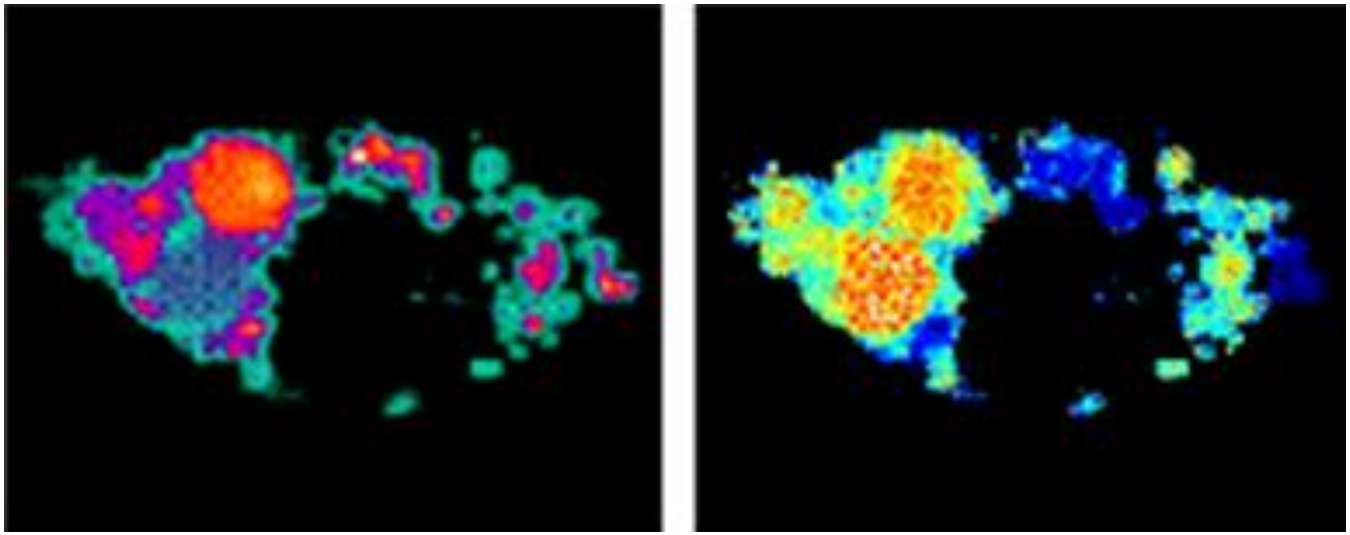
Fluorescence Spectroscopy



Fluorescence Spectroscopy

We have discussed quite extensively about white light microscope and the many contrast enhancement methods. All these methods are based on two properties of the specimen:

(1) Index of refraction – scattering, interference
(2) Birefringence – polarization

It is important to note that these are mostly macroscopic properties of materials (although ultra-thin objects such as microtubules that are on the order of tens of nanometer can be visualized by methods such as DIC).

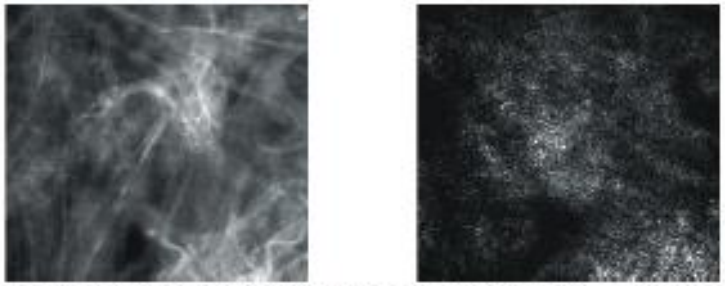
There are situations where we need addition contrast enhancement mechanism:

(1) The difference in indexes or birefringences of the object and the background is too small.
(2) There are many objects with the same index.
(3) The objects are too small as in the case of single proteins.
(4) The need to monitor biochemical states
(5) Need to resolve the position of chemically distinct objects that are below resolution limit of the microscope

Fluorescence microscopy

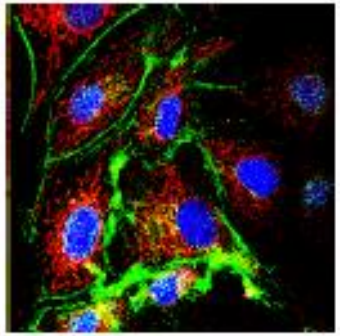
To address these limitation, new contrast enhancement techniques have been developed in microscopy. Among the many techniques, the most mature and common method is probably fluorescence microscopy. Let's look at what fluorescence microscope has to offer:

(1) New contrast enhancement mechanism



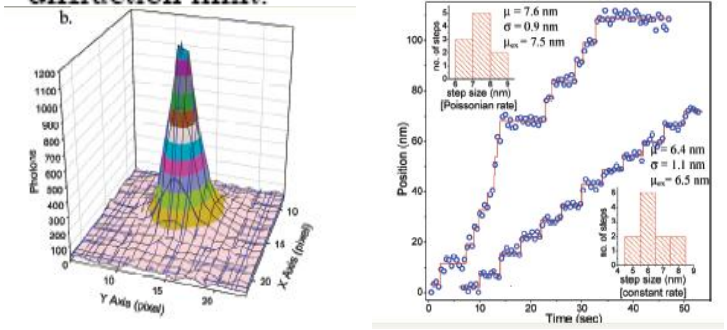
Imaging collagen/elastin fibers in dermis. Fluorescence image (left), scattered light image (right)

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



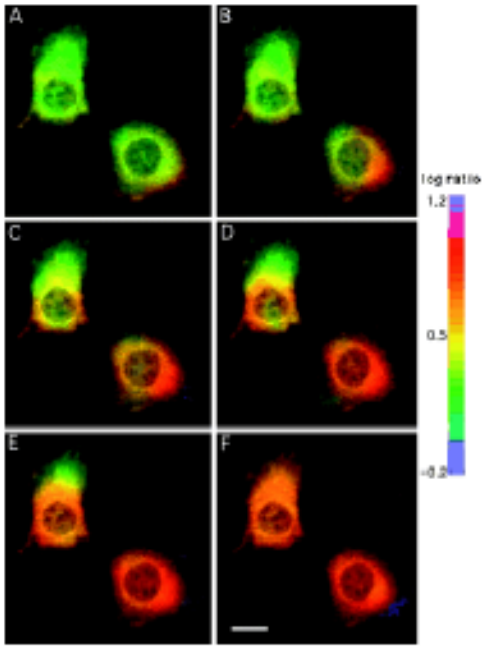
Nuclei (blue) is label with DAPI, Actin (green) is label with Bodipy phallodin, mitochondria (red) is label with MitoTracker.

(3) Image and resolve objects that are substantially below diffraction limit.



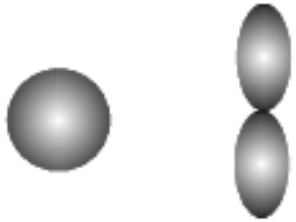
Yildiz, Acc. Chem. Res., 2005

(4) Image biochemical reactions/ Monitor microenvironmental changes



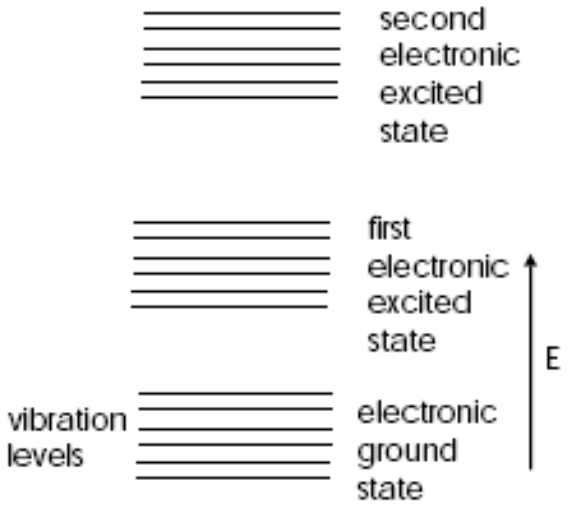
Fan et al. Biophys. J., 1999
Calcium wave in HeLa cells

The electron cloud of the molecule can assume a discrete set of configurations as the energy in the molecule increases (such as by the absorption of light). For people who remember basic chemistry, these are similar to the S-, P- and D- orbitals of atoms but is much more complicated in a molecule with multiple atoms. For a molecule, these are called different electronic states.



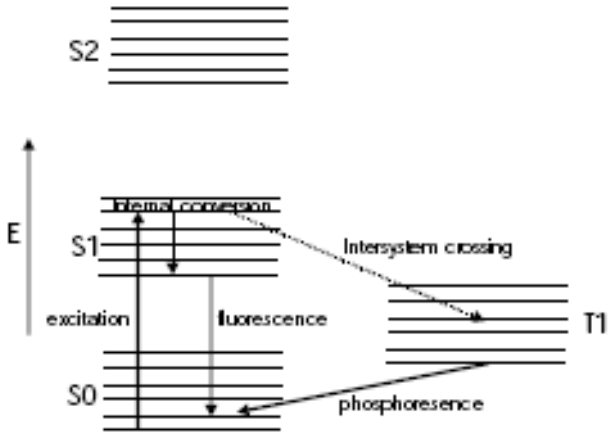
S P

With the same electron cloud configuration, the possible energy levels of a complex molecule is a bit more complicated. The nuclei are bound together as if linked by springs. This spring can vibrates at different modes at another discrete set of energy levels. This are called vibration level. Typical energy different between vibration levels are much smaller than that between electronic levels.



Fluorescence, phosphorescence and Jablonski Diagram

Typically the ground, first and second excited states of the molecules are called S0, S1, and S2. These are so called singlet states. The absorption of a photon maps a molecule from singlet state to singlet states with high efficiency. There are also triplet state electronic state, T0, T1, etc where the photonic transition between singlet and triplet state is very slow.



A description of this process is typically expressed as the Jablonski diagram. After fluorescence excitation, the molecule typically reaches the first or second excited singlet state. Internal conversion, a vibrational relaxation process, rapidly (within ps) relax the molecule back to the lowest vibrational energy level of S1. This process is called internal converion. Fluorescence occurs when a photon is emitted and the molecule return to the ground state. The typical residence time of the molecule in S1 is on the order of nanoseconds. After fluorescence excitation, there is a possibility that the molecule will relax to a triplet state (intersystem crossing). Since the coupling of T1 to S0 through photon emission is of very low probability, the residence time of the molecule in T1 is long (μs to ms). This emission process is called phosphoresence.

Basic properties of fluorescence

(1) 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.

(2) 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

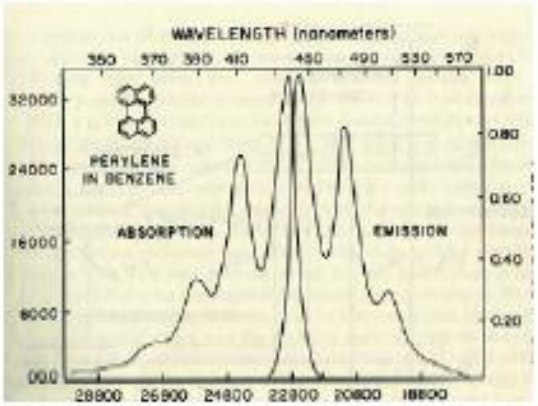
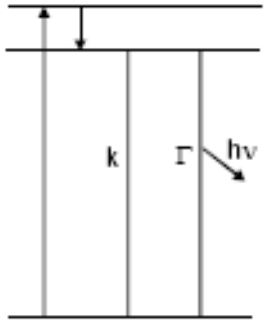


figure for perylene is extracted from Lakowicz, Principle of Fluorescence Spectrocpy, 1999.

(3) 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.

(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.

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

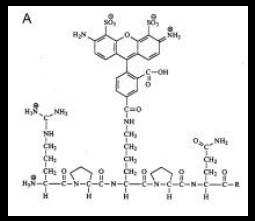
The "efficiency" of the fluorophore, the quantum efficiency, is defined as:

$$Q = \frac{\Gamma}{\Gamma + k} = \frac{\tau_0}{\tau}$$

Optical Microscopy Can Utilize a Variety of Molecular Probes

Fluorescent Probes

Organic Probes

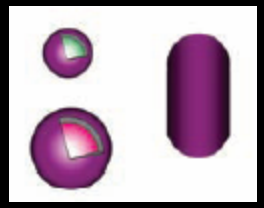


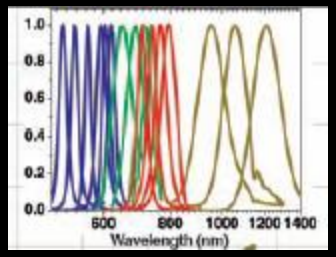
Molecular Probes, Oregon

Genetic Probes

Hoffmann et al, Nat. Meth, 2005

Quantum Dots

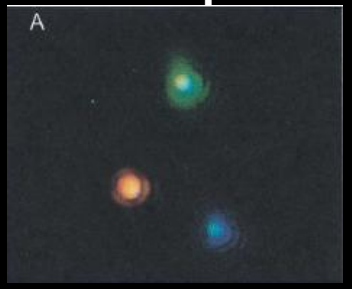




Michalet et al, Science, 2005

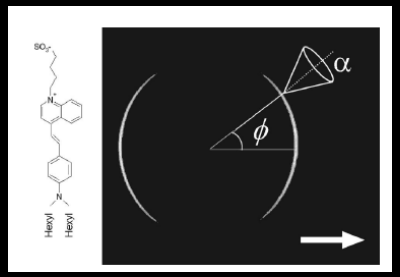
Non-fluorescent Probes

Metal Nanoparticles



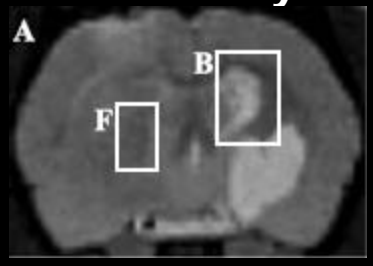
Schultz, PNAS, 2003

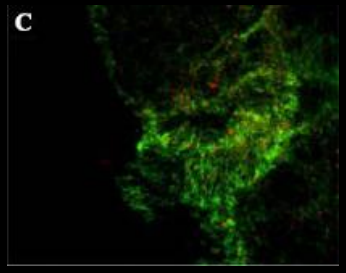
SHG Probes



Pons, JBO, 2003

Hybrid Probes





Modo et al, Neuroimage, 2004

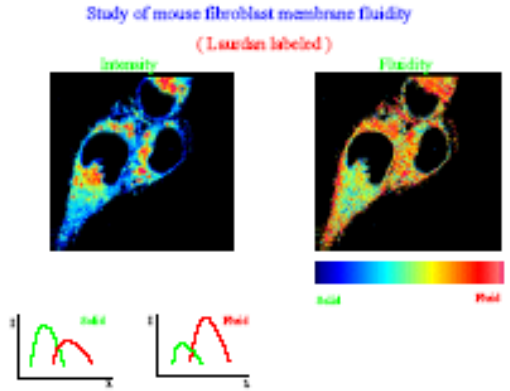
Basic Fluorescence Measurement

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:

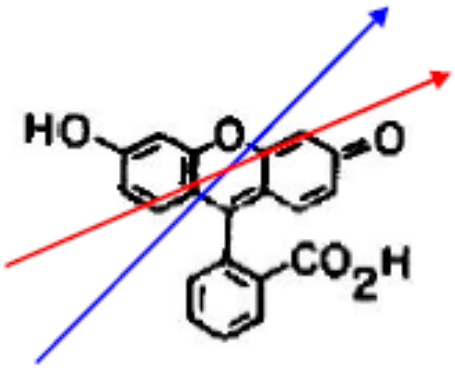
Spectral measurement is quite diagnostic. Most fluorophores has a fairly unique spectral pattern. Spectral measurement allows the experimenter to determine what fluorophores are present. In microscopy setting, the interaction of microscopic structures can be studied if they can be labeled with different 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.

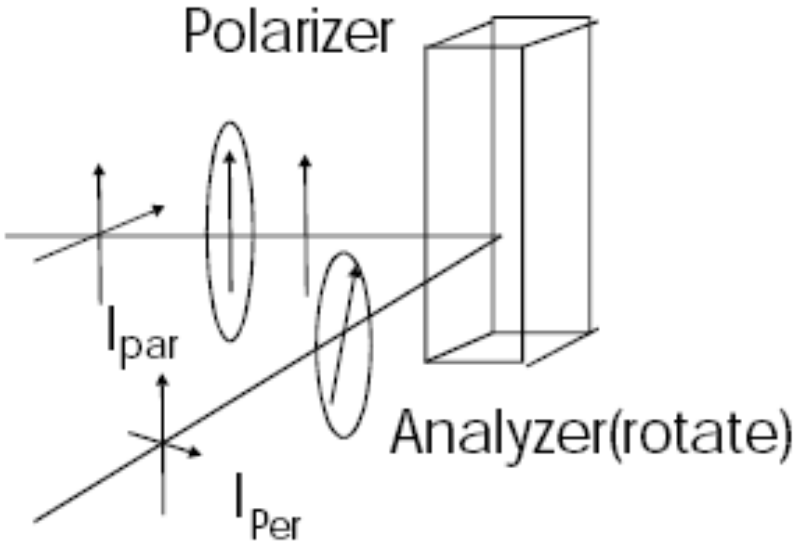


Emission spectra is defined as measuring emission intensity as a function of wavelength at a given excitation wavelength. Excitation spectra is defined as the measurement of emission intensity at a given emission wavelength as a function of excitation wavelength.

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.