

***Fluorescence Workshop***  
***UMN Physics***  
***June 8-10, 2006***

**Fluorescence Principles**  
**Joachim Mueller**

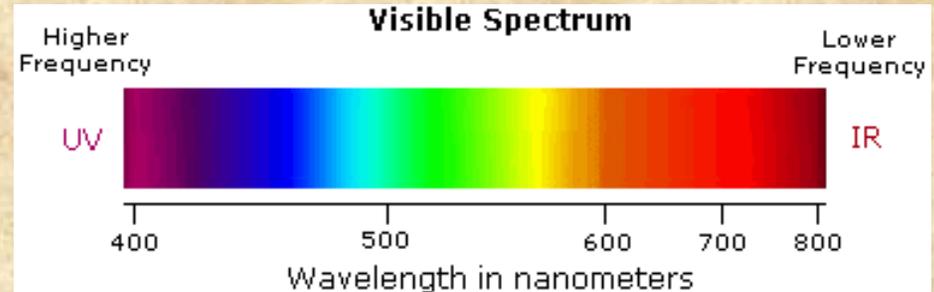
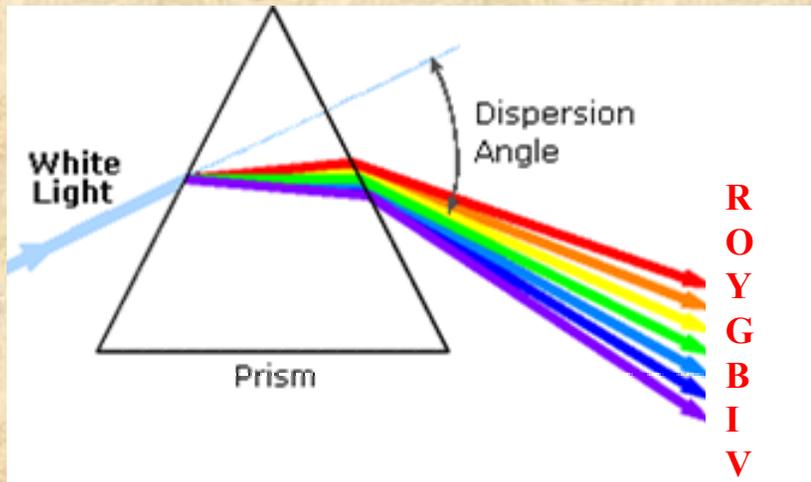
**Fluorometer, Excitation and Emission  
Spectrum, Mirror-image rule**



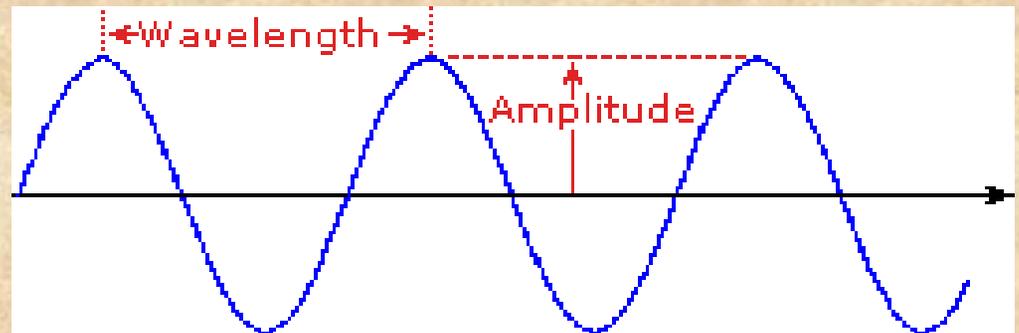
# Review: Light

## 1. Sir Isaac Newton 1672:

Showed that the component colors of the visible portion of white light can be separated through a prism, which acts to bend the light (refraction) in differing degrees according to wavelength. Developed a “corpuscular” theory of light.



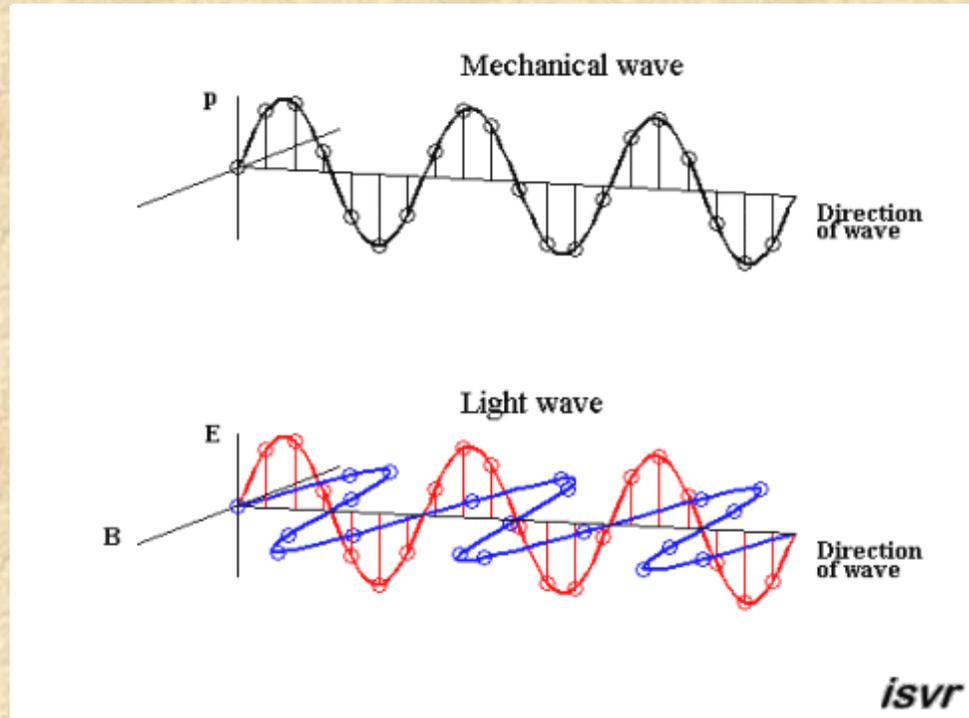
## 2. Christian Huygens 1692: Developed a wave theory of light



#### 4. Albert Einstein: 1905

He won the Nobel Prize for Physics in 1921 for his work on the photoelectric effect. This work proposed that light be considered as consisting of particles called photons. He also stated that the energy the photon contains is proportional to the frequency of the radiation.

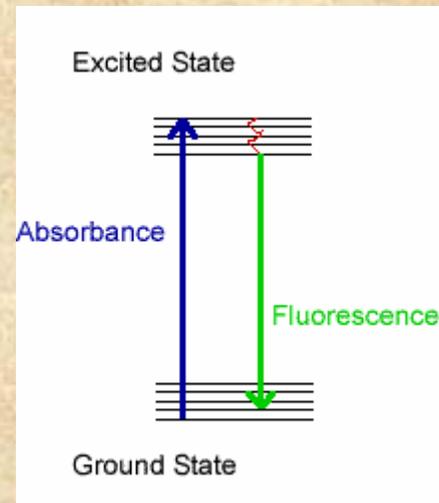
$$E = h f = hc / \lambda$$



We need to concern ourselves with how molecules interact with electromagnetic waves.

# What is fluorescence?

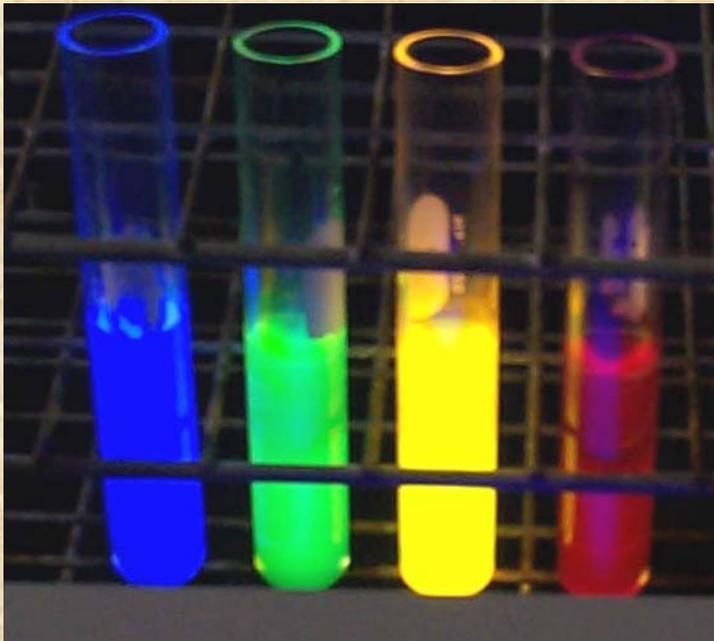
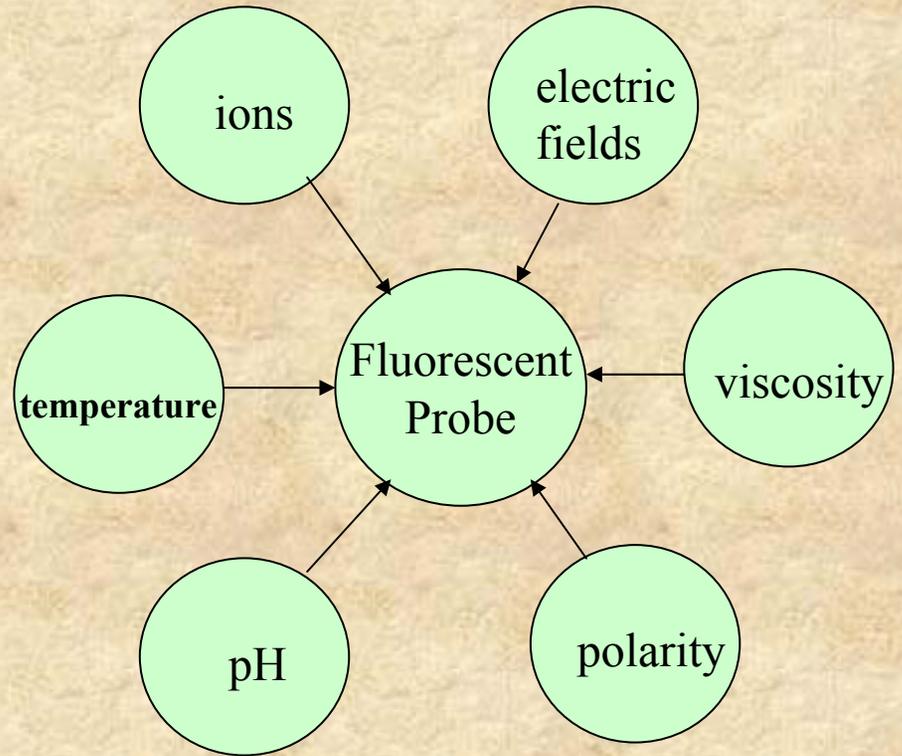
**FLUORESCENCE** is the light emitted by an atom or molecule after a finite duration subsequent to the absorption of electromagnetic energy. Specifically, the emitted light arises from the transition of the excited species from its first excited electronic singlet level to its ground electronic level.



The development of highly sophisticated fluorescent probe chemistries, new laser and microscopy approaches and site-directed mutagenesis has led to many novel applications of fluorescence in the chemical, physical and life sciences. Fluorescence methodologies are now widely used in the biochemical and biophysical areas, in clinical chemistry and diagnostics and in cell biology and molecular biology.

# Why fluorescence?

- its pretty!
- it provides information on the molecular environment
- it provides information on dynamic processes on the nanosecond timescale



Also fluorescence is very, very, very sensitive!

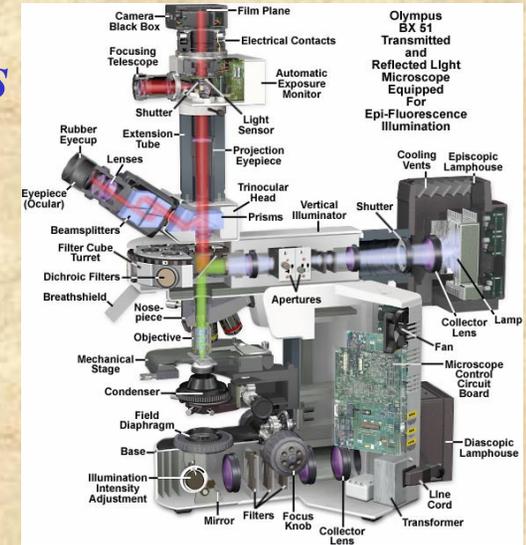
Work with subnanomolar concentrations is routine while femtomolar and even SINGLE MOLECULE studies are possible with some effort

# Fluorescence Instrumentation

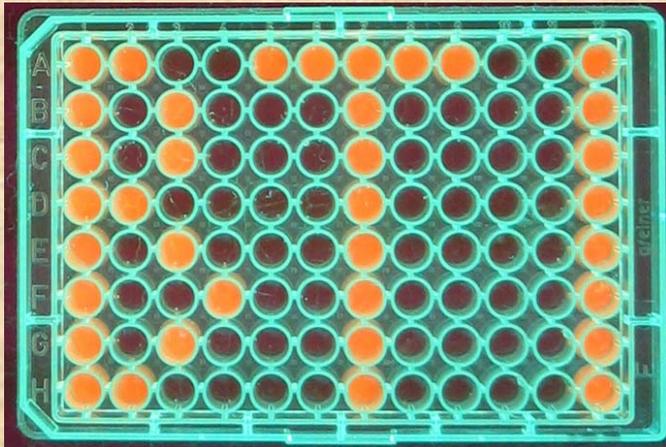
## Fluorimeters



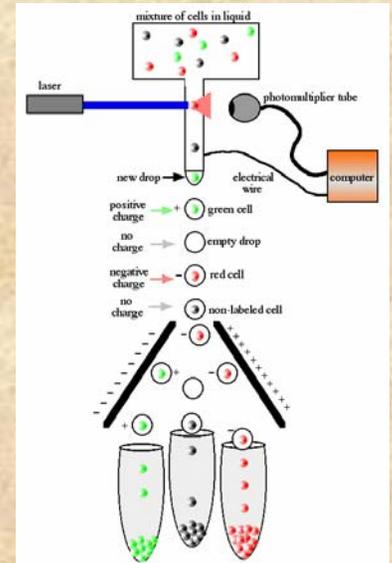
## Microscopes



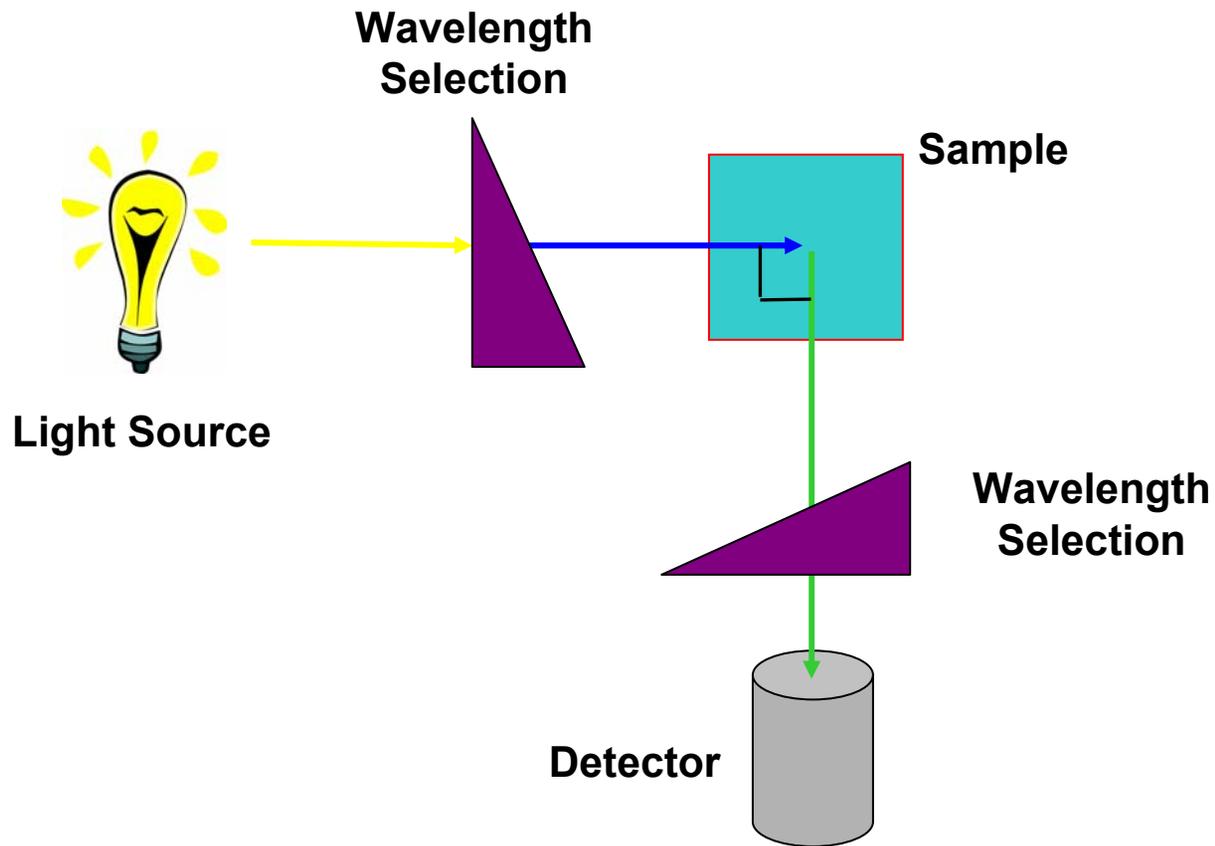
## High throughput Plateraders



## Fluorescence Activated Cell Sorting



# Fluorometer: The Basics



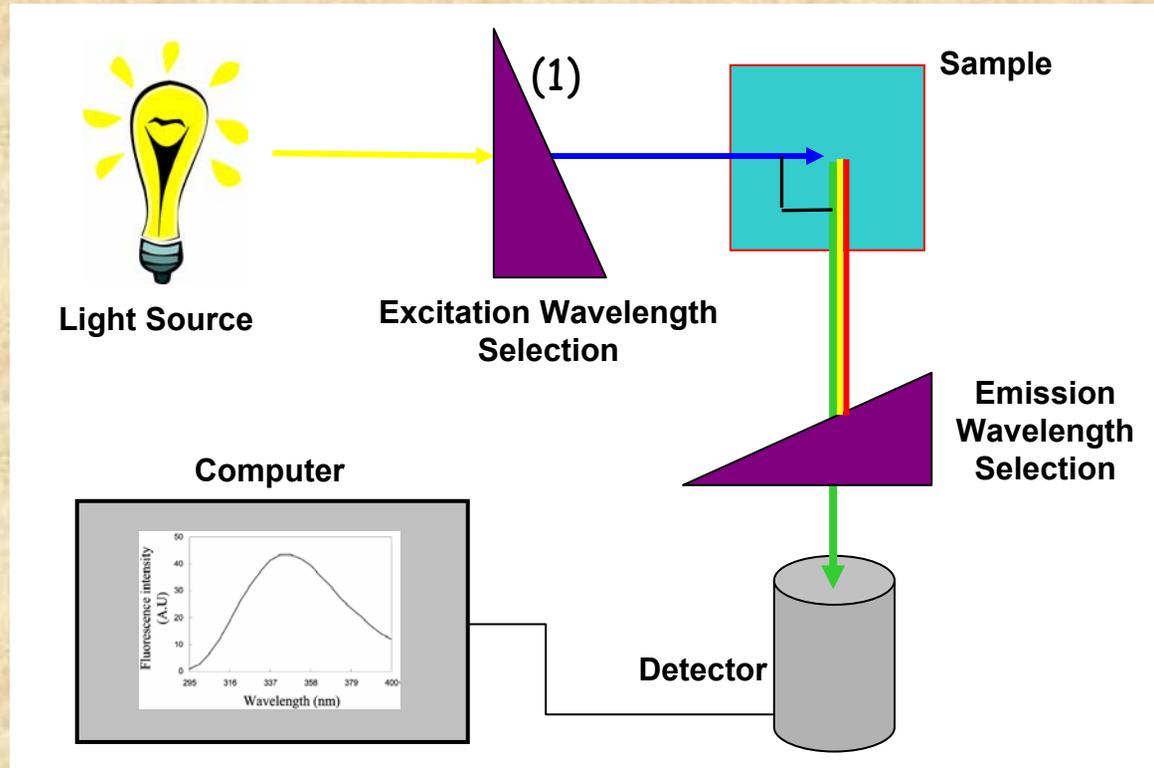
Virtually all fluorescence data required for any research project will fall into one of the following categories.

1. The fluorescence emission spectrum
2. The excitation spectrum of the fluorescence
3. The quantum yield
4. The polarization (anisotropy) of the emission
5. The fluorescence lifetime

In these lectures, we examine each of these categories and briefly discuss historical developments, underlying concepts and practical considerations

# Fluorescence Emission Spectrum

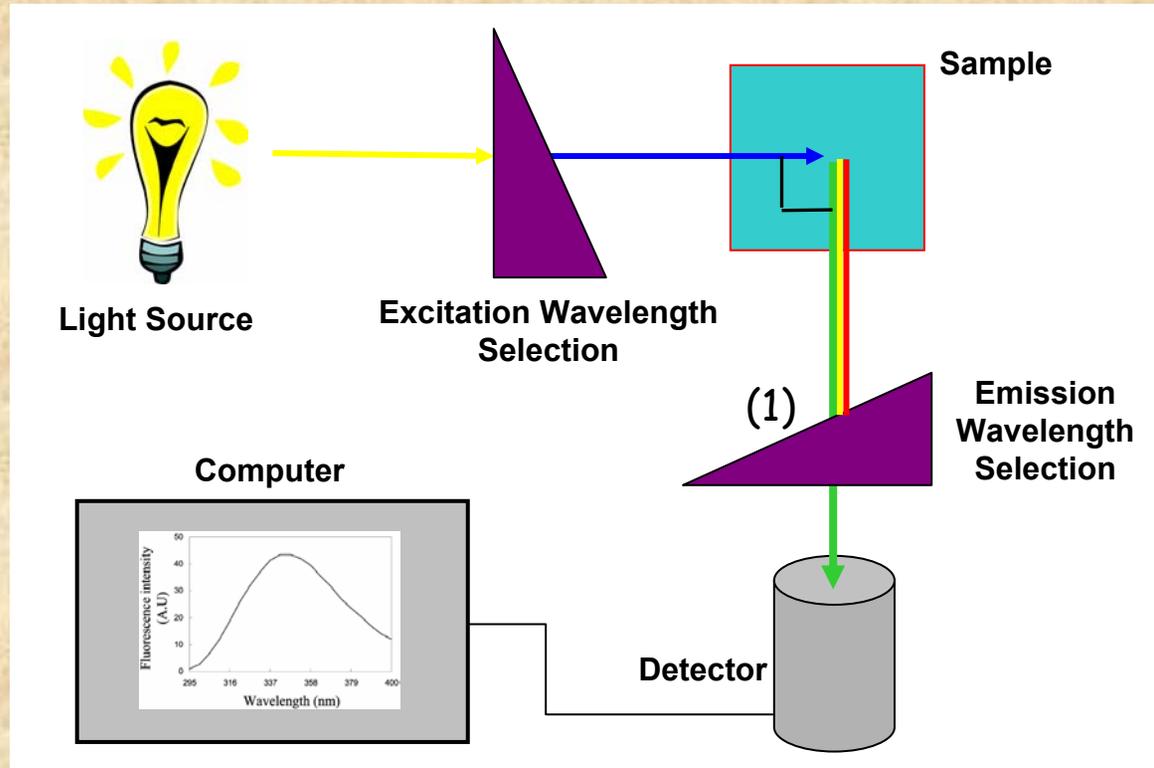
Measure the fluorescence emission spectrum for a fixed excitation wavelength



- (1) Select excitation wavelength
- (2) Illuminate sample with excitation light
- (3) The fluorescence emits in all directions
- (4) Collect fluorescence at  $90^\circ$  with respect to the exciting beam path
- (5) Select an emission wavelength and record the light intensity
- (6) Repeat (5) by scanning across a wide range of emission wavelengths

# Fluorescence Excitation Spectrum

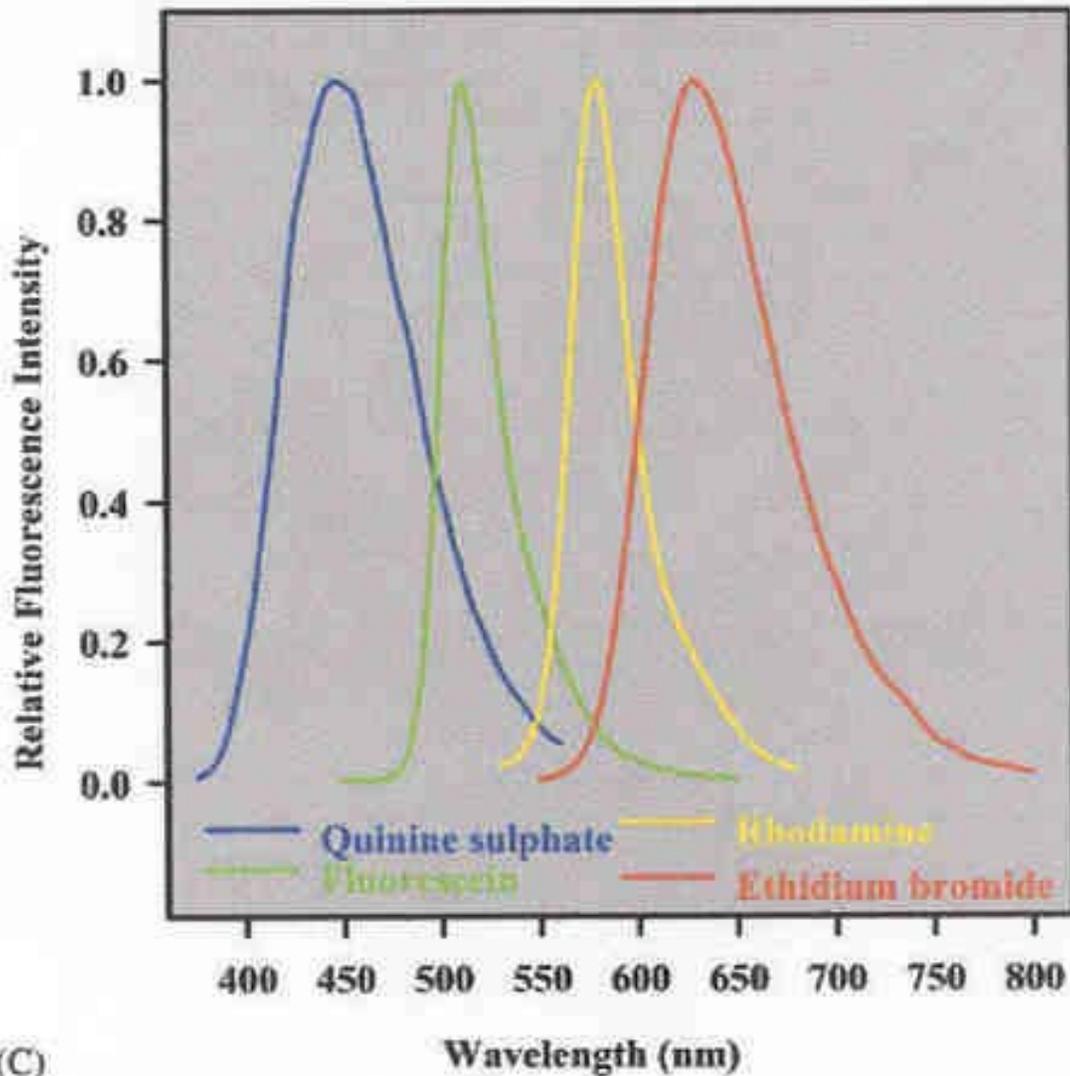
Measure the fluorescence emission at a fixed wavelength, while scanning the excitation wavelength.

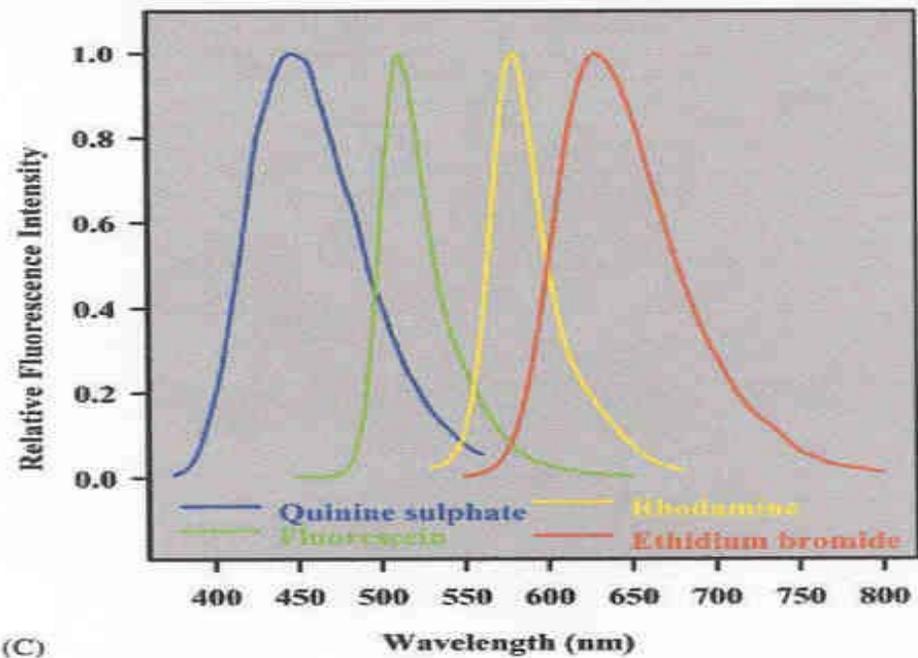
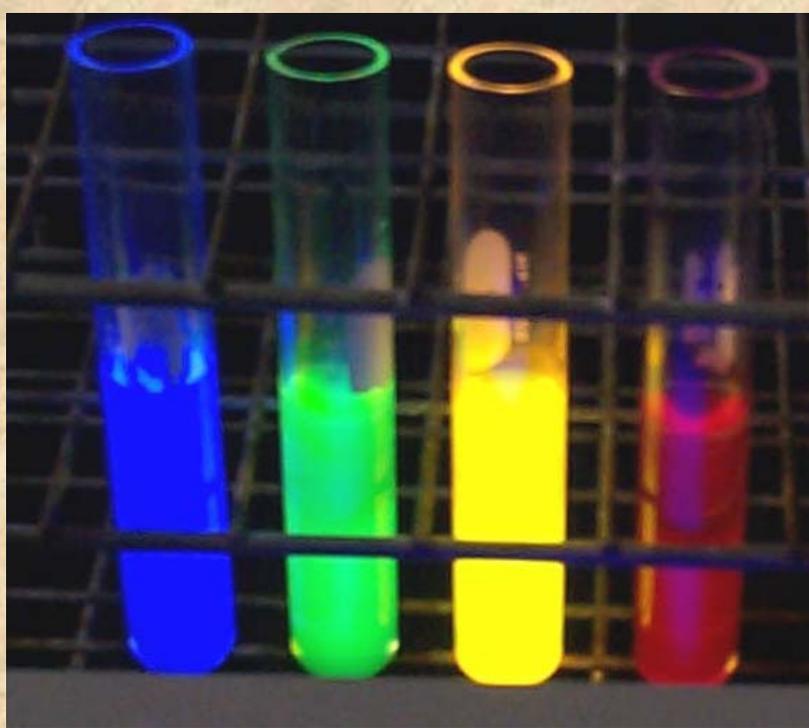


- (1) select fixed emission wavelength
- (2) Select an excitation wavelength and measure the intensity at the detector
- (3) Repeat (2) by scanning across a wide range of excitation wavelengths

# Examples of Fluorescence Emission Spectra

Remember: In a typical emission spectrum, the excitation wavelength is fixed and the fluorescence intensity versus wavelength is obtained





Early examination of a large number of **emission spectra** resulted in the formulation of certain general rules:

*1) In a pure substance existing in solution in a unique form, the fluorescence spectrum is invariant, remaining the same independent of the excitation wavelength*

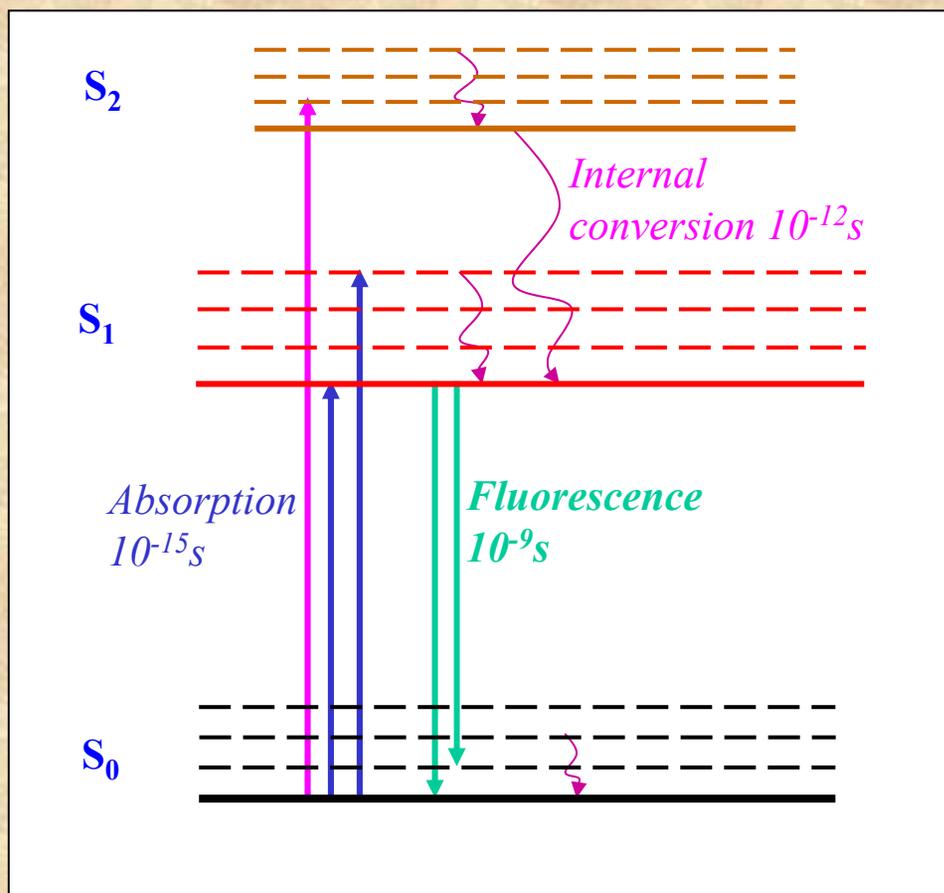
*2) The fluorescence spectrum lies at longer wavelengths than the absorption (Stokes' shift)*

*3) The fluorescence spectrum is, to a good approximation, a mirror image of the absorption band of least frequency*

These general observations follow from consideration of the Jablonski diagram shown earlier

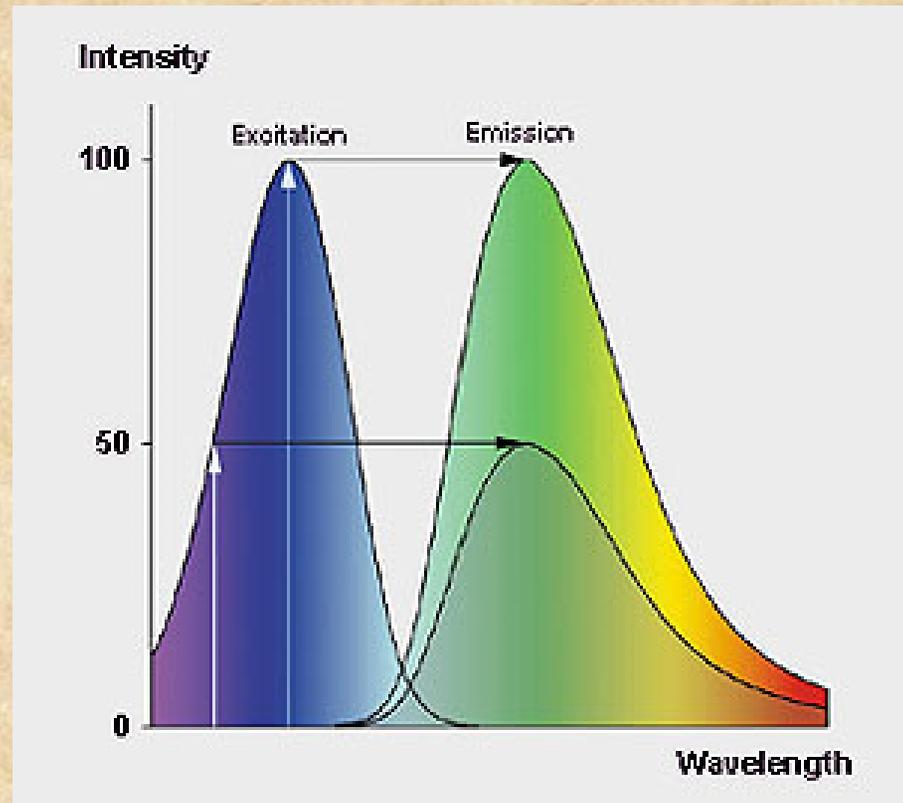
1) In a pure substance existing in solution in a unique form, the fluorescence spectrum is invariant, remaining the same independent of the excitation wavelength

Although the fluorophore may be excited into different singlet state energy levels (e.g.,  $S_1$ ,  $S_2$ , etc) rapid thermalization invariably occurs and emission takes place from the lowest vibrational level of the first excited electronic state ( $S_1$ ). This fact accounts for the independence of the emission spectrum from the excitation wavelength.



1) *In a pure substance existing in solution in a unique form, the fluorescence spectrum is invariant, remaining the same independent of the excitation wavelength*

## Schematic illustration



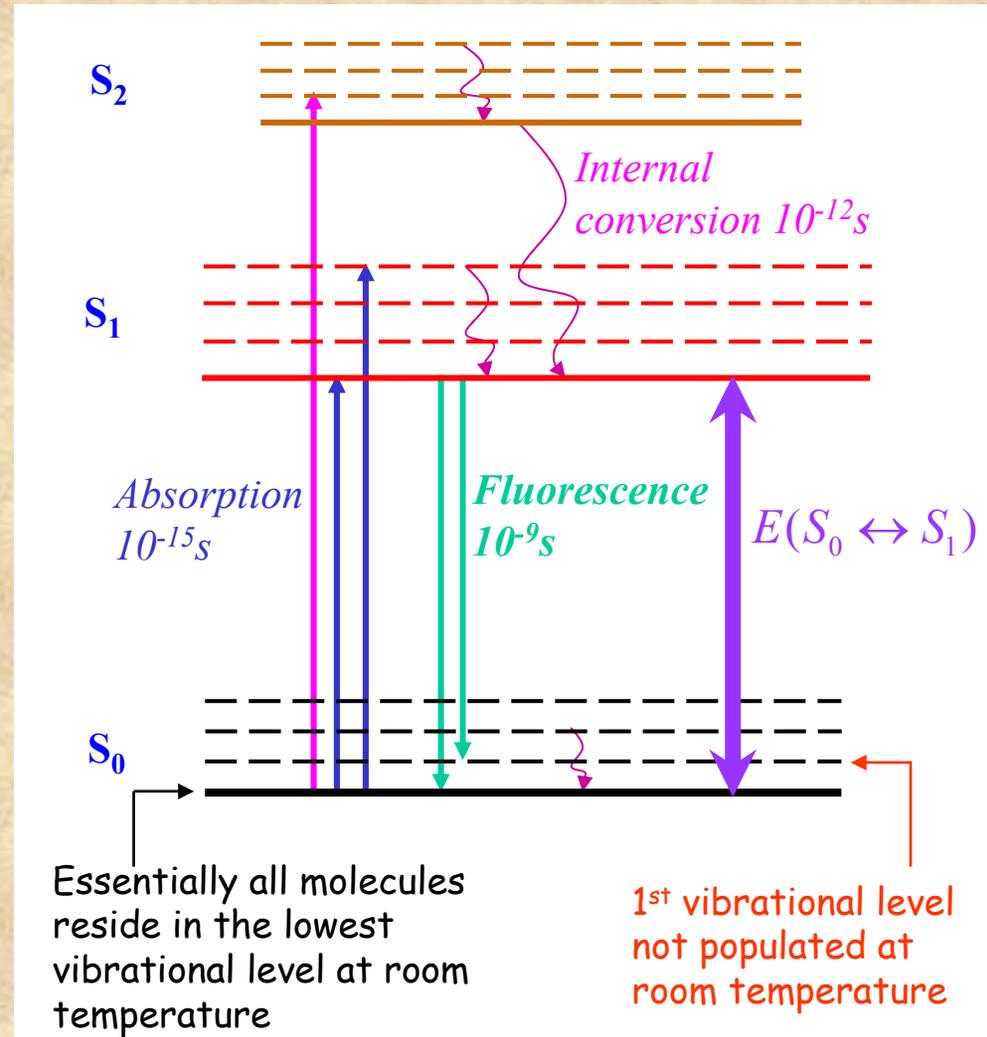
A fluorescence molecule is excited with two different wavelengths. The emission spectrum is measured for both cases. Note that the shape of the emission spectra is identical, reflecting the emission from the same state (vibrational ground state of  $S_1$ ). Note that the shape is the same, but the amplitude differs. The amplitude is determined by the intensity of radiation and the excitation efficiency, which is a function of the excitation wavelength

## 2) The fluorescence spectrum lies at longer wavelengths than the absorption (Stokes' shift)

(A) Fluorophores, at room temperature, are predominantly in the lowest vibrational level of the ground electronic state (as required from Boltzmann's distribution law). Thus the energy for absorption has to  $\geq E(S_0 \leftrightarrow S_1)$

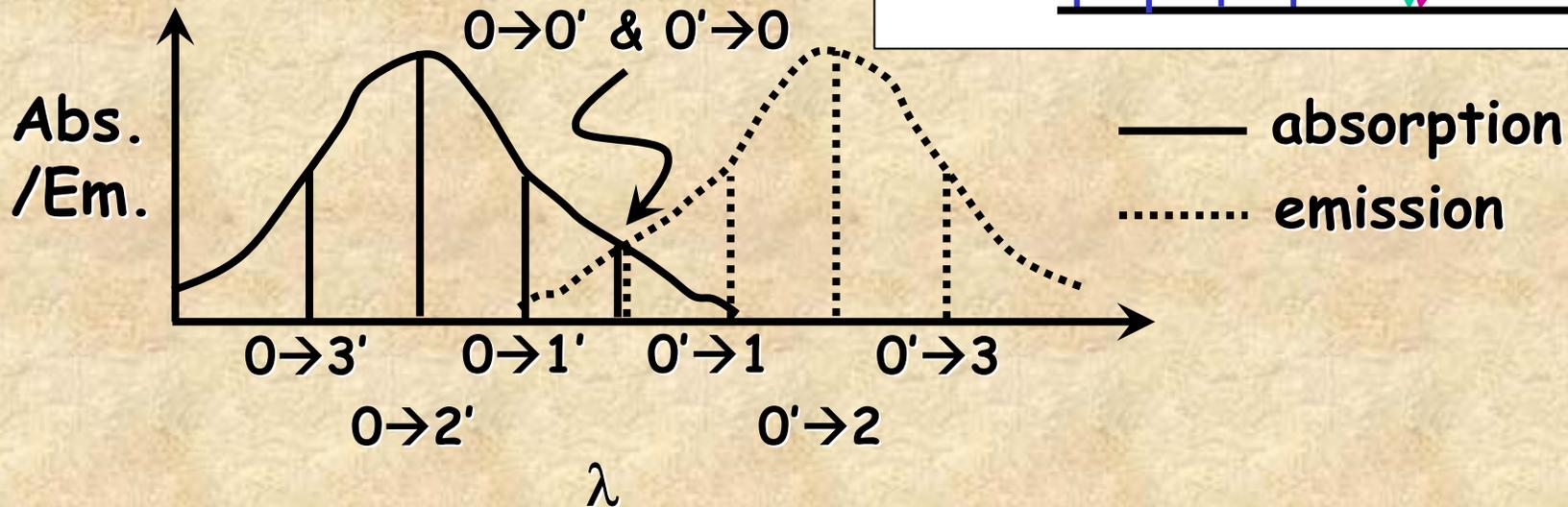
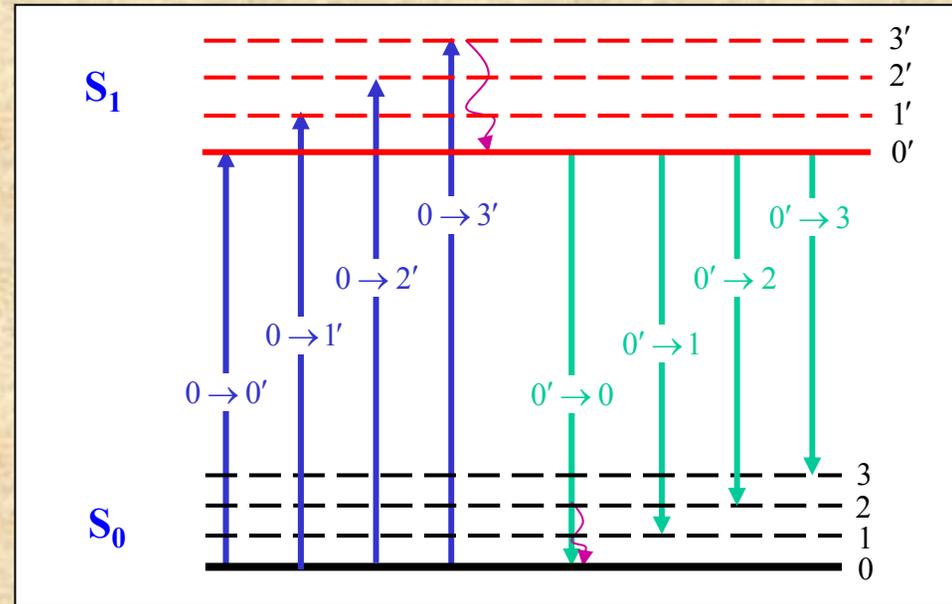
(B) Fluorescence emission occurs predominantly from the lowest vibrational level of the first excited electronic state ( $S_1$ ), and may reach any vibrational level of the electronic ground state ( $S_0$ ). Thus the energy of the emitted photon is  $\leq E(S_0 \leftrightarrow S_1)$

(A) together with (B) account for the Stokes shift.



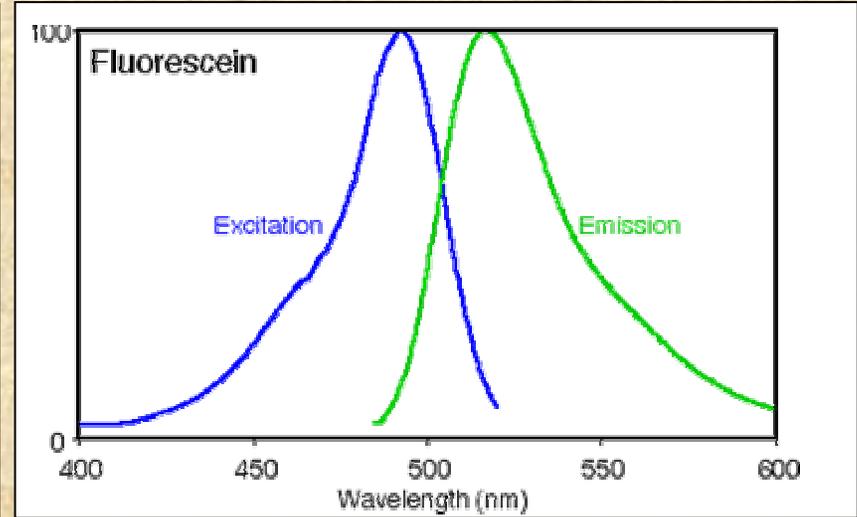
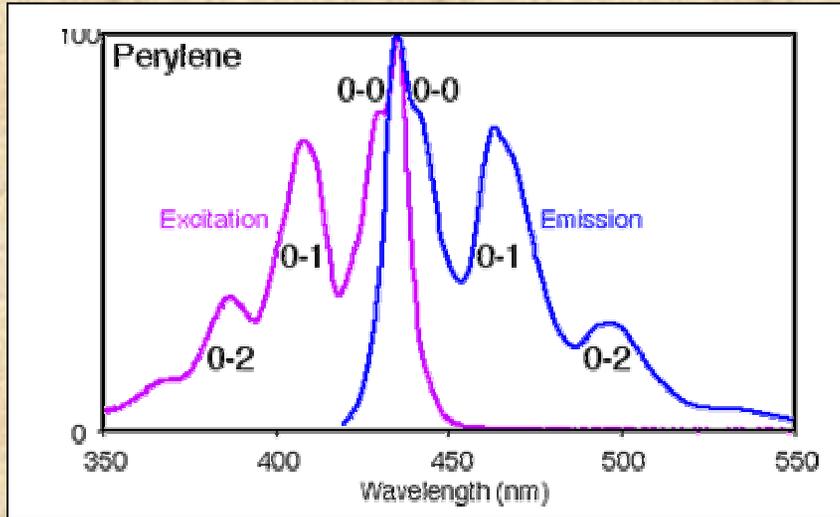
### 3) The fluorescence spectrum is, to a good approximation, a mirror image of the absorption band of least frequency

Finally, the fact that the spacings of the energy levels in the vibrational manifolds of the ground state and first excited electronic states are usually similar accounts for the fact that the emission and absorption spectra (plotted in energy units such as  $1/\lambda$ ) are approximately mirror images

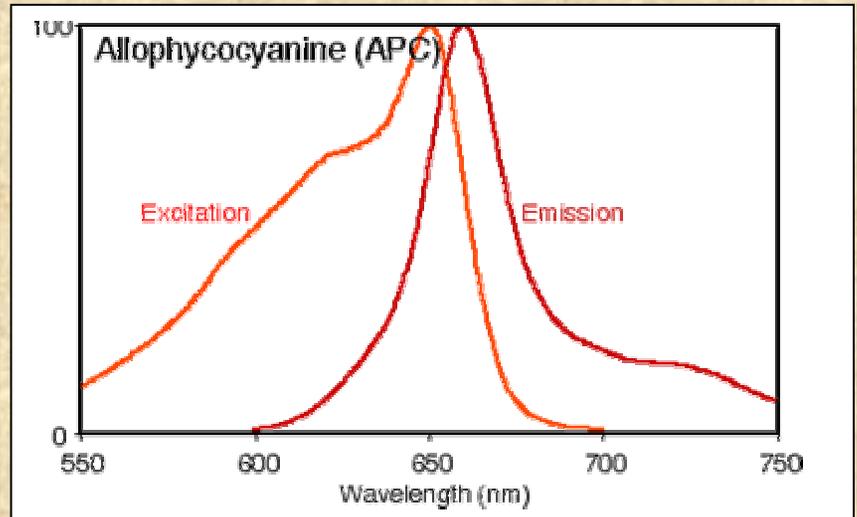
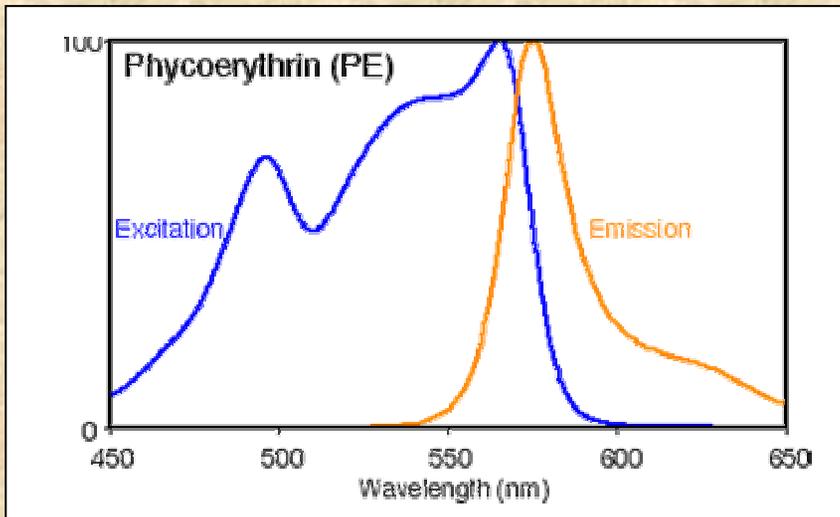


- note that in the above graph, the  $0 \rightarrow 0'$  and  $0' \rightarrow 0$  transitions occur at the same wavelength
- general solvents effects in water can change this...

# We do see mirror images...



# but not always...



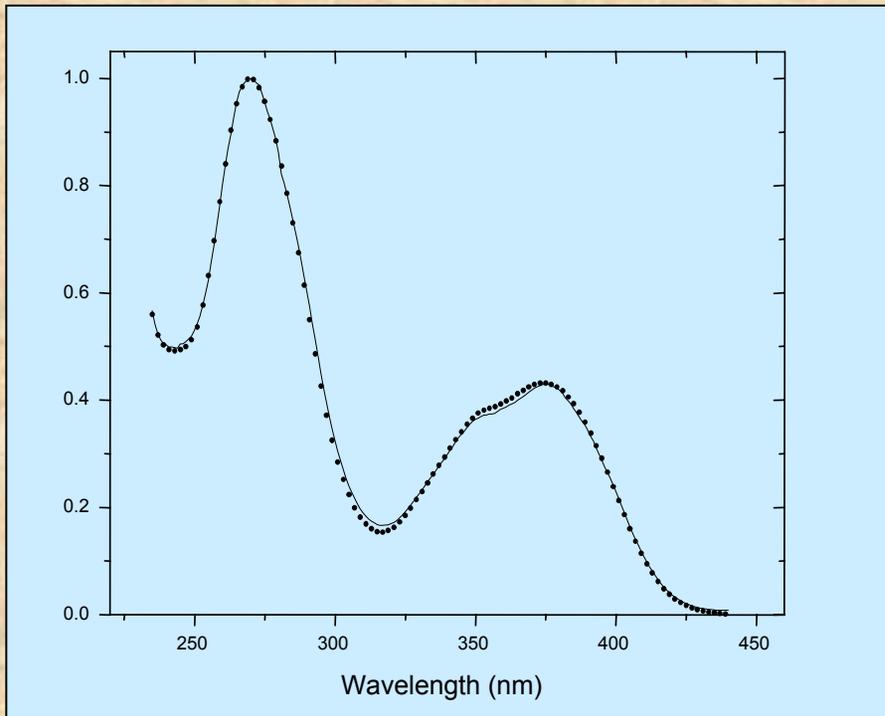
# The Fluorescence Excitation Spectrum

The relative efficiencies to excite a fluorophore at different wavelengths is determined by the excitation spectrum.

Remember: The observed emission wavelength is fixed, while the excitation wavelength is varied systematically.

If the system is "well-behaved", i.e., if the three general rules outlined above hold, one would expect that **the excitation spectrum will match the absorption spectrum.**

Example: Overlay of Absorption Spectrum and Corrected Excitation Spectrum for ANS in ethanol



Note that excitation (as well as emission) spectra require correction for instrumental factors.

We will cover corrections later in more detail.