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Enhanced Emission of Highly Labeled DNA Oligomers near Silver Metallic Surfaces

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Abstract

Fluorescein is a widely used fluorescent probe in DNA analysis. One difficulty with fluorescein is its self-quenching due to resonance energy transfer between the residues, which results in decreased intensities with increasing labeling density. We examined the emission spectral properties of DNA oligomers labeled with one or five fluorescein residues. The emission intensity of the more highly labeled oligomer was decreased due to self-quenching. The self-quenching was mostly eliminated when this oligomer was held ~90 Å from the surface of metallic silver particles. The intensities increased 7- and 19-fold for the oligomers with one or five fluoresceins, respectively. The increased intensity did not result in increased photobleaching. These results suggest the use of substrates coated with silver particles for increased sensitivity on DNA arrays or for DNA analysis.

Fluoresceins, rhodamines, cyanines, and other similar fluorophores are widely used in biotechnology. The favorable aspects of these probes include large excitation coefficients and high quantum yields. An unfavorable property of these probes is the small Stokes' shift and thus overlap of the absorption and emission spectra. As a result of the small Stokes' shift, these probes display homo resonance energy transfer (RET), which results in self-quenching. In fact, self-quenching of fluorescein (Fl) is among the earliest observations in fluorescence spectroscopy.[1–5] For fluorescein, the Forster distance for homo RET is ~42 Å,[6] which is comparable to the size of DNA oligomers with ~13 base pairs.

At the present time, fluorescence is the primary detection method for genetic analysis, DNA sequencing, and DNA arrays. These arrays are widely used for high-throughput studies of gene expression[7–9] and increasingly for medical testing, genetics profiling, or diagnostics.[10,11] In all these uses it is desirable to obtain the largest possible signal per target strand. One obvious approach for larger signals is to label the DNA with more fluorophores. Unfortunately, this approach often results in self-quenching and decreased intensities.

In the present report, we describe a new approach to avoid self-quenching and to increase the brightness of highly labeled oligomers. We examined DNA 23-mers labeled with one or five fluorescein residues. These labeled oligomers were hybridized with a complementary biotinylated oligomer, which in turn was bound to a quartz substrate coated with biotinylated albumin and avidin. The double-stranded oligomers were examined on quartz and on quartz coated with metallic silver particles. The subwavelength- size silver particles were deposited by chemical reduction of silver. These particles are referred to as silver island films (SIFs) and are commonly used in surface-enhanced Raman scattering.[12–14] In recent studies, we found that SIFs could result in increased intensities and increased photostability of nearby

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fluorophores.[15,16] In the present report, we found that the emission intensities were increased 7- and 19-fold for the oligomers labeled with one or five fluoresceins, respectively. Photostability was also increased near the silver particles. These results suggest the use of silver particles or silver colloids on DNA array substrates for increased sensitivity.

MATERIALS AND METHODS

Sample Preparation

All oligonucleotides were obtained from the Biopolymer Core Facility at the University of Maryland School of Medicine (Scheme 1). Silver island films on quartz slides were prepared as described previously.[15,17] Briefly, the quartz slides were soaked in a 10:1 (v/v) mixture of H_2SO_4 (95–98%) and H_2O_2 (30%) overnight before the deposition, washed with distilled water, and air-dried prior to use. Silver deposition was carried out in a clean beaker equipped with a Teflon-coated stir bar. Eight drops of fresh 5% NaOH solution were added to a rapidly stirred silver nitrate solution (0.22 g in 26 mL of water). Dark-brownish precipitates were formed immediately. Less than 1 mL of ammonium hydroxide was then added drop by drop to redissolve the precipitate. The clear solution was cooled to 5 °C in an ice bath, followed by placing the clean quartz slides in the solution. At 5 °C, a fresh solution of D-glucose (0.35 g in 4 mL of water) was added. The mixture was stirred for 2 min at that temperature. The beaker was removed from the ice bath and allowed to warm to 30 °C. As the mixture turned from yellow-greenish to yellow-brown, the color of the slides became greenish. The slides were removed, rinsed with water, and bath sonicated for 1 min at room temperature. After being rinsed with water, the slides were stored in water prior to the experiments. Each slide (12.5 mm × 45 mm, half-coated with silver island film) was covered with 250 μL of 10 μM biotinylated bovine serum albumin (BSA-biotin, Sigma) aqueous solution and placed in a humid chamber for 20 h (5 °C, cold room). After being washed 3 times with water, the slides were placed again in the humid chamber. A 250-μL sample of 5 μM avidin (egg white, Molecular Probes) in 0.1× PBS buffer was deposited on each BSA-biotin-coated surface for 40 min at room temperature. Slides were then washed 3 times with 0.1× PBS buffer. This procedure is thought to result in a monolayer of surface-bound BSA coated with a monolayer of avidin.[14,17]

Solutions of double-stranded DNA (ds-DNA) samples (Fl-DNA, Fl-DNA$(Fl)_4$, and biotin-DNA; Scheme 1) were prepared by mixing complementary oligonucleotides in 5 mM Hepes (pH 7.5), 0.1 M KCl, and 0.25 mM EDTA buffer to a final concentration of 2 μM, followed by very slow cooling after incubation at 70 °C for 2 min. A 250-μL solution of ds-DNA was deposited on each BSA-biotin–avidin- coated surface for 1 h at room temperature. The slides were then washed and placed in the buffer solution (5 mM Hepes (pH 7.5), 0.1 M KCl, and 0.25 mM EDTA) for 15 min. To protect from scratching during measurements, we covered each sample (ds-DNA on a layer of BSA-biotin–avidin) with a 0.5-mm demountable cuvette filled with above buffer, as shown in Scheme 1, bottom right. The cuvette was mounted on a translation stage.

Fluorescence Measurements

Emission spectra were collected in front face geometry on a SLM 8000 spectrofluorometer with excitation at 514 nm from a argon ion laser or 495 nm from a xenon lamp. Lifetimes were measured on a 10-GHz frequency-domain fluorometer[18] using mode-locked argon ion laser at 514 nm, 76-MHz repetition rate, and 120-ps pulse width. Excitation and emission polarizers were in the magic angle orientation. Emission was selected with combination of a 520-nm long-pass liquid chromate filter ($CrO_4^{2-}/Cr_2O_7^{2-}$, 0.3 M, pH 8) placed in a 2-mm, 1 in. × 1 in., quartz cuvette and an interference filter at 540 nm. Such combination of filters rejects efficiently scattered light and has minimal internal luminescence. We estimated ratios of the

emission signals from samples without and with fluorescein fluorophores (background). The background from BSA-avidin-coated SIF containing unlabeled biotin-DNA was less than 1% for both Fl-DNA and Fl-DNA(Fl)$_4$. Similarly, the backgrounds on quartz were less than 2 and 1% for Fl-DNA and Fl-DNA(Fl)$_4$, respectively.

The frequency-domain (FD) intensity decay were analyzed in terms of the multiexponential model

$$I(t)=\sum_i \alpha_i \exp(-t/\tau_i) \tag{1}$$

where τ_i are the lifetimes with amplitudes α_i and $\Sigma\alpha_i = 1.0$. Fitting to the multiexponential model was performed as described previously.[18]

Effect of SIFs on Fluorescence

In a close proximity, up to ~50-Å fluorophore emission is strongly quenched by metallic surface. The emission of fluorophores near SIF but outside the quenching region depends on two major factors, enhanced local field and an increase of intrinsic decay rate of the fluorophore. The first factor provides stronger excitation rates. The second factor changes quantum yield and lifetime of the fluorophore. The observed fluorescence enhancement

$$G \sim G_{ex}\, G_{QY} \tag{2}$$

where $G_{QY} = Q_m/Q_0$ is the increase in quantum yield of fluorophore near SIF.

Scheme 2 shows the energy diagrams for molecules in the absence and presence of SIF. In the absence of SIF, the quantum yield and lifetime are given by

$$Q_0=\Gamma/(\Gamma+k_{nr}) \tag{3}$$

$$\tau_0=1/(\Gamma+k_{nr}) \tag{4}$$

where Γ is a radiative rate and k_{nr} is a nonradiative decay rate. In the presence of SIF, the quantum yield and lifetime are given by

$$Q_m=\frac{\Gamma+\Gamma_m}{\Gamma+\Gamma_m+k_{nr}+k_m}=\frac{\Gamma(1+\gamma)}{\Gamma(1+\gamma)+k_{nr}+k_m} \tag{5}$$

$$\tau_m=\frac{1}{\Gamma+\Gamma_m+k_{nr}+k_m}=\frac{1}{\Gamma(1+\gamma)+k_{nr}+k_m} \tag{6}$$

where Γ_m and k_m are radiative and nonradiative rates induced by metal.

Increases in radiative rate near SIF results in increased quantum yields and decreased lifetimes. For more details, see ref [16].

RESULTS

Sample Configuration

The labeled oligomers are shown in Scheme 1. The first oligomer had one fluorescein residue on the 3′-end (Fl-DNA). The second labeled oligomer contained a fluorescein residue at the same 3′-end plus four additional fluorescein residues (Fl-DNA$(Fl)_4$) distributed along the sequence. These oligomers were tethered to the avidin-coated substrate by a complementary oligomer containing a biotin residue on the 5′-end.

Following hybridization of the fluorescein- and biotin-labeled oligomers the double-stranded DNA was bound to the slides via a double layer of proteins (Figure 1). The slides were first coated with a monolayer of biotinylated BSA[14] followed by avidin. The half of the slides coated with silver displayed the expected plasmon absorption (Figure 1, bottom). From previous studies, we found roughly the same surface concentation of albumin on the quartz and SIF surfaces.[14,17] Briefly, we examined the absorption of consecutive BSA-avidin protein layers on silvered and unsilvered surfaces and did not notice any significant difference in binding. Hence, it is likely that roughly the same amounts of double-stranded DNA are bound to both surfaces. The observed DNA should only be the fluorescein-labeled double-stranded DNA. Single-stranded biotin-DNA would not contribute to the signal, and single-stranded fluorescein labeled DNA without biotin is not expected to bind to the surfaces.

The albumin-avidin layer deposited on SIF and on quartz provides a spacer between metal and probe. The thickness of this layer, ~90 Å, gives us the opportunity to place our dye in the area of maximum enhancement.[17] Direct deposition of our fluorescein-labeled DNA on SIF could result in partial quenching, which occurs up to 50 Å from the metallic particles.

Spectral Properties of the Fluorescein-Labeled Oligomers in Solution

Prior to examining surface-bound DNA, we examined the double-stranded DNA in solution. The emission spectra of Fl- DNA$(Fl)_4$ showed a 2-fold decrease in intensity as compared to Fl-DNA (Figure 2, top). For these spectra, the optical densities at the excitation wavelength of 495 nm were identical. If the concentrations of the samples were adjusted to the same amount of DNA, the Fl-DNA$(Fl)_4$ would show only 2-fold higher brightness than Fl-DNA (Figure 2, middle), instead of the 5-fold expected for an unquenched system. This result shows that one cannot obtain significantly increased intensities from fluorescein-labeled DNA by increasing the extent of labeling for short oligonucleotides. The steady-state anisotropies of these oligomers were 0.13 and 0.085 for Fl-DNA and Fl-DNA$(Fl)_4$, respectively. This loss of anisotropy is consistent with RET between the fluorescein residues. Following the homotransfer event, emission occurs from fluorophores with a different orientation of transition moments; that is, fluorescence is depolarized. The loss of anisotropy is considered as a manifestation of energy transfer between identical molecules.[1–6] For accuracy, we note that we did not examine oligomers with just two or three fluoresceins, so we did not know the extent to which such oligomers would also be quenched.

We also examined the intensity decays of the double-stranded oligomers in homogeneous solution. The amplitude-weighted lifetime of Fl-DNA was near 3.6 ns, as expected for an isolated fluorescein residue. For Fl-DNA$(Fl)_4$, the mean lifetime was reduced to 1.5 ns (Figure 2, bottom). A decreased lifetime is an indication of self-quenching between the fluorescein residues.

Spectral Properties of Fluorescein-Labeled Oligomers Bound to Quartz and Silver Island Films

We examined the emission spectra of Fl-DNA and Fl-DNA$(Fl)_4$ when bound to quartz and silver island films, Figure 3 and Figure 4, respectively. When normalized, the spectra distributions on SIF and quartz are essentially identical. For the oligomer with a single fluorescein residue, the intensity increases 7-fold on SIF as compared to quartz. This intensity increase may be due to a combination of an increased quantum yield of Fl-DNA on the SIF surface, with a possible increased rate of excitation due to an enhanced local field near the metal particles.[16,19,20] Local field effect has been modeled for ellipsoidal particles and predicts maximum enhancement of ~140.[19] The enhancement due to an increase in quantum yield can be as large as $1/Q$.[21] The quantum yield of Fl-DNA in Hepes buffer, pH 7.5, is ~0.44. The increased quantum yield can be responsible for ~2.2-fold enhancement of Fl-DNA emission on SIFs. A more dramatic intensity increase of 19-fold was observed for Fl-DNA$(Fl)_4$ on the SIF as compared to quartz (Figure 4). The larger intensity increase observed for Fl-DNA $(Fl)_4$ as compared to Fl-DNA is consistent with the lower solution quantum yield of the more heavily labeled oligomer. Note that a mean lifetime of Fl-DNA$(Fl)_4$ on quartz is ~3.5-fold shorter than for Fl-DNA (Table 1 and Figure 2, bottom). An increased intensity with a decreased lifetime strongly suggests an increase in the radiative decay rate.[16] The increased intensity is nicely shown by a color photograph of the labeled DNA when illuminated with a laser beam at 440 nm (Figure 4, top). The laser beam was positioned either on the SIF (left), on the quartz (right), or on both (center image). This photograph has not been adjusted for intensity or contrast.

In previous reports, we noticed that the lifetimes of fluorophores near quartz surfaces are usually lower than the lifetimes in solution.[15,22,23] We believe the effect is partly due to the higher refractive index of quartz for part of the space near the fluorophore. Also, the density of labeled DNA could be higher on a surface monolayer than in solution, resulting in interaction with fluoresceins from other DNAs, which increases a homotransfer. We examined the lifetimes of both labeled oligomers on quartz (Figure 5, solid lines). The amplitude-weighed lifetimes were reduced ~2-fold relative to the lifetimes in solution (Table 1).

We believe that the increase in intensity on SIFs is due at least in part to increase in the radiative decay rate near the silver particles,[15] which would result in decreased lifetimes. For the singly labeled oligomer, the lifetime decreased 10-fold on silver as compared to quartz (Figure 5, top). For the heavily labeled oligomer, the amplitude-weighed lifetime decreased 6-fold (Figure 5, bottom) as compared to quartz (Table 1). A larger relative decrease in lifetime is expected for the higher quantum yield sample. This is because the same increase in the radiative decay rate will have a smaller effect when competing with the larger nonradiative rate for a more highly quenched fluorophore.

As described previously,[15] decreases in lifetimes are expected to result in increased photostability. This effect occurs because the shorter lifetimes result in less time in the excited state and, hence, less time for photochemical reactions. The decreased lifetime is not expected to change the probability of photobleaching per unit time, but it will reduce the probability per excitation-relaxation cycle. Hence, the emission intensity with continuous illumination should be more constant for a shorter lifetime but otherwise comparable fluorophore. On the other hand, fluorophores near SIF are exposed to the enhanced local field and should display more rapid photobleching. The measured photostability will be affected by these two factors.

We examined the steady-state emission intensity of the highly labeled oligomer with continuous illumination (Figure 6). When observed with the same incident intensity, the relative emission intensity for Fl-DNA$(Fl)_4$ decreased more rapidly on the SIF than on quartz (top). However, intensity on the SIF remained greater at all times up to 300 s. Suppose the

increased intensity seen on the SIF was due to an increased rate of excitation rather than an increase in quantum yield. In this case, the rate of photobleaching would increase by the same factor as the intensity increased, which did not occur. The number of photons detectable per fluorophore is expected to be proportional to the area under the curves in Figure 6. This suggests that for Fl-DNA$(Fl)_4$ the total detectable signal per fluorophore is increased 12-fold or more.

An alternative approach for examining photostability is to compare the samples on SIFs and quartz illuminated with adjusted laser power yield the same emission intensity at time zero. This comparison was accomplished by attenuating the intensity incident on the brighter region of the slide with the SIF. We found roughly equivalent relative rates of photobleaching (Figure 6, bottom). Since the same signal level is obtained with a lower excitation intensity, it is probable that background signal from the instrument and optics, or autofluorescence from the sample distant from the surface, will be decreased by the same attenuating factor. Hence, the SIFs can be used to increase the signal-to-background level on DNA arrays.

DISCUSSION

The results described in the previous paragraph show that it is possible to significantly increase the brightness of the sample near the SIF. Assuming arbitrarily the brightness of Fl-DNA on quartz as a 1, the brightness of Fl-DNA$(Fl)_4$ on quartz is only 2 instead of the expected 5, due to self-quenching. Taking into account measured enhancement, the brightness on SIFs is 7 (1× 7) and 38 (2 × 19) for Fl-DNA and Fl-DNA$(Fl)_4$, respectively. Assuming that all five fluoresceins in Fl-DNA$(Fl)_4$ were equal, the maximal brightness would be 5 × 7 = 35. This result suggests the flurorophore–SIFs spacing used in our experiment is close to optimal. In our earlier study, we investigated the dependence of fluorescence enhancement of SIF-cyanine dye separation by building consecutive BSA-avidin layers.[17] We found the strongest enhancement for one BSA-avidin spacer, e.g., for fluorophore-SIF distance of ~90 Å.

In our opinion, the results in this report can be immediately applied to DNA arrays. A wide variety of approaches are available to deposit silver on surfaces, including illumination of silver nitrate solutions or passage of current between silver electrodes.[24–27] Furthermore, we found that silver deposition occurs more rapidly on surfaces coated with amino groups,[27] which are commonly used in DNA arrays. Additionally, amine-coated surfaces spontaneously bind silver colloids from suspensions, and the bound colloids also enhance fluorescein. Given these procedures are not expensive, we expect silver-coated substrates to become widely used for DNA analysis.

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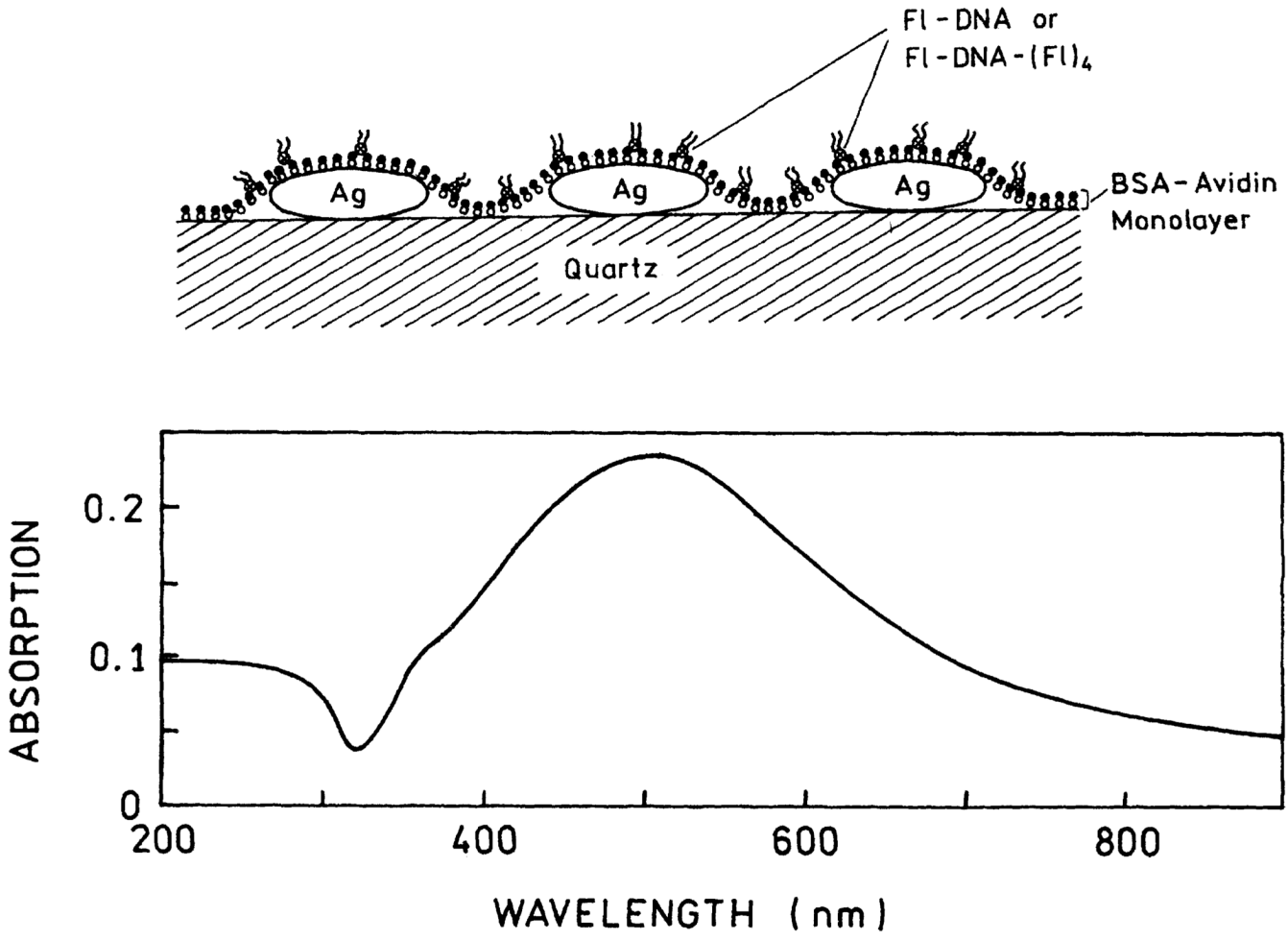


Figure 1.

Top: BSA-avidin monolayer deposited on SIFs. Fl-DNA or Fl-DNA$(Fl)_4$ were tethered to avidin using the complementary biotinylated oligonucleotide. Bottom: Absorption spectrum of SIFs used in this experiment.

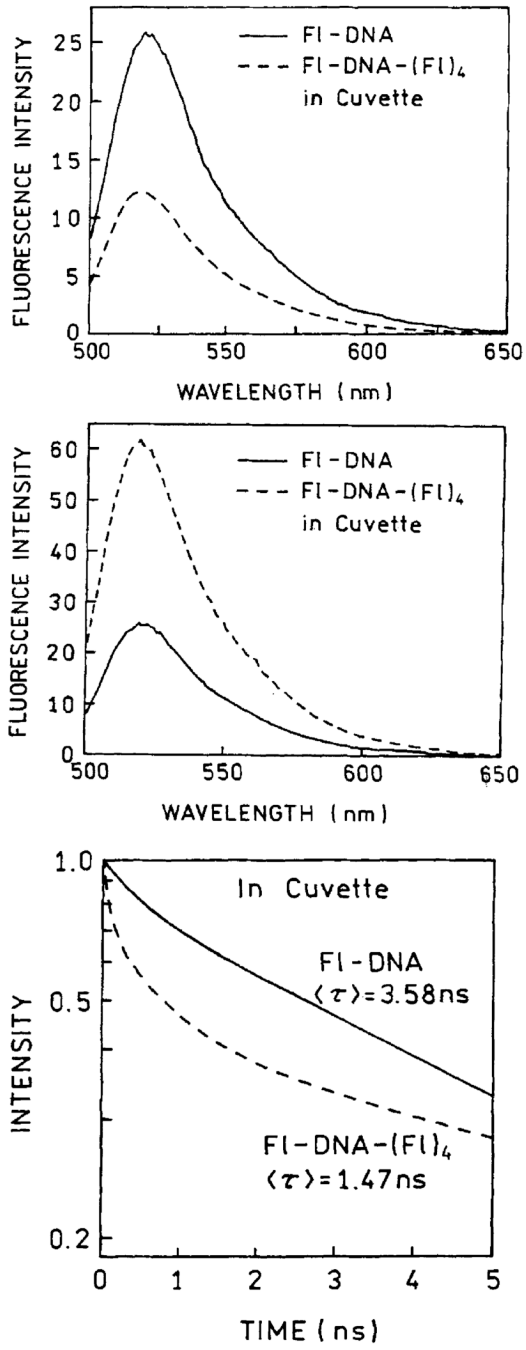


Figure 2.

Emission spectra of Fl-DNA and Fl-DNA$(Fl)_4$ solutions in a cuvette. The absorption of both samples were matched at the excitation wavelength of 495 nm (top). The emission anisotropies are 0.13 and 0.085 for Fl-DNA and Fl-DNA$(Fl)_4$, respectively. The middle panel shows the emission spectra of Fl-DNA and Fl-DNA$(Fl)_4$ with the same DNA concentration. The bottom panel shows the intensity decays. These lifetimes were measured in frequency domain. The decay parameters are in Table 1.

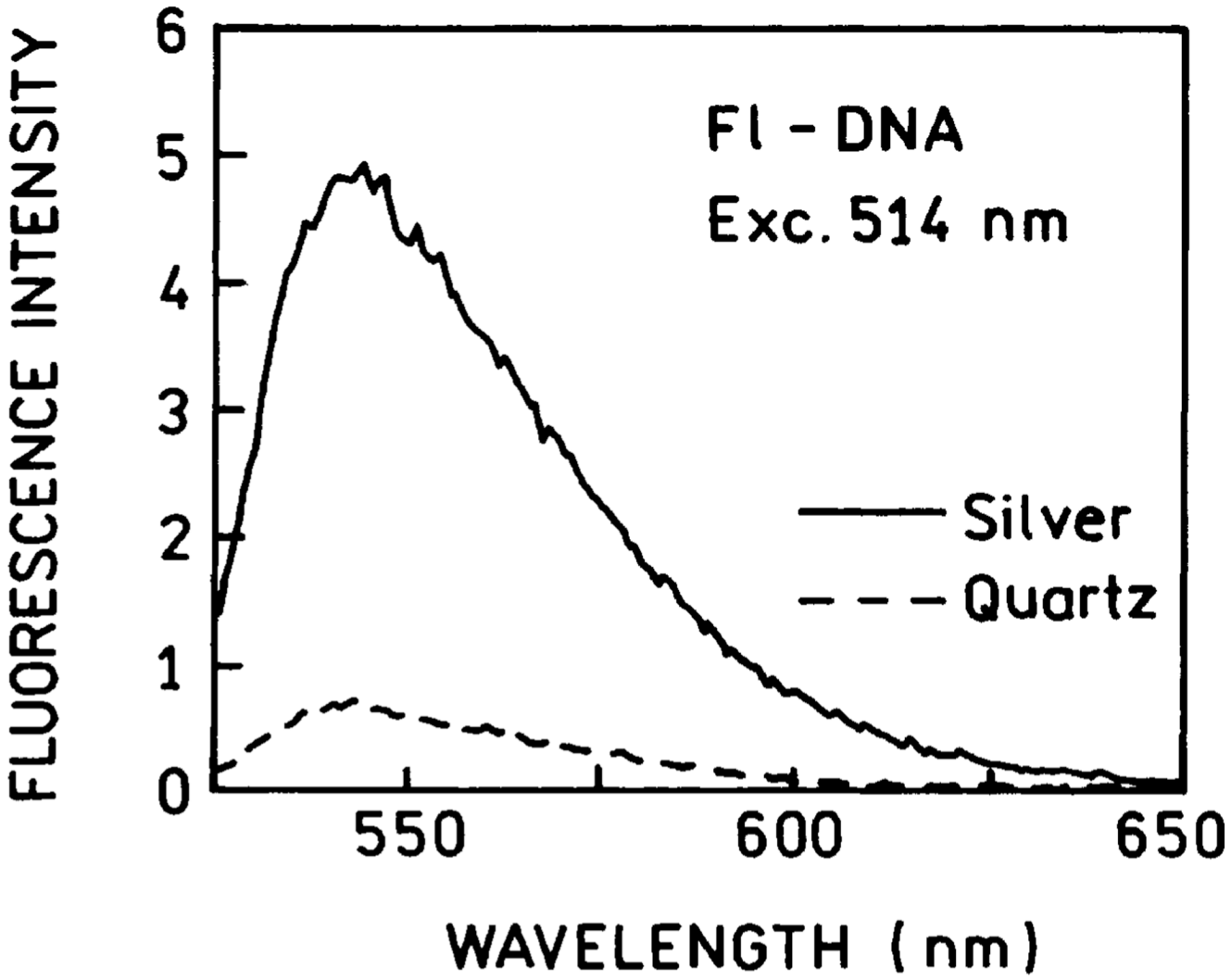


Figure 3.
Fluorescence emission spectra of Fl-DNA tethered to the BSA-avidin monolayer deposited on quartz and SIFs.

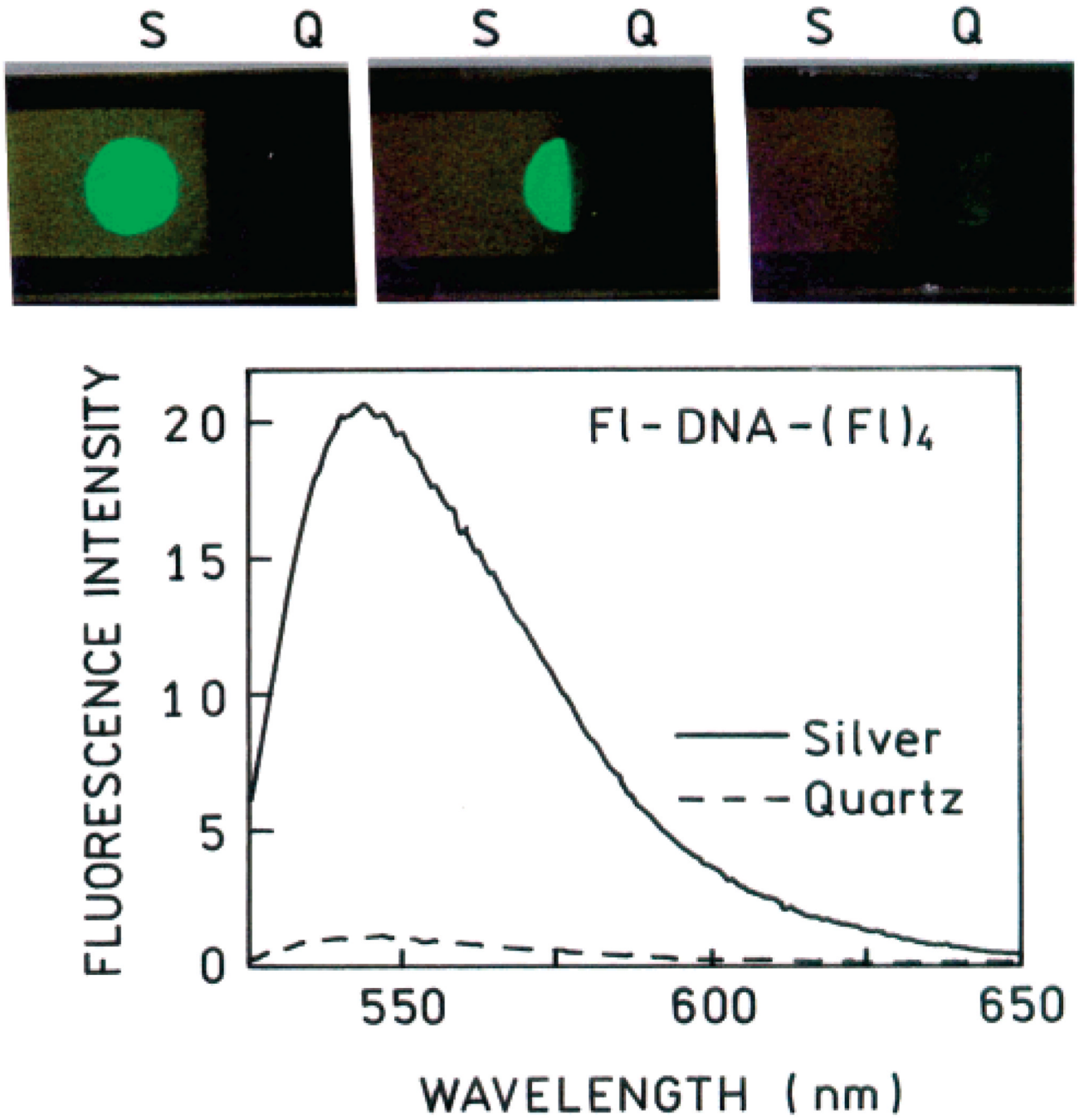


Figure 4.
Fluorescence emission spectra of Fl-DNA$(Fl)_4$ tethered to the BSA-avidin monolayer deposited on quartz and SIFs. Also shown is a photograph of Fl-DNA$(Fl)_4$ fluorescence.

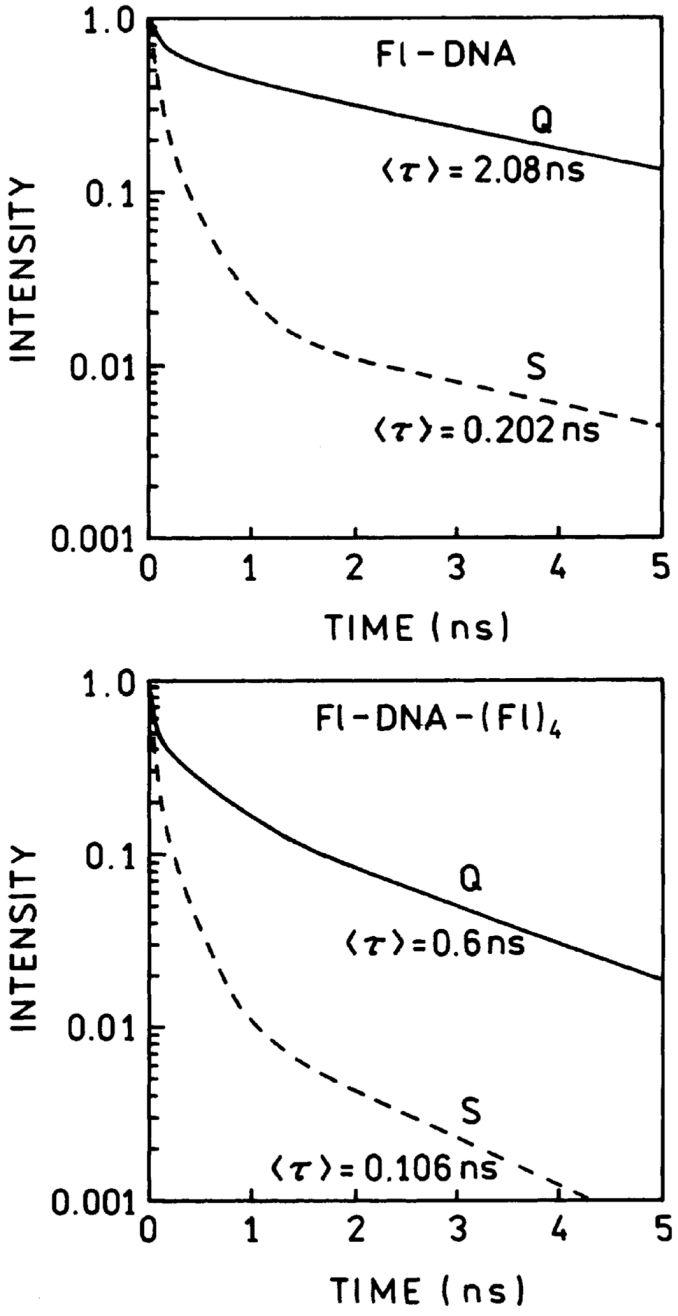


Figure 5.
Time-domain representations of intensity decays of Fl- DNA (top) and Fl-DNA$(Fl)_4$ (bottom) on quartz (Q) and SIFs (S). These lifetimes were measured in the frequency domain. The decays parameters are in Table 1.

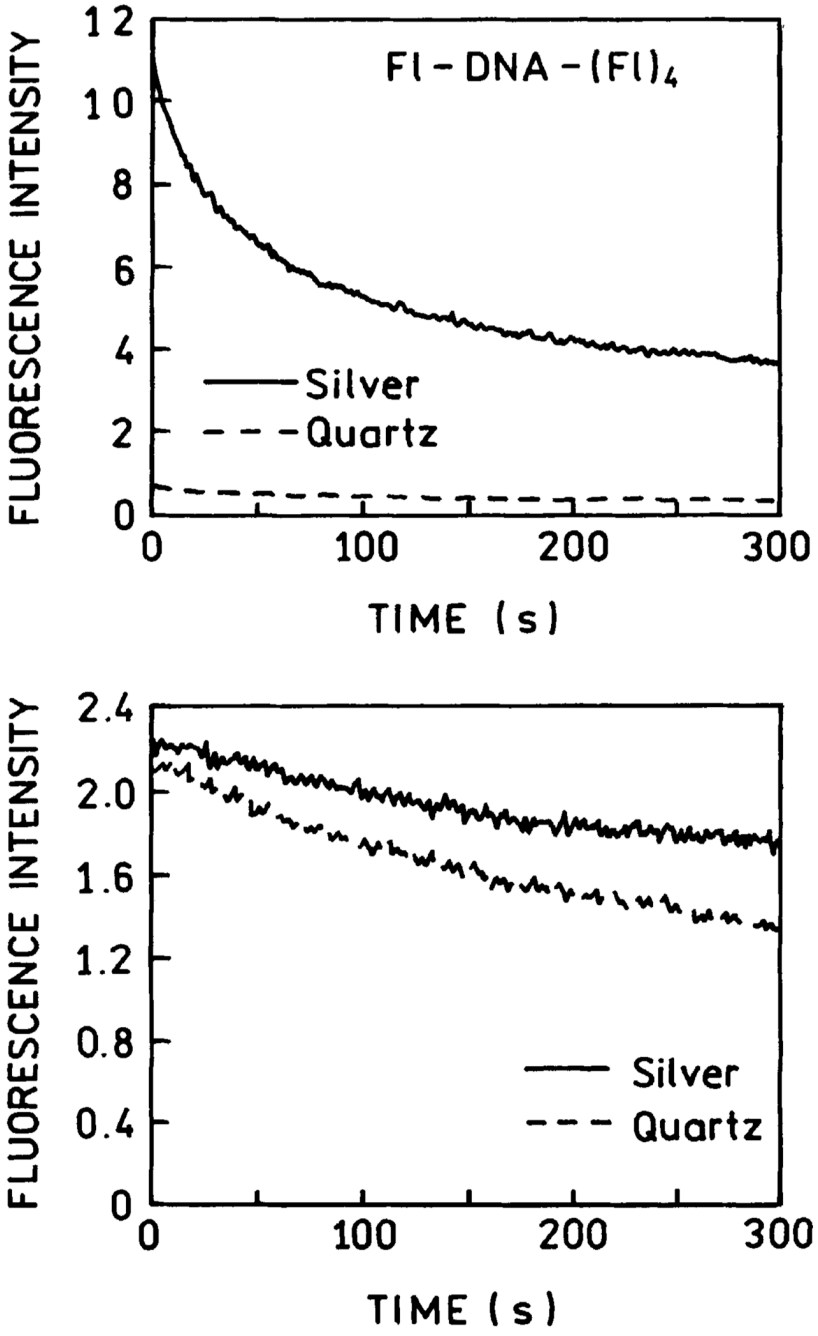


Figure 6.

Photostability of Fl-DNA$(Fl)_4$ tethered to the BSA-avidin monolayer deposited on quartz and SIFs. Top: measured with the same excitation power (514 nm, 5 mW focused to a diameter near 100 μm). Bottom: measured with the excitation intensities adjusted to yield the same emission intensity.

biotin-5'-TCC ACA CAC CAC TGG CCA TCT TC-3'
Fl-3'-AGG TGT GTG GTG ACC GGT AGA AG-5'

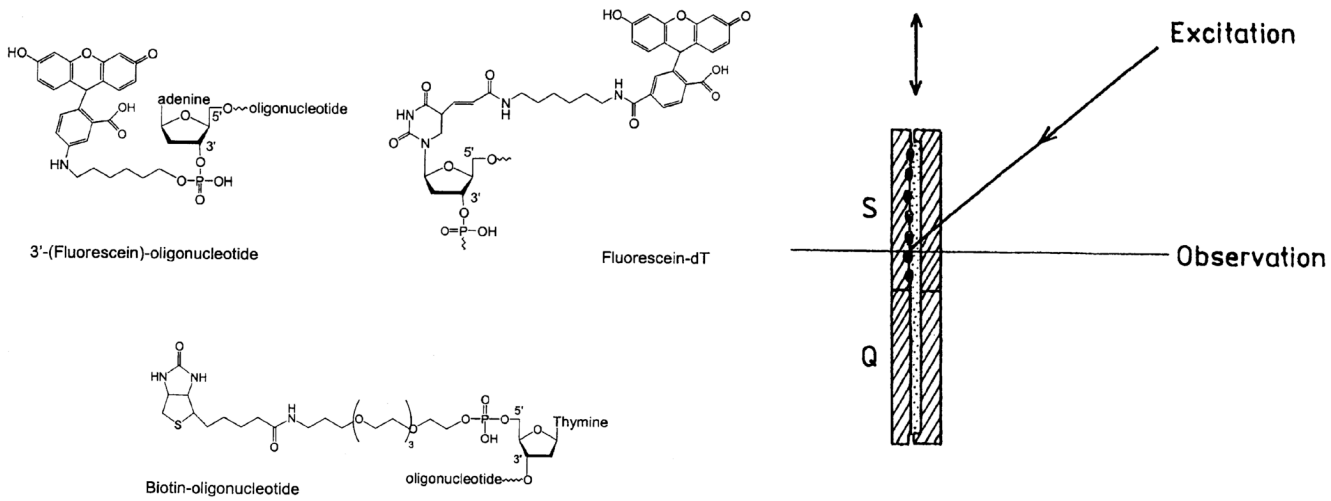
Fl-DNA

biotin-5'-TCC ACA CAC CAC TGG CCA TCT TC-3'
Fl-3'-AGG TGT GTG GTG ACC GGT AGA AG-5'
Fl **Fl** **Fl** **Fl**

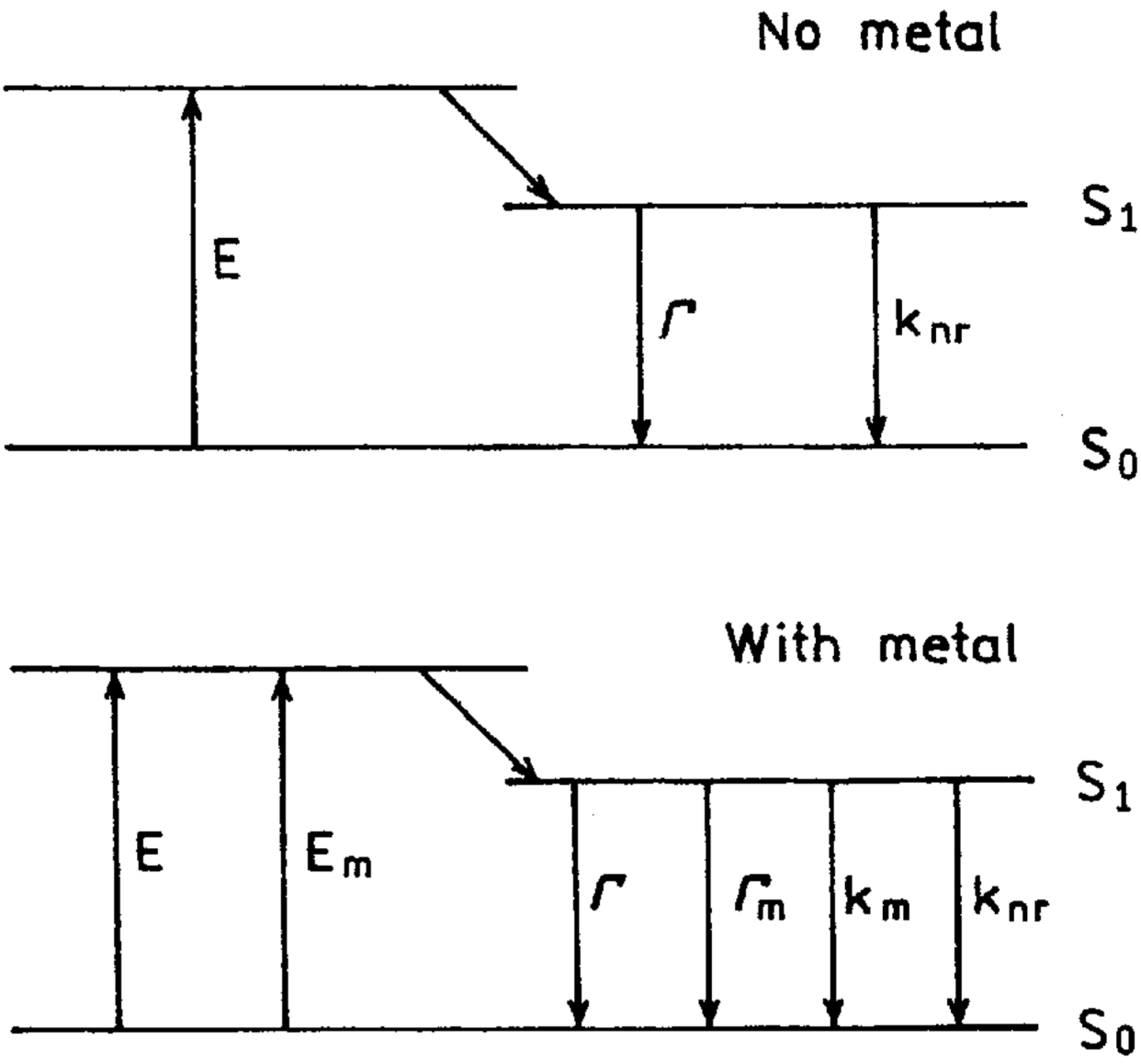
Fl-DNA(Fl)$_4$

biotin-5'-TCC ACA CAC CAC TGG CCA TCT TC-3'
3'-AGG TGT GTG GTG ACC GGT AGA AG-5'

DNA



Scheme 1.
Structures of Fl-DNA and Fl-DNA(Fl)$_4$a
[a] In the right bottom panel is also shown a sample geometry.



Scheme 2.
Jablonski Diagram for Free Fluorophores (Top) and on SIFs (Bottom)a
[a] The fluorescence occurs from a relaxed lowest excited singlet state (S_1).

Table 1

Multiexponential Analysis of Fluorescein-Labeled DNA Frequency-Domain Intensity Decays

compound/conditions	$\langle\tau\rangle$ (ns)	$\bar{\tau}$ (ns)	α_i	f_i	τ_i (ns)	χ^2_R
Fl-DNA, cuvette	3.58[a]	4.21[b]	0.188	0.024	0.46	
			0.812	0.976	4.30	1.0 (72.6)[c]
Fl-DNA(Fl)$_4$, cuvette	1.47	2.79	0.308	0.022	0.10	
			0.329	0.168	0.75	
			0.363	0.810	3.28	1.1 (511.5)
Fl-DNA, quartz	2.081	3.517	0.286	0.011	0.082	
			0.206	0.069	0.699	
			0.508	0.920	3.766	1.0 (325.6)
Fl-DNA(Fl)$_4$, quartz	0.596	1.648	0.492	0.036	0.043	
			0.300	0.232	0.460	
			0.208	0.732	2.098	1.0 (947.3)
Fl-DNA, silver	0.202	1.256	0.684	0.237	0.069	
			0.297	0.442	0.298	
			0.019	0.321	3.456	2.0 (1018.4)
Fl-DNA(Fl)$_4$, silver	0.106	0.412	0.757	0.321	0.045	
			0.231	0.502	0.229	
			0.012	0.177	1.610	1.1 (582.3)

[a] $\langle\tau\rangle = \Sigma_i \alpha_i \tau_i$.

[b] $\bar{\tau} = \Sigma_i f_i \tau_i$, $f_i = \alpha_i \tau_i / \Sigma_j \alpha_j \tau_j$

[c] For the best single decay time fit.

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