

# 1 Fluorescence Resonance Energy Transfer

FRET is nominally the non-radiative transfer of energy from a donor molecule to the acceptor molecule, therefore the signature of FRET is quenching of the low energy fluorophore followed by emission from the acceptor fluorophore of relatively high frequency of light.

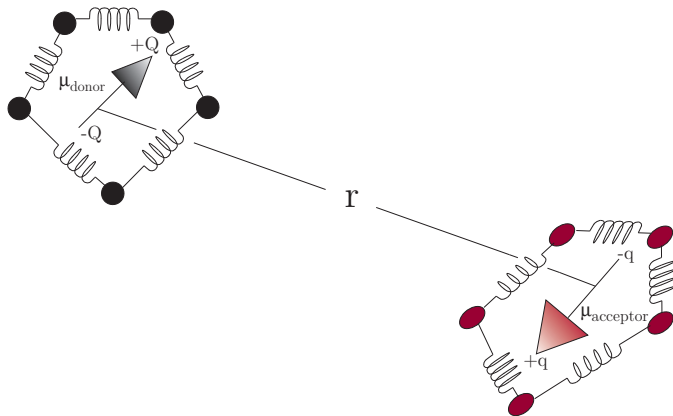


FIG. 1. Dipoles of two interacting fluorophores

Let us first analyze the name of this technique. What is the meaning of the word *fluorescence*? Fluorescence is a particular method of de-excitation of an excited molecule; there are, however, several such methods of de-excitation. Some of those methods of de-excitation involve emission of light others do not. And other pathways combine non-radiative transitions with radiative emission. Contrast fluorescence with: vibrational relaxation; phosphorescence; and internal conversion. Vibrational relaxation is merely a transition from one excited vibrational level within an excited electronic state to a lower energy vibrational level. Such a transition is a consequence of the fact that the vibrational states are not exactly harmonic; in other words the anharmonicities in vibrational potentials permit otherwise orthogonal states to exchange energy. Next let us consider phosphorescence.

Phosphorescence is similar to fluorescence in that both are types of luminescence. Fluorescence is light emitted from a *singlet* excited state and phosphorescence is light emitted from a triplet state. (Singlet and triplet states are described on p.166 of D. Griffiths, *Introduction to Quantum Mechanics* (Prentice Hall, Upper Saddle River, 1995). The basic idea concerns the number of ways that total spin of an atom—proton plus electron—can be achieved. There are three states with with  $s = 1$ :  $|1\ 1\rangle$ ,  $|1\ 0\rangle$ , and  $|1\ -1\rangle$ , whereas there is only one way to achieve the orthogonal state  $s = 0$ :  $|0\ 0\rangle$ .  $\hbar^2 s(s + 1)$  is the eigenvalue of the  $S^2$ . ) Since triplet states that are derived from a singlet state in the de-excitation process are of lower energy phosphorescence is of longer wavelength. The longer lifetime of phosphorescence is beyond the scope of the present treatment.

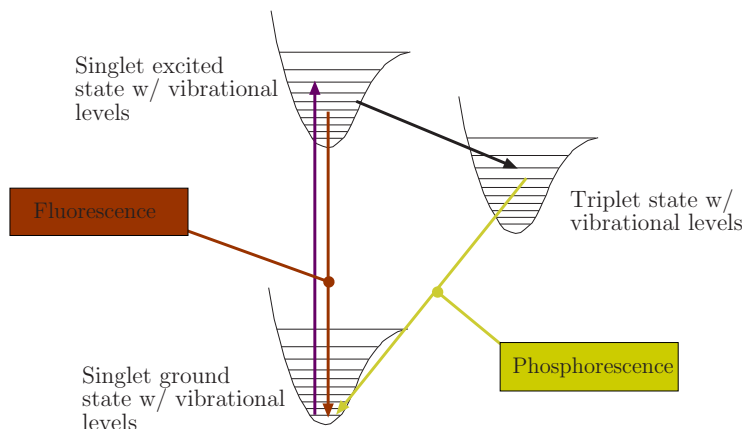


FIG. 2. The three methods of deexcitation explained quantum mechanically. (See R. M. Clegg, in *Fluorescence Imaging Spectroscopy and Microscopy* ed. Xue Feng Wang and Brian Herman, Chemical Analysis Series, Vol 137, p.196.)

Finally the de-excitation process can be accomplished by *internal conversion*. This process involves energy loss to the solvent through collisions and anharmonicities in vibrational states.

Having explained the first of the words in the acronym FRET, we can now concentrate on the the second word—*resonance*. The best and most commonly used metaphor is that of coupled pendula diagrammed below. If two pendula have a spring connecting their rods then when one pendulum is set swinging (the donor molecule absorbs a photon) the other pendulum of couple will begin swinging (the acceptor molecule emits a photon).

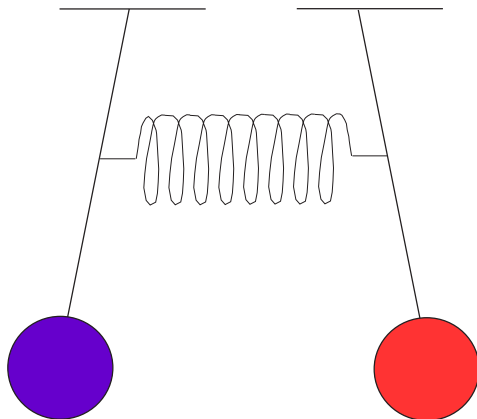


FIG. 3. The classical metaphor for resonance—two coupled pendulua

The baffling point about FRET is that for the non-radiative transfer of energy to occur there must be an overlap between the emission profile (the function of intensity versus wavelength) of the donor and the absorption profile of the acceptor. Yet these are precisely the conditions for radiative transfer, also. The point is that the two processes—radiative and non-radiative transfer—have very different dependences on distance.

## 1.1 Outline

1. Qualitative description of FRET in the context of a fascinating problem in neural physics—the association and dissociation of the core complex involved in synaptic vesicle docking.
2. Presentation of most relevant expressions associated with FRET: the derivation of the distance dependence and efficiency.
3. Derivation of the rate constant of non-radiatively exciting the acceptor molecule  $K_T$ .

## 1.2 Qualitative Description of FRET

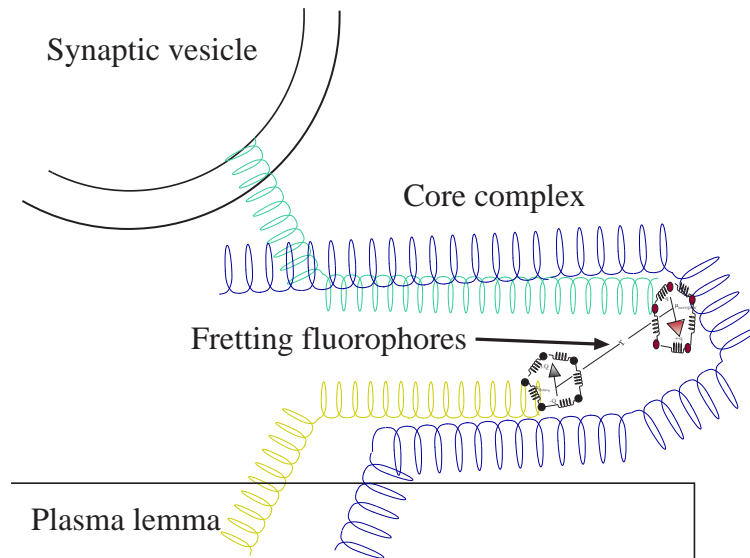


FIG. 4. FRET and the core-complex. Part of an experiment to prove that the core complex is intimately involved in some instances of synaptic vesicle fusion.

1. The high energy (low wavelength) dipole begins oscillating after absorption of and appropriate photon in addition to the fact that the donor is emitting a longer wavelength photon.
2. The low energy (longer wavelength) dipole feels the oscillations in the electric field and at the resonant frequency absorbs the energy.
3. Now the acceptor is excited and emits in the longest wavelength photon of the FRET event.
4. One of the key qualitative points is that the FRETing fluorophores have to be within, say,  $50 \text{ \AA}$ s or each other. This necessity of close proximity for the donor and acceptor is responsible for the fantastic utility of FRET in neural biology. FRETing fluorophores on proteins prove that the two proteins are close enough for biologically relevant interactions. Fig. 4 supplies an example of an experimental design that is close that the is in the process of being published by W. Almers and An, S. in 2003.
5. QED provides the ultimate theory of FRET. Therefore the virtual photons Feynman diagrams are ultimately responsible for the non-radiative transfer.

### 1.3 The Elusive Connection Between Experiment and Theory in FRET

Reference: J. R. Lakowicz, *Principles of Fluorescence Spectroscopy* (New York, Kluwer, 1999) p. 367 -371.

Efficiency  $E$  is defined as the fraction of energy (in photons) absorbed by the donor that was subsequently transferred to the acceptor. We will show the following;

$$\text{efficiency} = E = \frac{R_0^6}{R_0^6 + R^6} \quad (1)$$

where:

1.  $R_0$ : Förster distance,
2.  $R$ : the distance between the centers of the fluorophore dipole moments.

More relevant to experimental proof of the existence of FRET is the following expression;

$$E = 1 - \frac{F_{DA}}{F_D}, \quad (2)$$

where:

1.  $F_{DA}$ : fluorescence intensity of the donor in the presence of the acceptor,
2.  $F_D$ : fluorescence intensity of the donor when the acceptor is far away.

Equation (2) makes sense because, when the acceptor is close to the donor  $F_{DA}$  should be low and the efficiency should be close to one. When the acceptor is far away then  $F_{DA} = F_D$  and efficiency equals 0.

## 1.4 Quantum Mechanical Derivation

### 1.4.1 Introduction

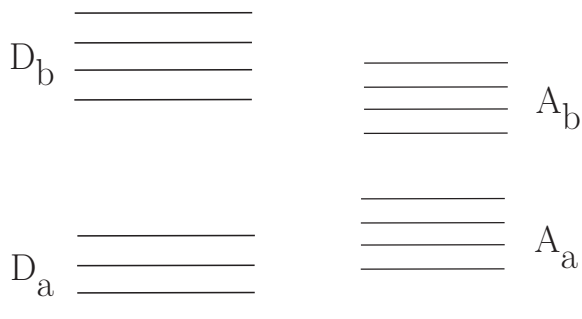


FIG. 5. Quantum mechanical levels of donor and acceptor. (Find the reference for this derivation. It is difficult to believe that this derivation comes from Clegg or Perisiamy. 07 Dec.2003. )

Presumably this derivation follows Clegg. (See note in the figure caption for Fig. 5) It can also be found in Andy Demond's thesis. A bit of a discussion concerning rate constants would be appropriate here because the quantum mechanical derivation starts from the assumption that knowing the rate constant will provide—among other important information—an expression for the distance dependence of FRET.

$$D_b + A_a \xrightarrow{\text{kappa (transfer)}} D_a + A_b$$

↓  
κ<sub>transfer</sub> versus κ<sub>ic</sub>, κ<sub>fluorescence</sub>, κ<sub>intersystem crossing</sub>

κ<sub>transfer</sub> : rate constant for non-radiative transfer.

κ<sub>ic</sub> : rate constant for internal conversion.

κ<sub>fluorescence</sub> : rate constant for fluorescence, *i. e.* radiative decay.

κ<sub>intersystem crossing;isc</sub> : rate constant for conversion from singlet to triplet.

The following expression is central to FRET theory and practice:

$$\kappa_{\text{transfer}} = \frac{1}{\tau_D} \left( \frac{R_0}{R} \right)^6,$$

where  $\tau_D$  : Lifetime of donor in absence of acceptor,

$R$  : The distance between the dipoles of donor and acceptor as in the above figure.  
 $R_0$  : A distance parameter of considerable importance in FRET literature.

$$R_0 \approx 10 \text{ s}\text{\AA}$$

Let us examine a few obvious consequences of the above expression for  $\kappa_{\text{transfer}}$  which we now abbreviate as  $\kappa_{\text{tr}}$

1. When  $R \rightarrow \infty$  then  $\kappa_{\text{tr}} = 0$
2. When  $R = R_0$ ,  $\kappa_{\text{tr}} = 1$ .

Since we want an expression for a rate constant our observable has to be a matrix element that connects  $\Psi_{D_a} \Psi_{A_b}$  to  $\Psi_{D_b} \Psi_{A_a}$ , that is to say our observable will be the perturbation ( $V$  with units of joules) which takes the system from donor in the excited state ( $\Psi_{D_b}$ —read psi sub D sub b) and acceptor in the ground state ( $\Psi_{A_a}$ ) to a quenched donor (donor in ground state— $\Psi_{D_a}$ ) and acceptor in the excited state ( $\Psi_{A_b}$ ). So this derivation recalls the quasi-classical derivation of the Einstein coefficients. As a reminder:

$\kappa_{\text{tr}}$  = The rate of non-radiative energy transfer.

$$\kappa_{\text{tr}} \propto \left| \left\langle \psi_{D_a} \psi_{A_b} \left| \underline{V} \right| \psi_{D_b} \psi_{A_a} \right\rangle \right|^2$$

↓  
 $V \propto \vec{\mu}_A \cdot \vec{E}_D$  (dipole of the acceptor in

E field of donor)

$$\begin{aligned} \downarrow \\ \underline{E}_D &= -\vec{\nabla} \phi_D \\ \downarrow \\ \phi_D &=? \end{aligned}$$

(As a reminder  $\phi_{\text{point charge}} = \frac{q}{4\pi\epsilon_0} \frac{1}{R}$ .)

We need the potential for a dipole.  
 Jackson's *Classical Electrodynamics*  
 discusses this problem nicely:

$$\phi_{\text{Dipole}} \propto \frac{\mu x}{R^3}$$

We will review this electrostatics later—for now.

This  $k$  is *not* a rate constant—rather a geometrical factor  
 ↓

$$V \propto \frac{k_{\text{geometrical}} \mu_A \mu_D}{R^3}$$

Substitute the above expression for  $V$  into the original expression for  $\kappa_{\text{tr}}$

$$\kappa_{\text{tr}} \propto \left| \left\langle \psi_{D_a} \psi_{A_b} \left| \frac{k_{\text{geometrical}} \mu_D \mu_A}{R^3} \right| \psi_{D_b} \psi_{A_a} \right\rangle \right|^2.$$

This result implies the crucial fact for the importance of FRET in neural biological physics that,

$$\kappa_{\text{non-radiative transfer}} \propto \frac{1}{R^6}.$$

Note that—by the inverse square law;

$$\kappa_{\text{radiative transfer}} \propto \frac{1}{R^2}.$$

Let us make the important assumption of monochromatic distribution of frequencies. (We will consider the case of an arbitrary distribution of frequencies later.) Since the coordinates of the two dipoles are clearly independent they can be separated and related to the Einstein coefficients.

$$\kappa_{\text{tr}} \propto \frac{k_{\text{geometrical}}^2}{R^6} \underbrace{\left| \left\langle \psi_{D_a} \psi_{A_b} \left| \mu_D \right| \psi_{D_b} \psi_{A_a} \right\rangle \right|^2}_{\text{emission}} \underbrace{\left| \left\langle \psi_{D_a} \psi_{A_b} \left| \mu_A \right| \psi_{D_b} \psi_{A_a} \right\rangle \right|^2}_{\text{absorption}}.$$

<p>emission</p> <p style="margin-left: 40px;">↙</p> <p style="margin-left: 40px;"><math>\nu^{-3} A_{ab}</math></p> <p><math>A_{ab}</math>: rate of spont. emiss.</p> <p style="margin-left: 40px;"><math>\nu^{-3} \tau_{\text{radiative}}^{-1}</math></p>	<p>absorption</p> <p style="margin-left: 40px;">↘</p> <p style="margin-left: 40px;">Simple case of mono- chromatic abs. @ <math>\nu</math></p> <p style="margin-left: 40px;"><math>\propto \epsilon_A \nu^{-1}</math></p> <p style="margin-left: 40px;"><math>\epsilon_A</math>: molar absorptivity</p>
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$$\kappa_{tr} \propto \frac{k_{\text{geometrical}}^2}{R^6} \nu^{-3} \tau_{\text{radiative}}^{-1} \epsilon_A \nu^{-1}$$

$$\tau_{\text{radiative}}^{-1} = \frac{\phi_{\text{donor}}}{\tau_D} \quad \text{Where } \phi \text{ is quantum yield.}$$

(Note: Quantum yield is best understood as the percentage of de-excitation energy in photons.  $\phi = \frac{\kappa_{\text{fluorescence}}}{\kappa_{\text{fluorescence}} + \sum_i \kappa_i}$ . Ref. A. Periasamy *Methods in Cellular Imaging*)

$$\kappa_{tr} \propto \frac{k_{\text{geometrical}}^2}{R^6} \frac{\phi_{\text{donor}}}{\tau_D} \epsilon_A \nu^{-4}$$

Next remove the restriction of monochromatic frequencies and molar absorptivity  $\epsilon_A$  becomes a function of frequency  $\nu$ . In order to successfully integrate over frequencies



we need to know what fraction of the total fluorescence of the donor that exists at each particular frequency. So let us define;

$f_D \equiv$  fraction of donor fluorescence at each frequency.

$$\kappa_{tr} \propto \frac{k_{\text{geometrical}}^2}{R^6} \frac{\phi_{\text{donor}}}{\tau_D} \underbrace{\int \epsilon_A(\nu) f_D(\nu) \nu^{-4} d\nu}_{\text{measure of overlap}}$$

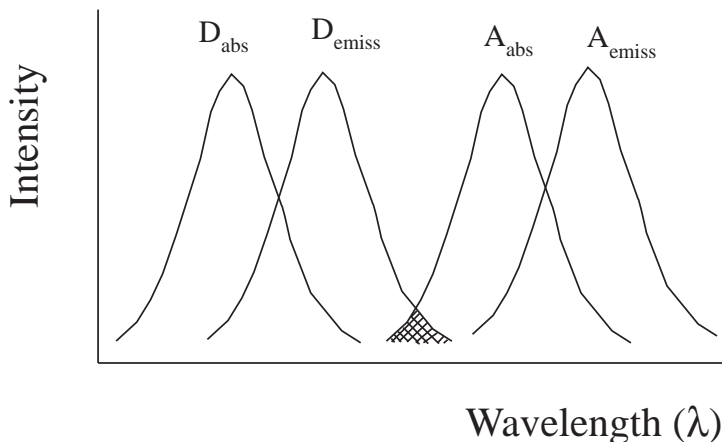


FIG. 6. Four fluorescence intensity bands associated with FRET: two absorption bands and two emission bands. Area of the cross-hatched region is calculated by the integral above.

## 2 Classical derivation

This section largely follows Clegg Chapter. 7, Fluorescence Resonance Energy Transfer in *Fluorescence Imaging Spectroscopy and Microscopy* Eds. Xue Wang and Brian Herman. Chemical Analysis Series , Vol. 137.

H. Kuhn, *J. Chem. Phys.* **53** Classical Aspects of Energy Transfer in Molecular Systems (1970).

The electric field of an oscillating dipole  $\mu$  (departing from Clegg's convention in order to prevent confusion with magnetic permeability  $\mu$ ) can be written in polar coordinates as follows

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Pedagogical questions and notes

- What is the history of the following Equations (3) and (4)?
  - In terms of the physics, what is the conceptual development of the notion that an oscillating electric is not necessarily associated with light. The near-field microscopy literature must have some pedagogically sound articles on this issue.
  - A very cool point about the signature of FRET can be derived from efficiency expression (in Lakowicz) and energy conservation. The formula for efficiency involves all energy transfer methods including radiative transfer, therefore if the FRET efficiency is one then no other mechanism can be contributing including the far-field interaction. The conclusion is—then—that at efficiencies of one there is NO radiative transfer. Supposedly at efficiencies different than one—by this argument—have radiative transfer mixed in with the non-radiative component.
  - The of units used by Clegg is Gaussian. The rules for switching between Gaussian and SI are given in the appendices of both Griffiths's and Jackson.
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$$E_\theta = \frac{1}{n^2} \left\{ \frac{1}{R^3} - i \frac{\kappa}{R^2} + \frac{\kappa^2}{R} \right\} \sin\theta \cdot \mu \cdot e^{i[\omega(t-R\frac{n}{c})]} \quad (3)$$

$$E_r = 2 \frac{1}{n^2} \left\{ \underbrace{\frac{1}{R^3}}_{\text{Near-field}} - i \frac{\kappa}{R^2} + \underbrace{\frac{\kappa^2}{R}}_{\text{far field}} \right\} \cos\theta \cdot \mu \cdot e^{i[\omega(t-R\frac{n}{c})]} \quad (4)$$

$\downarrow$                        $\downarrow$   
 Near-field          far field  
 non-radiation field      radiation field

- $\theta$  is the angle between the dipole axis and the direction vector between the two dipoles.
- $n$  is the index of refraction

(Because only the  $E_\theta$  term is perpendicular to the direction of the propagation of light only  $E_\theta$  contributes to the radiation field.) FRET occurs due to the near-field (both  $E_\theta$  and  $E_r$ ) and that is why FRET is referred to as a *non-radiative* transfer of energy. From (1) and (2) it is clear that both  $E_\theta$  and  $E_r$  contribute to the dipole-dipole interaction in the near field. The electric field of the donor is

$$\vec{E}^{donor} \approx \vec{E}_0^{donor} e^{i\omega(t-R\frac{n}{c})} \quad (5)$$

Where  $\vec{E}_0^{donor}$  merely indicate the amplitude. The sign indicating an approximate relation exists because we are only taking the non-radiative term.

$$\vec{E}_0^{donor} = E_r \hat{r} + E_\theta \hat{\theta} \quad (6)$$

Where  $E_r$  and  $E_\theta$  are given by the non-radiative parts of (1) and (2) apart from the time dependence.

$$E_\theta = \frac{1}{n^2} \left\{ \frac{1}{R^3} \right\} \sin\theta \cdot \mu \quad (7)$$

$$E_r = 2 \frac{1}{n^2} \left\{ \frac{1}{R^3} \right\} \cos\theta \cdot \mu \quad (8)$$

(5) and (6) in (4) which subsequently goes into (3) gives,

$$\vec{E}^{donor} \approx 2 \frac{1}{n^2} \left\{ \frac{1}{R^3} \right\} \cos\theta \cdot \mu \hat{r} + \frac{1}{n^2} \left\{ \frac{1}{R^3} \right\} \sin\theta \cdot \mu \hat{\theta} \quad (9)$$

$$\vec{E}^{donor} \approx \frac{1}{n^2} \frac{1}{R^3} \mu \left\{ 2\cos\theta \hat{r} + \sin\theta \hat{\theta} \right\} e^{i\omega[(t-R\frac{n}{c})]} \quad (10)$$

$$|E^{donor-acceptor}| = \vec{\mu}^{acceptor} \cdot \vec{E}^{donor} \quad (11)$$

$$|E^{donor-acceptor}| = \frac{1}{n^2} \frac{1}{R^3} \mu \left\{ 2\cos\theta \hat{r} + \sin\theta \hat{\theta} \right\} \quad (12)$$

- It is fair to question the units of  $\mu$  because this symbol also represents magnetic permeability. Harking back to (1) we can consider the dimensions of  $\mu$ : the exponential, the  $\sin\theta$ , and the  $\frac{1}{n^2}$  are all dimensionless. The curly bracket terms presumably all have the same dimension, so let's just consider the easiest one  $\frac{1}{R^3}$ . Since  $E$  must have dimensions of  $\frac{charge}{length^2}$ , the  $\mu$  factor in (1) must have the dimensions of charge  $\times$  length  $\rightarrow$  the dimensions of electric dipole *not* magnetic permeability.
- the transition from (9) to (10) needs some explanation. For example, why are there not two  $\mu$ 's in (10)?
- At this juncture in the derivation I part from Clegg's path. I move directly to Kuhn's derivation of absorbed intensity in a Beer-Lambert-like situation.

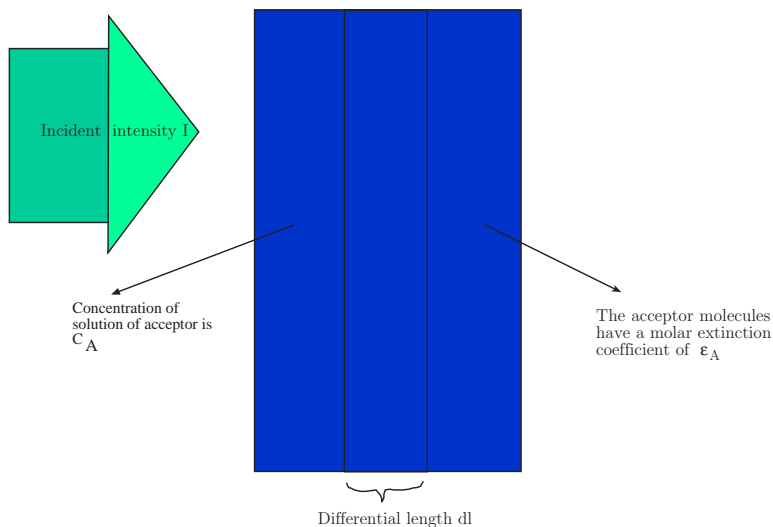


FIG. 7. The Kuhn derivation of a Beer-Lambert-like law.

$$-dI = I\epsilon_A C_A (\ln 10) dl \quad (13)$$

The above expression is new for me because of the  $\ln 10$ . Where does  $\ln 10$  come from?

$$-dI/I = \epsilon_A C_A (\ln 10) dl \quad (14)$$

$$\int -dI/I = \int \epsilon_A C_A (\ln 10) dl$$

$$\ln I = -\epsilon_A C_A (\ln 10) l$$

$$I = e^{-\epsilon_A C_A (\ln 10) l}$$

$$I = e^{-\epsilon_A C_A l (\ln 10)}$$

What is the Beer-Lambert Law exactly?

$$A = \epsilon Cl$$

$$A \equiv -\log_{10} \frac{I_{transmitted}}{I_{incident}}$$

So the question appears to be what is the  $I$  in Kuhn's derivation?  $dI$  might be interpreted as  $I_{incident} - I_{transmitted}$  in which case  $dI/I$  might be the fractional decrease. On the otherhand—and much more sensibly,  $dI$  is probably the decrease in intensity

due to the differential of length  $dl$ . So it seems reasonable to assert that  $I$  for Kuhn's derivation is  $I_{incident}$  in the above statement of the Beer-Lambert Law.

From standard E&M the incident intensity  $I$  depends on the square of the modulus of the relevant electric field:

$$I = \frac{cn}{8\pi} |E^{donor-acceptor}|^2. \quad (15)$$

$E^{donor-acceptor}$  is the electric field of the oscillating donor dipole at the position of the acceptor—that is what is meant by the superscript.

(13) in (11) gives:

$$-dI = \frac{cn}{8\pi} |E^{donor-acceptor}|^2 \epsilon_A C_A (\ln 10) dl. \quad (16)$$

Divide by the expression that provides the number of acceptor fluorophores. Such an expression is obtained by considering a unit area so that apparently the units of the following expression are incorrect.

$$\text{Number of fluorophores} = C_A N_{Av} dl \quad (17)$$

The units of the RHS are

$$\frac{\text{moles}}{\text{liter}} \times \frac{\text{number of fluorophores}}{\text{mole}} \times \text{length}$$

$$\frac{\text{moles}}{\text{length}^3} \times \frac{\text{number of fluorophores}}{\text{mole}} \times \text{length}$$

which clearly gives number of fluorophores per unit area. So that is why we are considering the  $I$  as incident on a unit area.

$$\frac{\text{moles}}{\text{length}^3} \times \frac{\text{number of fluorophores}}{\text{mole}} \times \text{unit area} \times \text{length}$$

$$\frac{\text{moles}}{\text{length}^3} \times \frac{\text{number of fluorophores}}{\text{mole}} \times \text{length}^2 \times \text{length}.$$

The final expression above provides the number of acceptors.

RHS of (14) divided by (15) gives a LHS of (14) that can be interpreted as an absorption per molecule which is very good because we are ultimately interested in is the distance at which the donor and acceptor are absorbing equal amounts of energy—that assertion cannot be correct, but mathematically it appears that way.

$$-dI_{\text{per molecule}} = \frac{\frac{cn}{8\pi} |E^{donor-acceptor}|^2 \epsilon_A C_A (\ln 10) dl}{C_A N_{Av} dl} \quad (18)$$

$$-dI_{\text{per molecule}} = \frac{cn}{8\pi} |E^{\text{donor-acceptor}}|^2 \epsilon_A(\ln 10). \quad (19)$$

(10) into (17) gives:

$$-dI_{\text{per molecule}} = \frac{cn}{8\pi} \left| \frac{1}{n^2} \frac{1}{R^3} \mu \left\{ 2\cos\theta\hat{r} + \sin\theta\hat{\theta} \right\} \right|^2 \epsilon_A(\ln 10). \quad (20)$$

- Some very objectionable math is going on here; for example the equation above has an isolated differential on the LHS. One way to eliminate the difficulty is to integrate at some point close to (11) and then divide by the full number of fluorophores in the total volume instead of dividing by a differential volume.
- The next part is unclear because

In order to define a fiducial distance mark

$$-dI_{\text{per acceptor}} = -dI_{\text{per donor}} \quad (21)$$