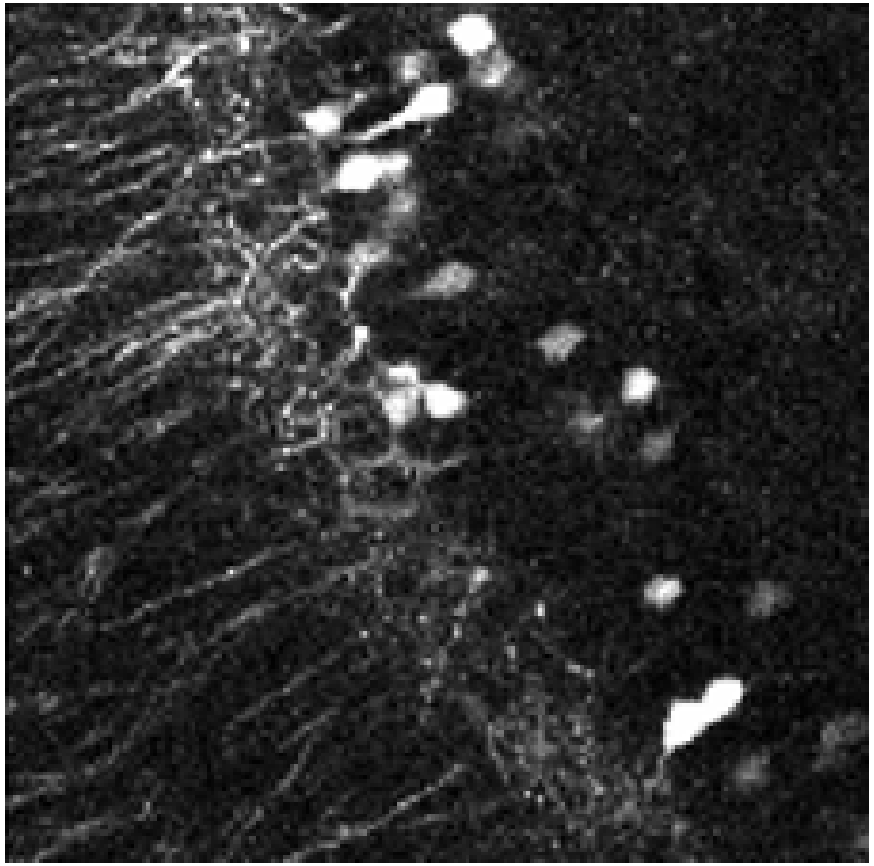
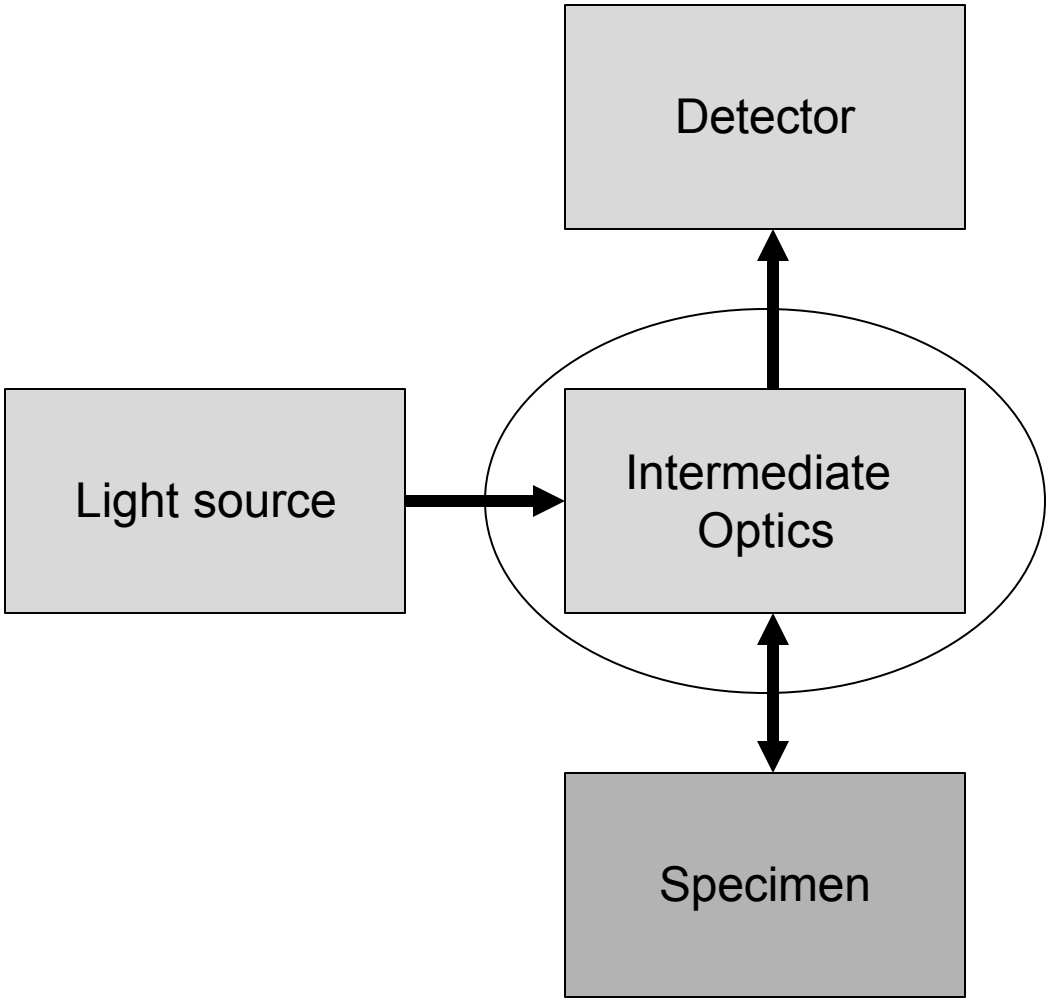
Fluorescence Spectroscopy & Microscopy



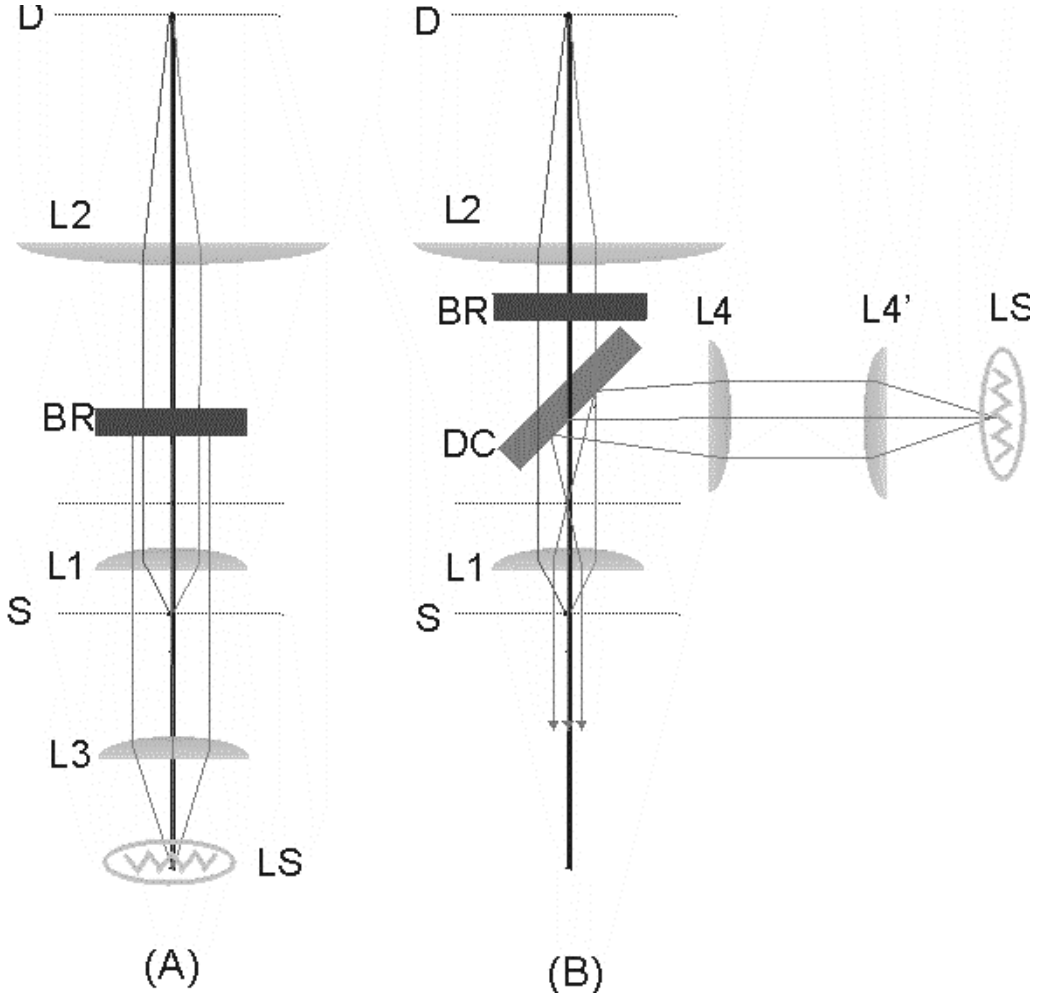
A typical biomedical optics experiment



Microscope Configurations

Trans

Epi



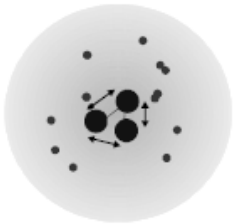
Fluorescence is fundamentally a quantum phenomena

Light ray can be thought of as a stream of photons each having energy:

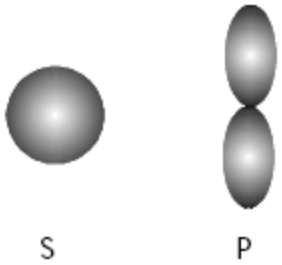
$$E = hu = h\frac{c}{l}$$

Light-Molecule Interaction

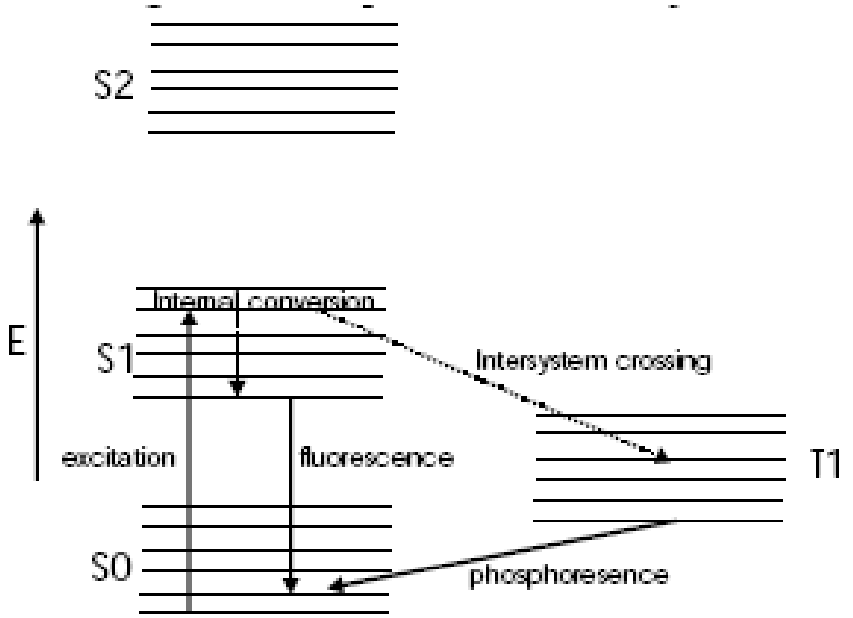
Polyatomic molecules



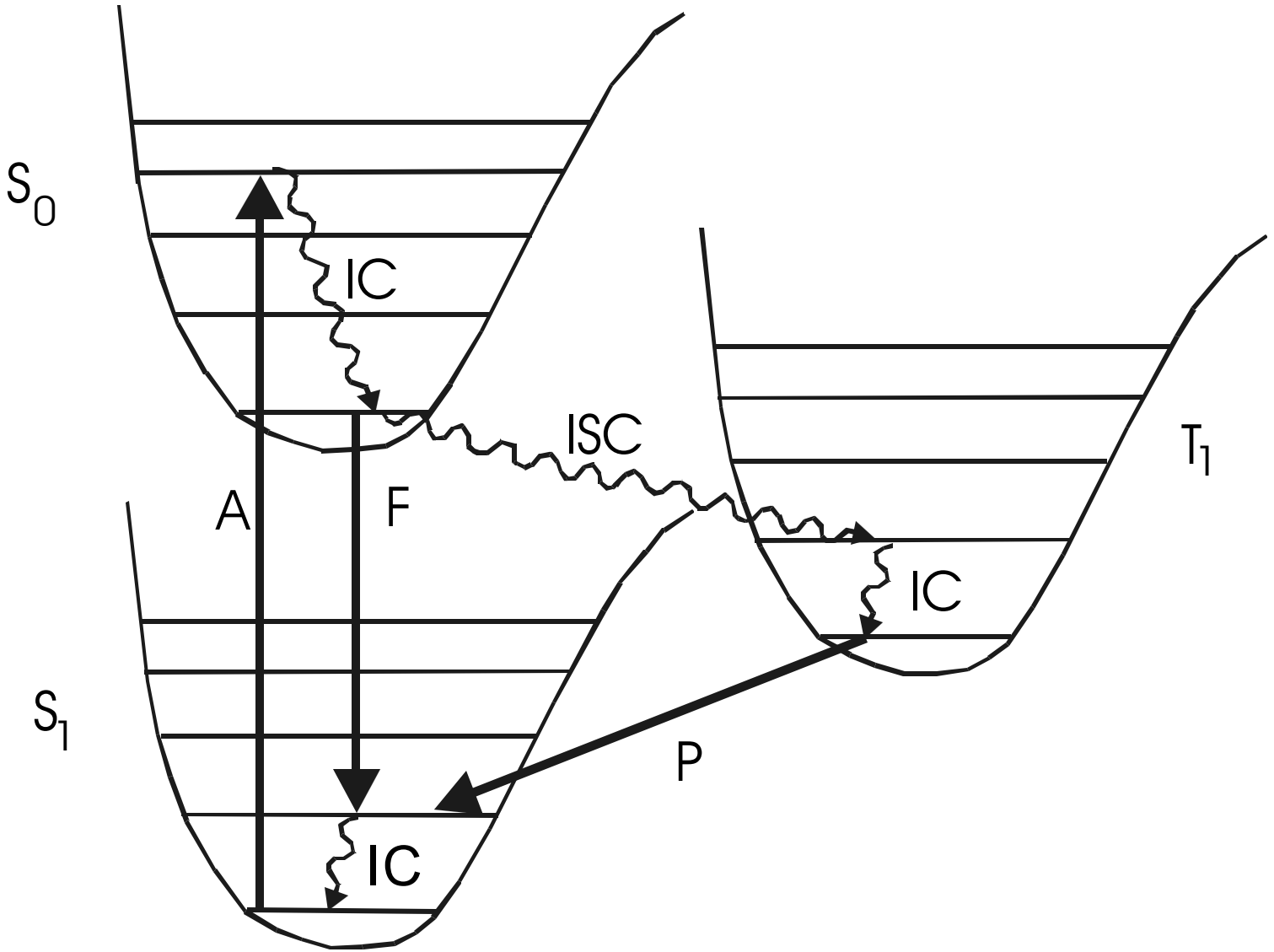
Simple orbitals



Jablonski Diagram



Jablonski Diagram



Basic properties of fluorescence

Stokes' shift. This refers to the observation that fluorescence (phosphorescence) always occur at a longer wavelength (lower photon energy) as compared with the excitation process. This fact is immediately obvious from the Jablonski diagram. Fluorescence (phosphorescence) does not emit from the excited vibrational level originally reached during the excitation process but occur in a lower energy state due to internal conversion and intersystem cross.

Invariance of emission with excitation: In general, emission spectrum is roughly (with some exceptions) independent of excitation wavelength. This fact is a direct result of internal conversion will put the molecule to the bottom of S1 independent of excitation process.

Basic Properties of Fluroescence

Mirror rule: The fluorescence absorption and emission spectra reflects the vibrational levels in the ground and electronic states. In general, the vibrational levels are not significantly altered during electronic excitation and the absorption and emission spectra has mirrored feature. There are, however, plenty of exceptions to this rule. The following

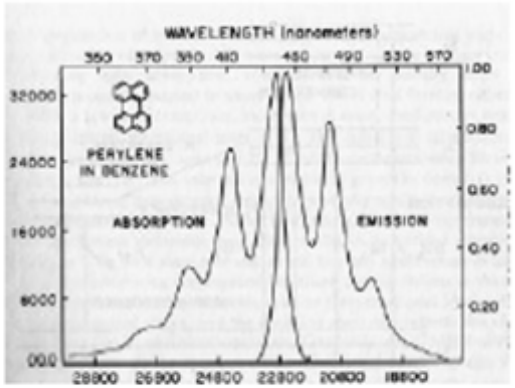
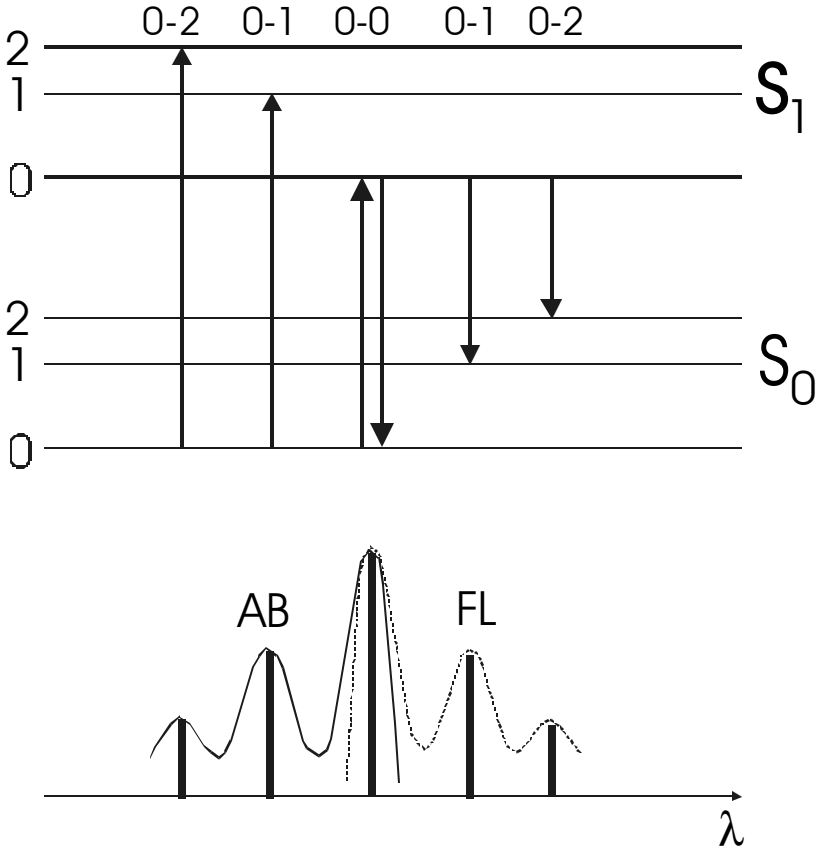
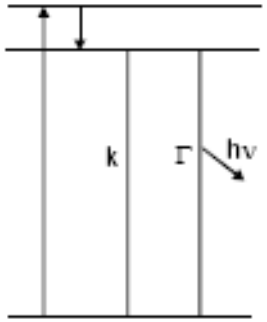


figure for perylene is extracted from Lakowicz, Principle of Fluorescence Spectrocpy, 1999.



(4) Lifetime and quantum yield

Fluorescence molecule does not have to relax by emitting a photon (radiative decay) but they can also relax by thermal process without emitting a photon (non-radiative decay). The quality of a fluorophore is clearly related to the rates of these two decay modes.



In this simplified Jablonski diagram, the radiative decay is denoted by Γ and the non-radiative decay is denoted by k.

The residence time of the molecule in the excited state (S1), lifetime, is affected by both radiative and non-radiative rates. In particular,

$$\tau = \frac{1}{\Gamma + k}$$

In the absence of non-radiative decay processes, the lifetime measured is called the intrinsic lifetime of the fluorophore.

$$\tau_0 = \frac{1}{\Gamma}$$

The "efficiency" of the fluorophore, the quantum efficiency, is defined as:

$$Q = \frac{\Gamma}{\Gamma + k} = \frac{t}{t_0}$$

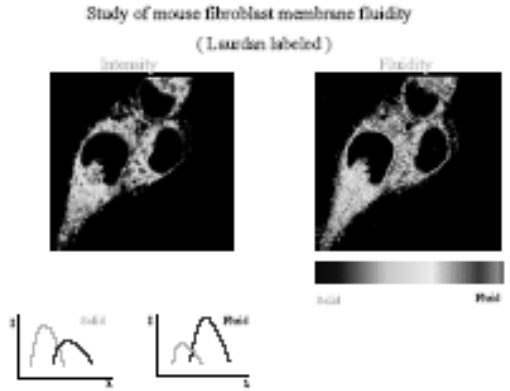
Basic Fluorescence Measurement

Intensity measurement:

This is the most basic measurement. It is not very diagnostic and it mainly reflects the presence or absence of fluorophore and their concentration. Note that quantitative intensity measurement is very hard as many factors affect the excited state of the fluorophore and will modify its intensity especially in biological systems.

Spectral measurement:

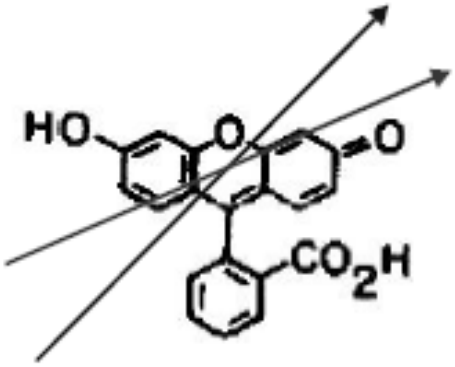
Spectral measurement is quite diagnostic. Most fluorophores has a fairly unique spectral pattern. Spectral measurement allows the experimenter to determine what fluorophores are present. In microscopy setting, the interaction of microscopic structures can be studied if they can be labeled with different color fluorophores. Equally important, many fluorophores changes color (excited state vibrational level shifts) as a function of biochemical environment. This allow a sensitive monitoring of intracellular or tissue biochemical state. The calcium probe described earlier is a good example. Another example is this membrane probe Laurdan which changes color as a function of the fluidity of the membrane.

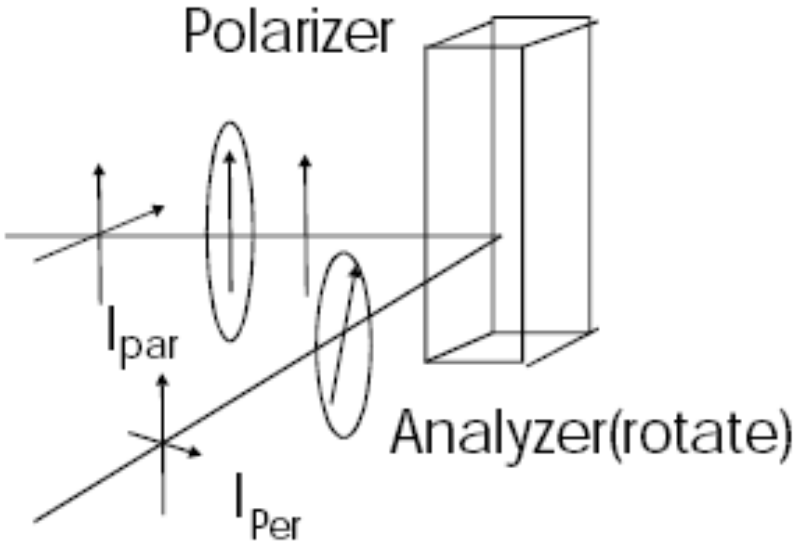


Emission spectra is defined as measuring emission intensity as a function of wavelength at a given excitation wavelength. Excitation spectra is defined as the measurement of emission intensity at a given emission wavelength as a function of excitation wavelength.

Polarization and Isotropy

Polarization is also another useful property of fluorescence. All fluorescence molecules have a preferential direction of excitation (excitation dipole) and emission (emission dipole). Note that the excitation and emission dipoles do not have to coincide in general. The probability of exciting a molecule depends on the relative orientation of the molecular excitation dipole and the polarization of light. Let θ be the angle between the light polarization and the molecule excitation dipole. The probability of excitation is: $P \propto \cos^2\theta$. This is similar to what we see for the transmission of a polarizer. One can also see that exciting molecules with polarized light selects a sub-population of molecule that are oriented close the polarization of light.





The measurement of polarization of aqueous specimen is typically performed using the above geometry. Excitation light is first polarized. The emission light is analyzed for its polarization parallel and perpendicular to the excitation direction.

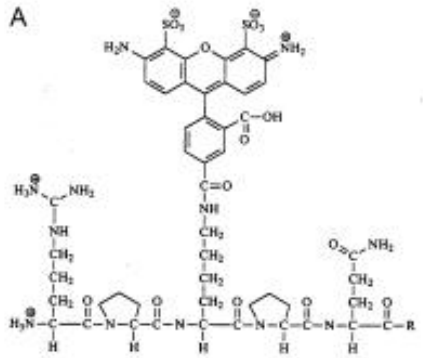
The result is expressed in terms of polarization, P, or anisotropy, r:

$$P = \frac{I_{par} - I_{per}}{I_{par} + I_{per}}, \quad r = \frac{I_{par} - I_{per}}{I_{par} + 2I_{per}}$$

Note that the steady state polarization is high with rotation diffusion rate slow compared with its lifetime but its polarization is low if diffusion is fast compared with its lifetime. This is very useful for measuring the binding of small ligand to large molecules or surfaces. Polarization is also often used to measure the mean orientation of molecules.

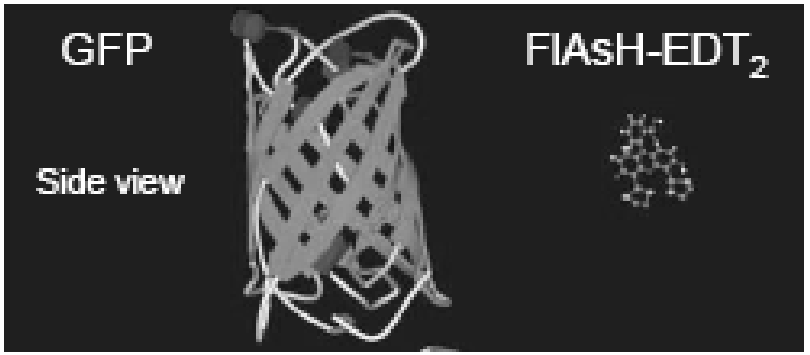
Fluorescent Probes

Organic Probes



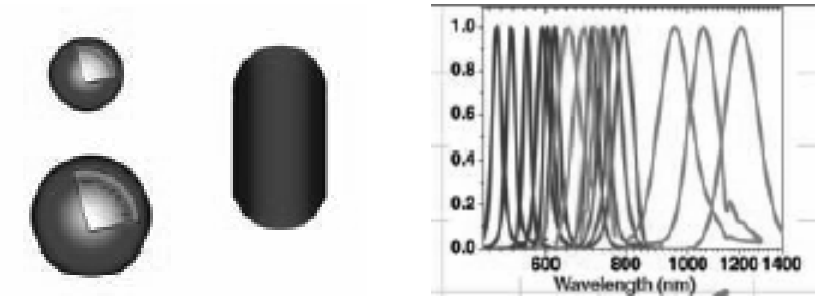
Molecular Probes, Oregon

Genetic Probes



Hoffmann et al, Nat. Meth, 2005

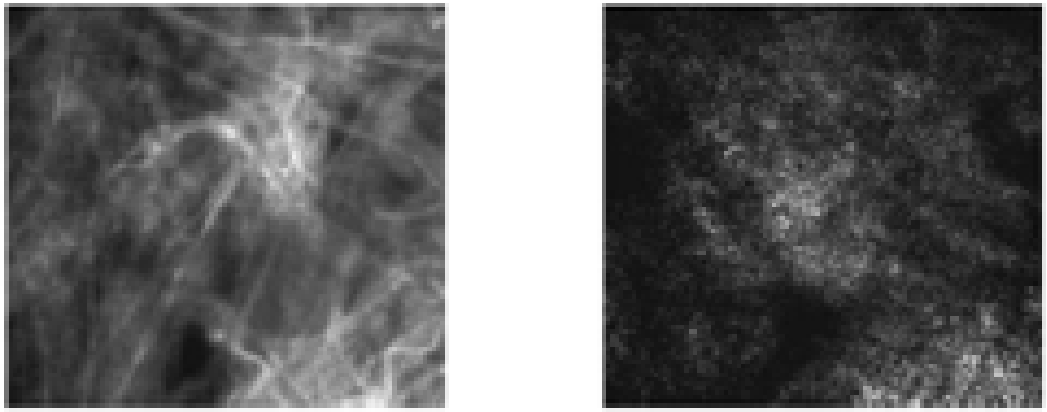
Quantum Dots



Michalet et al, Science, 2005

Strengths of Fluorescence Microscopy

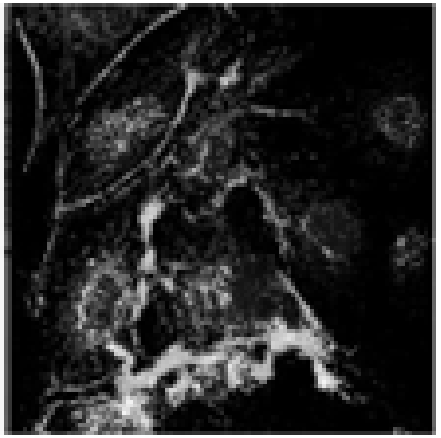
(1) New contrast enhancement mechanism



Imaging collagen/elastin fibers in dermis. Fluorescence image (left), scattered light image (right)

Strengths of Fluorescence Microscopy

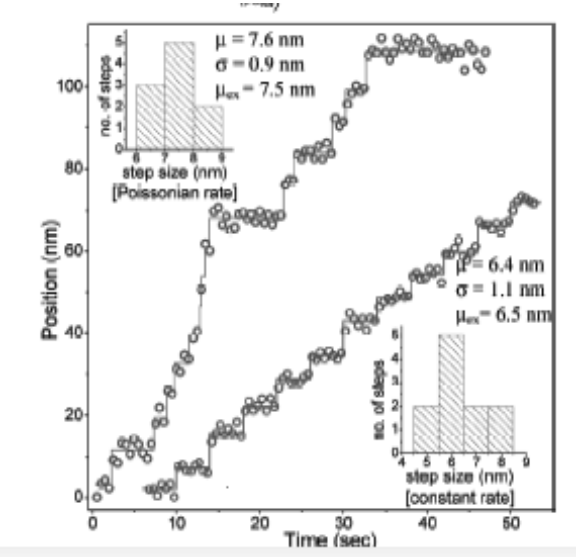
(2) Specificity – individual structural components can be tagged based on their biochemical difference



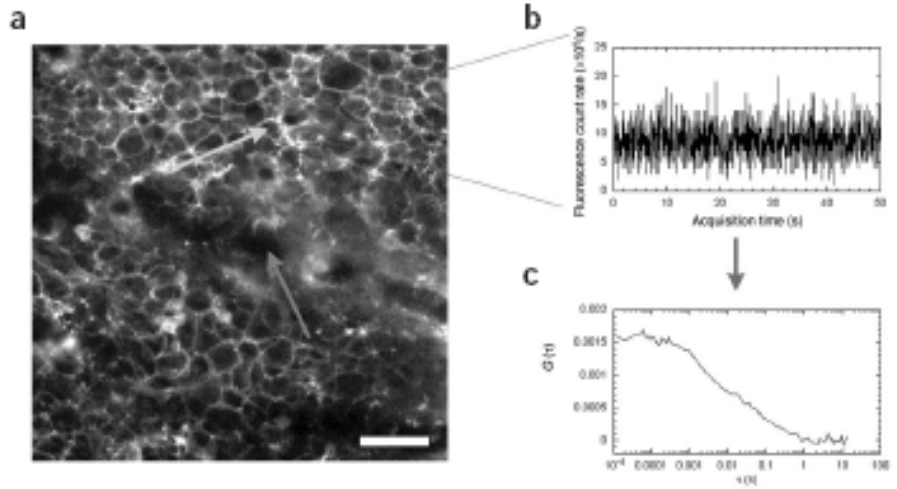
Nuclei (blue) is label with DAPI, Actin (green) is label with Bodipy phallodin, mitochondria (red) is label with MitoTracker.

Strengths of Fluorescence Microscopy

(3) Molecular Sensitivity



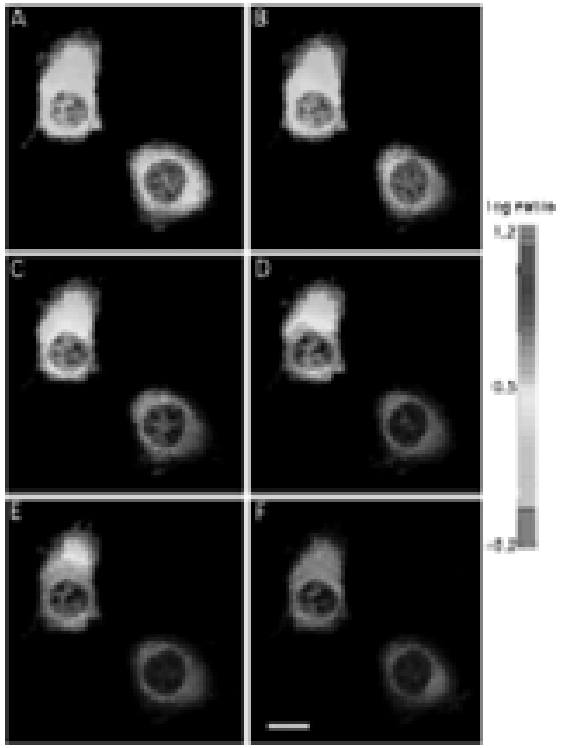
Yildiz, Acc. Chem. Res., 2005



Alexandrakis, Nat. Med., 2004

Strengths of Fluorescence Microscopy

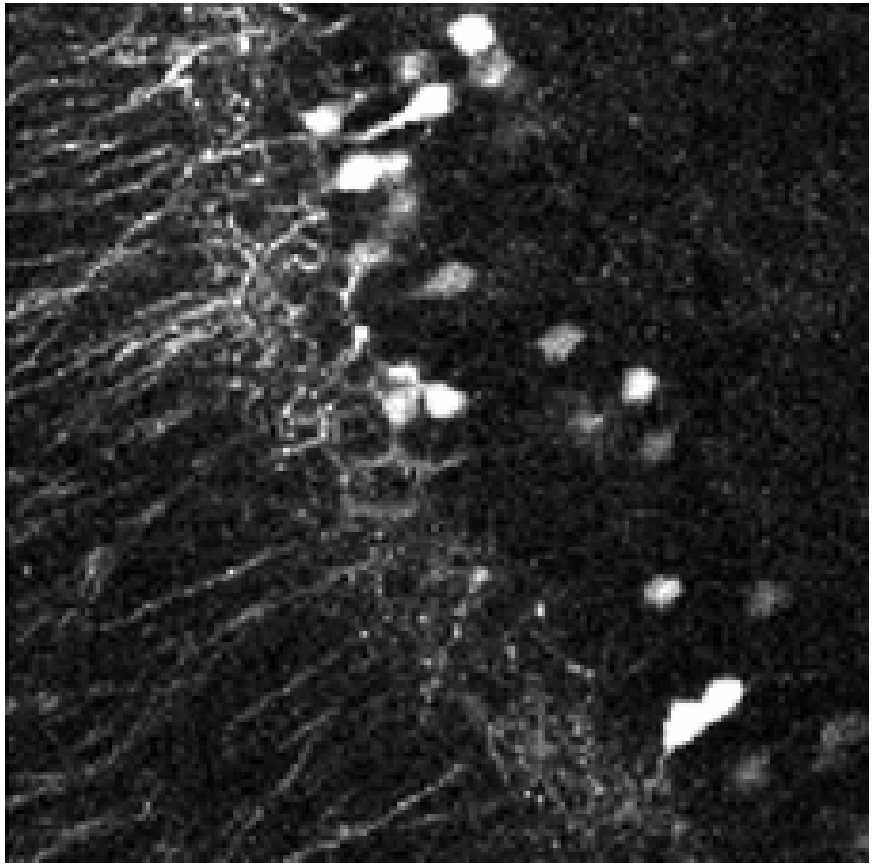
(4) Image biochemical reactions/ Monitor microenvironmental changes



Fan et al. Biophys. J., 1999
Calcium wave in HeLa cells

Strengths of Fluorescence Microscopy

(5) Monitors genetic expression



Advanced fluorescence measurement

Fluorescence lifetime

Fluorescence lifetime is a complimentary measurement to spectral measurement. Most fluorophores has a signature lifetime as well as spectral. More important, some fluorophores have lifetimes tha are more sensitive to environmental factors than their spectra.

The fluorescence decay of a fluorophore is governed by the following equation:

$$\frac{dN_e}{dt} = -(k + \Gamma)N_e$$

where N_e is the number of molecules in the excited state which is proportional to the fluorescence intensity. This differential equation can be easily solved:

$$F = F_0 e^{-(k+\Gamma)t} = F_0 e^{-t/\tau}$$

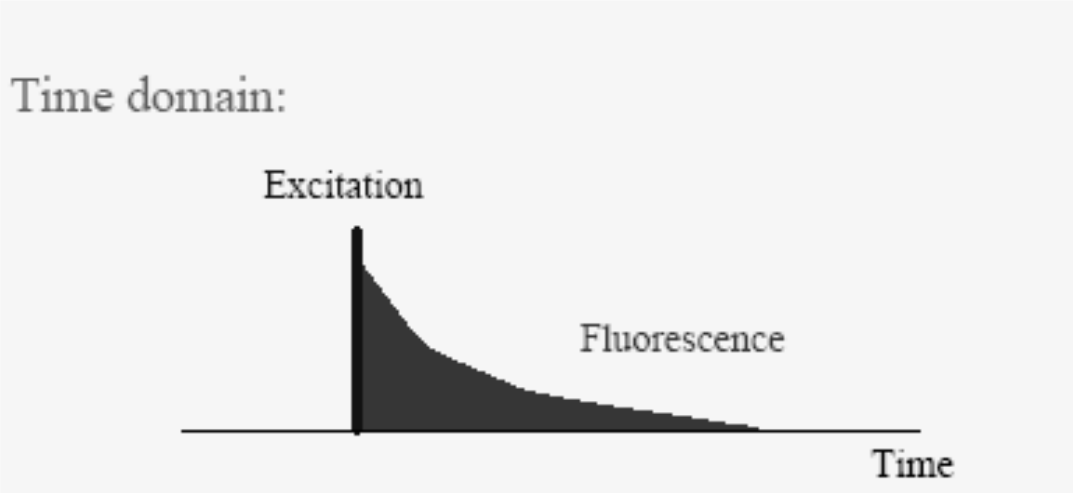
where F and F_0 are the instantaneous and initial fluorescence intensity. Therefore, we can see that fluorescence emission is a statistical process that is characterized by exponential decays. What if there are multiple decay pathways and multiple rates? In this case, the fluorescence decay will be multiple exponential:

$$\frac{dN_e}{dt} = -(\frac{1}{\tau_1} + \frac{1}{\tau_2} + ...)N_e$$

$$F = F_0 e^{-\Sigma t/\tau_i}$$

As a matter of fact, fluorescence decay of most fluorophores in biological system often has multiple exponential decay that is characteristic both of the probe and its environment.

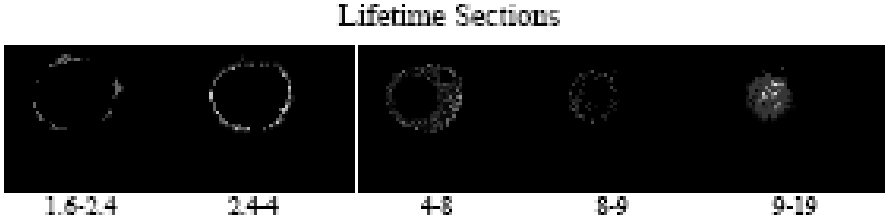
While fluorescence lifetime is a very useful parameter, it is however difficult to measure. As we have discussed before, fluorescence lifetime is typically on the time scale of nanoseconds. We therefore require very fast optics electronic to measure these events. Conceptually, the measurement can be done in the following way:



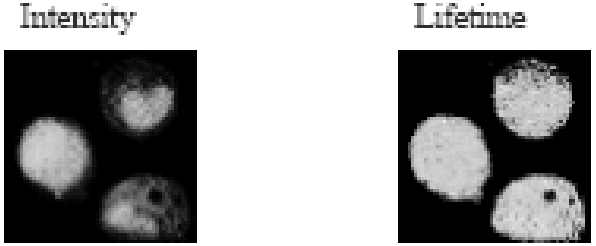
We will use a laser that can generate pulses that are very short compared with the fluorescence decay time (fs or ps). The fluorescence lifetime can then be measured by determining the time lapse between the excitation light and the first emission photon detected. One important catch to this scheme is that the photon detected for each excitation pulse has to be less than one.

Lifetime imaging and biological functions

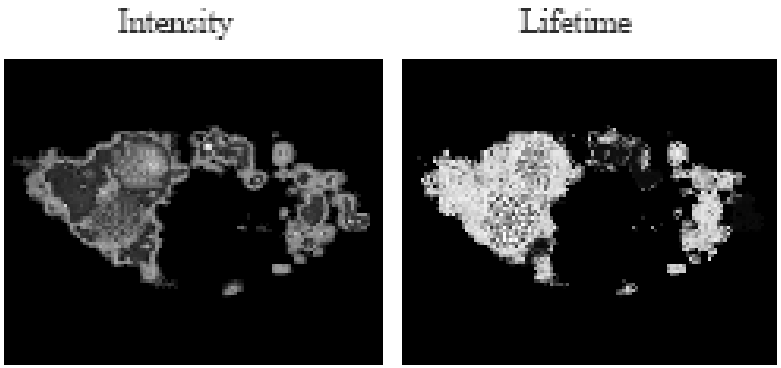
(1) Distinguish cellular organelles by multiple lifetime imaging



(2) Monitor metabolite concentration (Ca, pH etc)



(3) Monitor cellular processes such as proteolytic activity



Fluorescence Quenching

It is often very useful to measure the diffusion of small metabolites in a biological system; oxygen is a good example. As it turns out, a number of fluorophore, such as pyrene, with sufficiently long lifetime can be quenched by the presence of metabolite such as oxygen due to molecular collision. Upon collision, the fluorophore is de-excited non-radiatively.

The collision frequency is proportional to the concentration of the quencher and the rate equation in the presence of the quencher can be expressed as:

$$\frac{dN_e}{dt} = -(k_o[Q] + \Gamma)N_e$$

where [Q] is the concentration of the quencher and k_0 is a proportionality constant related to the diffusivity of the reactants.

$$k_0 \propto (R_f + R_q)(D_f + D_q)$$

where R_f, R_q are the "collision" radii of the fluorophore and the quencher and D_f, D_q are the diffusion coefficients of the fluorophore and the quencher

Therefore, we have:

$$\tau^{-1} = k_0[Q] + \Gamma = k_0[Q] + \tau_0^{-1} = \tau_0^{-1}(1 + k_0\tau_0[Q])$$

Therefore, by measuring fluorescence lifetime, we can determine quencher concentration as long as the natural lifetime and the proportionality constant k can be calibrated.

The effect of quencher can also be studied by monitoring the steady state fluorescence emission. We will add a constant illumination term, I, to the fluorescence rate equation:

$$\frac{dN_e}{dt} = -(k_o[Q] + \Gamma)N_e + I$$

In the steady state, $\frac{dN_e}{dt} = 0$, and we have the fluorescence, F, signal:

$$(k_o[Q] + \Gamma)F = I$$

We can re-write this equation in the absence of quencher. The steady state fluorescence in the absence of quencher, F_0, is:

$$\Gamma F_0 = I$$

Combining the last two equations, we get the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + k_o \tau_0 [Q]$$

For dynamic (collision) quenching process, the steady state fluorescence intensity is a linear function of quencher concentration.

The quenching process that we have described previously is called dynamic quenching where a fluorophore is de-excited by collision process in the excited state. For dynamic quenching, both the steady state fluorescence intensity and the fluorescence lifetime changes linearly with quencher concentration.

A molecule can also be quenched by a ground state process where the molecule is chemically bound to a quencher to form a "dark complex" – a reaction product that do not fluoresce. The ground state reaction can be described by the standard chemical kinetic rate equation where Ks is the association constant, [F] is the concentration of the un-complexed fluorophores, [F-Q] is the concentration of the complexes.

$$K_S = \frac{[F-Q]}{[F][Q]}$$

The total concentration of fluorophore, $[F]_0$, is given by:

$$[F]_0 = [F] + [F-Q]$$

$$K_S = \frac{[F]_0 - [F]}{[F][Q]} = \frac{[F]_0}{[F][Q]} - \frac{1}{[Q]}$$

$$\frac{F_0}{F} = \frac{[F]_0}{[F]} = 1 + K_S[Q]$$

Therefore, in static quenching, the steady state fluorescence again decreases linearly with quencher concentration.

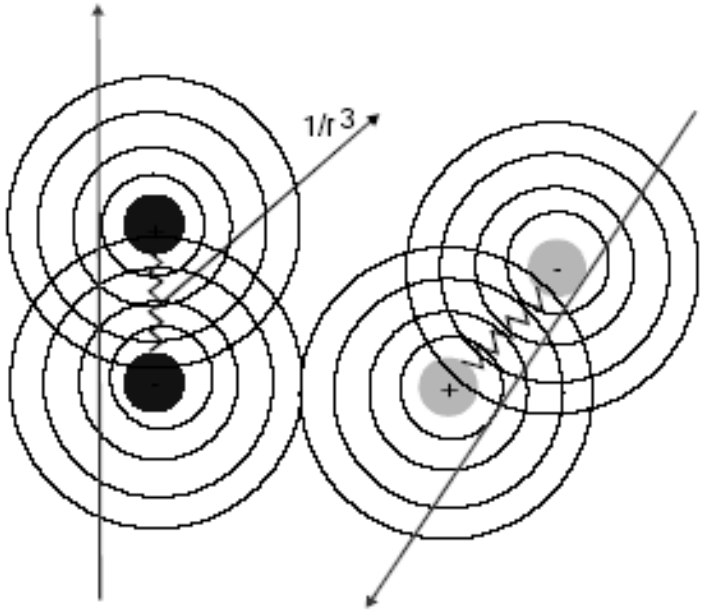
However, it is important to note that steady state quenching does NOT affect fluorescence lifetime as it does not affect the excited state and its effect is mainly the reduction of available fluorophores to be excited.

Fluorescence Resonance Energy Transfer (FRET)

FRET is a very "cool" technique. It is an optical ruler with resolution that is better than angstroms. The theory of FRET is rather complicated and we will not go into the details here. Instead, I will provide a qualitative description about what is FRET, what are the relevant experimental parameters, and what it can measure.

FRET is a process where energy is transferred from one excited fluorophore (the doner) to a second fluorophore (the acceptor). After energy transfer, the acceptor is excited and will emit a photon in the emission spectrum of the acceptor. As an example, fluorescein (a green dye) and rhodamine (a red dye) make a good FRET pair. A fluorescein molecule is normally excited by blue light and emits green light. A rhodamine is be excited by green light and emits red. Without FRET, the fluorescence of the composite system will emit green. In the presence of FRET, the energy of the fluorescein is transferred to the rhodamine under appropriate conditions. In this case, the emission of the composite system will be red.

It should be pointed out that FRET itself is a non-radiative process, i.e. no real photon is transferred between the doner and the acceptor. Instead, the transfer is via a dipole-dipole coupling of the doner and acceptor molecule. The excited doner can be modeled as a vibrating electrical dipole. The energy is "transferred" to the acceptor through the fluctuating dipole field.



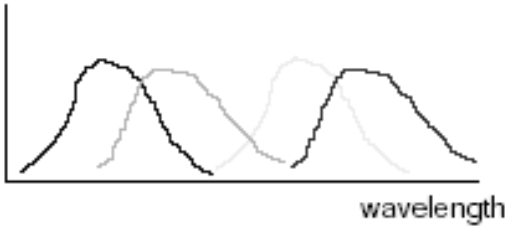
The dipole electric field has a spatial dependence of r^{-3} (Recall that electric monopole, an isolated charge, has an electric field that has a spatial dependence of r^{-1}. Since the dipole has no net charge, its field should decay faster).

The rate of FRET is described by the following equation:

$$k_T \propto [\frac{\kappa^2 \phi_d}{n^4 \tau_d} \int_0^\infty F_d(\lambda)\varepsilon_d(\lambda)\lambda^4 d\lambda]\frac{1}{r^6} = \frac{\kappa^2 \phi_d J}{n^4 \tau_d}\frac{1}{r^6}$$

The deviation of this formula is quite involved but it is not hard to see why these parameters come into play. First, κ^2 is a measure of the relative orientation of the doner emission and the acceptor excitation dipole. Only when the dipole are correctly aligned would FRET be efficient. n is the index of refraction of the medium in between. A large n corresponds to larger shielding

effect of the medium and decreases FRET. Φ_d is the quantum yield of the doner. A large quantum yield indicates there are less non-radiative decay mechanisms to compete with FRET. τ_d is the lifetime of the doner. A larger lifetime implies a more stable excited state and less probability of energy transferring. The integral contains two terms: $F_d(\lambda)$is the fluorescence emission spectrum of the doner as a function of wavlength, λ, and $\varepsilon_d(\lambda)$ is the absorption spectrum of the acceptor. This integral, J, represent their overlap.



The spectra with better overlap will results in more efficient energy transfer. This is reasonable as overlap implies the energy transferred from the doner is sufficient to put the acceptor molecule into the excited state.

Finally, the r^{-6} spatial dependence comes from the fact that FRET is a combined process of two dipoles and each dipole has a r^{-3} dependence.

By combining most of the molecular parameters into a distance parameter, R_0, the Froster distance, the equation can be rewritten as:

$$k_T = \frac{1}{\tau_d}\left(\frac{R_0}{r}\right)^6$$

This is called Froster equation. Typical value of R_0 is from 5-10 nm. Therefore, FRET does not happen if the doner and acceptors are very farther apart than atomic dimensions.

One important quantity to be determined is the efficiency of the FRET, E:

$$E = \frac{k_T}{k_T + \tau_d}$$

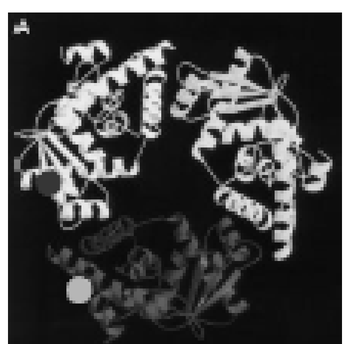
When the concentration of the doners and acceptors are fixed, the efficiency FRET can be calculated (derivation is similar to quenching):

$$E = 1 - (\frac{\tau_{da}}{\tau_d})$$

$$E = 1 - (\frac{F_{da}}{F_d})$$

where τ_{da}, F_{da} are the lifetime and the steady steady fluorescence with the presence of both doner and acceptor and are τ_d, F_d the lifetime and the steady state fluorescence of the doner only.

Resonance energy transfer is commonly used to measure distance of different part of a biological molecule, such as a single protein, by putting a doner and an acceptor fluorophore on it. Since the motion (dynamics) of many protein are of interest (such as in force generation), FRET is also used to monitor the change in protein conformation as it work.



Kovall & Matthews, Science 1997