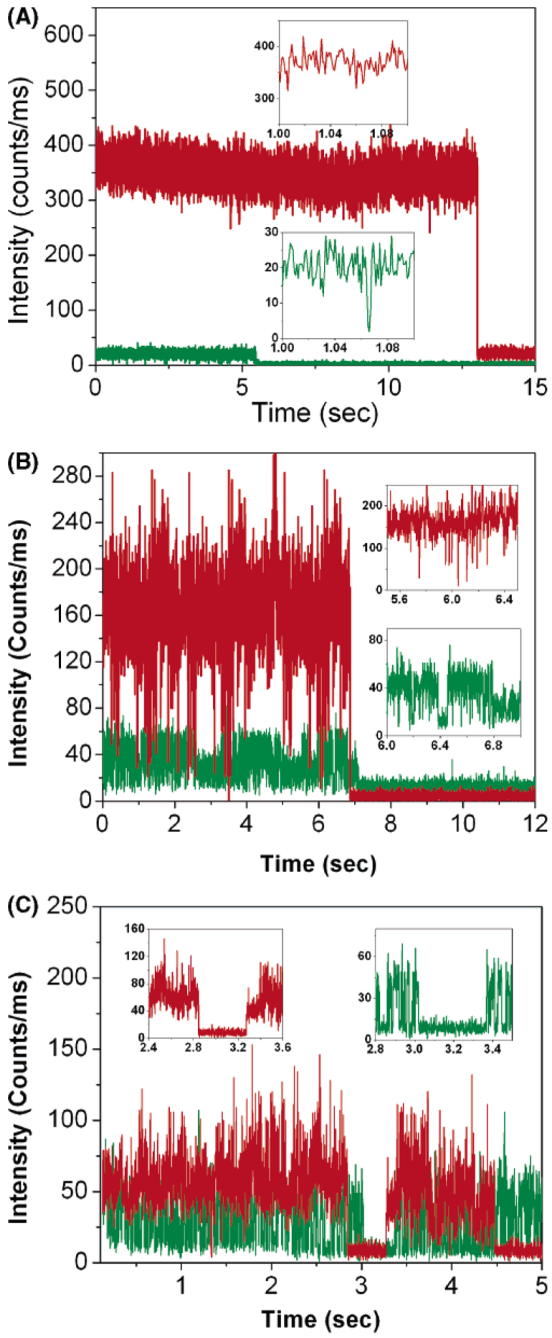


Figure 2.

(A) Representative fluorescence time traces and their expanded sections recorded for Cy5-labeled oligonucleotides bound to single BSA-biotin/avidin layers on glass substrate (green) and on SIF (red). (B) Representative fluorescence time traces and their expanded sections recorded for Cy5-labeled oligonucleotides bound to double BSA-biotin/avidin layers on glass substrate (green) and on SIF (red). (C) Representative fluorescence time traces and their expanded sections recorded for Cy5-labeled oligonucleotides bound to triple BSA-biotin/avidin layers on glass substrate (green) and on SIF (red).

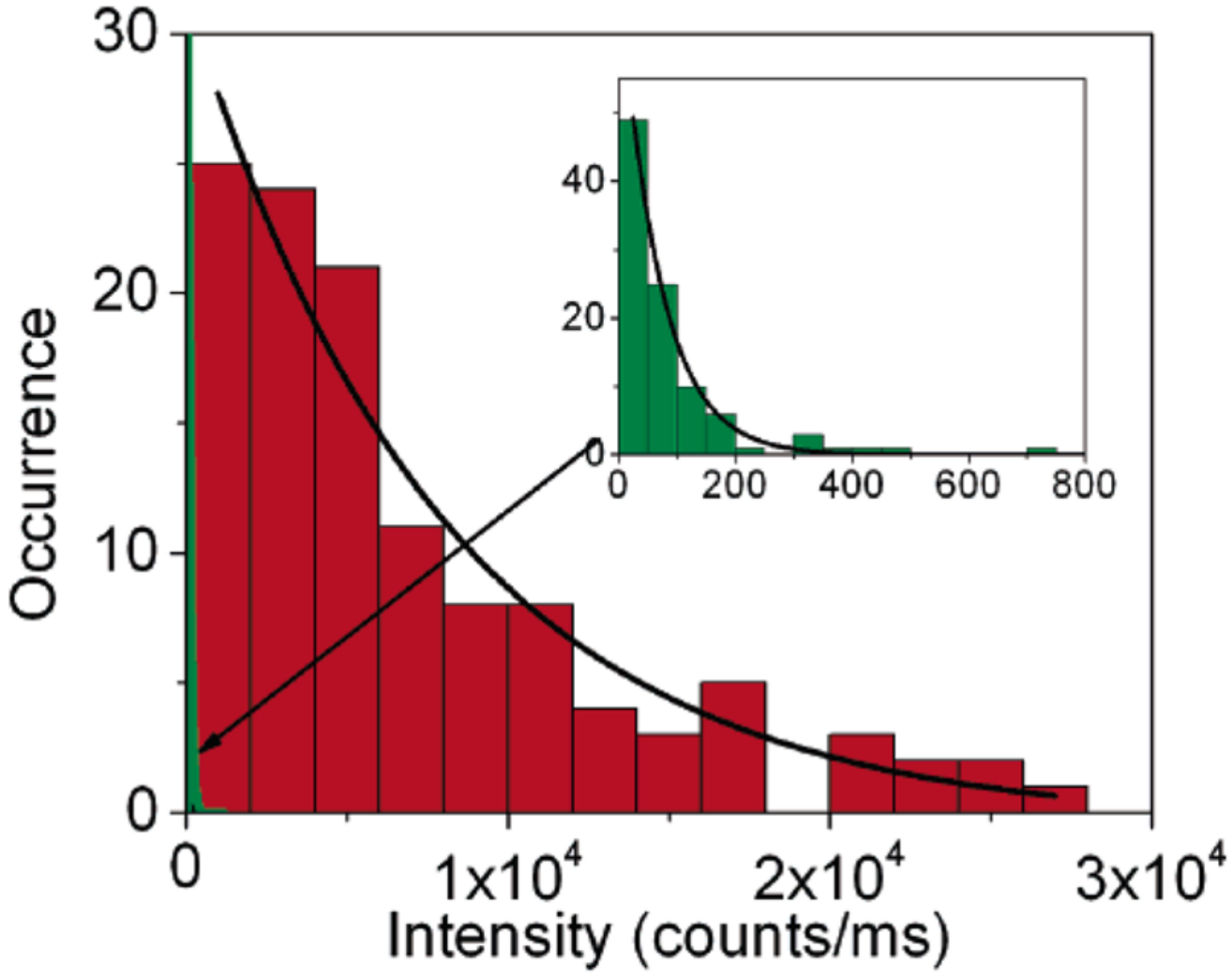


Figure 3.
Normalized histograms showing distributions of fluorescence intensity levels recorded from a single BSA-biotin/avidin layers on glass (green) and silver island films (red), respectively. (Black lines: single-exponential fits).

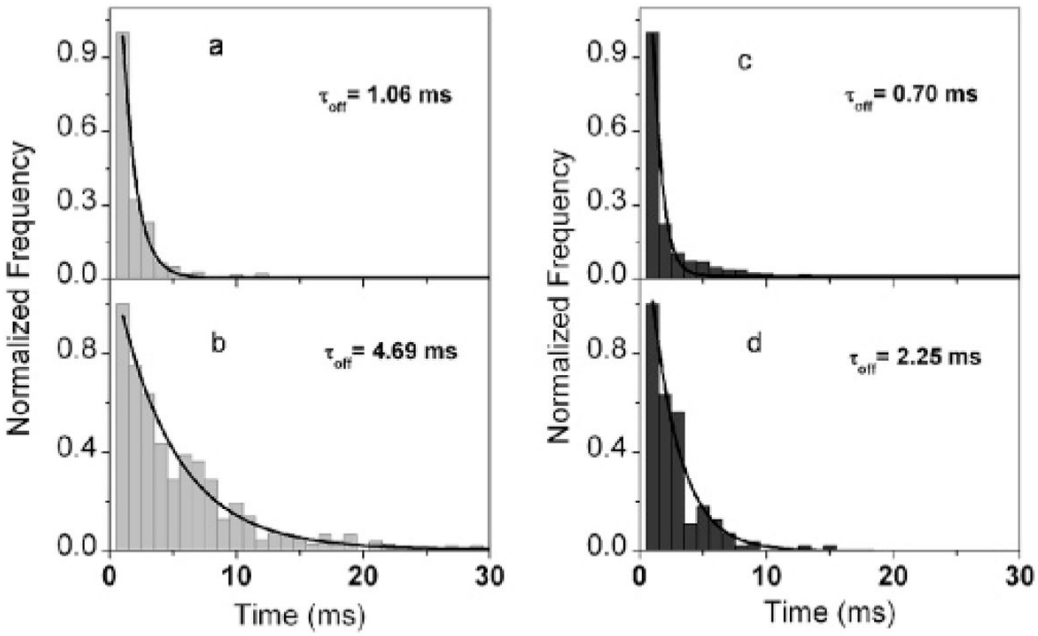


Figure 4.

"Off" time histograms compiled from single molecules. The solid lines show the single-exponential fits. Averaged "off" times (τ_{off}) for single Cy5 molecules immobilized on multiple BSA-biotin layers on glass: a. τ_{off} = 1.06 ms (double layers); b. τ_{off} = 4.69 ms (triple layers). Averaged "off" times (τ_{off}) for single Cy5 molecules immobilized on multiple BSA-biotin layers on SIF: c. τ_{off} = 0.70 ms (double layers); d. τ_{off} = 2.25 ms (triple layers).

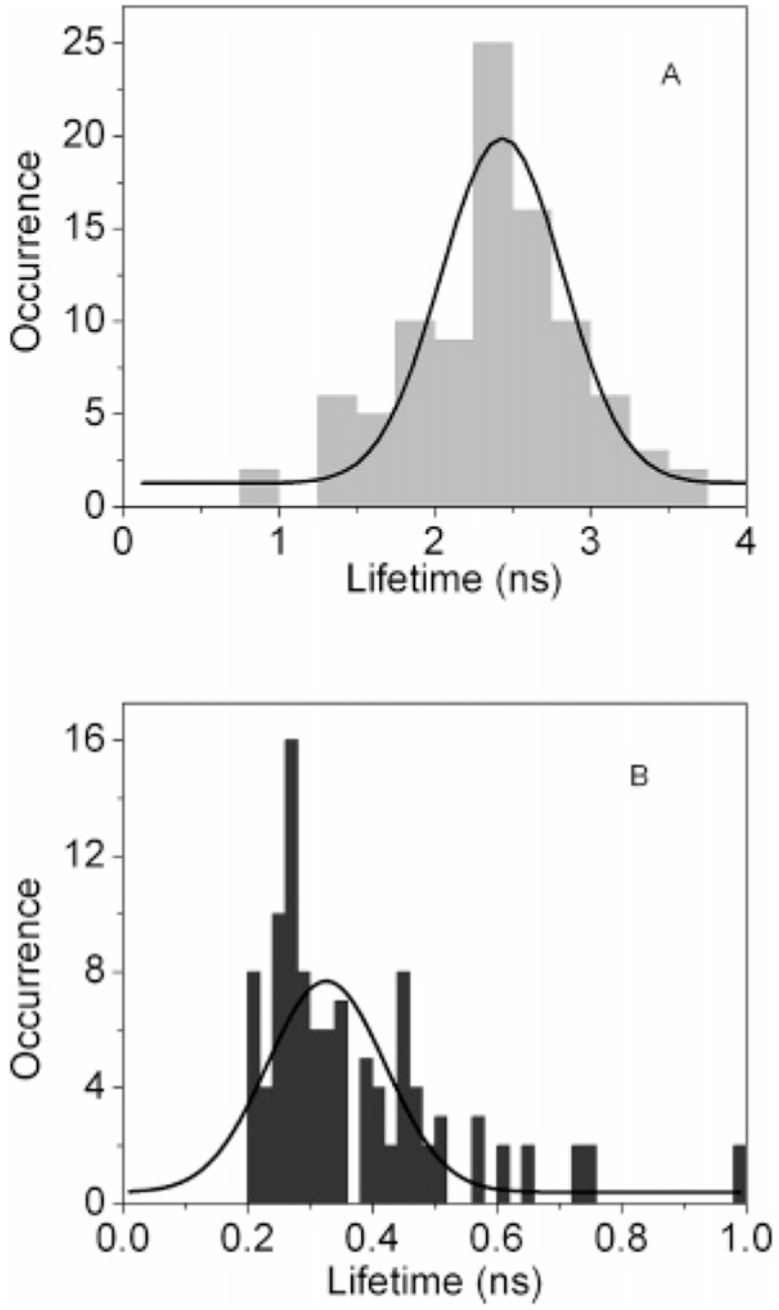
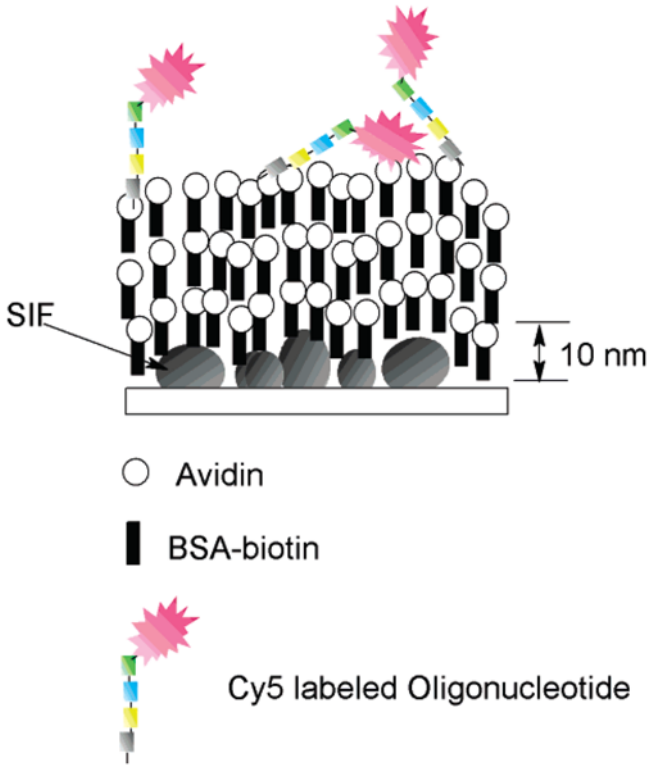


Figure 5.
(A) A histogram of lifetime distribution of Cy5-labeled oligonucleotides bound to a single BSA-biotin/avidin layer on the bare glass surface. (B) A histogram of lifetime distribution of Cy5-labeled oligonucleotides bound to a single BSA-biotin/avidin layer on SIF. (Black lines: Gaussian fits).



SCHEME 1.
Cy5-Labeled Oligonucleotides Were Bound to a BSA-Biotin/Avidin Multilayer Deposited on Silver Island Film

TABLE 1

Mean Values of Brightness and Normalized Fluorescence Enhancement Ratios Derived from Intensity Distributions of Various BSA-Biotin/Avidin Layers on Glass Surfaces and Silvered Surfaces, Respectively (original graphic distributions are provided in Supporting Information)

		number of BSA-biotin layers		
		1	**2**	**3**
mean values derived from intensity distribution (counts/ms)	on glass	68	54	56
	on SIF	7500[a]	190	110
relative enhancement on SIF with respect to glass surface		110[a]	3.5	2.0

[a]The estimated intensity and enhancement were obtained by correcting with incident laser power.

TABLE 2

Mean Values of Cy5 Lifetimes Derived from Lifetime Distributions of Various BSA-Biotin/Avidin Layers on Glass Surfaces and Silvered Surfaces, Respectively

	number of BSA-biotin layers		
	1	**2**	**3**
τ_F on glass (ns)	2.43	2.20	2.11
fwhm on glass (ns)	0.74	0.71	0.80
τ_F on SIF (ns)	0.31	0.68	1.92
fwhm on SIF (ns)	0.17	0.23	0.56