TOPICAL REVIEW

Radiative decay engineering: the role of photonic mode density in biotechnology

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Abstract
Fluorescence detection is a central technology in biological research and
biotechnology. A vast array of fluorescent probes are available with diverse
spectral properties. These properties were 'engineered' into fluorophores by
modification of the chemical structures. Essentially, all present uses of
fluorescence rely on the radiation of energy into optically transparent media,
the free space which surrounds the fluorophores. In this paper, we
summarize an opportunity for novel fluorescence technology based on
modification of the photonic mode density around the fluorophore and thus
control of its spectral properties. This modification can be accomplished by
proximity of fluorophores to metallic particles of gold, silver and possibly
others. By engineering the size and shape of the metal particles, and the
location of the fluorophores relative to the surfaces, fluorophores can be
quenched, display increases in quantum yield, and changes in lifetime.
Fluorophore–metal surface combinations can even display directional rather
than isotropic emission. We describe recent experimental results and suggest
potential biomedical applications of fluorophore–metal particle interactions.

1. Introduction

Control of photonic mode density (PMD) will soon make
important contributions to biotechnology. This is a remarkable
assertion which must be justified. The authors of this paper
have decades of experience in fluorescence, and have always
accepted the spectral properties of fluorophores radiating into
free space. Until about two years ago we did not anticipate the
remarkable potential available by controlling the PMD near
the fluorophore. Control of PMD can be accomplished by
placing fluorophores near conducting metallic surfaces. In
this review, we describe the numerous potential applications
of metal-modified fluorescence.

Fluorescence detection is now a central technology
for research in medicine, biology and biotechnology. As

examples, DNA sequencing by fluorescence was first reported
in 1987 [1, 2], resulting in near completion of the sequence of
the human genome by 2001 [3, 4]. Fluorescence detection has
replaced radioactivity in most medical testing [5, 6]. More
recently, fluorescence methods have been extended to high
throughput studies of gene expression using the so-called
gene chips which can contain over 30 000 individual DNA
sequences [7, 8]. These applications rely on the free-space
spectral properties of the fluorophores.

The usefulness of fluorescence detection has been driven
in part by rapid advances in optics, detectors and electronics.
However, the defining technology for fluorescence is probe
chemistry. During the past 20 years there has been extensive
development of new fluorophores, mostly organic molecules
[9–13] with absorption and emission ranging from the
deep UV to the near infrared (NIR). Near UV and visible
fluorophores are widely used as biophysical probes for studies

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of biomolecules. Red and NIR probes has been developed for detection in biological samples where the autofluorescence decreases progressively with increasing wavelengths [12, 13]. Additionally, the longer wavelength probes were useful with inexpensive solid state lasers.

The desired spectral properties of fluorophores are obtained by the combination of general intuitive principles and organic synthesis, with only modest use of quantum mechanical theory. One can usually predict the absorption and emission maxima of probes by analogy with known fluorophores and some general principles [14]. However, it is more difficult to predict which molecules will be strongly, weakly or non-fluorescent. Also, even if a fluorophore displays useful spectral properties in solution these may change when covalently linked to biomolecules. The design and synthesis of useful fluorophores is long-standing and time-consuming process, resulting in as many failures as successes.

In this paper, we describe a physical rather than chemical approach to controlling the spectral properties of fluorophores. This approach relies on the interactions of fluorophores with metallic particles and surfaces. Theoreticians and physicists describe these effects as due to changes in the PMD around the fluorophores. In our opinion, control of PMD will have profound implications for the use of fluorescence in biotechnology and will result in many novel and unanticipated applications.

Fluorescence is due to spontaneous emission of photons by molecules in the excited state. One of the most fundamental equations in quantum dynamics is Fermi's Golden Rule. This rule states the transition probability Γ_{ij} to the ground state is given by

$$\Gamma_{ij} = \frac{2\pi}{n}|M_{ij}|^2\rho(v_{ij}) \qquad (1)$$

where M_{ij} is the matrix element, determined by the wave functions, which connect the initial (i) and fixed (j) state and $\rho(v_{ij})$ is the photon mode density for the transition frequency v_{ij}. Almost all biochemical experiments are done using fluorophores in transparent media where the photon mode density is not very different from a vacuum. However, the PMD can be changed by metallic surfaces and particles, which in turn changes the decay rate [15–17]. We refer to this phenomenon as radiative decay engineering (RDE) because we are primarily interested in the radiative decay rate which results in detectable photons.

It is informative to start with an experimental example of RDE. Figure 1 is a photograph of a protein labelled with the commonly used probe fluorescein. The labelled protein was bound to either a quartz surface or a quartz surface coated with silver particles. The emission is nearly invisible on quartz, and is brightly visible on the silver particles. This difference in intensity is due to an increase in the PMD near the fluorophore, which in turn results in an increase in the radiative decay rate and quantum yield of the fluorophores. Other effects may be present, such as quenching or increased rates of excitation. However, our spectral data indicate the increase in intensity is due in large measure to an increase in the radiative rate near the silver particles.

2. Biochemical fluorescence

Prior to describing the usefulness of PMD control it is informative to consider typical uses of fluorescence in biochemistry. These uses are shown in the Jablonski diagram shown in figure 2. The information content of fluorescence is derived from the processes which occur following excitation. A fluorophore in the excited state can lose energy by the solvent relaxation (Stokes shift), can emit a photon, with a rate Γ, can undergo non-radiative decay to the ground state (k_{nr}), can be quenched by solvent or other molecules (k_q) or can transfer energy by dipolar interaction (k_T) to acceptors (Förster energy transfer). Fluorophores also undergo rotational diffusion which affects the emission anisotropy (not shown).
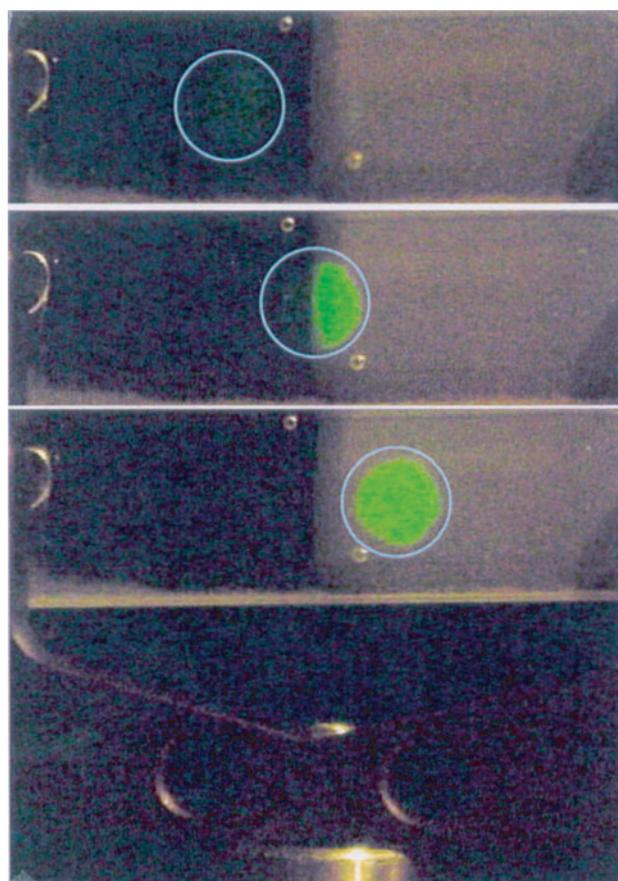

Figure 1. Photograph of fluorescein-labelled human serum albumin (HSA) on quartz and SIFs.
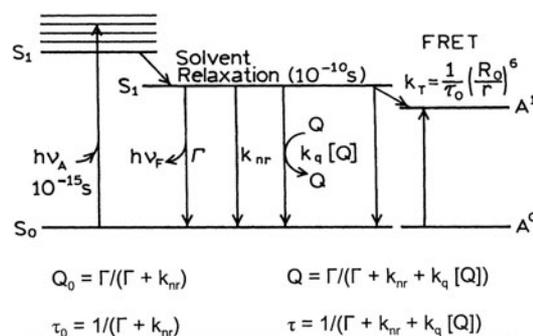

Figure 2. Jablonski diagram for biochemical fluorescence.

These interactions affect the emission spectra, quantum yield, lifetime and anisotropy, which are used to infer the properties of the target molecules.

While the spectral properties mentioned above change dramatically in various solutions, one spectral property, the radiative decay rate Γ, remains nearly constant. This rate is determined by the oscillator strength of the optical transition, or equivalently the extinction coefficient [18]. Hence, the information content of the spectral measurements is governed by changes in the non-radiative, quenching and energy transfer rates.

3. Metallic particle effects on radiative decay rates

The proximity of fluorophores to metallic particles provides an opportunity to modify the radiative decay rates. While seemingly a simple concept, this opportunity has not been available in fluorescence spectroscopy. This concept is shown in the simplified Jablonski diagram in figure 3, for a fluorophore in free space (top) and for a fluorophore perturbed by a metallic particle (bottom). In free space the quantum yield is given by the fraction of the molecules which decay by the radiative pathway

$$Q_0 = \frac{\Gamma}{\Gamma + k_{nr}} \qquad (2)$$

The lifetime is the reciprocal of the rates which depopulate the excited state

$$\tau_0 = \frac{1}{\Gamma + k_{nr}} \qquad (3)$$

Suppose the metallic particles result in an increase in the radiative decay rate to $\Gamma_m = \gamma\Gamma$ where γ is a factor greater than unity. One immediate consequence of increasing the radiative rate Γ is an increase in the quantum yield to

$$Q_m = \frac{\gamma\Gamma}{\gamma\Gamma + k_{nr}} \qquad (4)$$

In writing this equation, we assumed that the metal did not result in a new quenching pathway ($k_m = 0$). In free space, increases in quantum yield are typically accompanied by increased lifetimes due to decreased non-radiative decay
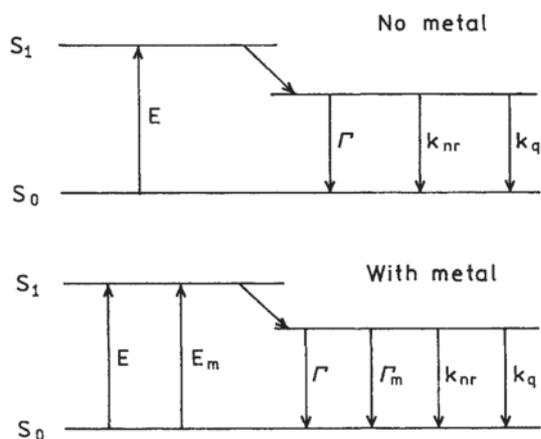
rates. In contrast, a metal-induced increase in the radiative rate results in a decreased lifetime to τ_m near the metal,

$$\tau_m = \frac{1}{\gamma\Gamma + k_{nr}} = \frac{1}{\Gamma_m + k_{nr}} \qquad (5)$$

Fluorophores near metallic particles may show the unusual behaviour of increased quantum yields and decreased lifetimes due to the increase PMD near the fluorophore.

In reality, the situation is considerably more complicated. Several fluorophore–metal interactions are known to occur. At short distances from the surface fluorophores are quenched (k_m in figure 3). The rates of excitation can also be increased by the enhancements of the electric field which occurs when light is incident on the particles (not shown). This area of electrodynamics and photophysics is rich in theory and experiment [19–22] and it is not practical to summarize the complex physics within this paper. Instead we will focus on our recent experimental results with the implications of these interactions yielding new types of experiments and analytical methods.

The effects of metal colloids on fluorophores are due to the surface plasmon resonances (SPRs). Small metallic particles of silver and gold display vivid colours which may not have been expected from their atomic properties. An interesting example of metallic colloid colours is the Lycergus chalice dating from the fifth century, which contains gold nanoparticles. Ruby glass which contains gold colloids is another example [23]. These colours are due to electron oscillations induced in the particles by the incident light resulting in the characteristic surface plasmon absorption as shown for silver colloids in figure 4 [24]. The extinction spectra can be calculated using Mie theory, at least for simple shapes, using the dielectric constants of the metals. The extinction is due to a combination of absorption and scatter and depends on the shape of the particle as well as the properties of the surrounding media [25]. These light–metal interactions are at the origin, at least in part, of the larger increase in Raman scattering observed on rough silver surfaces, which resulted in the field of surface enhanced Raman spectroscopy (SERS) some twenty years ago. In the case of SERS, the enhanced scattering may involve molecular contact with the surface. In the case of surface-enhanced fluorescence (SEF) the effect appears to be due to a through–space interaction. Effects on fluorophores directly on the metallic surface will not be observed if their emission is quenched. A through–space
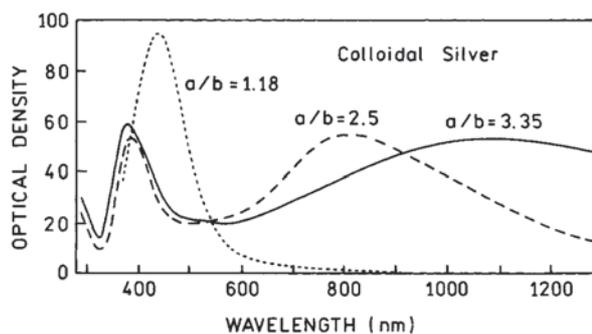

Figure 3. Simplified Jablonski diagram for a fluorophore in free space (top) or with a modified PMD and increase radiative rate.

Figure 4. Absorption spectra of silver colloids with various average aspect ratios (*a/b*).
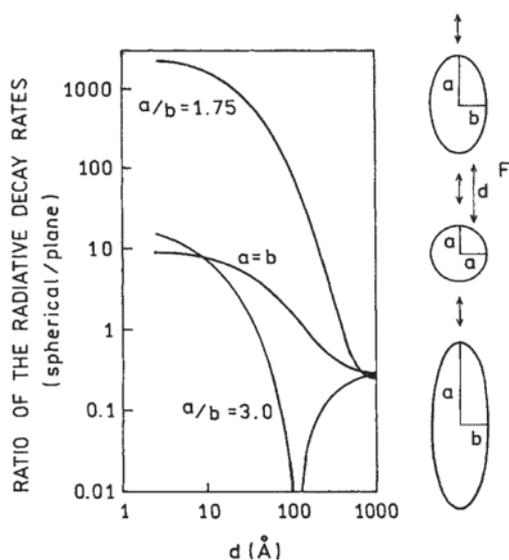
Figure 5. Effect of silver spheroid on the radiative decay rate of a model fluorophore. The resonant frequency of the dye is assumed to be $25\,600\,\text{cm}^{-1}$, approximately equal to 391 nm. The volume of the spheroids are equal to that of a sphere with a radius of 200 Å.

interaction will probably be more consistent than a contact interaction and thus easier to develop analytical applications.

This paper will not present the physics theory, but one theoretical calculation is particularly informative. Figure 5 shows the increase in the radiative decay rate for a fluorophore near silver spheroids with various aspect ratios [26]. For an elongated resonant shape and fluorophore distance from the surface the radiative rate can be enhanced 2000-fold. For a sphere or a non-resonant shape, the change in radiative rate is less pronounced and can even be decreased (figure 5). Other calculations and experiments have shown the changes in lifetime expected for planar metallic surfaces, periodic surfaces and between particles [27–29]. For the moment the behaviour shown in figure 5 is adequate to anticipate useful applications of fluorophore–metal interactions. For example, consider a fluorophore with a free-space quantum yield of 0.001, meaning k_{nr} is about 1000-fold larger than Γ (equation (2)). Now assume Γ is increased 1000-fold by a nearly metallic particle. The quantum yield increases to 0.5, a 500-fold increase. Since the sensitivity of fluorescence detection is proportional to the quantum yield, such an increase has enormous potential for biology and biotechnology.

4. Effects of PMD on biochemical fluorophores

Intrinsic fluorophores are those which occur naturally in biomolecules. These molecules include fluorescent tryptophan residues in proteins, enzyme cofactors like flavin and NADH, collagen and green fluorescent protein [30]. In DNA, each nucleotide residue contains a UV absorbing ($\approx$260 nm) base which might be expected to display fluorescence. However, the intrinsic fluorescence of the bases is exceedingly weak in DNA or in the isolated bases [31, 32]. The emission is so weak that there are no practical uses of intrinsic DNA emission. For this reason a vast array of fluorophores have been developed which bind to DNA and display UV, visible to NIR fluorescence
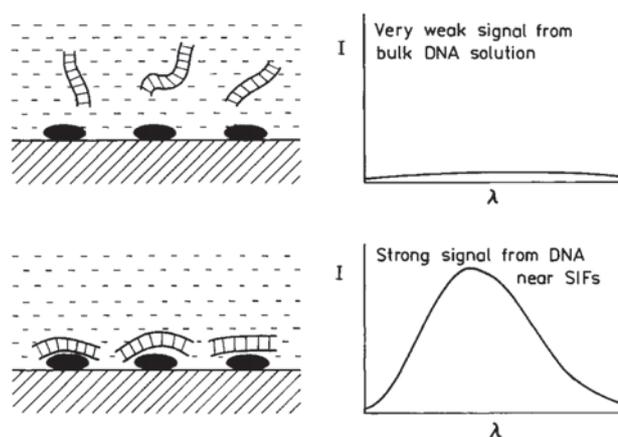

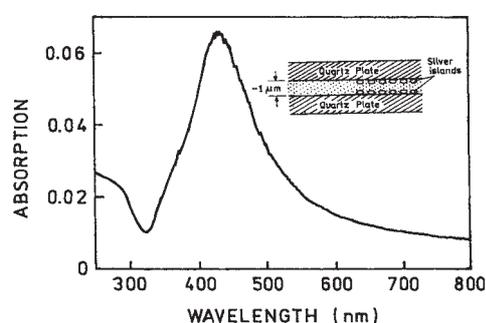**Figure 6.** Schematic for metal-enhanced intrinsic DNA emission.

Figure 7. Absorption spectra of SIFs. The inset shows the sample geometry between two films.

[33–36]. The difficulty with DNA is not that the radiative rates are slow, but that the non-radiative rates are exceedingly fast, so that the bases return to the ground state prior to emission. That is, fluorescence is a competitive process between radiative and non-radiative decay.

In the case of DNA, the low quantum yields are due to non-radiative rates which are much larger than the radiative rate. Suppose that the PMD near the DNA can be increased so that the radiative rate increases. This may be accomplished by bringing the DNA into proximity of silver particles which display plasmon resonance suggesting usefully high intrinsic fluorescence from DNA, as is shown as a predictive schematic in figure 6.

We examined the emission of DNA in micron thick samples between quartz plates and between silver island films (SIFs). These films consist of sub-wavelength size silver particles formed by chemical reduction of silver, and are widely used in SERS and metal-modified fluorescence [37, 38]. These films display a characterstic plasmon absorption near 400 nm due to free electron oscillations (figure 7). When a solution of DNA is examined between two quartz slides the emission is barely detectable (figure 8). There is a dramatic increase in intrinsic emission for the DNA between SIFs [39]. In this experiment, the information is only qualitative because the DNA is not bound to the surfaces and only a small fraction is near the silver particles. Hence, the increase in quantum yield for those molecules near the silver particles is likely to be larger than seen in figure 8.
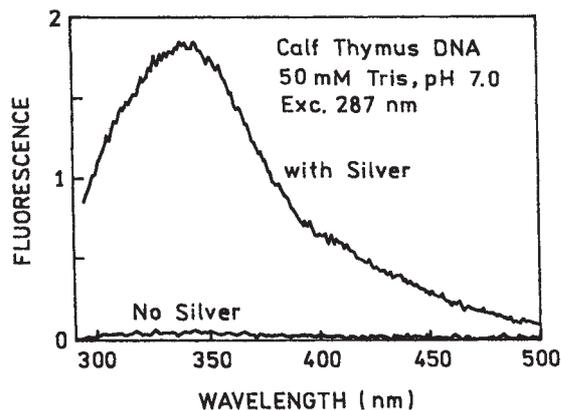
Figure 8. Emission spectra of calf thymus (ct) DNA in solution and near SIFs.
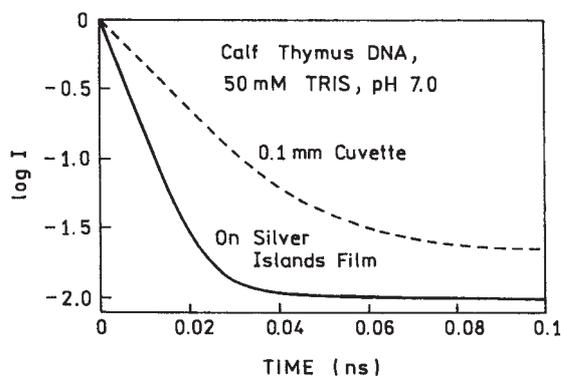

Figure 9. Time-domain intensity decays of ctDNA in solution and near SIFs.

The emission spectra in figure 8 do not demonstrate an increase in the radiative rate. However, such an increase can be demonstrated by measurement of the intensity decays. These results show that the intensity decay is more rapid near the SIFs (figure 9). We interpret this decrease in lifetime as due to the increased PMD near the DNA bases.

Most DNA analyses use extrinsic probes which bind covalently or non-covalently to DNA. The cyanine dyes are widely used in DNA analysis because of their good quantum yields. We examined DNA oligomers labelled with the cyanine dyes Cy3 or Cy5 (figure 10) [40]. The complementary strand was an oligomer containing biotin, which binds strongly to avidin. The labelled oligomer was held at known distances from the surface by binding to avidin, which in turn was bound to biotinylated albumin which forms a monolayer on surfaces. Multiple layers of BSA–biotin–avidin, about 90 Å each layer, were used to vary the distance from the surface. The highest intensity was observed for the labelled oligomers bound to the first layer. We also examined the intensity decays of the labelled DNA. The most rapid intensity decay were found for the cyanine dyes bound to the first monolayer. Using additional results for thinner protein layers, and for DNA bound directly to the surface [41], we concluded that the maximal enhancements and most rapid decays are found 50–90 Å above the silvered surface (figures 11 and 12). This is a convenient distance for biochemistry since it corresponds to the thickness of a modest size protein such as an antibody.
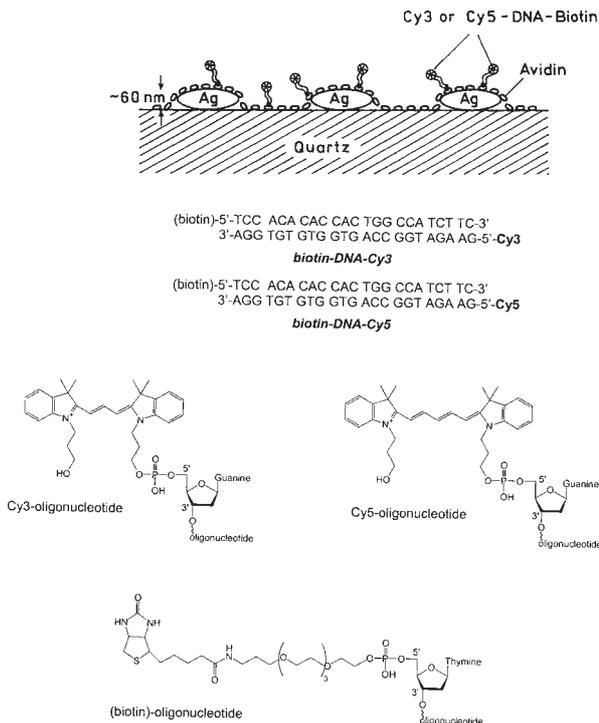

Figure 10. Chemical structures of DNA oligomers covalently labelled with Cy3 or Cy5 and biotin. Also shown is a schematic of labelled DNA bound to monolayers of avidin on the SIFs.
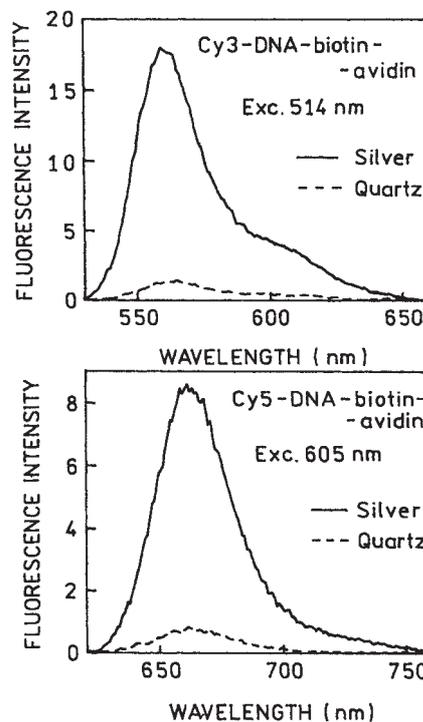

Figure 11. Emission spectra of Cy3 or Cy5-labelled oligomers on avidin monolayers.

Proteins covalently labelled with fluorophores are widely used as reagents, such as immunoassays or immunostaining of biological specimens with specific antibodies. In this application, fluorescein is one of the most widely used probes.
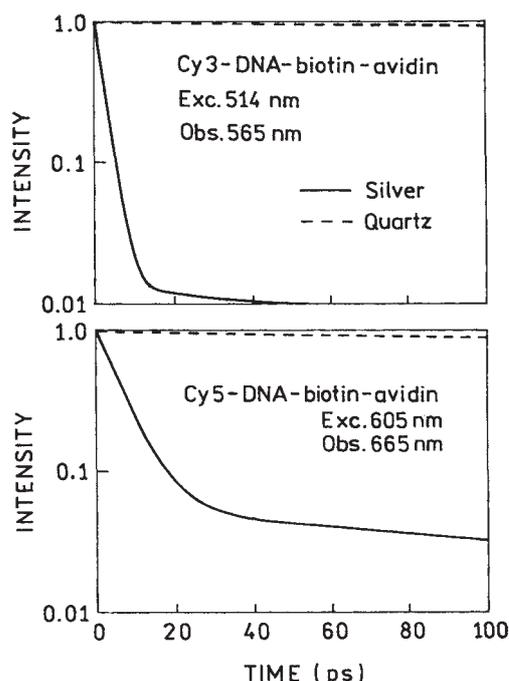
Figure 12. Time-dependent intensity decays of Cy3 or Cy5-labelled oligomer on avidin monolayers. These decays were measured in the frequency-domain [41] but are presented as the recovered time-dependent decay.
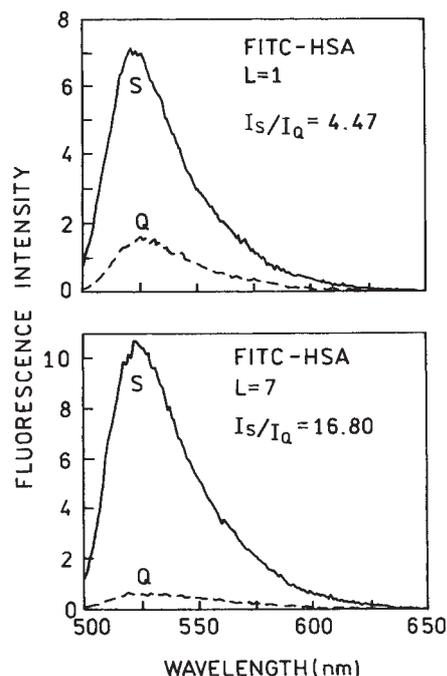

Figure 13. Emission spectra of HSA covalently labelled with fluorescein isothiocyanate (FITC).

Figure 14. Emission spectra of FITC–HSA on quartz and SIFs. The average labelling was $L = 1$ (top) or $L = 7$ (bottom).
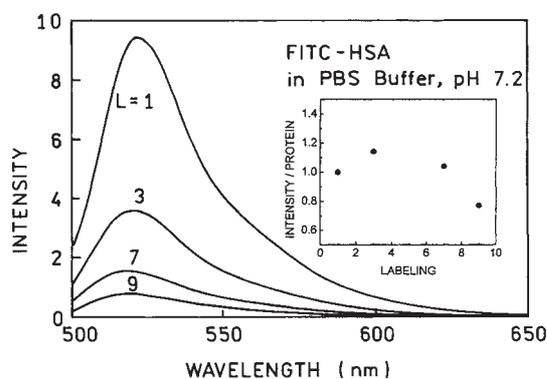

Figure 15. Emission intensities of FITC–HSA on quartz and SIFs for various degrees of labelling.

An unfortunate property of fluorescein is self-quenching, which is due to Forster resonance energy transfer between nearby fluorescein molecules [42]. As a result, the intensity of labelled protein does not increase with increased extents of labelling, but actually decreases (figure 13). We found that the self-quenching could be largely eliminated by proximity to SIFs [44], as can be seen from the emission spectra for labelling ratios of 1 and 7 (figure 14) and from the dependence of the intensity on the extent of labelling (figure 15). We speculate that the decrease in self-quenching is due to an increase in the rate of radiative decay. This result suggests the possibility of ultra bright labelled proteins based on high labelling ratios and metal-enhanced fluorescence.

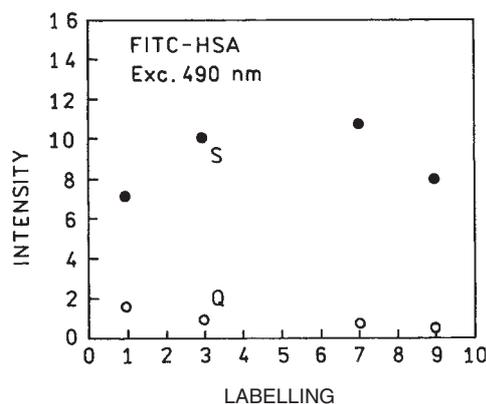As a final experimental result, we show the effects of silver particles on resonance energy transfer [45]. Theory has suggested that, depending on geometry, energy transfer can be either increased or decreased [46, 47]. Such transfer is due to dipolar coupling of a donor and acceptor as discussed by Forster. An oligonucleotide was labelled with both a fluorescein donor and a Tamra acceptor (figure 16). We found no significant change in energy transfer between two quartz plates as compared to one quartz and one SIF surface. However, we observed an increased in energy transfer between two SIFs (figure 16). In our experimental configuration the SIFs are about $1 \, \mu$m apart. We suspect that the increase in energy transfer is due to microcavity effects between the two SIFs. Distances of $1 \, \mu$m and less are easily manipulated with piezoelectric devices or with atomic force microscopes. Hence, the results in figure 16 suggest the possibility of mechanically controlling energy transfer by the distance between metallic surfaces.
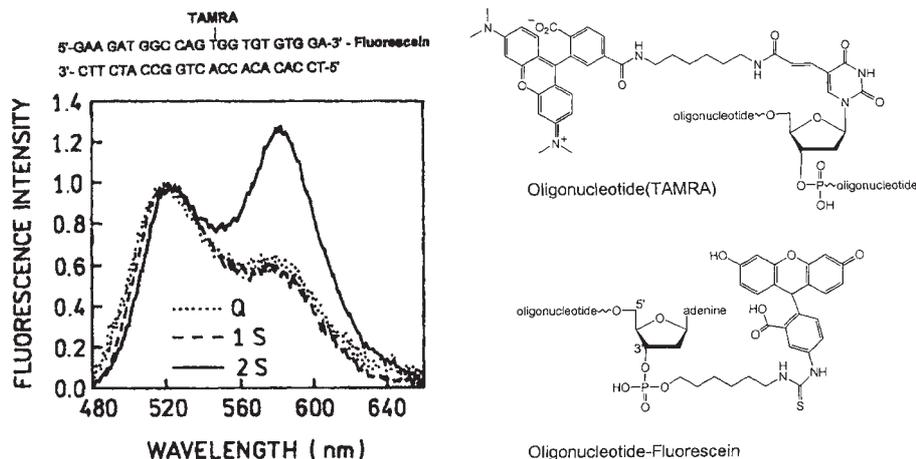
TAMRA
5'-GAA GAT GGC CAG TGG TGT GTG GA-3' - Fluorescein
3'- CTT CTA CCG GTC ACC ACA CAC CT-5'

Oligonucleotide(TAMRA)

Oligonucleotide-Fluorescein

Figure 16. Emission spectra of DNA labelled with both fluorescein (donor) and Tamra (acceptor) between quartz plates and one or two SIFs.

5. Future applications of metal-enhanced fluorescence

We could describe additional experiments on metal-enhanced fluorescence. However, it is informative to use the known physics and experimental results to predict future possibilities. One important possibility is sequencing of a single strand of DNA. Work is already underway to accomplish this task [48, 49]. The basic idea of single strand sequencing is to bind the DNA strand to a bead, progressively cleave each nucleotide from one end by an exonuclease, detect and identify the released nucleotides. Single strand sequencing will require that the released nucleotide be reacted with a fluorescent probe because the intrinsic fluorescence of the nucleotides is too weak. Labelling of the nucleotides will almost certainly require one excess of the reactive label, and the presence of unreacted probe will interfere with detection of the single labelled nucleotide. The use of a detection chamber with metallic particles may avoid the need for chemical labelling (figure 17). The flow chamber may contain metallic particles to increase the intrinsic emission of the nucleotides. It is possible that the emission spectra of the nucleotides will be sufficiently distinct that the base could be identified by the ratio of two intensities at different excitation or emission wavelengths.

As described below the configuration of the chamber may be further designed to result in directional emission or multi-photon excitation. The use of metallic surfaces and particles provides another remarkable opportunity. It is well known that semi-transparent metallic surfaces display surface plasmon absorption at the appropriate incident angle [50, 51]. However, there are other features of surface plasmon absorption which are less well known. First, at the SPR angle the evanescent wave in the distal medium has an approximate 16-fold increase in intensity, which in turn can result in an increased rate of excitation of fluorophores within the evanescent field (figure 18) [52, 53]. Additionally, energy from the excited fluorophores can couple back into the metal surface and emerge at the plasmon angle for the emission wavelength [54, 55]. This directional emission is important because fluorescence is usually emitted isotropically so that only a small fraction of the total emission can be captured with practical optics, so that the detection efficiency can
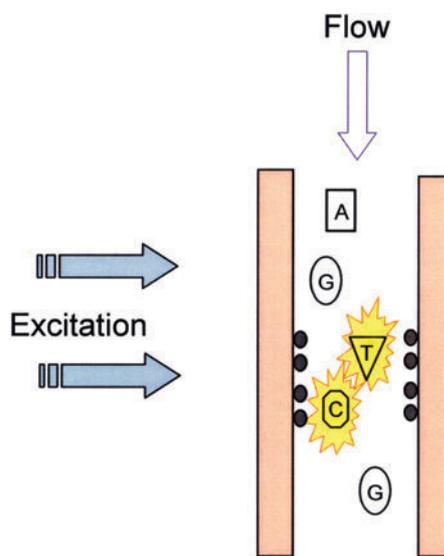
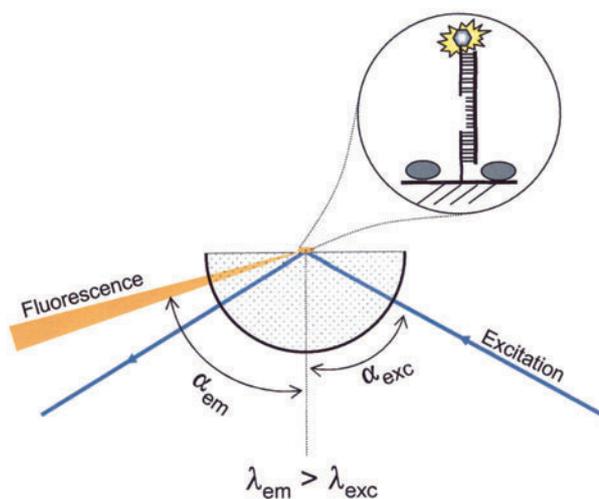**Figure 17.** Detection and identification of single DNA nucleotides.

Figure 18. Biological assays based on evanescent wave surface plasmon excitation and directional emission.
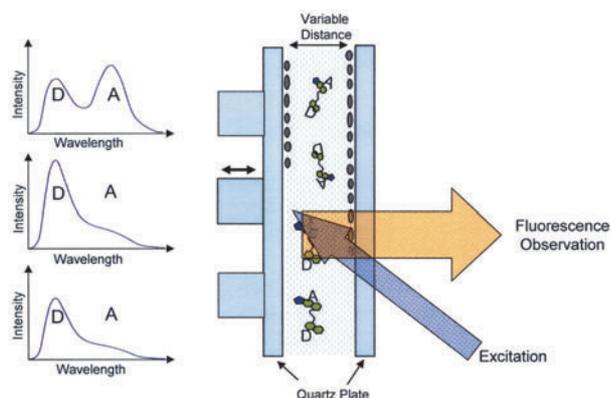
Figure 19. Flow or mechanical control of resonance energy transfer.

Figure 20. Localized cellular emission with spatially-localized colloids.

Figure 21. Multi-photon excitation of a surface-bound fluorophore near metallic particles.

be improved. Additionally, biological samples typically display significant autofluorescence which limits detectability of the desired signal. The combination of surface-localized chemistry, the increased evanescent field strength, and capture of emission mostly from the interface region, can result in less interference from unwanted autofluorescence. The concept of directional emission can also be applied to DNA sequencing. Directionally can be accomplished using either the SPR conditions [54, 55] or by the use of a periodic metallic surface [56, 57].

Another possibility of metal–fluorophore interactions is the mechanical control of energy transfer. Theoretical studies have predicted that resonance energy transfer will be affected by metallic particles and periodic surfaces [58–61]. As shown above (figure 16), RET can be decreased or increased depending on the experimental geometry and/or distance between the metallic surfaces. This suggests the use of flow chambers for analysis in which the metallic surfaces are at various distances, forming or not forming micro-cavities (figure 19). Based on the preliminary results in figure 16, a single metallic surface might be used to increase the donor emission, or two surfaces may be used to increase RET and/or the acceptor emission. The distance between metallic surfaces could also be varied using the nano-manipulation capability of an atomic force microscope.

The interactions of metallic particles with fluorophores can also have applications for intracellular measurements. A variety of approaches are possible. The metal particles could be used as a source of localized excitation of intrinsic fluorophores in the cell, extrinsic fluorophores added to the cell, or sensing fluorophores localized on the particle. This particle could be moved to desired locations in the cell using a micromanipulator or an atomic force microscope (figure 20). This application will require identification of wavelengths and conditions where the metal particle can be spatially manipulated without significant heating [62].

A final biological research possibility using metallic particles is multi-photon excitation. MPE is now widely used in optical microscopy [63–65] because the quadratic or higher dependence on the local intensity provides localized excitation in diffraction limited spots. Additionally, the longer wavelengths used for MPE result in less heating and less photodamage of the sample. Increased multi-photon processes are expected from the non-linear properties of metallic particles [66]. In fact, we have already observed
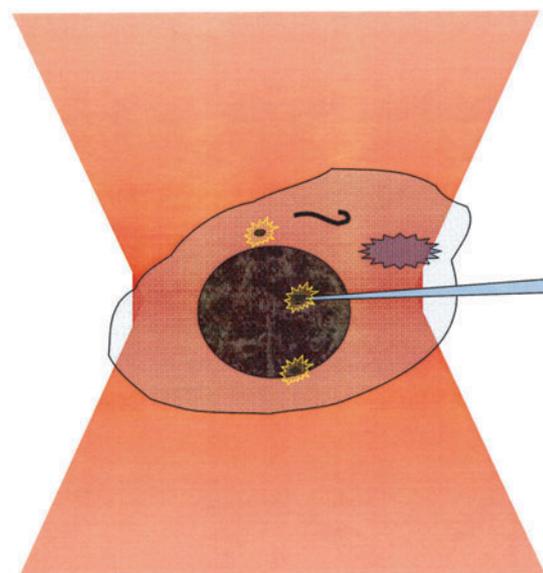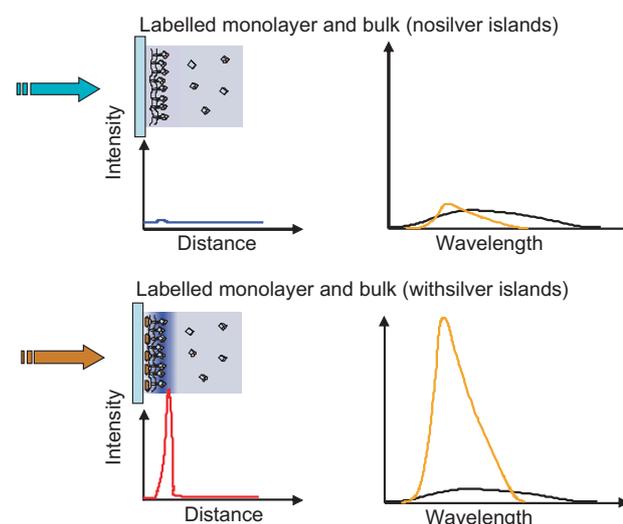
selective MPE near silver particles [67], and recently another laboratory reported even larger MPE enhancements [68]. Because of the enormous increases in the local field it seems possible that MPE imaging might be accomplished with wide field optics rather than the usual scanning of a forward beam. Additionally, the MPE enhancement from metal particles may allow the use of solid state lasers rather than the usual fs titanium–sapphire lasers. If the sample contains both surface-bound and free fluorophores, it should be possible to selectively excited the surface-bound species using multi-photon excitation (figure 21).

6. Conclusion

The interactions of fluorophores with metallic particles is likely to become an active area of research. These studies will include a wide range of scientific disciplines, including optical physics

and electrodynamics to obtain a molecular and/or physical understanding of these effects. Sophisticated chemistry will be needed for preparation of metalized surfaces and particles for the desired effects. And finally, biologists will develop the applications of these effects to medical diagnostics, cellular imaging and molecular biology.

Acknowledgments

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