

SibFU-4
February 2012

QUANTITATIVE SPECTROSCOPY

Much unfinished business

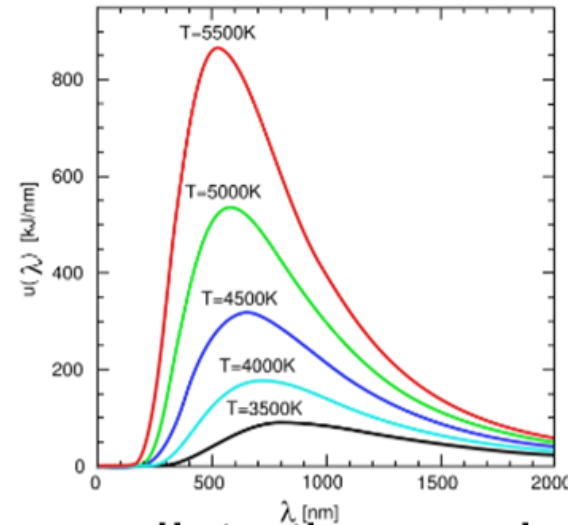
Lecture 4. **Quantitative Spectroscopy**

- Bioluminescence is a chemiluminescent oxygen oxidation of a reduced organic molecule, luciferin, catalyzed by an enzyme, luciferase.
- The emission spectrum corresponds to fluorescence from the excited singlet state S_1 of that product.
- It is essential for solving the chemical mechanism, to establish the **stoichiometry** i.e., how many molecules react and how many correspondingly produced.

Bioluminescence Basics

1. Measuring photons.
2. Calibrations of spectrographs and measurement corrections.
3. Luminol chemiluminescence standard
4. Quantum yields and spectral correction
5. Origin of spectral perturbations.

Black-Body Radiation



Wien's Displacement Law predicts the maximum for a “perfect” **Black Body**.

$$\lambda_{\max} = 3 \times 10^6 / T \text{ nm-K}$$

The Sun is an approximate Black Body, 5700K

$\lambda_{\max} = 530 \text{ nm}$ (is also our visual sensitivity maximum)

Planck's Radiation Law

Wien (1895) fitted the black-body spectrum by classical theory (frequency, $\nu = c/\lambda$):

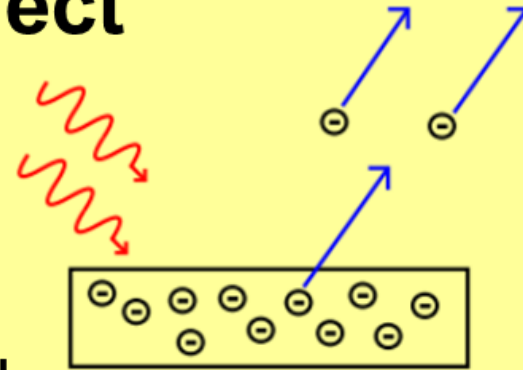
$$I(\nu)d\nu = B\nu^3 \exp(-A\nu)$$

This **fails** for $\nu > \nu_{\max}$ “The Ultraviolet Catastrophe”

Planck (1900) postulated that the radiating atoms could only emit “packets” of energy (quanta); then:

$$I(\nu)d\nu = 2h\nu^3/[c^2 (\exp(h\nu/kT) - 1)]$$

The Photoelectric Effect



Einstein (1905) proposed that light is composed of **particles** to explain the paradox that in the photoelectric effect:

electron energy \propto frequency (ν)
NOT intensity

Einstein's particles \equiv Planck's quanta

E = h. ν h = Planck's constant

Law of Photochemistry

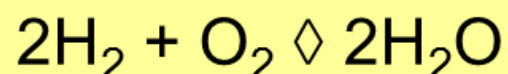
Grothus and Draper (1818)

- “Only **absorbed** light produces chemical change”
- Number of product molecules
 \propto number of photons absorbed
(the **quantum yield**)
- *Contrariwise for bioluminescence*

The number of photons emitted \propto number of molecules changed (the **quantum yield**)

High School Chemistry

Although by pencil and paper a balanced chemical equation can be written

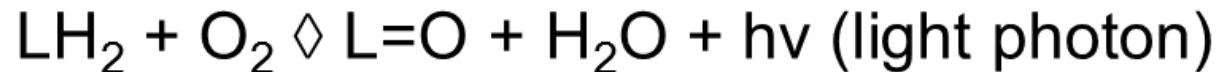


the **material** balance must be established experimentally, i.e., by “weighing” the reactants and products:

4.0 g hydrogen + 32.0 g oxygen, yields 36.0 g water

Photon Chemistry

We write a bioluminescence reaction (or chemiluminescence) like this



where the luciferin, LH_2 , reaction with oxygen is catalyzed by luciferase to produce the oxidized product L=O and light.

The symbol $h\nu$ is often used to represent a photon from the **Planck** equation for the energy of a photon:

$$\mathbf{E = h\nu}$$

PHOTON MOLES

It is seen that one of the products ($h\nu$) is a light photon so the **photon stoichiometry** must also be measured.

In establishing chemical stoichiometry the number of **moles** of each participant must be determined, not their mass in grams.

EINSTEINS

A “mole” of photons is called an “**einstein**” and is given by

$$N \cdot h\nu = N \cdot h \cdot c / \lambda$$

where λ is the wavelength of the light (e.g., blue light is around 450 nm), and N , Avogadro’s number, is 6.025×10^{23} molecules/mole.

Weighing Photons

1 **einstein** = $(2.86 \times 10^4 \text{ kcal-nm}) / (450 \text{ nm})$
= 64 kcal. for a “mole” of **blue** light.

The value is given in energy units since the fundamental determination of the photon is through a heat measurement. The instrument used is called a **thermopile**.

Notice also that this is a lot of energy to be encountered in a biochemical process, the reason that these bioluminescent reactions are oxygen oxidations.

Absolute Calibration

The standard photon is determined by reference to the Planck radiation law for the emission flux, F , from a perfect black body at a known absolute temperature, T :

$F = \sigma \cdot T^4$ where σ is a constant.

Black body standards are very complicated and are constructed and maintained usually at **National Standard Laboratories**.

Visual Sensitivity

The distribution of photon flux over the black-body spectrum is:

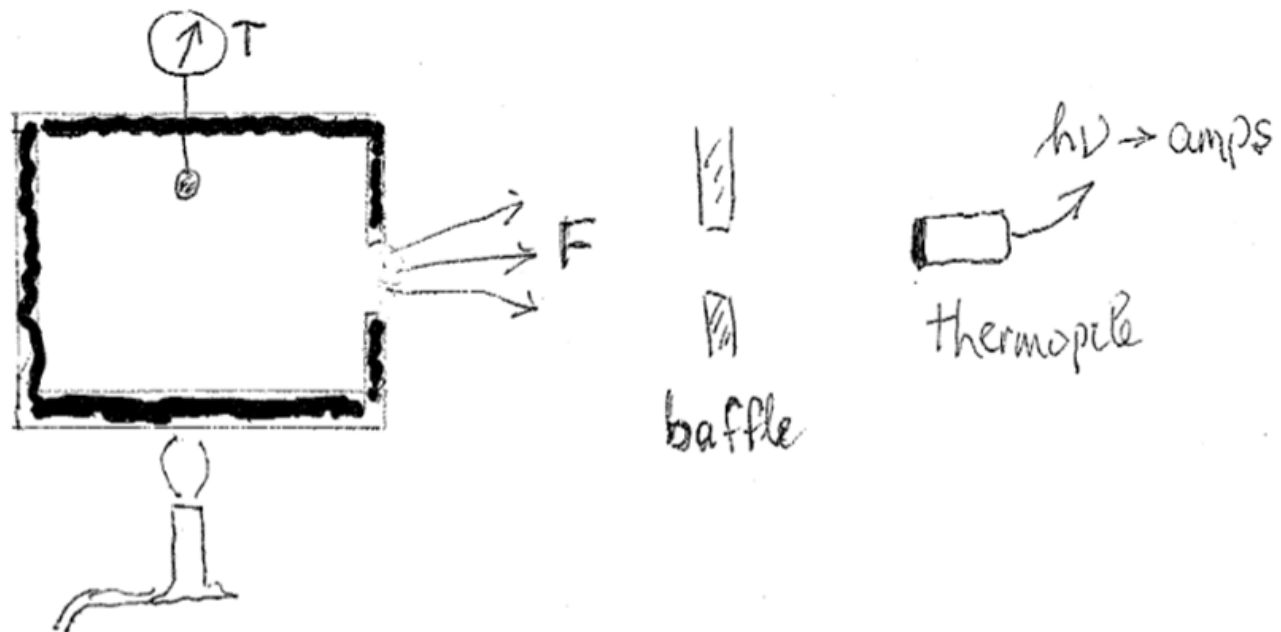
$$I(\lambda) = A \lambda^{-5} [\exp(B / \lambda T) - 1]^{-1}$$

where A, B = constants.

For an incandescent lamp, $T = 3000 \text{ K}$ gives $\lambda_{\text{max}} \sim 1000 \text{ nm}$. It is no coincidence for example, that the 530 nm spectral maximum for sunlight is similar to our visual sensitivity maximum.

CALIBRATION of a PHOTODETECTOR

Black-body Primary Calibration

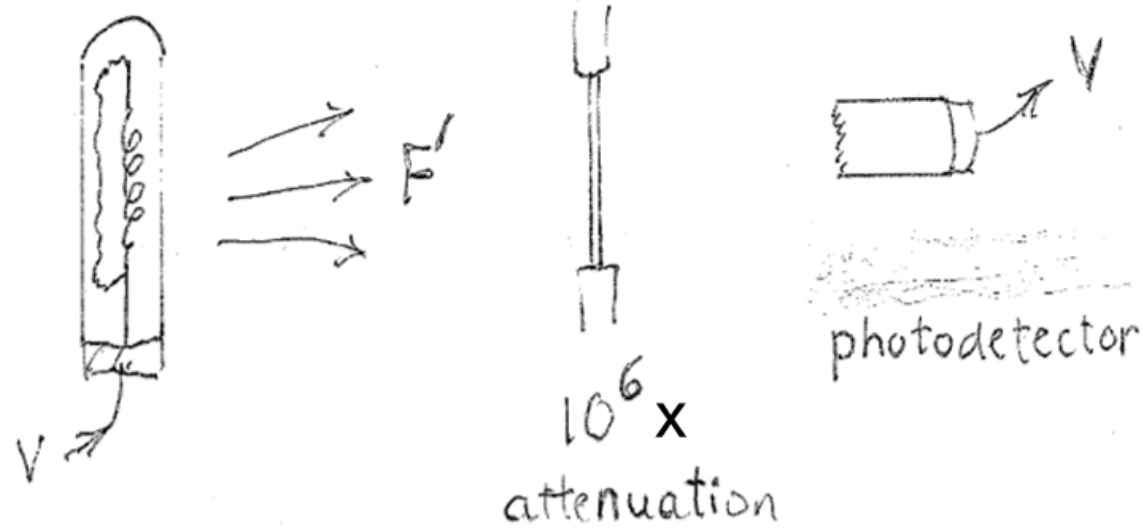


Secondary Standards

A secondary standard is available consisting of a calibrated incandescent lamp from which the absolute calibration of light detecting devices such as light meters, **luminometers**, etc., can be determined. Great care must be taken to accurately reduce the intensities from these lamps by several orders of magnitude, in order to calibrate the highly sensitive luminometers used in bioluminescence studies.

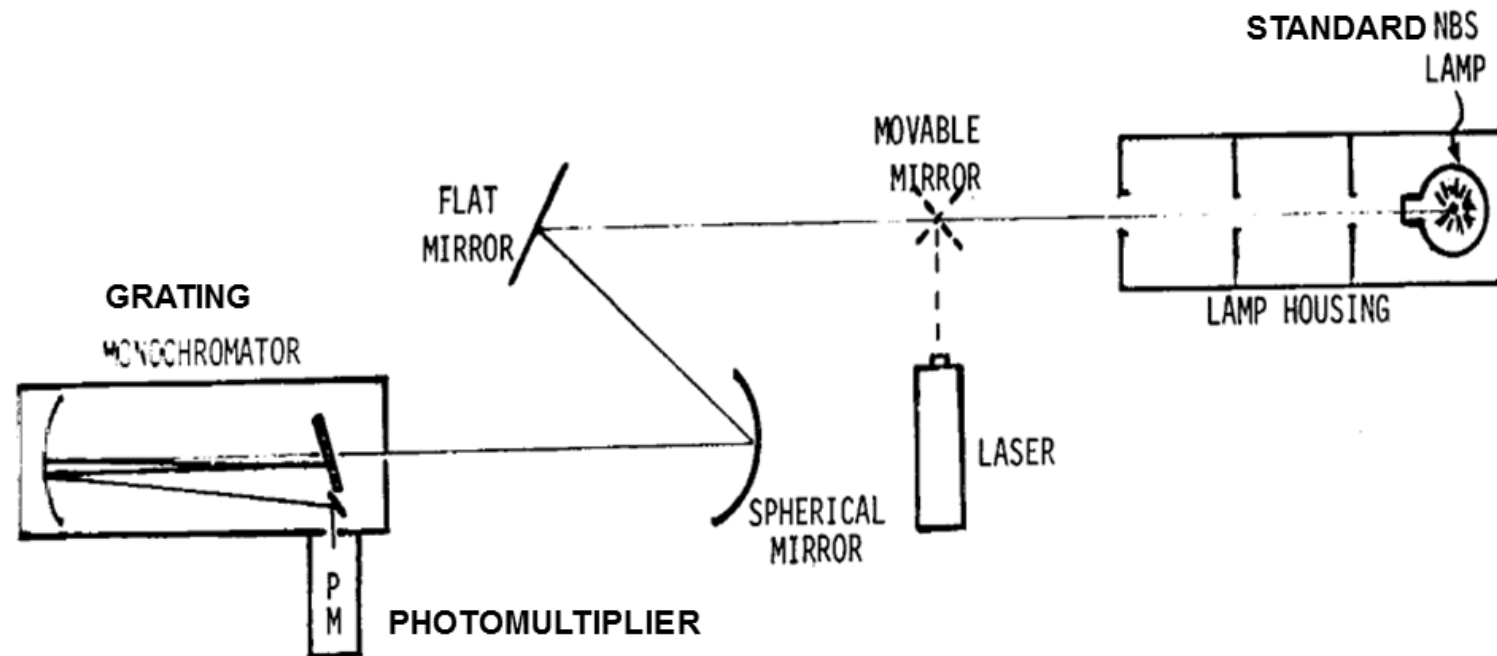
Incandescent lamp

Secondary calibration



Grating Spectrometer-Phototube

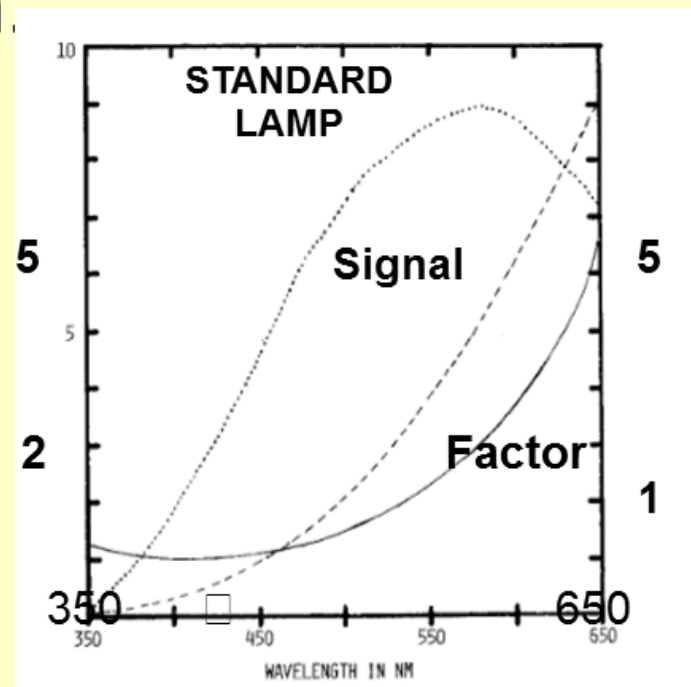
Calibration of absolute response



Correction Factors

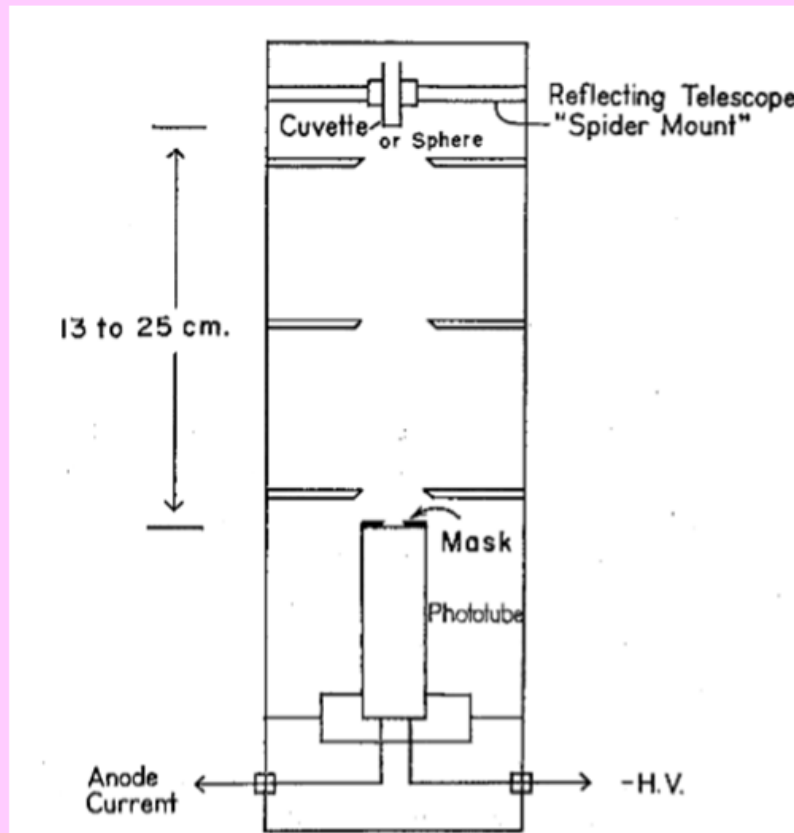
The transmission of the grating and the **Photoelectric Effect** efficiency of the photomultiplier, both depend on wavelength.

Correction
factor



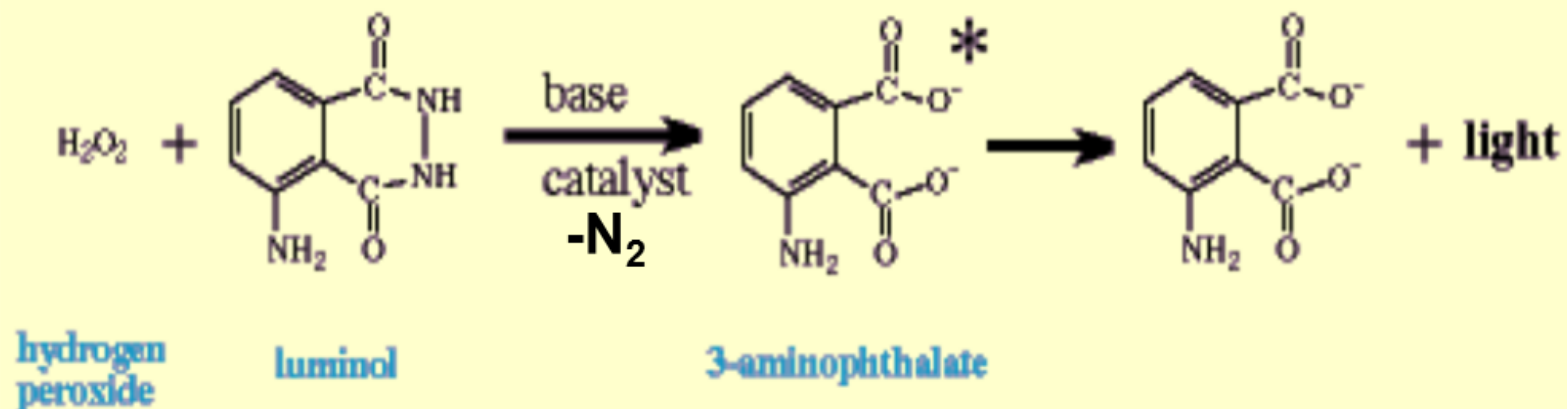
Basic Luminometer

↓ Inject reaction



Luminol Chemiluminescence

The chemiluminescence of luminol is a **calibration** method for luminometers **traceable** to the black body. The CL spectrum is in a convenient range and the optical geometry and phototube spectral properties are folded in to the measurement.



Luminol Protocol

1. Aqueous reaction, pH=11.6, H₂O₂, hemoglobin catalysis, maximum $\lambda_m = 420$ nm

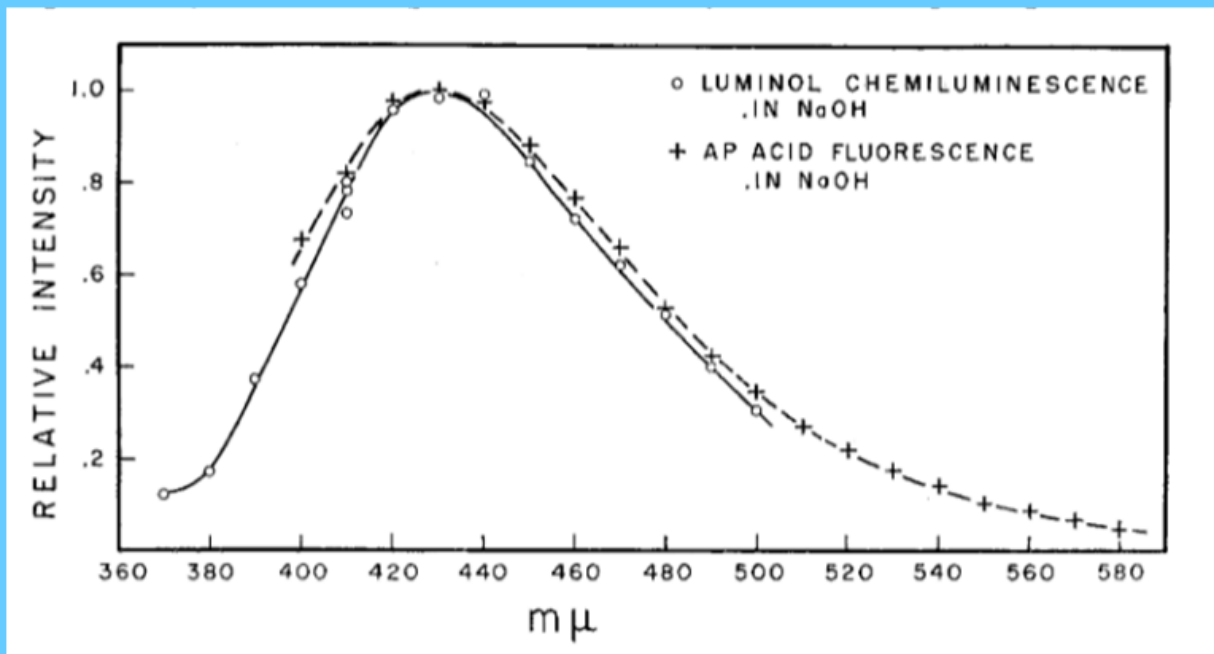
2. DMSO, t-butoxide, $\lambda_m = 480$ nm

For both reactions the **Qc** = 0.0124
±0.001
einsteins/mole of luminol

Radioactive light sources are available: ³H (liquid) and ⁶³Ni (solid).

Luminol Spectrum

Luminol chemiluminescence emission is in a convenient range for calibration of a luminometer.



How to prove that a chemical reaction is the light path

Oxidation reactions are very exogenic and are prone to side reactions. Also the eye is very sensitive so we can be easily misled if the light path comes from a very minor chemical side reaction.

Assume the reaction goes with
 $Q_y = 100\%$ chemical yield.

Abbreviations

Fluorescence quantum yield

Q_f = photons emitted / photons absorbed

Bioluminescence quantum yield

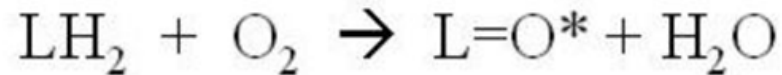
Q_b = photons emitted / molecules reacted

or, **einsteins/mole**

(Q_c = chemiluminescence quantum yield)

Q_y = chemical product yield

EVIDENCE



REQUIREMENTS

1. $Q_b > 0.01$
2. $\text{BL } \lambda_m \approx \text{FL } \lambda_m$
3. $Q_b < Q_f$

The reaction product L=O , is in its excited* (fluorescent) state from which the bioluminescence originates.

Fluorescence and Bioluminescence

- Fluorescence yield (Q_f) is always $< 100\%$
- Quantum yield of bioluminescence

$$Q_b = Q_e \times Q_f$$

- Q_e is the yield of the **excited** state product, also $< 100\%$.

Practical Result

In a favorable case we might measure

1.0 mole $\text{LH}_2 + \text{O}_2 \rightarrow 0.75$ moles L=O
+ 0.4 einsteins
+ 0.25 moles other products

Qb = 0.4 (einsteins/ mole of LH_2)
= $Q_f \times Q_e \times Q_y$

and if we measure $Q_f = 0.7$ for L=O , then
together with spectral match, it can be concluded
that this equation is the light path, with **Qe = 0.76**.

Side Pathway

If we find only $Q_b = 0.13$, then there could be a minor pathway for luminescence.

$$(Q_f, 0.7) \times (Q_e, 0.76) \times (Q_y, 0.25)$$

$$= 0.13$$

Bioluminescence Quantum Yields

The quantum yield is defined as

$Q_b = \text{einsteins /mole luciferin reacted}$

firefly $Q_b = 0.41 - 0.49 \pm 17\%$

Cypridina $0.30 \pm 7\%$

Oplophorus 0.34

Aequorin 0.16

Mnemiopsin 0.12

bacteria (aldehyde) 0.05-0.13

Pholasin 0.09

Renilla 0.07-0.15

Spectral Match

- The fluorescence **spectrum** of authentic L=O must be close to the same as the BL and in addition, the $Q_b < Q_f$.
- If the fluorescence and bioluminescence spectra do not **match**, this would need to be explained.

Luminol spectra match product fluorescence

Reaction

	chemiluminescence max (nm)	fluorescence of 3-AP
aqueous	431	431
DMSO	502	495
DMF	499	497
AN	500	500
THF	496	500

Fluorescence Calibration

The fluorescence spectral distribution and quantum yield of quinine sulfate are accurately known and are used as calibration standards.

$$Q_f = 0.55$$

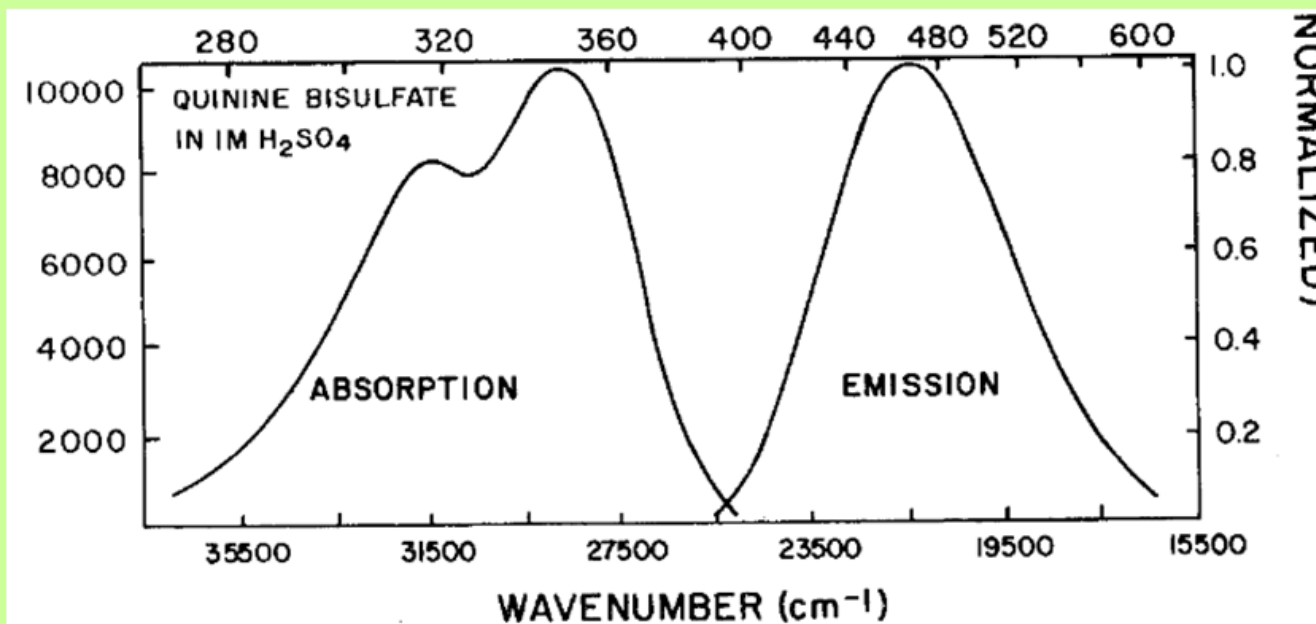
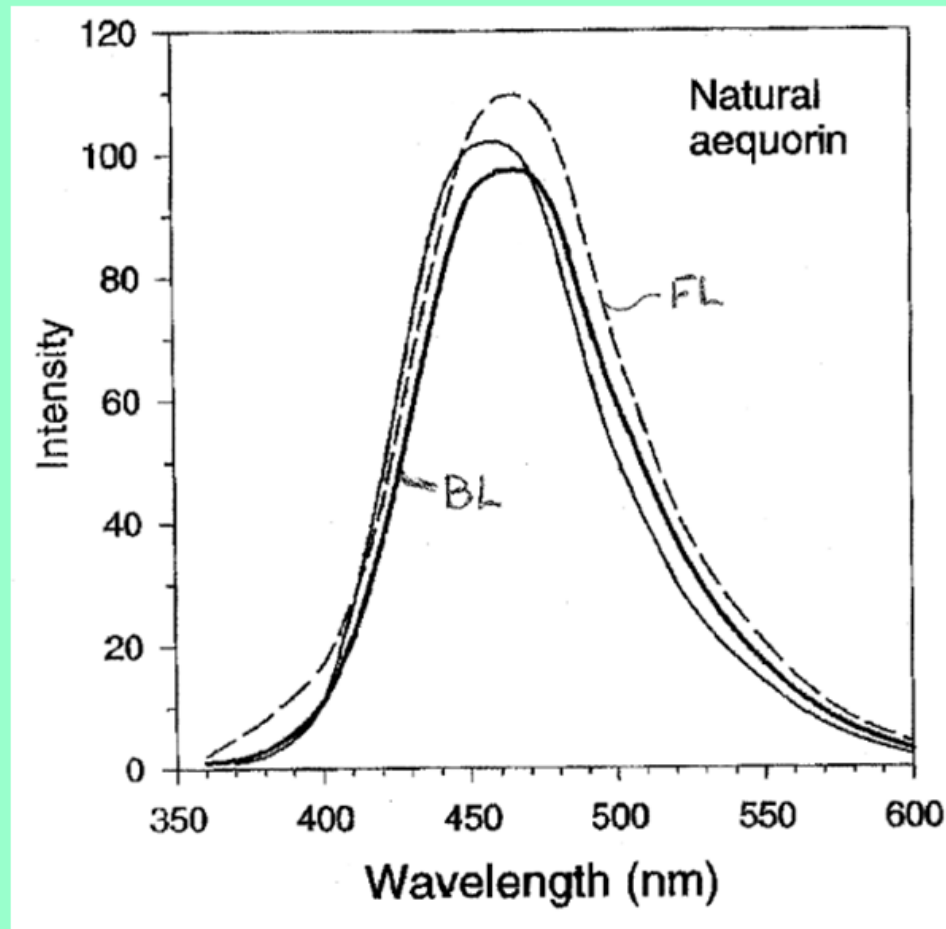
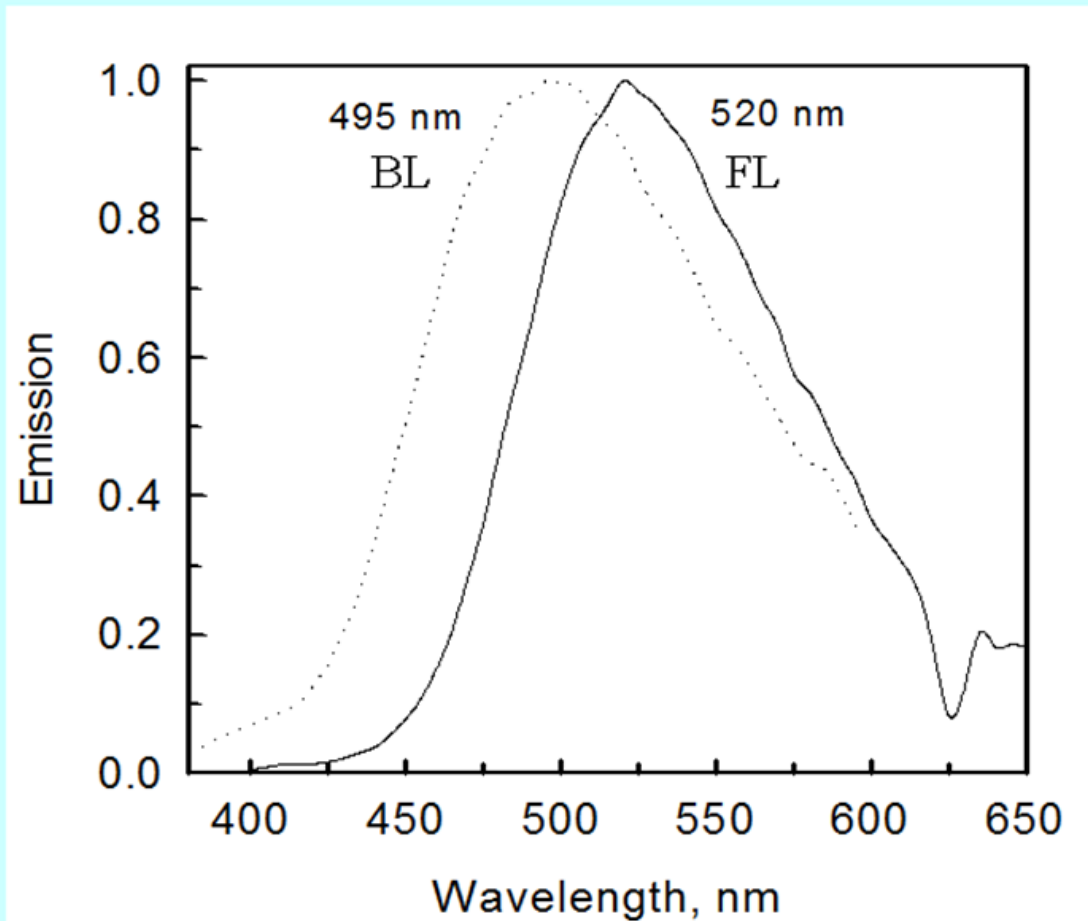


Figure 1.2 Absorption and fluorescence emission spectra of perylene and quinine. Emiss

Aequorin Bioluminescence = Product Fluorescence



Obelin Bioluminescence does not = Fluorescence



Spectral Maxima

<u>BL</u>	<u>(nm)</u>	<u>FL</u>
aequorin	465	465
OG-obelin ¹	475 -	
OL-obelin	485	510
OG-obelin ²	495	520

¹Pacific ²White Sea

Spectral Variations

Hypothesis

Differences among photoprotein bioluminescence spectra and of obelin bioluminescence and product fluorescence, arise from various protein binding site residue interactions with the emitting species.

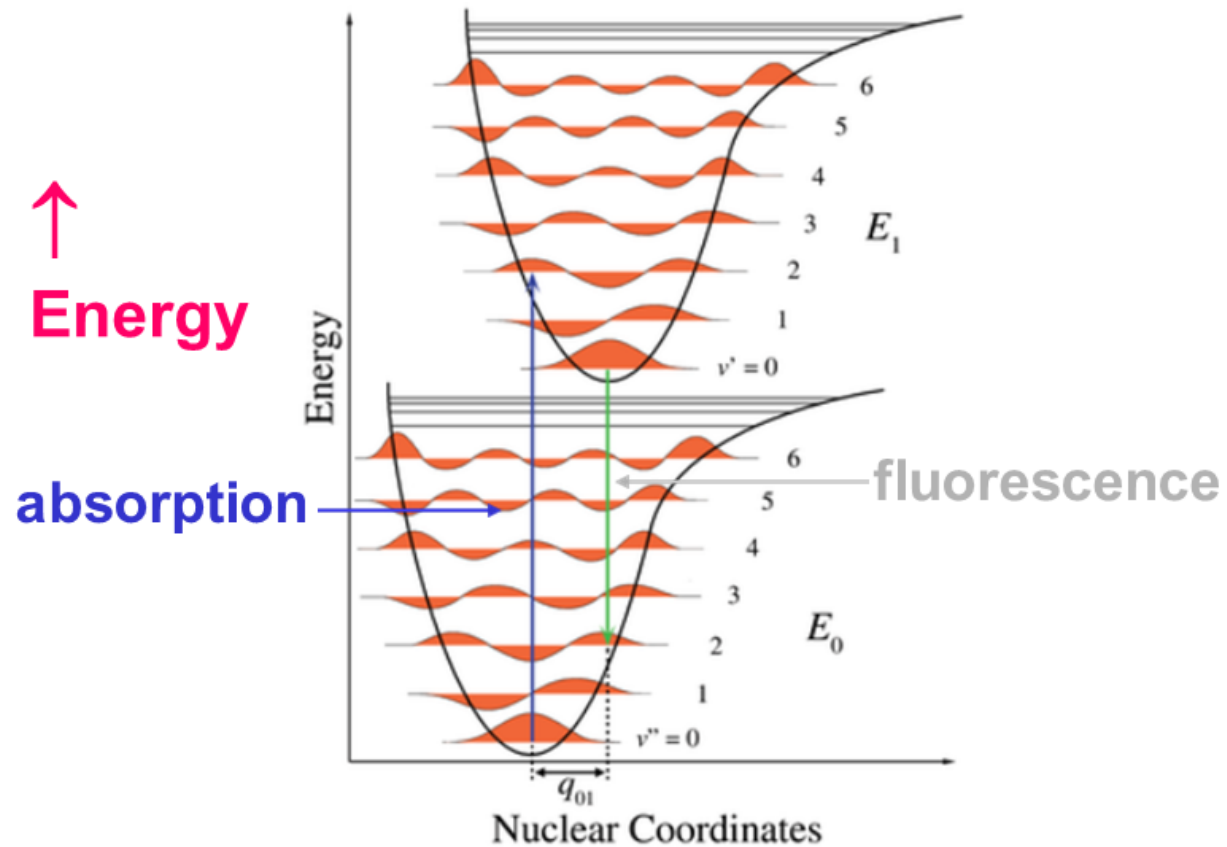
Fluorescence Spectroscopy

1. Franck-Condon principle
2. Inhomogeneous broadening
3. Absorption to fluorescence spectral shift
4. Gaussian band shape approximations
5. Grating spectrometer

Franck-Condon Principle

1. Electronic transitions are essentially **instantaneous** (10^{-16} s) on the time-scale of nuclear movement (10^{-12} s).
2. These are called **vertical** transitions, from the ground to the Franck-Condon State.
3. Transition probability \propto **overlap** of respective vibrational wave-functions.

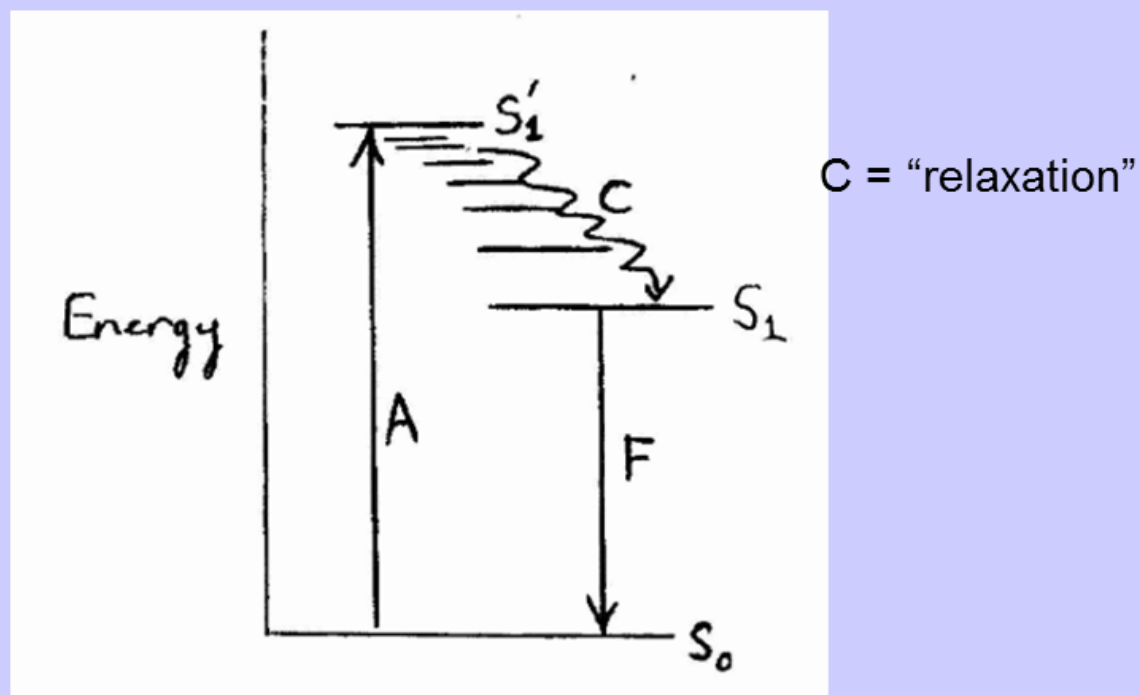
Vertical Transitions



Molecular dimensions →

Jablonski Diagram

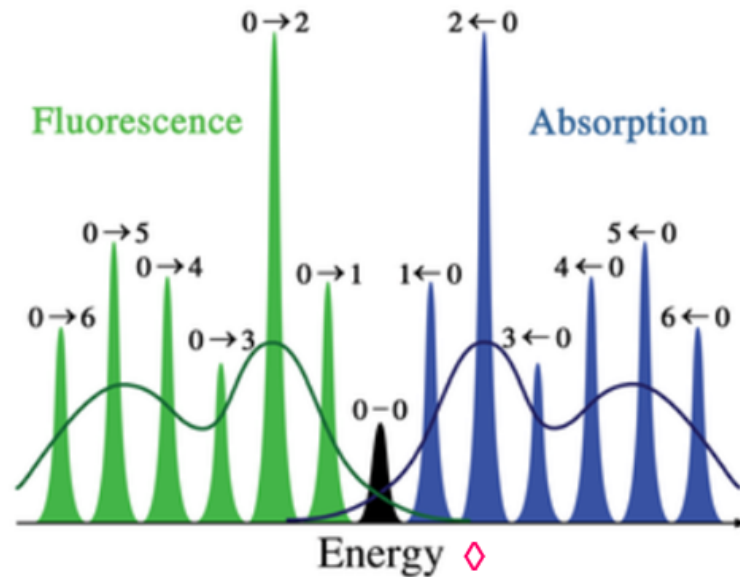
This is a convenient picture to rationalize vibrational and electronic properties.



Potential Wells

1. In the gas phase the potential well represents a quantum-mechanical oscillator with vibrational wave-functions.
2. In the condensed state the energy curves represent the distribution of environmental interactions. Spectra in the condensed state are therefore “inhomogeneously broadened”.

Inhomogeneous Broadening

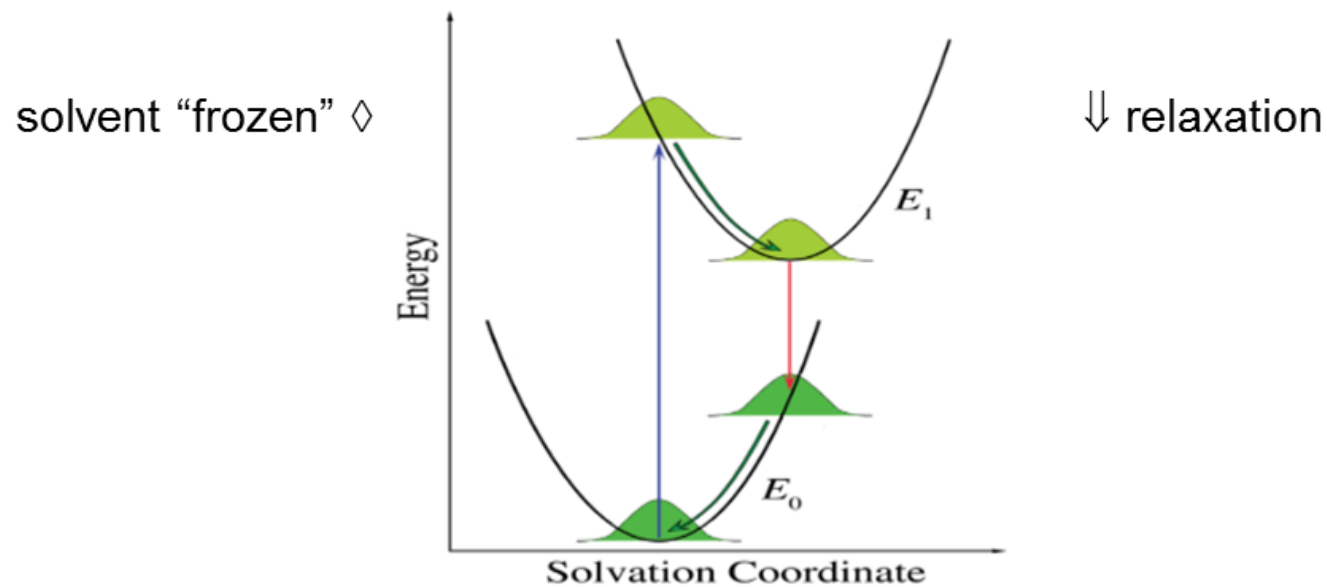


Gas phase \diamond narrow bands

Liquid or solid \diamond broad spectra

Solvation Energy Well

The Franck-Condon principle is extended to general interactions.



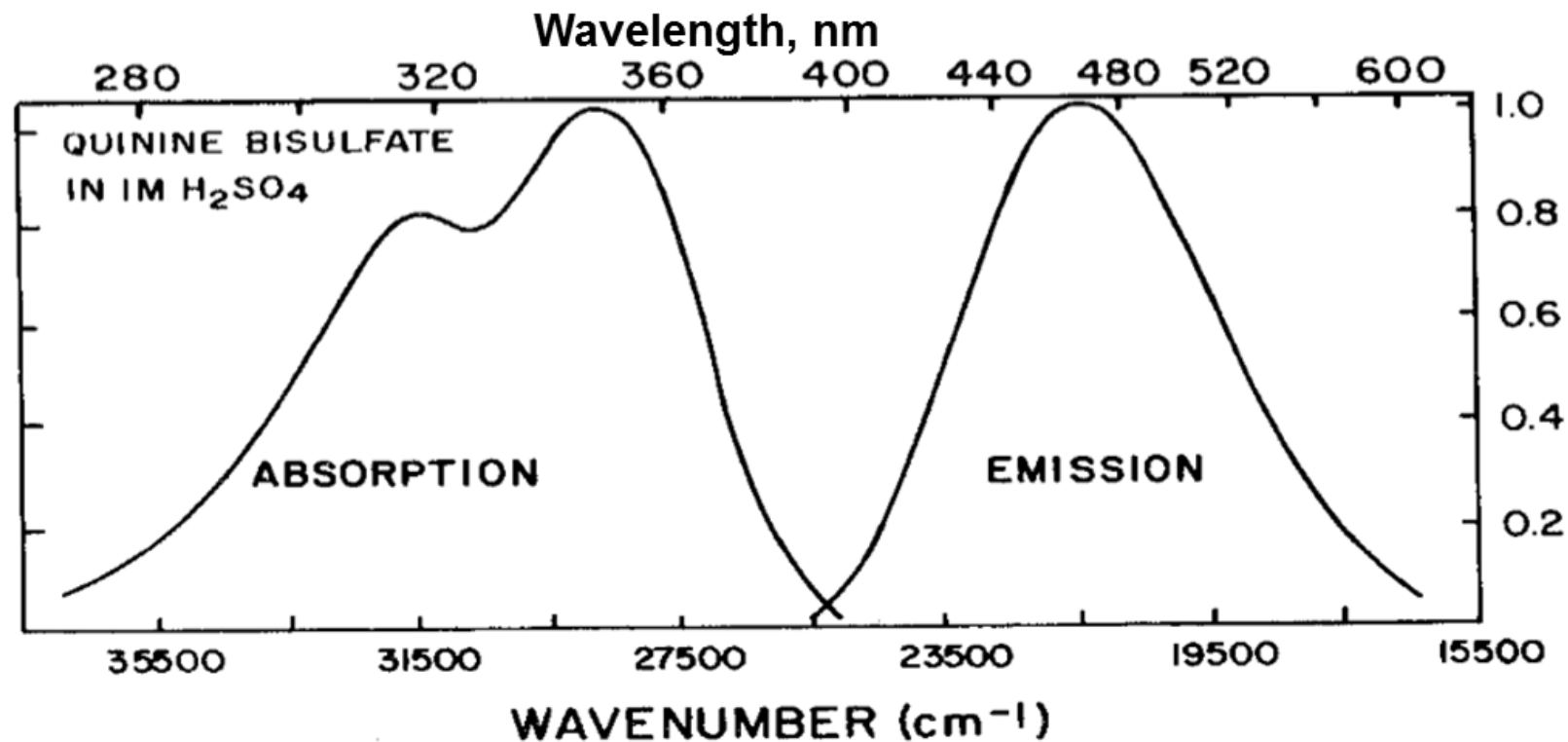
Stokes Shift

1. Electron or other interactions in the excited state **increase** the molecular dimensions so that the excited state well shifts.
2. This shifts fluorescence spectra to **lower** energy than absorption.
3. The energy distributions are **mirror images**.

Absorption -> Fluorescence Shift

299nm 101nm

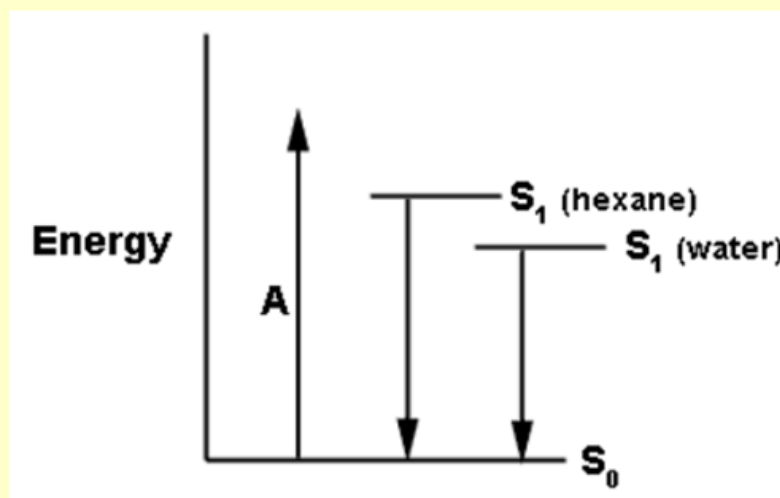
<- Stokes shift ->



Polarity

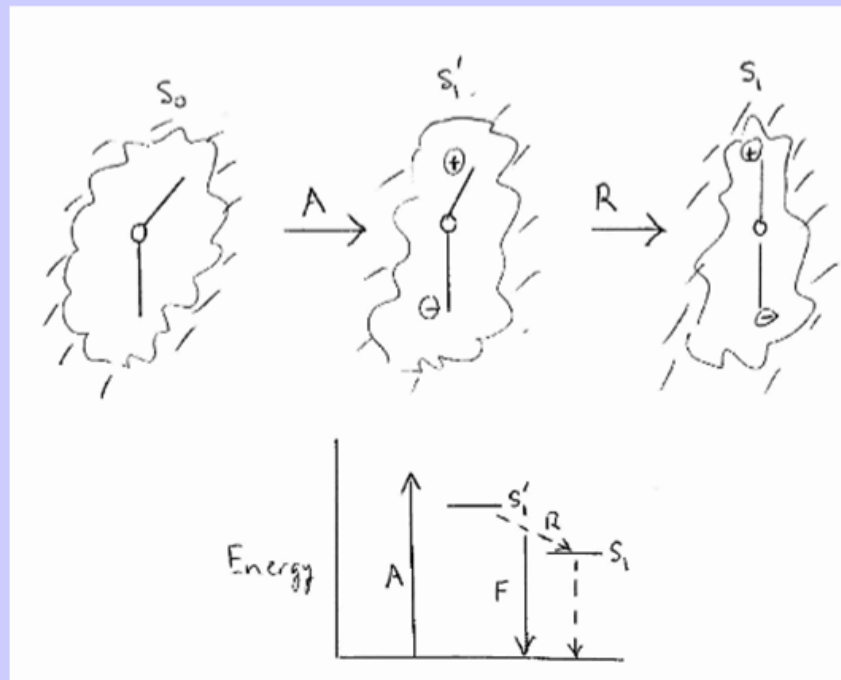
Environmental Perturbation

- Dipole moment of S_1 excited state usually $>$ S_0 ground state – due to electron redistribution.
- Fluorescence shifts to lower energy with increase in solvent polarity.



Rigidity

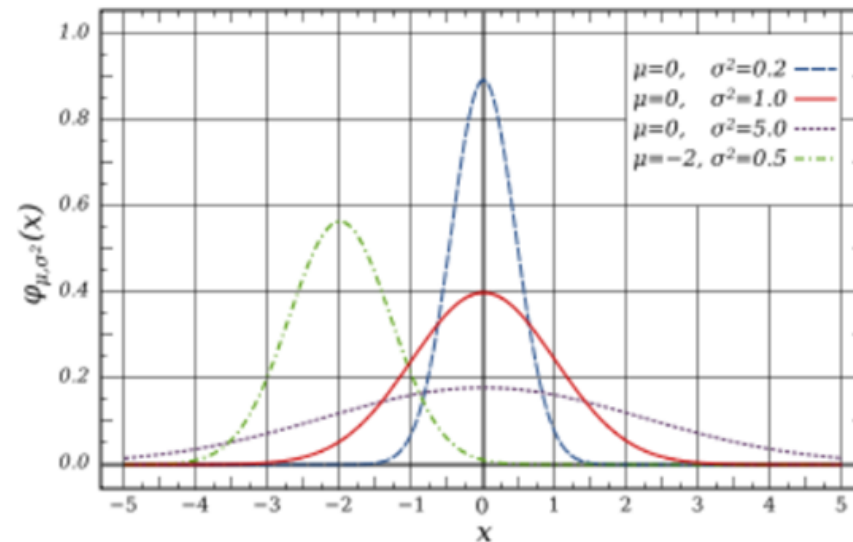
Higher solvent viscosity shifts fluorescence to higher energy, because radiation takes place before collisional relaxation.



Spectrum Analysis

1. The harmonic oscillator model for the potential well predicts the transition probabilities to be statistical, i.e., Normal or Gaussian distributions.

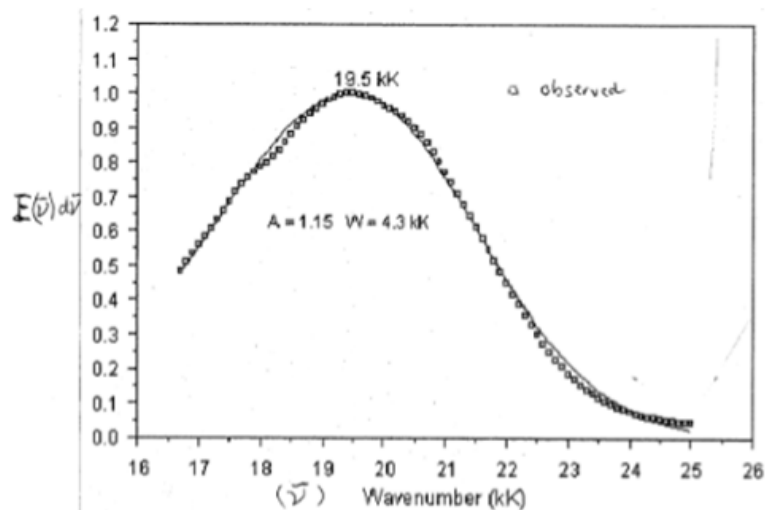
$$\frac{1}{\sigma\sqrt{2\pi}} \exp\left(-\frac{(x-\mu)^2}{2\sigma^2}\right)$$



Skewed Gaussians

In reality the well functions are anharmonic due to dissociation at the highest energies and are better approximated by the “Morse Function”.

Asymmetric Gaussian fits are required.



Literature

Seliger and McElroy (1965) *Light: Physical and Biological Action*, 417 pp.(Academic press, New York).

Seliger (1978) Excited states and absolute calibrations in bioluminescence. *Methods Enzymol.* LVII: 560-600 (M. DeLuca, Ed.).

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Lee and Seliger (1965). Absolute Spectral Sensitivity of Phototubes and the Application to the Measurement of the Absolute Quantum Yields of Chemiluminescence and Bioluminescence. *Photochem. Photobiol.* 4: 1015.

Seliger and Morton (1968). A Physical Approach to Bioluminescence. *In* *Photophysiology* (A.C.Giese, Ed.) Academic Press, NY.