

# Using Quantum Dots for a FRET Based Immunoassay Technique

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## Introduction

This proposal outlines the construction and functioning of a quantum dot based assay technique which would use fluorescence resonance energy transfer (FRET) to study both qualitative and quantitative features of macromolecular interactions. More specifically the proposal considers the use of this method to improve a homogenous immunoassay technique for human serum albumin (HSA).

## Organic Fluorophores

The study of cellular and subcellular interactions have traditionally been carried out with help of fluorescent tags such as fluorescein, rhodamine and green fluorescent protein (GFP). Macromolecules are tagged with organic fluorophores and are traced using fluorescence spectroscopy. However, this method has certain disadvantages. First, the attachment of the fluorophore must not alter the three-dimensional structure of the protein. For large proteins this is not a significant problem, but small changes in the conformation might alter the natural behavior of the protein. Furthermore different fluorophores require different methods of conjugation to the target macromolecule. Quenching via intra- and inter-molecular energy transfers can reduce the mean lifetime.<sup>1</sup> Another important feature that affects organic fluorophores is the fact that most of them have a narrow excitation spectra and broad emission bands. As a result spectral overlap occurs when several species in a sample are tagged with different fluorophores.<sup>2</sup> Finally, due to the short lifetime of fluorescence of organic fluorophores, rejection of the autofluorescence signal is usually not possible and hence limits sensitivity.

## Quantum Dots

The term quantum dot is generally applied to semiconductor particles whose sizes are of the order of a few to hundreds of angstroms and exhibit a hybrid combination of molecular and bulk properties. The use of semiconductor quantum dots provides us with a useful alternative to organic fluorophores. Properties<sup>3</sup> such as high emission quantum yield, sharp emission spectra, chemical and photostability, long lifetimes and tunability from the red to the blue ends of the

spectrum make them a useful tool for labelling purposes. One problem with quantum dots is that they are in general water- insoluble. However there are several techniques available to allow quantum dots to be used in biological applications. One method is to cap the quantum dot with an organic layer such as mercaptoacetic acid<sup>3</sup> or trioctyl phosphine/trioctyl phosphine oxide mixture<sup>2</sup> (TOP/TOPO). Substitution of the organic cap with other ligands allows for the functionalization of quantum dots for various purposes such as conjugation to biological macromolecules. One problem that such organic capping faces is the possibility of the capping material being hydrolysed or oxidized. A technique which resolves this problem to some extent is the encapsulation of quantum dots within silica spheres,<sup>3</sup> liposomes or water soluble block copolymers.<sup>4</sup>

## Fluorescence Resonance Energy Transfer (FRET)

As mentioned before, spectral overlap of different fluorophores can lead to problems during imaging. However such overlap can be used constructively with the help of fluorescence resonance energy transfer analysis. FRET is a distance dependent interaction between the excited electronic states of fluorescent molecules. In this phenomenon, energy is transferred from one of the molecules (donor) to the other (acceptor) non-radiatively. The efficiency of FRET between a donor and acceptor molecule is dependent on the inverse sixth power of their separation, and thus it is an useful tool in studying spatial interactions between biological molecules.

An important parameter involved in FRET analysis is the Förster radius ( $R_o$ ) which is defined as the distance at which energy transfer is 50% efficient. Table 1 contains the Förster radii for some common donor acceptor combinations. Using the Förster radius it is possible to determine the rate of energy transfer using the equation

$$k = \frac{1}{\tau_D} \left( \frac{R_o}{r} \right)^6 \quad (1)$$

where  $\tau_D$  is the donor lifetime in the absence of the acceptor, and  $r$  is the donor acceptor distance. However a more useful parameter is the energy transfer efficiency,  $E$ , defined as

$$E = 1 - \frac{I_{DA}}{I_D} \quad (2)$$

where  $I_D$  and  $I_{DA}$  are the donor intensities in the absence and presence of the acceptor respectively. This equation allows us to monitor the quenching of the donor in the presence of the acceptor.

## Combining Quantum Dots and FRET

FRET studies have traditionally been carried out using a wide variety of organic fluorophores though inorganic fluorophores have also been used.<sup>1,5</sup> Several features need to be taken into account such as the effect of intramolecular energy transfers between macromolecule and donor and conformational changes of the macromolecule. Furthermore, different fluorophores require different methods to conjugate with a given macromolecule.

The long term stability of quantum dots, together with the ease with which their emission frequencies can be tailored (see Figure 1) suggests the use of quantum dots in sensor or assay

applications. Furthermore when capped inorganically, creating what are known as core shell quantum dots, the resultant increase in band gap implies that quenching from intra-molecular energy transfers can be reduced or even prevented. Such core shell quantum dots also have enhanced photochemical stability and resistance to photobleaching<sup>2</sup> due to the fact that holes are confined to the core of the structure.<sup>6</sup> These properties suggest that the replacement of organic fluorophores in FRET studies with quantum dots could lead to an experimental setup which would be cheap and easy to configure as well as provide the capability to make simultaneous measurements of different macromolecular systems.

The first step in setting up the assay would be the creation of a suitable quantum dot. Several techniques are available such as arrested precipitation,<sup>7</sup> zeolite based ion exchange<sup>8</sup> and cluster fusion<sup>9</sup> (whereby small single sized quantum dots are enlarged by addition of reagents which would cement the smaller units). These techniques have been successfully used to generate CdS quantum dots of varying sizes. However, the methods described do not always lead to monodisperse systems of quantum dots. An alternative method that does lead to monodisperse quantum dot systems is thermal decomposition of organometallics in coordinating solvents. This method has been used to generate monodisperse CdE (E = S,Se,Te) quantum dots.<sup>10</sup>

After synthesis, the quantum dots would have to be capped. Though quantum dots have been studied in their native state, the capping process is vital if the synthesized quantum dots are required to interact with macromolecules. As mentioned before the capping procedure also inhibits aggregation and hence enhances the shelf life of the capped quantum dots. Common capping reagents include polyphosphates and thiols. For this application, a moderately long chain acid containing a thiol group would be required. The reason for long chains rather than short chains is to reduce anisotropy of the quantum dot fluorescence as well as make sure that the attachment of the dot to the macromolecule does not cause significant deformation of the tagged molecule. Examples of such capping reagents include mercaptobutanoic acid and dihydrolipoic acid (DHA). A schematic figure of DHA capped quantum dot (CdSe - ZnS core shell dot) is shown in Figure 2.

After synthesis and capping, the quantum dot must be conjugated to the macromolecule in question. The conjugation could be non-covalent or covalent in nature. One covalent method described by Hermanson<sup>11</sup> used 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) condensation to conjugate nanoparticles to IgG. In this case mercaptoacetic acid (MCAA) was used as the capping molecule. It has been reported<sup>2</sup> that this method leads to extensive protein aggregation when dihydrolipoic acid is used as the capping layer. A possible explanation is that the long chains of DHA lead to interlinking and resultant aggregation, indicating that shorter capping groups would be preferred. Another possibility is that the density of DHA groups per unit area is smaller than when mercaptoacetic acid is used. This could result in larger surface charge density on the DHA capped quantum dot leading to electrostatic aggregation. An alternative method of conjugation involves electrostatic attraction. This method has been used by Mattoussi<sup>2</sup> where quantum dots were electrostatically combined with a recombinant protein which was attached to a biologically relevant domain. This method appears to be relatively involved since it requires the creation of a recombinant linking protein and fusion with the actual protein under study. It would probably be more fruitful to consider other capping reagents which do not exhibit aggregation. For the moment it is assumed that mercaptopropanoic or mercaptobutanoic acid is the capping reagent.

The final step would be to conjugate the second macromolecule with a quantum dot. In this

case techniques similar to those described above could be used. However, it would be required that the fluorescence of this system be different from that of the substrate. As mentioned in the introduction, tuning emission wavelengths of quantum dots is a simple matter of modifying the synthesis environment to generate quantum dots of different radii.

The actual assay would involve mixing the two systems and studying the interactions between the labelled macromolecules via FRET. Before the actual experiment is carried out a few preliminary measurements would be required.<sup>12</sup> These include the fluorescence intensity of the donor in the absence of the acceptor and determining the emission spectrum of the acceptor. The former measurement provides a value of the quantum yield and the latter is important since it would help in choosing an appropriate emission wavelength for the study of donor emission properties. In the present proposal, the macromolecules would consist of an antigen and antibody pair. After tagging and mixing, the antibody and antigen will bind to each other thus, bringing the quantum dot tags in close proximity to each other. As a result FRET is expected to occur. FRET can be observed by exciting the system with a laser pulse of frequency  $\nu$  such that only the donor quantum dot is excited. It should be noted that even if the acceptor does get excited it would not hinder the monitoring process since we would be observing the quenching of donor fluorescence. It would however affect the energy transfer from donor to acceptor and thus should be minimized. By monitoring the quenching of donor fluorescence various details of the binding process can be studied. These include binding kinetics and spatial details of the binding process as well as quantitative estimation of antigen concentration.

A specific application of this procedure would be to use quantum dot labelled human serum albumin (HSA) and anti human serum albumin (AHA) in a homogenous immunoassay technique which could be extended to other antibody-antigen pairs. FRET based immunoassays using organic<sup>13</sup> and inorganic<sup>1</sup> fluorophores have been developed in the past and a schematic diagram of the process can be seen in Figure 3. Youn<sup>1</sup> showed that the use of a ruthenium ligand complex as the donor fluorophore with Reactive Blue 4 (RB4) as acceptor (see Figure 4) led to improved sensitivity due to increased lifetimes over that of organic donor fluorophores. However the method described has some disadvantages - the time required for the synthesis of the Ru complex is relatively long and the materials required are expensive. The quantum yield of the Ru complex is low (of the order of .05 to .04) thus leading to a small value of the Förster radius, which places a limit on the size of antigens that can be tested with this assay method. In addition, the acceptor RB4 was prone to hydrophobic interactions with HSA.

Using quantum dots rather than the inorganic or organic fluorophores would enhance this assay technique. As described above, the synthesis of quantum dots is relatively easy, rapid and can be carried out cheaply. In this case a core shell dot would be preferred for its enhanced stability and resistance to bleaching, thus increasing lifetime. The donor and acceptor quantum dots would need to be tuned to the correct wavelengths so that there is sufficient overlap between the emission and absorption spectra of the donor and acceptor, respectively. Conjugation of the dots with the antigen and antibody could be carried out by the EDC condensation method described above with mercaptoacetic acid as the capping reagent, though other capping reagents could also be investigated. The problem of hydrophobic interactions of the quantum dot with HSA would be alleviated by using a core shell quantum dot.

In the assay itself the advantages provided by the ruthenium complex would be maintained in the case of the quantum dot system. However certain aspects would be improved. For example there is a distinct possibility of intra molecular energy transfer from HSA to the Ru complex re-

sulting in a decrease of mean lifetime. In contrast, due to the large bandgap in the quantum dot, such transfers would not be able to affect mean lifetime of the donor significantly. Furthermore, due to the increased lifetimes of quantum dot fluorescence the problem of autofluorescence can be ignored (since its time scale is on the order of nanoseconds). Another important feature is the high quantum yield of quantum dot systems (ranging from 10%<sup>6</sup> to 50%<sup>6</sup>). In the original work<sup>1</sup> the Förster distance for the donor-acceptor energy transfer was calculated to be 30.1 Å. The Förster distance is defined as<sup>14</sup>

$$R_o^6 = 8.785 \times 10^{-5} \frac{\kappa^2 \phi_D J}{n^4} \quad (3)$$

where  $\kappa^2$  is the orientation factor,  $\phi_D$  is the quantum yield of the donor,  $n$  is the refractive index of the medium (usually taken as 1.4 for aqueous mediums in biological studies) and  $J$  is the normalized overlap integral<sup>12</sup> (see Figure 5) between the donor and acceptor,

$$J = \frac{\int F_D(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda}{\int F_D(\lambda) d\lambda} \quad (4)$$

$F_D$  is the peak-normalized fluorescence spectrum for the donor and  $\epsilon_A$  is the molar absorption coefficient for the acceptor. Since the value of  $\phi_D$  for a quantum dot is 2 to 2.5 times greater than for the Ru complex this would allow for larger separations between the donor and acceptor, implying that antibodies or antigens larger than HSA could be successfully used.

The very sharp sharp emission spectra of quantum dots allows for a modification of the above technique. Rather than a single antibody-antigen combination one could test for multiple antigen- antibody reactions simultaneously by tagging the different antigens (or antibodies) with quantum dots of different sizes producing different colors. In this case one would simple have to monitor the quenching of donor fluorescence at different wavelengths. This would lead to a high-throughput version of the single antigen-antibody setup described above.

## Expected Problems

The technique described here faces a few problems. First, intensity and lifetime calculations depend on knowledge of the Förster radius. The Förster radii for quantum dot systems are not well defined. As a result, before the technique can be put to use, values of  $R_o$  must be determined. As given by Equation 3 the Förster radii for a given donor acceptor pair depend on the three factors: quantum yield of the donor, fluorescence emission spectrum of the donor and molar absorption coefficient of the acceptor. Equation 4 can be converted to a summation and measuring the emission spectrum of the donor (correcting for a wavelength-dependent instrument response, either automatically or by performing measurements with a reference compound with known spectrum) allows one to obtain a value of  $J$ . The quantum yield can also be measured with reference to a compound of known yield such as quinine<sup>15</sup> ( $\phi = 0.55$  between 400 and 600 nm) or 2 aminopyridine<sup>16</sup> ( $\phi = 0.6$  between 300 and 450 nm). With the development of large-scale preparative techniques it is expected that properties such as quantum yields and emission spectra will be standardized for quantum dots of a specific composition and size. As a result much of the preliminary measurements would be avoided.

An important aspect of the above discussion that must be considered in a little more detail is the value of the orientation factor,  $\kappa^2$ . Mathematically it is defined<sup>12</sup> as

$$\kappa^2 = (\cos \theta_T - 3 \cos \theta_d \cos \theta_a)^2 \quad (5)$$

where  $\theta_T$  is the angle between the emission dipole of the donor and absorption dipole of the acceptor and  $\theta_d$  and  $\theta_a$  are the angles between the vector joining the donor and acceptor and emission and absorption dipoles respectively. In many cases it is assumed that the donor and acceptor molecules randomly sample all the possible orientations during energy transfer and hence Equation 5 gives an average value of 2/3. However, for the assay technique described here the quantum dot itself is large (compared to the Ru complex in the original work) and is attached to a large molecule and hence its rotation would be restricted and thus would be unable to sample all available orientations resulting in an increase in anisotropy. As a result  $\kappa^2$  is restricted to a range of values which must be estimated or minimized. One method to reduce the anisotropy to some extent is to use longer capping molecules. This would, however, lead to the possibility of multiple macromolecules being attached to a single quantum dot. Other techniques such as the depolarization factor method described by Dale<sup>17</sup> and Haas<sup>18</sup> provide a range of  $\kappa^2$  values and could possibly provide a better method to account for anisotropy.

A feature that has been observed in FRET studies of antibody-antigen reactions<sup>1</sup> is that an increased density of acceptor molecules on the antibody improves energy transfer, but an increased density of donor molecules on the antigen decreases energy transfer. Thus multiple labelling of the antigen with the donor quantum dots must be controlled. This could be achieved by maintaining the proper ratio of quantum dot to antigen molecule during preparation. Another approach (for smaller antigen-antibody pairs) is to use larger quantum dots so that steric crowding would inhibit multiple attachment. The fluorescence efficiency of multiple donor molecules on an antibody has been analyzed statistically by Ullman<sup>13</sup> and this could be used as a rough guide to deciding on how to prepare the sample so as to prevent (or minimize) multiple labelling.

## Conclusion

This report describes the use of quantum dots coupled with FRET analysis to act as an accurate and wide ranging immunoassay technique. Traditionally, quantum dots have been used in imaging methods acting as fluorescent labels. At the same time FRET has been used to study the interaction of macromolecules labelled with organic fluorophores. The combination of these two techniques has several advantages in the study of macromolecular interactions. First, the synthesis of quantum dots is a relatively simple process and once synthesized they have a relatively long shelf life. Minor modifications to the synthetic method allow for a wide variation in the dot size and resultant optical properties. It is these optical properties that enhance the capabilities of FRET analysis. The long lifetimes of quantum dots overcome the problem of autofluorescence. The high quantum yields of quantum dots make energy transfer very efficient. As a result the study of molecular interactions over larger distances should be possible using FRET and quantum dots. This allows the investigation of spatial features of macromolecules (via multiple quantum dot labelling) as well as long range kinetics.

The homogenous antigen-antibody assay technique described in this proposal has several advantages over that described in the literature.<sup>1,13</sup> These include easy and rapid setup, handling

of a large variety of antigen-antibody pairs with little change in preparative method and the possibility of measuring responses from different pairs simultaneously, resulting in high throughput.

However, as described in a previous section, traditional FRET analysis uses organic fluorophores whose FRET characteristics (such as Förster distance) are well known. This is not the case when using quantum dots as the fluorophore. As a result preliminary work would be required to analyze the range of distances over which FRET would occur and the resultant characterization of the Förster distance.

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Table 1: Förster radii for some donor acceptor combinations<sup>14</sup>

Donor	Acceptor	$R_o$ (Å)
Naphthalene	Dansyl	22
Pyrene	Coumarin	39
CPM <sup>a</sup>	Fluorescein	47
Proflavin	ETSC <sup>b</sup>	46
CF <sup>c</sup>	TR <sup>d</sup>	51

<sup>a</sup> 7 diethylamino-3-(4'- maleimidylphenyl)-4-methylcoumarin

<sup>b</sup> eosin thiosemicarbazide

<sup>c</sup> carboxyfluorescein succinimidyl ester

<sup>d</sup> Texas Red

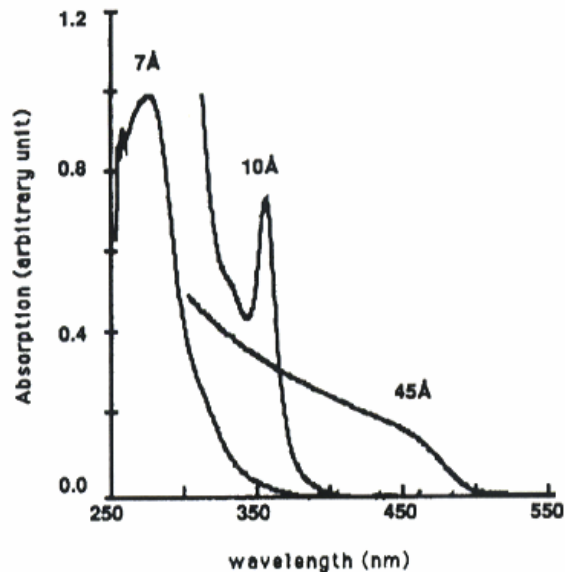


Figure 1: Absorption spectra of 7 Å  $(\text{Cd}_{10}\text{S}_4(\text{SPh})_{16})^{-4}$  clusters, 10 Å  $(\text{Cd}_{20}\text{S}_{13}(\text{SPh})_{22})^{-8}$  clusters and 45 Å CdS clusters in Nafion film<sup>19</sup>

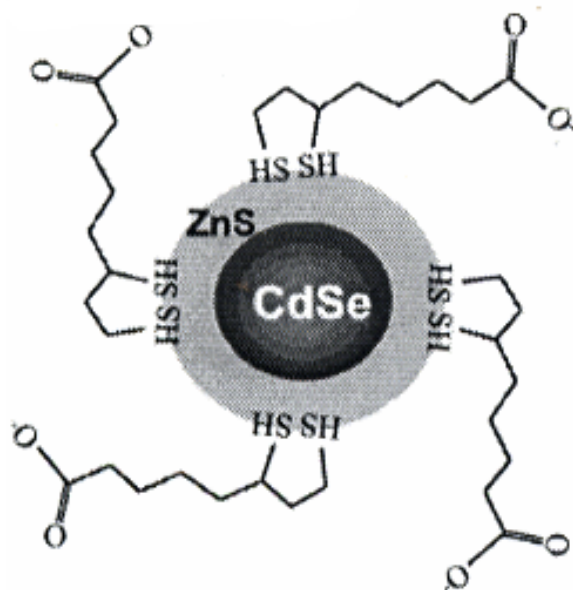


Figure 2: Schematic representation of a dihydrolipoic acid capped quantum dot<sup>2</sup>

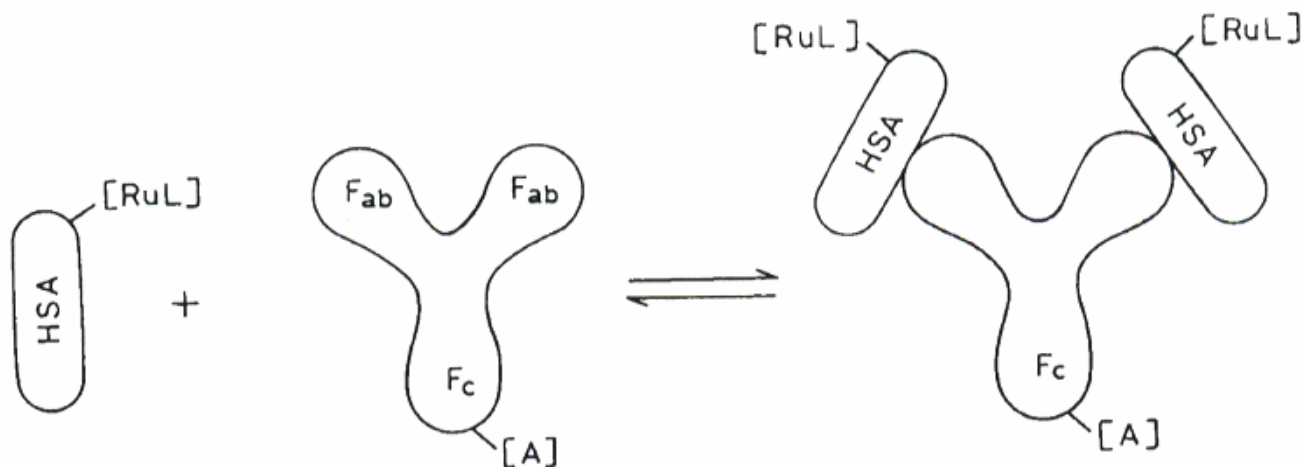


Figure 3: Schematic diagram of the FRET based immunoassay of Ru labelled human serum albumin and Reactive Blue 4 labelled anti human serum albumin as donor and acceptors respectively<sup>1</sup>

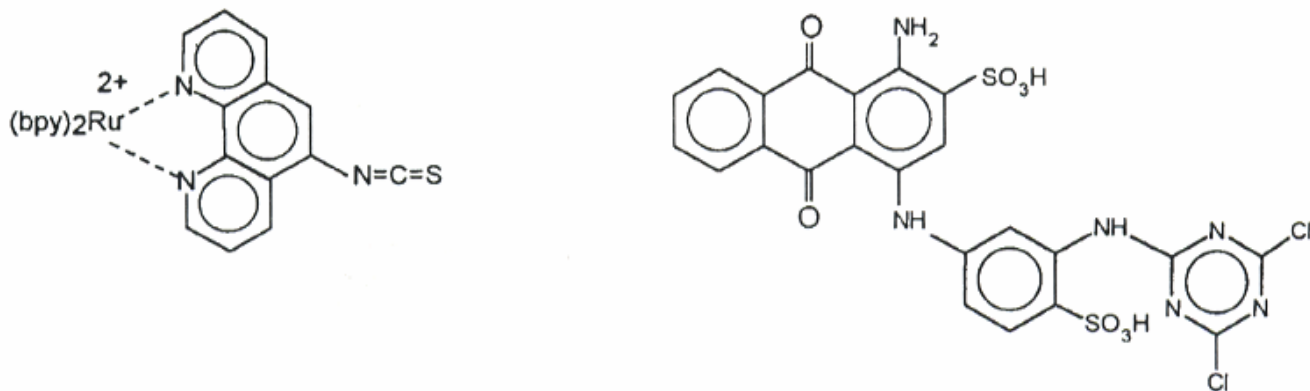


Figure 4: Ruthenium ligand complex (donor) and Reactive Blue 4 (acceptor)<sup>1</sup>

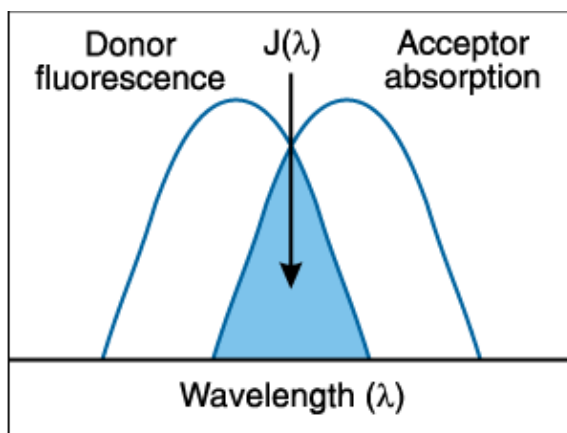


Figure 5: Schematic representation of the FRET spectral overlap integral