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# Effect of plasmonic nanostructures and nanofilms on fluorescence resonance energy transfer

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# Abstract

We investigate the effect of silver nanostructures and nanofilms on fluorescence resonance energy transfer (FRET). Thin layers of poly (vinyl alcohol) of 10 or 20 nm with homogenously distributed donor (Cy3) and acceptor (Cy5) molecules were coated on silver islands and continuous silver films. Several geometrical configurations were studied with multiple donor-acceptor (D-A) samples with various acceptor concentrations. The acceptor concentrations were adjusted to yield FRET efficiencies from about 10 to 92% on bare glass surfaces. Time-resolved fluorescence spectroscopy was used to determine the energy transfer efficiency and the relative transfer rate. It was found that the relative transfer rate increased 2-fold in the presence of continuous silver films and about 25-fold in presence of silver island films. The largest enhancements in the energy transfer efficiency, up to 90%, were observed for low acceptor concentrations (large donor-acceptor separations). To a first approximation, observed changes in FRET efficiencies were explained by an increase of the quantum yield of the donor molecules in the presence of silver islands.



FRET in the presence of plasmonic nanostructures

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#### Keywords

FRET; metal-enhanced fluorescence; plasmon resonance; fluorescence lifetime; decay rate

# 1. Introduction

Recent strong interest in exploring the interaction between fluorescence materials and plasmonic nanostructures is driven mostly by the need for new ultra-sensitive methods for biotechnology applications. Plasmonic nanostructures in the form of metallic particles provide a means to greatly enhance fluorescence intensities and reduce lifetimes due to the increased excitation field and increased radiative decay rate [1–8]. These effects are frequently known as metal-enhanced fluorescence (MEF).

Additionally, continuous metallic nanofilms, such as gold and silver with a thickness of about 50 nm, provide a means to enhance fluorescence by directional emission. In this case, the fluorescence is coupled to surface plasmons and is emitted at a narrow angle into the glass substrate [9–10]. This phenomenon is called surface-plasmon coupled emission (SPCE).

Both phenomena, MEF and SPCE, of many fluorophores have been observed [3–10] and theoretical calculations have been reported [11,12]. In the case of FRET, the interaction between plasmonic nanostructures and donor-acceptor (D-A) systems is more complex than in the case of a single fluorophore. The environment with metallic particles can play a very active role in energy transfer between donor and acceptor molecules. The positions of D and A molecules relative to the surface of metallic particles is an important factor as is the size and shape of metallic particles. Theoretical calculations predict substantial enhancement in energy transfer rate and efficiency for D-A systems nearby plasmonic nanostructures [13–15].

FRET is widely used in fluorescence sensing and imaging modality in life sciences including biomedical applications [16–19]. The usual drawback of FRET in biosensing is the limited distance of less than 10 nm between donor and acceptor molecules for efficient energy transfer. Initial experimental results for FRET systems near the silver particles indicated that there is indeed a strong enhancement in energy transfer efficiency, determined as several-fold increase in apparent Förster distance  $R_0$  [20–23]. These experiments were carried out using liquid samples of about 1 µm thick with D-A pairs placed between two surfaces without (control sample) and with the silver island films [20–22]. The time-resolved data were analyzed for two populations of D-A pairs; one unaffected by metallic nanoparticles described by critical energy transfer distance  $R_{01}$  and the second population of D-A pairs in proximity to the metallic surface described by  $R_{02}$ . The  $R_{02}$  values of three systems were from 1.46 to 4.6 times larger than of  $R_{01}$ . A FRET experiment using single silver particles and a single D-A pair resulted in about a 1.6-fold increase in Forster distance [23].

In the present report, we describe experiments with a FRET system where donor and acceptor molecules are placed in close proximity to silver nanostructures and nanofilms. The geometry of D-A and metallic surface systems mimics surface-based configurations used in biosensing for biomolecule interaction studies where one labeled biomolecule is immobilized on the substrate surface (glass, polystyrene, etc.) and the second labeled biomolecule binds to the surface. The biomolecule binding is studied by monitoring the energy transfer rate and efficiency. In our studies we chose a randomly distributed donor and acceptor molecules in a thin 10 and 20 nm poly(vinyl alcohol) (PVA) layer coated onto silver nanoparticles or nanofilms. The thin PVA layer allows for most of the donor and acceptor molecules to be involved in interactions with the surface plasmons. The use of PVA layers with various acceptor

concentrations also allowed us to study the effect of plasmonic nanostructures over a wide range of the energy transfer efficiencies.

# 2. Materials and methods

#### 2.1 Substrates and sample preparation

MEF substrates were prepared using wet chemical deposition of silver on glass slides as described earlier [4,24]. The optical density of silver island films (SIFs) was about 0.6 with extinction maximum at about 450 nm. Wet chemical deposition results in plurality of particle sizes and shapes as is shown using imaging with field emission scanning electron microscopy (Hitachi SU-70 SEM) (Figure 1). Silver films for SPCE measurements were fabricated using thermal vacuum deposition (Edwards Model 506) of 43 nm thick followed with silica layer of 5 nm thickness using microbalance control without breaking vacuum.

The FRET system consisted of the Cy3 as donor and Cy5 as acceptor molecules embedded into a poly(vinyl alcohol) (PVA) film. The PVA films with a fixed Cy3 donor concentration of 0.5 mM and various concentrations of the Cy5 acceptor from 0 to about 8 mM were spin coated over the substrates with silver islands and silver nanofilms. Concentrations of acceptor were determined from the known Cy5 concentrations and the volume change from liquid PVA to a solid film. The control samples were prepared on bare glass slides (uncoated areas of substrates with Ag nanostructures) at the same donor/acceptor concentrations and spin coating conditions. A scheme of geometrical configurations investigated is shown in Figure 2.

#### 2.2 Spectroscopic measurements and FRET calculations

Steady-state intensity measurements were performed using fiber optic fluorometer (SD 2000 Ocean Optics, FL) configured for MEF and SPCE acquisition. Time-resolved measurements were carried out using frequency-domain fluorometry with excitation source of mode-locked argon ion laser, 514.5 nm at 76,236 MHz, and K2 fluorometer (ISS, Champaign, IL) modified with microchannel plate photomultiplier (R2566U, Hamamatsu). Intensity decays of donor (Cy3) were measured for each D-A system and data was analyzed using multi-exponential intensity decay model. Frequency-domain data were acquired up to 2 GHz, thus allowing an accurate determination of the intensity decays of donors whose lifetimes are strongly shortened by the silver islands and by energy transfer.

A two- or three-exponential model was used to fit the intensity decay of donors

$$I(t) = \sum_{i=1}^{n} \alpha_i \exp\left(-t/\tau_i\right) \tag{1}$$

where  $\tau_i$  is the decay time and the  $\alpha_i$  is the amplitude  $\left(\sum_i \alpha_i = 1.0\right)$ . The multi-exponential intensity decay model was used to determine the extent of energy transfers in the presence of silver particles and solid silver films as well as for control samples on bare glass. The transfer efficiencies (*E*) were calculated from the average lifetimes of the donor [25]

$$E=1-\frac{\langle \tau_{\rm DA}\rangle}{\langle \tau_{\rm D}\rangle} \tag{2}$$

where the average lifetimes  $\langle \tau_D \rangle$  and  $\langle \tau_{DA} \rangle$  are amplitude weighted lifetimes given by the sum of the  $\alpha_i \tau_i$  products. The lifetimes were calculated for each sample in the absence of acceptor ( $\langle \tau_D \rangle$ ), and in the presence of acceptors ( $\langle \tau_{DA} \rangle$ ). These average lifetimes are proportional to the steady-state intensities.

# 3. Results

#### 3.1 Effect of silver islands and continuous films on donor properties

The energy transfer efficiency of any D-A system strongly depends on spectral properties of donor and acceptor molecules. Close proximity of metallic nanoparticles causes the spectral properties of fluorophores to be modified due to the increased absorption probability and changes in radiative and non-radiative decay rates. Thus the energy transfer efficiency and transfer rates are also modified. The effect of silver particles on fluorophore spectral properties strongly depends on the distance between the fluorophore and the metal surface [4,8,10,26, 27]. We used spacer layers as shown in Figure 2 to facilitate various degree of interaction between donor molecules and silver islands. Our approach uses the randomly distributed donor and acceptor molecules in a solid matrix where the FRET efficiency is easily varied by the acceptor concentration. Table 1 summarizes the effect of SIFs and silver nanofilm on the donor (Cy3) intensity and lifetime in each configuration. The fluorescence of Cy3 in the presence of SIFs was enhanced several fold and the lifetime was substantially decreased.

The effects of the metallic particles and continuous nanofilms on fluorescence are very different. These differences arise from the defined geometry of the metal film and from the various natures of surface plasmons on sub-wavelength sized particles and planar surfaces. The excited donor molecules in thin PVA films that are in near-field create plasmons in the silver film, and these plasmons in turn radiate into the substrate at the surface plasmon angle [9,10]. The donor lifetime in the SPCE is moderately affected by the silver nanofilm (Table 1). We have chosen the thickness of the PVA layer to be 20 nm and the spacer layer of SiO<sub>2</sub> to be 5 nm to maximize the potential effect of surface plasmon on FRET efficiency in SPCE configuration. It is known from several reports that the effective fluorescence volume in SPCE experiments is limited by distance dependent coupling of excited fluorophores to the surface plasmons. Close proximity to the metal surface of less than 10 nm results in quenching of fluorescence [10,27]. The average distance of donor and acceptor molecules from metal surfaces is therefore about 15 nm.

## 3.2 Effect of silver islands and silver nanofilm on FRET efficiency

The FRET efficiencies were determined from the intensity decays of donor at various concentrations of the acceptor. Figures 3 and 4 show the frequency-domain intensity decays of donor (Cy3) at several acceptor (Cy5) concentrations in a 10 nm PVA film coated on glass and directly on SIFs, respectively. The intensity decays of donor for samples in the absence (Figure 3) and presence of silver nanoparticles (Figure 4) display expected shifts of phase and modulation responses towards higher modulation frequencies, indicating a decrease in the average lifetime with increased acceptor concentration. The observed phase and modulation shifts towards higher frequencies are a direct indication of progressive increase of FRET efficiency with increased acceptor concentration. One should notice that the presence of silver island films caused the intensity decays of Cy3 to be significantly faster compared to that on bare glass (about 10-fold, Table 1). In order to accurately determine the intensity decay of the donor, we used a fast frequency-domain fluorometer to acquire phase-modulation data for up to 2 GHz. We acknowledge that despite our state-of-the art frequency-domain instrument, we are not able to precisely measure the intensity decays of fluorescence in the presence of plasmonic nanostructures. This would require ultra fast instrumentation beyond the 10 GHz range to obtain larger changes in phase shifts and modulation. However, frequency-domain

fluorometry provides the best accuracy for measurement of very fast decays and determination of average lifetimes. This is because the information on short lifetime component (can be in range of 10 ps in the presence of plasmonic structures) is acquired over all modulation frequencies. A more commonly used time-domain fluorometry is prone to underestimate the contribution of short lifetime component because of its time-scale nature and convolution procedure. Cut-off of first couple channels during analysis might result in substantial underestimation of short decay time component. Our approach of using multiple samples with various degrees of FRET, parallel experimentation using identical samples on bare glass, and using three geometrical configurations allowed us to obtain conclusive data on FRET in the presence of SIFs and silver nanofilms. The parallel procedure assures us that the direct comparison of FRET efficiencies is not affected by potential variations in the spin coating procedure. The intensity decays were fit to a multi-exponential model to determine the decay times and their amplitudes (Eq. (1)) and the average lifetime for each measured sample were calculated. We carefully investigated the confidence of intervals and found that the shortest components are better than  $\pm 20\%$  and amplitudes within  $\pm 10\%$ . Based on the calculated average lifetimes, efficiencies of FRET were calculated (Eq. (2)). Similarly as shown in the Figures 3 and 4, the intensity decays were measured for other configurations (SIFS with 10 nm PVA spacer layer and for continuous Ag film) and the calculated amplitude weighted lifetimes and FRET efficiencies are summarized in Table 2. Figure 5 illustrate the FRET efficiencies for D-A systems in various geometrical configurations. The FRET data determined on glass substrates are represented by open points, each related to respective data point on SIFs or Ag film.

To facilitate the comparison between FRET efficiencies determined on the glass with plasmonic nanostructures  $(E_m)$  and on bare glass (E), we calculated the FRET enhancement defined as the ratio of  $[(E_m - E)/E]$ . Figure 6 shows that FRET enhancements can be positive or negative and this strongly depends on the acceptor concentrations. These observations indicate that careful consideration must be given in design of FRET systems combined with plasmonic nanostructures.

For D-A samples deposited directly on the SIFs, the FRET efficiency is lower compared to that on the glass as shown with negative enhancement. This indicates that strong interaction between donor molecules and particle plasmon resonance decreases the donor ability for energy transfer to the acceptor molecules. The observed enhanced intensity enhancement of donor alone (Table 1) is due to strong effect of the enhanced electromagnetic field at excitation wavelength thus enhancing absorption probability. However, it is known that increasing the intensity of donor by increasing excitation intensity does not lead to higher energy transfer efficiency. The decreased lifetime of the donor alone is due to combined effects of surface plasmons on radiative and nonradiative decay rates. It is known that close proximity of the fluorophore to the metal surface leads to energy transfer to the metal, known commonly as nanometal surface energy transfer (NSET) [28–31]. Therefore, the competitive energy transfer to the metal leads to decreased FRET to Cy5. This can also be explained by the decreased effective quantum yield of the donor.

For D-A samples deposited on the spacer layer of 10 nm PVA over the SIFs, the relative FRET is enhanced and the enhancement decreases with the increased acceptor concentration. For low acceptor concentration, the FRET efficiency is enhanced by more than 2-fold (Table 2). This can be explained by the increased effective quantum yield of the donor because of enhanced radiative decay rate. The important fact is that the largest FRET enhancement is observed for low acceptor concentration which is beneficial for FRET systems with large separation between donor and acceptor molecules. In this regard one can expect more sensitive FRET in studies of biological systems. For example, FRET application to surface-based assays is currently limited to relatively small biomolecules, however using plasmonic structures, interactions

between dye labeled antibodies will likely produce easily detectable FRET thus expanding the applicability of FRET into broader range of immunoassays.

Time resolved measurements for samples deposited on continuous silver films were performed using direct excitation of the sample (reverse Kretschmann configuration) and observed donor emission in the SPCE mode [9]. In contrast to the results on SIFs, the intensity decays of donor alone (Cy3) on solid Ag films were modified moderately compared to that on glass (Table 1). The observed enhancement of FRET is less than for SIFs with spacer layer of 10 nm but display a similar trend; larger effects at lower acceptor concentrations. The small changes in lifetime indicate small modifications of radiative and non radiative decay rates, thus minimally affecting the quantum efficiency of donor. The possible explanation of enhanced FRET at lower acceptor concentrations present on continuous metallic films.

The common approach of interpreting the FRET efficiency is use of energy transfer rate. The theoretical works on effects of metallic particles considered the changes in the energy transfer rate which can increase many folds compared to that in free space [13–15]. The rate of energy transfer from a donor to an acceptor ( $k_T$ ) is given by

$$k_T = \frac{1}{\tau_{\rm D}} \left(\frac{R_0}{r}\right)^6 \tag{3}$$

where  $\tau_D$  is the decay time of the donor in the absence of acceptor,  $R_0$  is the Förster distance, and *r* is the donor-to-acceptor (D-A) distance. It should be noted that in the presence of metallic nanoparticles the energy transfer rate increase is due to decreased lifetime of the donor. The value of  $R_0$  is also modified by plasmonic structures and may increase or decrease the energy transfer rate (more detailed discussion is below). The efficiency of energy transfer is the fraction of the photons absorbed by the donor that are transferred to the acceptor and can be described by

$$E = \frac{k_T}{\frac{1}{\tau_{\rm D}} + k_T} \tag{4}$$

In order to estimate the relative change in transfer rate at each acceptor concentration, one can use the ratio of  $k_{Tm}/k_T$ , where the subscript *m* relates to the D-A system in the presence of metallic nanostructures

$$\frac{k_{Tm}}{k_T} = \frac{\langle \tau_{\rm D} \rangle}{\langle \tau_{\rm Dm} \rangle} \frac{E_m}{E} \frac{1 - E}{1 - E_m} \tag{5}$$

Eq. (5) can also be expressed directly with the calculated average lifetimes in the absence (D) and the presence (DA) of acceptor (see Eq. (2))

$$\frac{k_{Tm}}{k_T} = \frac{\langle \tau_{\rm D} \rangle}{\langle \tau_{\rm Dm} \rangle} \frac{\langle \tau_{\rm DA} \rangle}{\langle \tau_{\rm DAm} \rangle} \frac{\langle \tau_{\rm Dm} \rangle - \langle \tau_{\rm DAm} \rangle}{\langle \tau_{\rm D} \rangle - \langle \tau_{\rm DA} \rangle} \tag{6}$$

The plots of relative transfer rates  $k_{Tm}/k_T$  for the three investigated geometrical configurations are shown in Figure 7. While the real situation is likely more complex and average lifetimes are not the best parameters to draw quantitative conclusions, we believe that the presented data

illustrate the important aspects of the FRET systems in the presence of plasmonic nanostructures. The first term in Eq. (6) describes the proportionality of the transfer rate to the decay rate of the donor (about 10 in presence of SIFs and 1.3 for SPCE, see Table 1). The dashed lines in the Figure 7 illustrate this effect. This term is the same as in classical Förster energy transfer where the energy transfer rate is always proportional to the radiative rate of the donor. This is the reason that the FRET efficiencies and Förster distances are the same for organic fluorophores, metal-ligand complexes, and lanthanides, which have lifetimes of nanoseconds, microseconds, and milliseconds, respectively. The second and third terms imply that additional changes are from parameters that contribute to  $R_0$ . For dipole-dipole interaction where the energy transfer efficiency is dependent on the inverse sixth power of the distance between donor and acceptor, the critical distance is defined as [25]

$$R_0^6 = 8.79 \times 10^{-5} [\kappa^2 n^{-4} Q_{\rm D} J(\lambda)] \tag{7}$$

where  $\kappa^2$  is the average relative orientation between the transition dipole moments of the donor and acceptor, *n* is the refractive index of medium,  $J(\lambda)$  is the spectral overlap between donor emission and acceptor absorption, and  $Q_D$  is the quantum yield of fluorescence of donor molecules. To the first approximation, the obvious parameter that changes value is the quantum efficiency of the donor when the fluorophore is in close proximity to the metallic nanostructures. It is also possible that the average orientation factor,  $\kappa^2$  might change also. This is because the donor dipole and the dipole induced in silver particles radiate out of phase for donor molecules oriented tangentially to the silver islands surface. Such a possibility has recently been reported using time-resolved spectroscopy, gold nanoparticles, and organic dyes [28,32]. Thus the interaction with surface plasmon may lead to the different average value of  $\kappa^2$  than in case of bare glass.

The large increase in transfer rates (more than 20-fold, Figure 6) as observed for samples with 10 nm PVA spacer layer resulted in about a 2-fold increase (about 92%, Figure 6) in energy transfer efficiency. A similar increase in transfer rate of 21-folds and increase in critical distance  $R_0$  of 1.6-fold has been observed in studies of single D-A pairs on silver colloids [23]. The single molecule studies provide the opportunity to examine the D-A system in a statistical format, thus revealing the D-A pairs with relatively high energy transfer efficiencies. Single molecule detection includes observation of heterogeneity of D-A pairs and their geometrical orientations relative the metallic surfaces while our observations are based on the averaging over the large number of D-A pairs. Nonetheless, averaging over the randomly distributed molecules provides the insights for design of FRET systems for biological applications.

Based on previous experiments from our group, we expected to see substantial increases in FRET near metal particles [20–23]. However, there is some difficulty in the direct comparison of our present data with that obtained previously. For several D-A systems investigated before, thick solution layers of D-A pairs (approximately 1  $\mu$ m) were used and only a small fraction (about 4%) was in the proximity of 20 nm from silver islands. In such cases, the data were analyzed using a FRET model with two Förster distances  $R_{01}$  and  $R_{02}$  to describe the two-component system, D-A pairs in solution and D-A pairs in close proximity to silver islands. It was also demonstrated that the enhanced FRET occurred if the solution (thickness of 1  $\mu$ m) was placed between two silver island films but not in the case of single silver island film [20–22]. Large enhancements in FRET for configuration with two layers of SIFs implies that it is likely that microcavities could be created facilitating a much more active role on FRET than our configurations where D-A system is placed above the single layer of SIFs. In another

report, the effects of microcavities on FRET were investigated and it was found that dipoledipole interactions can be modified [33–36].

The important observation from steady state intensity measurements is that the measurements of D-A systems with high energy transfer can be easily accomplished with plasmon resonance. It was found that the fluorescence enhancement (donor intensity ratio on SIFs to intensity on glass) of donor intensities increase with increased energy transfer efficiency. Figure 8 shows that intensity enhancements for donor increased from about 5.5-fold (in the absence of acceptor) to 20–30 folds when quenched by acceptor. This observation is in agreement with the expected higher fluorescence enhancements of low quantum yield fluorophores. This can be an important advantage when investigating highly FRET-efficient D-A systems (e.g. above 95%) because it would provide highly enhanced signals compared to free space systems.

To the best of our knowledge, our data demonstrates the first systematic studies of D-A systems with various acceptor concentrations placed in the near-fields of plasmonic structures. Overall, the presented data illustrate that the presence of metallic nanostructures allows FRET studies to be extended. For systems where the average distances between D and A are large (low acceptor concentration), the plasmonic nanostructures substantially enhance FRET. On the other hand, for systems with very high FRET, the presence of metallic nanostructures allows detection of highly quenched donors because of efficient signal enhancement. It is anticipated that the D-A systems with low quantum yield donors will result in substantially improved FRET efficiencies due to increased radiative rate.

Theoretical predictions indicate possible large increase in FRET efficiency if the D and A molecules positions are well defined relative to metallic particles. In such case the metallic particles can play an active role in mediating energy transfer from D to A, e.g. placing D and A on opposite side of the metallic particles which provides the FRET to occur on several fold of  $R_0$  [13–15]. Such geometrical configurations are not easy to construct, therefore the verification of high energy enhancements and transfer over distances of more than 50 nm has not been experimentally confirmed since early theoretical works in the late 1980's [13–15]. However, the question will arise if such systems can be described by Förster mechanism because no direct interaction between D and A molecules. To verify such mechanism, excitation of the donor and observation of acceptor fluorescence is desired.

# 4. Conclusion

Using several configurations of donor-acceptor systems, we have demonstrated that the energy transfer rate can be substantially increased in the presence of silver islands. This observation is in agreement with previous theoretical calculations and experimental observations. While the transfer rate increased substantially, the energy transfer efficiency changes are moderate compared to the standard samples coated on the glass substrate. The largest FRET enhancements were observed for low acceptor concentrations (large donor acceptor separations). We estimated that the changes in energy transfer efficiency (enhancement or decrease) for the D-A system placed above metallic nanostructures can be explained in first approximation, by changes in quantum yield of the donor molecules in the presence of MEF substrates. The advantage of using plasmonic nanostructures is expected for high efficiency FRET systems because the signal of the donor is highly enhanced. The methodology and observations reported here could be relevant for the design and construction of surface-based biochemical sensors that exploit the advantage of using FRET configurations with a monolayer of biomolecules labeled with a donor that bind biomolecules labeled with an acceptor.

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# Biographies

**Henryk Szmacinski** received his M.S. and Ph.D. in physics from the University of Gdansk, Poland in 1973 and 1980, respectively. Since 1986, he has been associated with University of Maryland at Baltimore, currently as an Associate Professor in the Department of Biochemistry and Molecular Biology. His major research interest is application of fluorescence spectroscopy to chemical sensing, fluoroimmunoassays and cellular imaging.



**Krishanu Ray** received his M.S. in Physics from I.I.T. Kharagpur and Ph.D. from Jadavpur University, India. Presently he is an Assistant Professor at the Department of Biochemistry and Molecular Biology, University of Maryland, Baltimore. His current area of research focuses on the application of single molecule detection and fluorescence spectroscopy in biomedical research.



**Joseph R. Lakowicz** is Professor at the University of Maryland at Baltimore and Director of the Center for Fluorescence Spectroscopy. He received his M.S. and Ph.D. in biochemistry from the University of Illinois, Urbana. His research interest is in broad aspects of fluorescence spectroscopy. He is the author of more than 600 publications and the book on Principles of Fluorescence Spectroscopy.





**Figure 1.** Absorption spectrum and field emission image (inset) of silver island films.



#### Figure 2.

(online colour at: www.biophotonics-journal.org) Geometrical configurations of D-A samples in the PVA films (red top layers) spin coated on substrates with silver nanostructures and respective control bare glass: **a**) 10 nm PVA with D-A directly on silver island films, **b**) 10 nm PVA with D-A on SIFs with 10 nm PVA spacer, **c**) 20 nm PVA with D-A on silver nanofilm with 5 nm SiO<sub>2</sub> spacer. The schemes are not in scale.



# Figure 3.

(online colour at: www.biophotonics-journal.org) Intensity decays of Cy3 in the (A) absence and presence of acceptor, Cy5, in 10 nm PVA film deposited on glass at concentrations (B) 1.56 mM, (C) 3.92 mM, (D) 7.84 mM.



#### Figure 4.

(online colour at: www.biophotonics-journal.org) Intensity decays of Cy3 in the absence and presence of acceptor, Cy5, in 10 nm PVA film deposited on silver islands.



# Figure 5.

(online colour at: www.biophotonics-journal.org) FRET efficiency for D-A systems determined from intensity decays of Cy3 (donor) in presence of various concentrations of Cy5 (acceptor) for three geometrical configurations. Detailed values of average lifetimes and calculated FRET efficiencies are in Table II. The empty symbols indicate the values obtained on bare glass substrates (one line is for clarity of plot).



#### Figure 6.

(online colour at: www.biophotonics-journal.org) FRET efficiency enhancement for D-A systems in close proximity to silver islands and silver continuous film (SPCE).



#### Figure 7.

(online colour at: www.biophotonics-journal.org) Relative increase of the energy transfer rate in the presence of silver islands and silver continuous film. Dashed lines indicate the anticipated increases of the transfer rates due to decreases in the lifetime of the donor.



#### Figure 8.

(online colour at: www.biophotonics-journal.org) Fluorescence enhancement of Cy3 (donor) is dependent on FRET efficiency to Cy5 (acceptor).

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Table 1Intensity and lifetime characteristics of Cy3 in 10 nm PVA film coated on glass and on SIFs. NIH-PA script

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Configuration	Intensity	Intensity Decays				
		$r_i$ (ns)	$a_i$	$f_i$	$\langle \tau_{\rm D} \rangle$ (ns)	$ au_{\mathrm{D}m}$ (ns)
Glass	1.0	2.34	0.736	0.911	1.89	2.19
		0.63	0.264	0.009		
Directly on SIFs	5.4	1.87	0.027	0.280	0.18	0.72
		0.45	0.153	0.382		
		0.07	0.820	0.338		
Spacer of 10 nm PVA	5.8	2.01	0.025	0.281	0.18	0.73
		0.34	0.223	0.422		
		0.07	0.752	0.297		
SPCE	N/A	2.06	0.538	0.777	1.43	1.75
		0.69	0.462	0.223		
	$\langle \tau_{\rm D} \rangle = \overline{\sum_{a;r_i}^{a_i r_i}}$		$\tau_{Dm} = \overline{\sum_{f_{itri}}^{J_i \tau_i}}$			
$\langle \tau D \rangle$ -amplitude weighted lifetime	e( <u>{</u> <u></u>	Dm-intensity weighted lifetime	- [ <sup>2</sup> , <sup>1</sup> ]			

Table 2	
FRET efficiency between Cy3 and Cy5 in	10 nm PVA film deposited on SIFs.

Acceptor [mM]	$\langle \tau \rangle$ (ns)		FRET Efficiency			
	glass	SIFs	glass	SIFs		
Directly on SIFs						
0	1.89	0.18	0	0		
0.4	1.42	0.16	0.247	0.117		
0.8	1.06	0.13	0.440	0.300		
1.56	0.80	0.10	0.575	0.444		
3.92	0.37	0.06	0.803	0.661		
7.84	0.17	0.035	0.908	0.806		
With 10 nm PVA Spacer on SIFs						
0	1.87	0.179	0	0		
0.18	1.64	0.137	0.123	0.235		
0.60	1.24	0.092	0.337	0.486		
1.80	0.77	0.058	0.588	0.676		
3.60	0.36	0.029	0.807	0.838		
7.20	0.17	0.015	0.909	0.916		
With 5 nm SiO <sub>2</sub> Spacer on Continuous Silver Film						
0	1.623	1.427	0	0		
0.3	1.285	1.021	0.208	0.285		
0.6	0.990	0.773	0.390	0.458		
1.45	0.638	0.547	0.607	0.617		
2.9	0.391	0.368	0.759	0.742		
9.0	0.170	0.111	0.895	0.922		

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