Metal-Enhanced Fluorescence: Potential Applications in HTS

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Abstract: Metallic surfaces and particles can have dramatic effects on fluorescence, including localized excitation, increased quantum yields, increased photostability and increased distances for resonance energy transfer (RET), and directional emission. While all these effects have not yet been realized in a single system, metal-enhanced fluorescence promises to provide the next generation of high sensitivity fluorescence assays for low copy number detection of biochemical species.

INTRODUCTION

Fluorescence detection is the basis of most assays used in drug discovery and high throughput screening. In almost all the current assays fluorophores are in the "free-space condition," in which they emit energy into a homogeneous transparent environment such as aqueous solutions and solvents. The sensitivity of fluorescence to the local environment is due to chemical interactions which result in quenching, changes in viscosity or in the local polarity. These interactions do not change the most fundamental property of a fluorophore, which is its rate of radiative decay. This rate can be changed by modifying the photonic mode density around the fluorophore so that it is no longer in the free-space condition.

In this article we describe the possibility of modifying the spectral properties of fluorophores by changing the "free space" condition. This can be accomplished by proximity of the fluorophore to a conducting metallic surface or particle, which we will call a metal. To understand the effects of metals we need to consider the definitions of the fluorescence quantum yield Q_0 and lifetime τ_0. Figure **1** (top) shows the classical Jablonski diagram for excitation (E) and emission. Photon absorption results in a fluorophore in the first singlet state S_1. The fluorophore can then emit a photon or radiate energy with a rate constant Γ, which is called the radiative decay rate. The fluorophore can also return to the ground state by non-radiative decay with a rate (k_{nr}) or due to some other quenching process (k_q). The quantum yield (Q_0) of a fluorophore reflects a competition between emission of a photon and the non-radiative decay process:

$$Q_0 = \frac{\Gamma}{\Gamma + k_{nr} + k_q} \quad (1)$$

The fluorescence lifetime or decay time is the mean time a fluorophore remains in the S_1 state and is given by:

$$\tau_0 = \frac{1}{\Gamma + k_{nr} + k_q} \quad (2)$$

If a fluorophore has a quantum yield of unity then the lifetime is given by $\tau_N = \Gamma^{-1}$, which is often called the natural lifetime.

It is well known that fluorescence intensities and lifetimes are strongly influenced by the environment surrounding the fluorophore, which is due to changes in k_{nr} and k_q. For example, molecules which display low quantum yields and lifetimes in solution often display high quantum yields and longer lifetimes in frozen solution or when bound to macromolecules. These spectral changes are due to changes the non-radiative decay rate k_{nr} and k_q. The quantum yield of a fluorophore is determined by the relative values of Γ and $k_{nr} + k_q$. The important point is that Γ is essentially a constant for each fluorophore and determined by the extinction coefficient [1]. The radiative rate is not significantly changed by conditions which alter its quantum yield and lifetime.

The unique opportunities of metals to modify fluorescence is due to changes in the rates of excitation and emission. Several effects are possible. One effect is the so called "lightning rod effect." A metal particle can amplify the incident light field by interactions of the light with the freely mobile electrons in the metal. This effect is shown in Figure **1** (bottom) by an additional excitation field E_m. This effect can be dramatic. For example, the local electric field can be increased by a factor of 140 near a silver particle [2], which is a 20,000-fold increase in the local intensity and rate of excitation. This suggests that one can obtain localized excitation of fluorophores near a metal surface. Surface localization is a common feature of many assays, such as immunoassays and DNA arrays.

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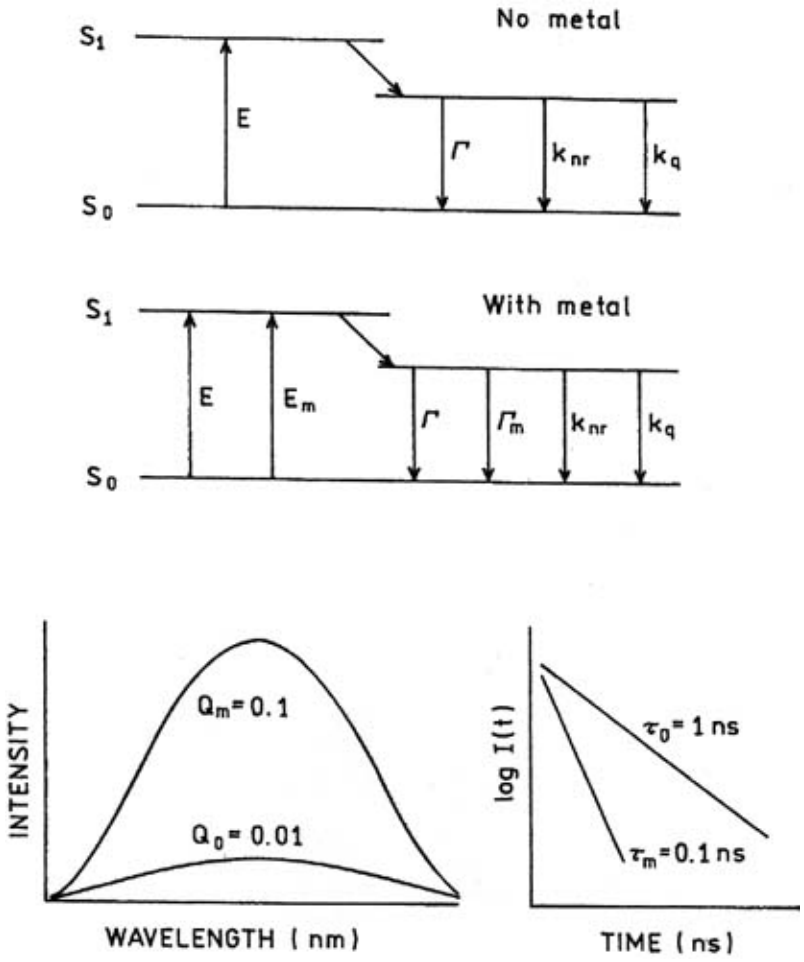

Fig. (1). Top: Modified Jablonski diagram for metal-enhanced fluorescence. Bottom: Increased quantum yield and decrease lifetimes for a fluorophore near a metallic (m) surface.

Another effect of metals is to increase the radiative decay rate. This is an unusual effect which is not encountered for fluorophores in the absence of metallic surfaces, for which the intensities and lifetimes typically increase or decrease in unison. The consequences of increasing the radiative decay rate Γ_m can be understood from a modified Jablonski diagram which includes the metal-induced radiative rate Γ_m (Figure **1**). If Γ_m increases, weakly fluorescent molecules can become fluorescent while the lifetime decreases, opposite of that found for decreases in k_{nr} and k_q. For a fluorophore at an appropriate distance from a metal surface the quantum yield (Q_m) and lifetime (τ_m) are given by:

$$Q_m = \frac{\Gamma + \Gamma_m}{\Gamma + \Gamma_m + k_{nr} + k_q} \quad (3)$$

$$\tau_m = \frac{1}{\Gamma + \Gamma_m + k_{nr} + k_q} \quad (4)$$

Suppose a fluorophore displays a low quantum yield. The experimental conditions determine k_{nr} and k_q. The quantum yield cannot be increased because the radiative decay rate cannot be changed. However, under the same solution conditions, proximity of a fluorophore to a metal can increase the radiative rate due to addition of Γ_m . This increases the quantum yield and decreases the lifetime (Figure **1**, bottom). Theory suggests that the radiative rates can be increased over 1000-fold near a metal [3], which is adequate to change a quantum yield from near zero to near unity. The interactions of fluorophores with metals is complex and depend on the size and shape of the metal. Metallic particles typically have larger effects than continuous metallic surfaces.

Localized excitation and increased quantum yield near metals are desirable effects in fluorescence assays. However, there are additional reasons to consider the use of metallic particles. By a combination of effects, metals can increase the number of detected photons per fluorophore by a factor of 10^5 or larger. These effects are summarized in Figure **2**, with conservative estimates of 10-fold used for each effect. As described above, metal particles can increase the local intensity by 10^4. This means that the same intensity could be observed with 10^4 -fold lower incident power, resulting in less photochemical damage to other parts of the sample. Since maximal effects may not be realized in practice, we

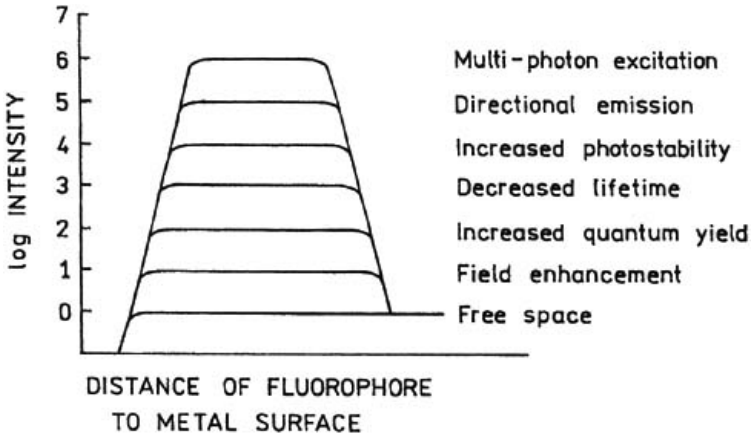

Fig. (2). Potential increases in intensity due to fluorophore-metallic surface interactions.

used a factor of 10 in Figure **2**. The quantum yield can be increased by a factor of $1/Q_0$ [2].

In the absence of metals decreased fluorophore lifetimes are usually an undesired effect associated with lower quantum yields and lower intensities. In the case of fluorophore-metal interactions decreased lifetimes can be advantageous and result in a higher photon flux and increased photostability. The maximum number of photons/sec emitted by a fluorophore is roughly limited by the inverse lifetime. For instance, a nanosecond lifetime can yield about 10^9 photons/sec/molecule. Hence if the metal reduces the lifetime 10-fold, the incident intensity can be increased 10-fold prior to depletion of the ground state. While ground state depletion is usually not obtained in a spectrofluorometer, it can occur with the higher local illumination intensities used in microscopy, flow cytometry and spot scanning imagers.

It is often desirable to observe single molecules. Single molecule detection (SMD) is usually limited by the photon emission rate of the molecule and its photostability [4-5]. Even the most photostable fluorophores photodegrade after 10^5 or 10^6 excitation emission cycles. Since fluorescence emission is usually isotropic, and optical elements have limited collection efficiencies, one can usually observe less than 10,000 photons per fluorophore. Since the extent of photochemical degradation depends on the amount of time a fluorophore is in the excited state, a 10-fold decrease in lifetime can result in a 10-fold increase in the number of photons emitted by a fluorophore prior to decomposition. If the lifetime of the fluorophore is decreased 10-fold it can emit 10-fold more photons/sec, which can be advantageous if the observation time is limited, which is typically the case in flow cytometry or with laser scanning imaging.

Additional effects are also possible. Two-photon excitation has become widely used in cellular imaging

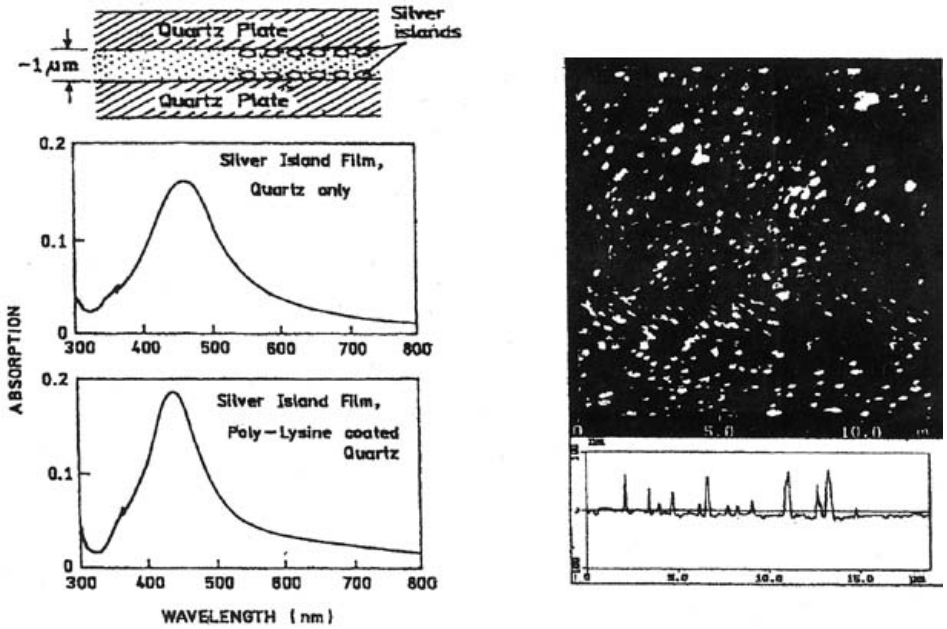

Fig. (3). Experimental system for metal-enhanced fluorescence. Top left: Liquid sample between silver island films (SIFs). Bottom left: Absorption spectra of SIFs on quartz or poly-lysine coated quartz. Right: Atomic force microscope image of SIFs.

because of localized excitation at the focal spot [6-7]. Recalling that metal particles can increase the local intensity by 10^4, and that photon excitation depends on the square of the intensity, two-photon excitation may be dramatically enhanced near metallic particles. Additionally, proximity of a fluorophore to a periodic metal surface can result in directional emission, rather than the usual isotropic emission under free space condition. This effect could increase the fluorescence collection efficient by a factor of 10 or more.

These effects can have a dramatic effect on fluorophore detectability, perhaps increasing the over intensity by a factor of a million or more (Figure **2**). To achieve these effects it will be necessary to locate the fluorophore near the metal surface, probably in the range of 100 to 1000 A. It will also be necessary to keep the fluorophore 50 A or more from the metal surface to avoid quenching at these shorter distances. There will be a zone near the surface where the effects are maximal. It will also be necessary to control the size and shape of the particle.

Fluorescence resonance energy transfer (RET) is widely used to measure macromolecular interactions, binding assays and DNA hybridization. Hence it is important to note that metal particles can also increase the distances over which RET occurs. The characteristic free-space Forster distances of 20-50 A may be increased to 500 A near metals, allowing RET to be used to detect widely separated fluorophores on large biomolecules.

Recent Results on Metal-Enhanced Fluorescence

The preceeding paragraphs contain surprising predictions which require experimental verification. We expect considerable development will be needed to fabricate fluorophore-metal systems with the optimal size, shape and distance for a desired application. To determine whether such effort was justified we chose a simple experimental system to test these predictions. Silver particles deposited on a quartz substrate. These so-called silver island films consist

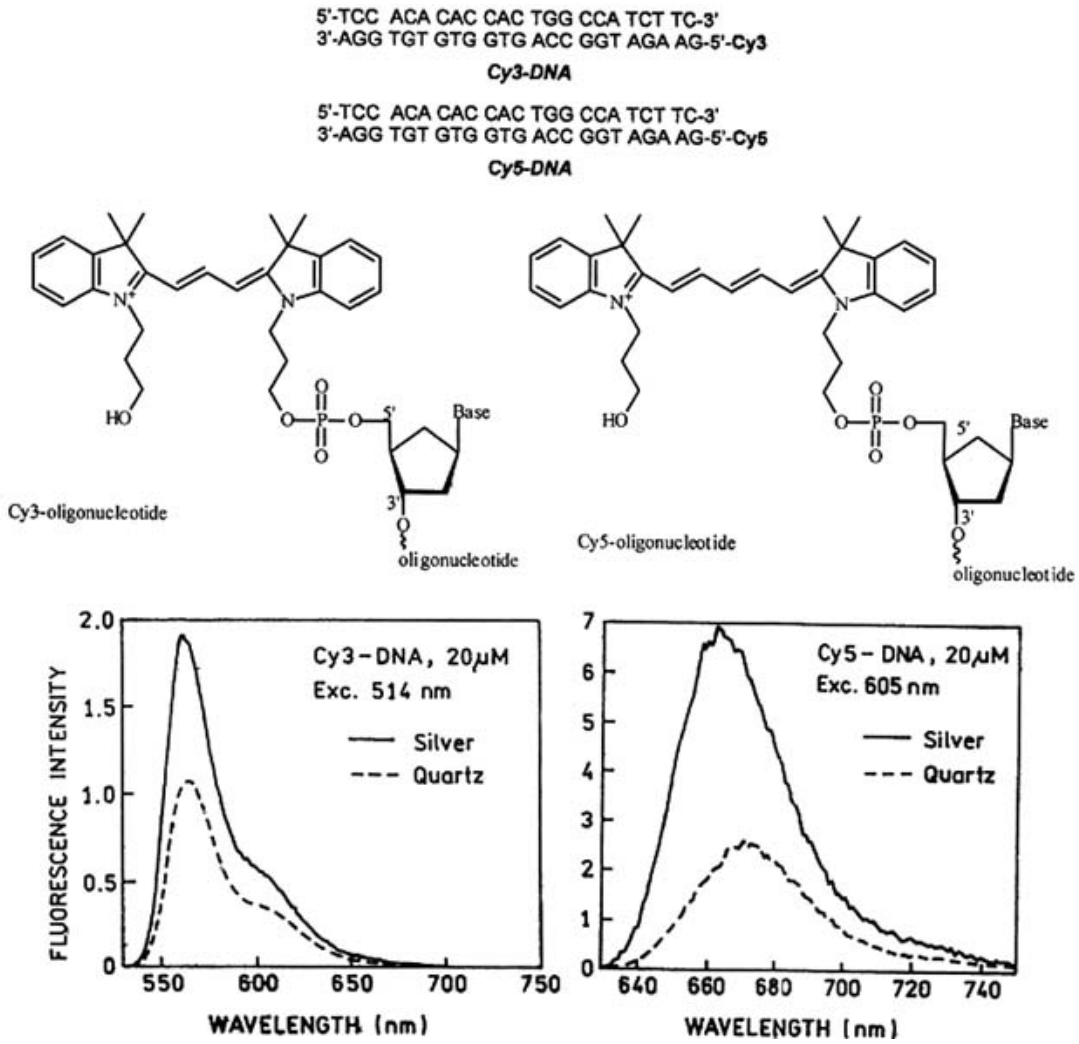

Fig. (4). Emission spectra of Cy3 and Cy5-labeled DNA oligomers between quartz and silver island films.

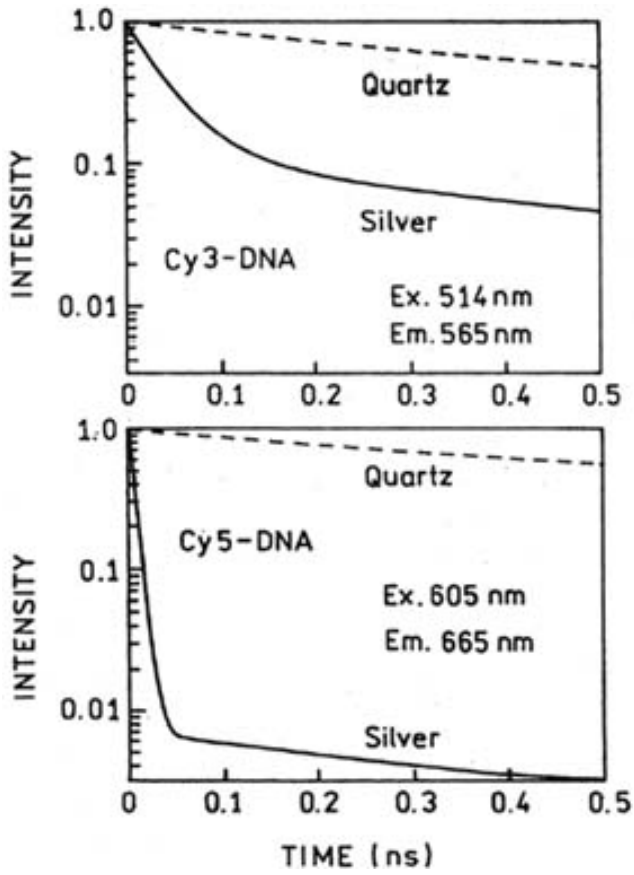

Fig. (5). Time-dependent intensity decays of Cy3-DNA and Cy5-DNA between quartz plates and silver island films.

of irregular flat particles ~ 300 A in size (Figure **3**, right). These particles display a surface plasmon absorption centered near 450 nm which is typical of subwavelength colloidal metal particles [8]. Fluid samples were placed between two such island coated slides positioned about 1 μ apart (Figure **3**, top left). This sample configuration results in a heterogeneous population of fluorophores. Much of the liquid sample is too distant from the silver to be affected. We estimate only ~ 10% of the sample is within the active volume affected by the metal. Hence the observed effects will greatly underestimate the effects possible for a thinner fluorophore film or fluorophores covalently attached to the silver by a spacer.

We questioned whether silver particles could increase the apparent quantum yields of fluorophores. We use the phrase "apparent quantum yield" because the measured intensity is affected by both the lightening rod effect and the increased quantum yield. We chose to examine DNA oligomers labeled with Cy3 or Cy5 [9] because of the widespread use of such molecules with DNA arrays [10-11]. These samples are double helical DNA with either Cy3 or Cy5 covalently linked to the 5' end of one of the strands. Figure **4** shows the emission spectra of Cy3-DNA and Cy5-DNA between unsilvered quartz plates or between two silver island films. In both cases the emission is ~ 2-fold higher between the silver island films. The silver enhancement is somewhat greater for Cy5-DNA than for Cy3-DNA, which is consistent with the lower quantum yield of Cy5 [2,12]. Our experimental systems make it difficult to determine the enhancement possible for those fluorophores which are ideally distant from the metal. In other experiments with monolayers of fluorophores ~ 100 A from the metal we observed 20-fold or larger intensity increases. The increases seen in Figure **4** and other recent experiments are probably due to both the lightening rod effect and the increased quantum yields. At this time we do not know the relative contribution of these effects to yield the observed intensity increases.

We measured the intensity decays of Cy3-DNA and Cy5-DNA. The decays were measured using the frequency-domain method [13], and the reconstructed time domain decays are shown in Figure **5**. The lifetime measurements are informative because an increased lifetime indicates less quenching and a decreased lifetime indicates an increase in the radiative decay rate. The intensity decays were multi-exponential and described by:

$$I(t) = \sum \alpha i \exp(-t / \tau i) \quad (5)$$

The amplitude weighted decay times

$$< \tau > = \sum \alpha i \tau i \quad (6)$$

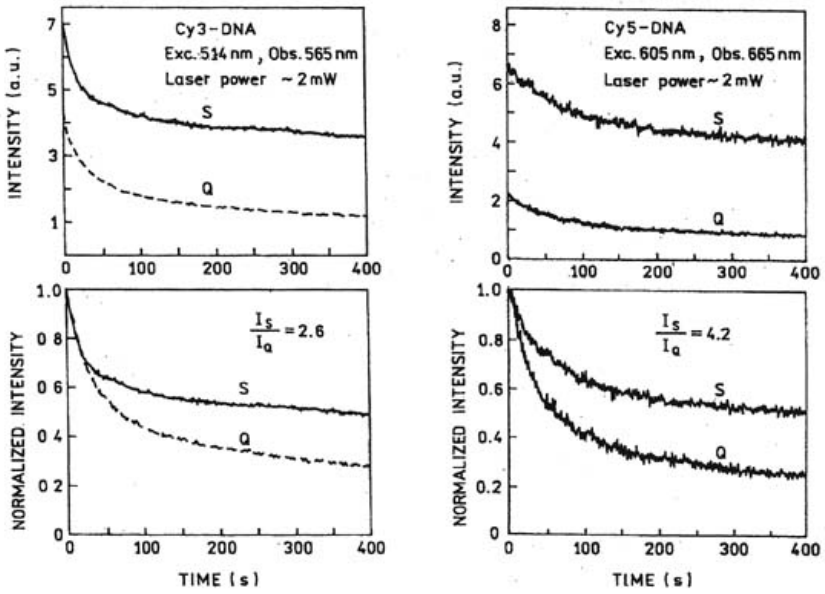

Fig. (6). Effect of silver island films on the photostability of Cy3 and Cy5 bound to a DNA oligomer.

decrease dramatically near the silver islands. The decreased lifetimes (Figure **5**) and increased intensities (Figure **4**) are consistent with an increase in the radiative decay rates of Cy3 and Cy5. The decrease of lifetime suggests increased photon emission fluxes at saturating illumination conditions. It is thought that photobleaching or photodecomposition only occurs during the time a fluorophore is in the excited state. Hence a fluorophore is expected to be more stable if it has a shorter lifetime [14]. Because of the spatial heterogeneity of our samples, and the freely diffusing oligomers, it is difficult to determine the photostability. We examined the intensities of the labeled oligomers with continuous illumination (Figure **6**). The time-zero intensities are higher as described above. The rates of photobleaching appears to be slightly slower with the silver islands, but quantitative interpretations await further experimentation. From the areas under the curves (bottom) we estimate that 2 and 4-fold more emission can be obtained from Cy3-DNA and Cy5-DNA, respectively, as compared to samples not near silver particles. Recalling that the initial intensities of the samples are 2-fold higher between the silver island films (top), then these films provide a 4- and 8-fold increase in the observable emission from these samples.

In these experiments the samples were spatially heterogeneous with only a minor fraction of the fluorophores located at the ideal distances from the particles for the maximal increase in the radiative rate. It is of interest to consider the increase in detected emission if the fluorophores were ideally located near the metal particles. In typical DNA array scanners the excitation intensity can be adequate to deplete the ground state population of the fluorophores. Under conditions of strong illumination the maximum emission rate is approximately given by the inverse lifetime. Using the short amplitude-weighted decay times as representing the ideally located fluorophore, the decreased lifetimes of Cy3-DNA and Cy5-DNA are expected to result in 8 and 38-fold increases in the maximum emission rate of these fluorophores. Since the effects on photostability and maximal emission rate are expected to be independent, we can expect 32 and 300-fold increase in detectable signal for Cy3 and Cy5-labeled DNA on substrates coated with silver particles. These considerations suggest the use of substrates containing silver particles for increased detection sensitivity on DNA arrays.

It is of interest to examine the effects of silver particles on resonance energy transfer (RET), which is widely used to measure biomolecules associations. RET is a useful

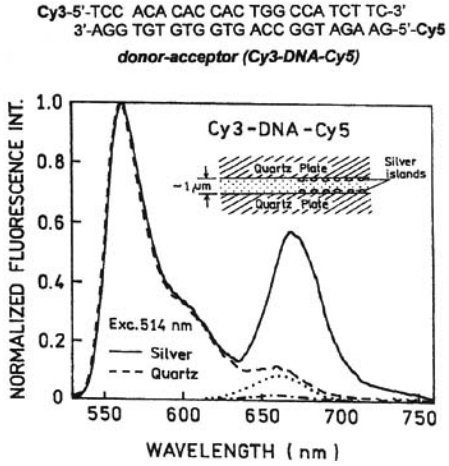

Fig. (7). Effects of silver islands on resonance energy transfer in a DNA oligomer labeled with both Cy3 and Cy5. Also shown are the emission spectra of Cy5 labeled DNA on both silver and Quartz, see figure 4 right also.

phenomenon because it occurs over relatively large distances up to about 60 A [15-16]. An advantage of RET is that it is mostly independent of the solvent or biomolecule between the D-A pair. Additionally, the Forster distances (R_0) can be calculated on first principles from the emission spectrum and quantum yield of the donor and the absorption spectrum of the acceptor [17-18]. If the D-to-A distance is comparable or shorter than R_0 then RET will occur. A disadvantage of RET is that 60 A is a small distance relative to the size of long DNA oligomers or large protein clusters such as antigen-antibody complexes. At present, RET over longer distances is not used because it does not occur. If RET could occur over longer distances then we believe applications will be developed. We found that spatial proximity to metallic silver particles results in a dramatic increase in the apparent Forster distances [19], in agreement with earlier theoretical predictions [20-21].

Emission spectra of a DNA oligomer labeled with both Cy3 and Cy5 are shown in Figure **7**. In the absence of silver the extent of energy transfer is small, consistent with the size of the oligo and the Forster distance near 54 A. Increased energy transfer was found with the silver island films, which can be seen from the increased acceptor emission near 670 nm. We analyzed the time-resolved donor decays (not shown) to determine the Forster distance of two populations, the molecules distant from the sample with R_0 = 54 A and the molecules close to the silver with an unknown R_0 value. The analysis revealed a R_0 value of 119 A, which can be seen from the increased acceptor emission near 670 nm. In other studies we observed 5-fold increases in the apparent Forster distance [22]. These results suggest development of protein or DNA assays based on long range RET occurring in large protein complexes as long nucleic acid sequences.

Applications of Metal-Enhanced Fluorescence to Drug Discovery

Metal-enhanced fluorescence appears to be readily adapted to the fluorescence assays used in drug discovery and DNA analysis. Silver can be readily deposited on glass or polymers substrates by a variety of methods. Silver colloids are easily prepared and readily attached to amine or sulfhydryl coated surfaces. We can imagine the bottom of multi-well plates or DNA arrays being coated with silver particles. A variety of new assay formats are possible. Assays could be based on the lightening rod effects. The biochemical affinity interactions could bring the fluorophore close to the metal surface, for localized excitation, eliminating the washing steps. In fact, one such report has

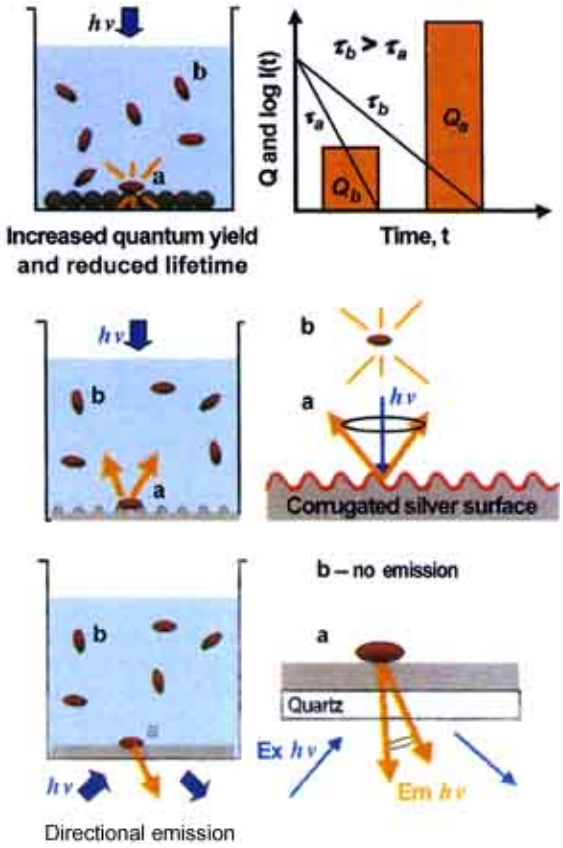

Fig. (8). Potential uses of metal-enhanced fluorescence in drug discovery based on local increases in quantum yield (top) or directional emission (bottom).

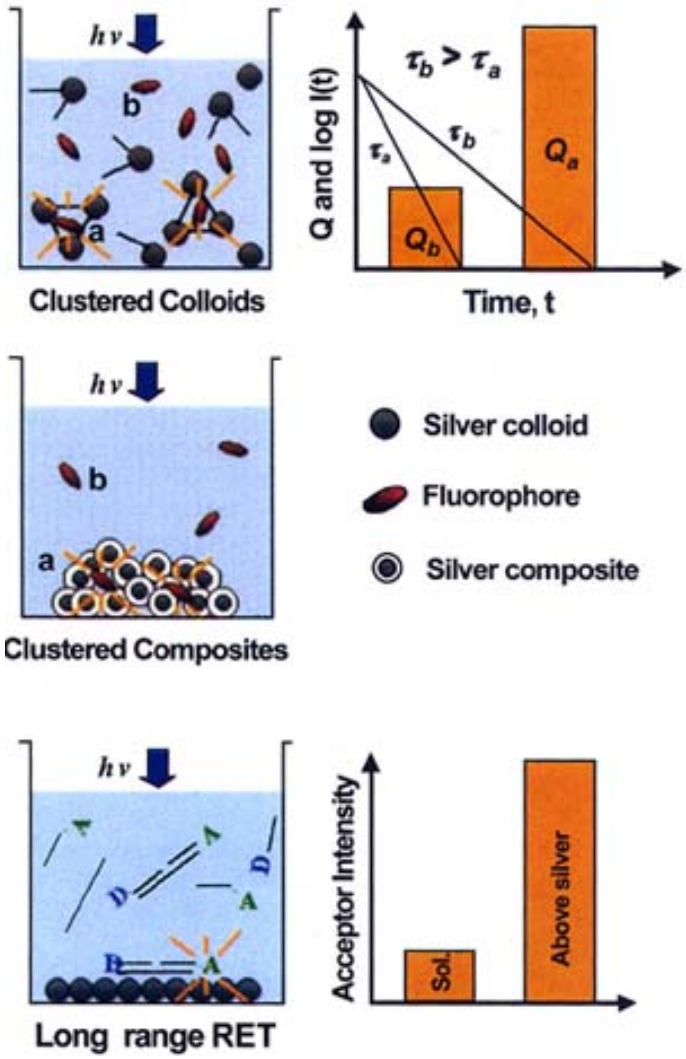

Fig. (9). Potential uses of metal-enhanced fluorescence based on colloid clustering multi-photon excitation (top) or long range RET (bottom).

already appeared [23]. Another approach could be to use low quantum yield fluorophores, and the increased quantum yield of fluorophores near the metal to obtain selective observation of fluorophores brought into close proximity with the metal [24] (Figure **8**, top). These effects might be coupled with another remarkable property of metal-fluorophore interactions. If the fluorophore is close to a semi-transparent metallic surface the emission can couple into the metal and become directional rather than isotropic (Figure **8**, bottom) [25]. With a suitable geometry up to 85% of the emission can couple into the metal surface and exit at the surface plasmon angle.

There are still additional ways to use metal enhanced fluorescence. Theory has predicted that the lightening rod effect is much stronger between two metallic spheres than for isolated spheres [26]. If the biochemical affinity reaction brings particles into closer proximity then excitation may be increased in the spaces between the particle (Figure **9**, top). Multi-photon excitation is also known to be increased near metallic surfaces [27] and may be even more efficient between metal particles. And finally, proximity to the particles can result in long range energy transfer (Figure **9**, bottom), which would allow selective detection of macromolecules complexes.

The application of metal-fluorophore interactions is in its infancy. Development of these applications will be facilitated to the extensive theory which was developed mostly to explore surface enhanced Raman scattering [28-31]. Additionally, there is presently ongoing development of metal particles of known size and shape, and predictable optical properties [32-34]. For example, elongated particles have been developed which display absorption of polarized light in one direction but not the orthogonal direction [35]. This suggests the use of polarized excitation to turn on and off the metal-enhanced excitation. We predict the widespread applications of metal-enhanced assays within the near future.

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