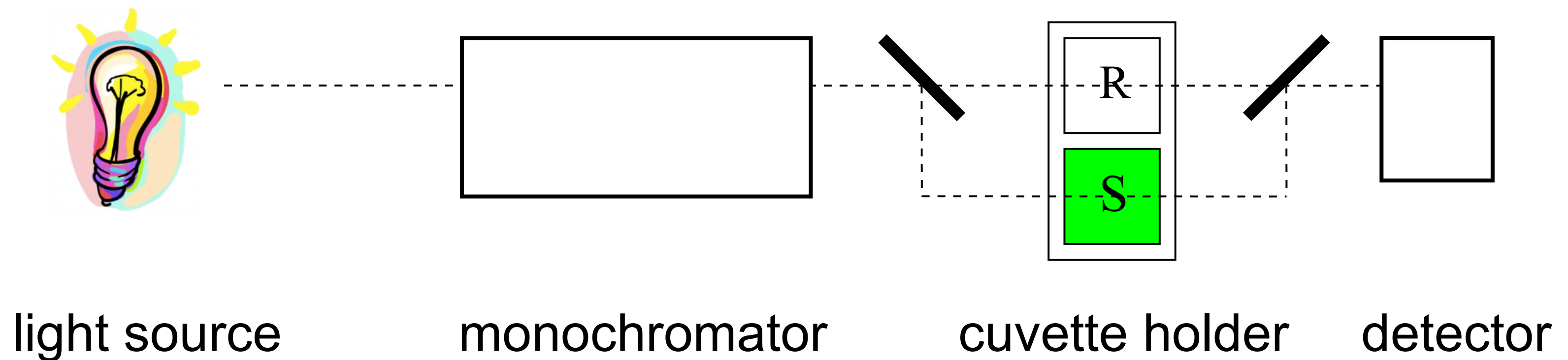
Spectroscopy

Absorption

Reported by:

1) percent transmission ($\%T_\lambda = 100 \times I_\lambda / I_{0,\lambda}$)
2) absorbance ($A_\lambda = \log \{I_{0,\lambda}/I_\lambda\} = \varepsilon_\lambda\, c\, l$)

Measured by: single or double beam spectrophotometer

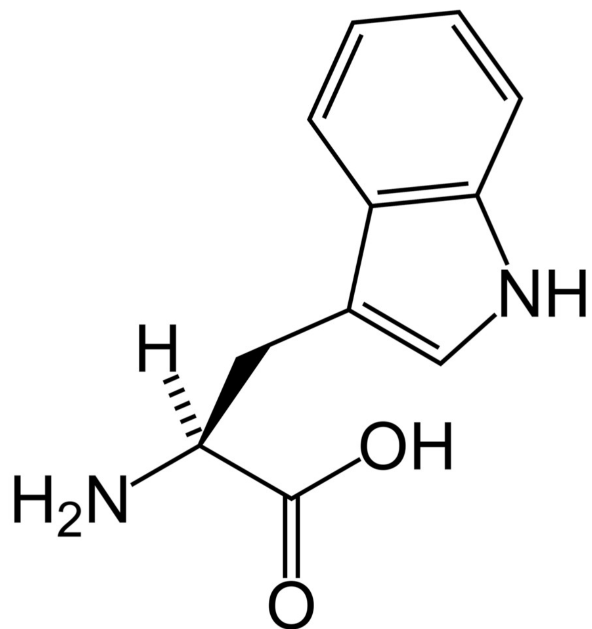


Significance of deviations from the Beer-Lambert Law:

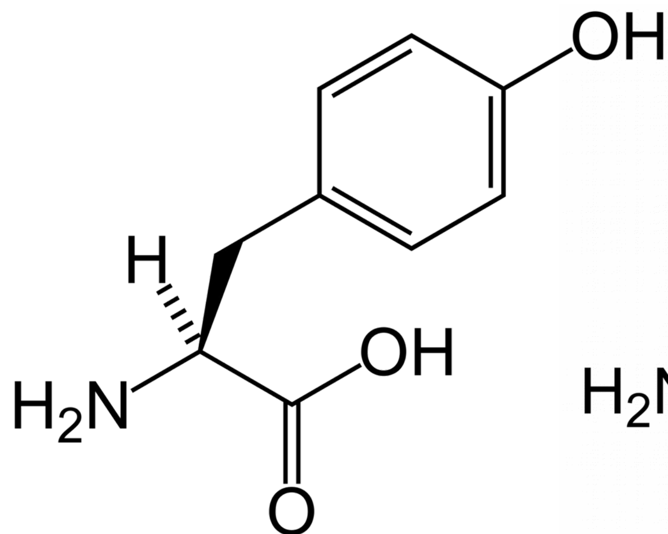
If nothing happens in a solution as a function of concentration of the component solutes, that is there are no concentration-dependent interactions, then the OD should increase linearly with increases in concentration of the components (this requires that all solute components have proportional increases in concentration or that different components have equivalent extinction coefficients.

Deviations mean interactions. The change in absorbance versus concentration will follow the thermodynamics of the system, reflecting cooperativity, positive or negative, or lack thereof.

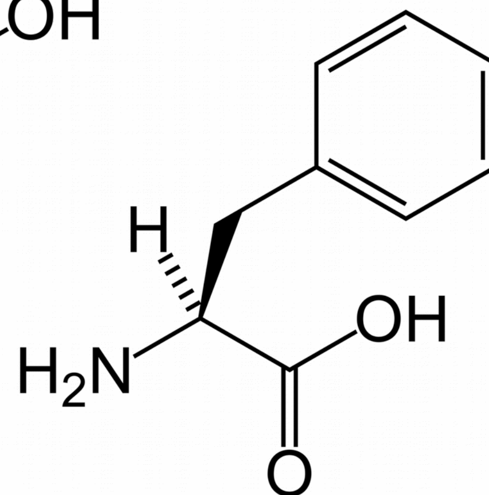
Aromatic Amino Acids



Trp (W)



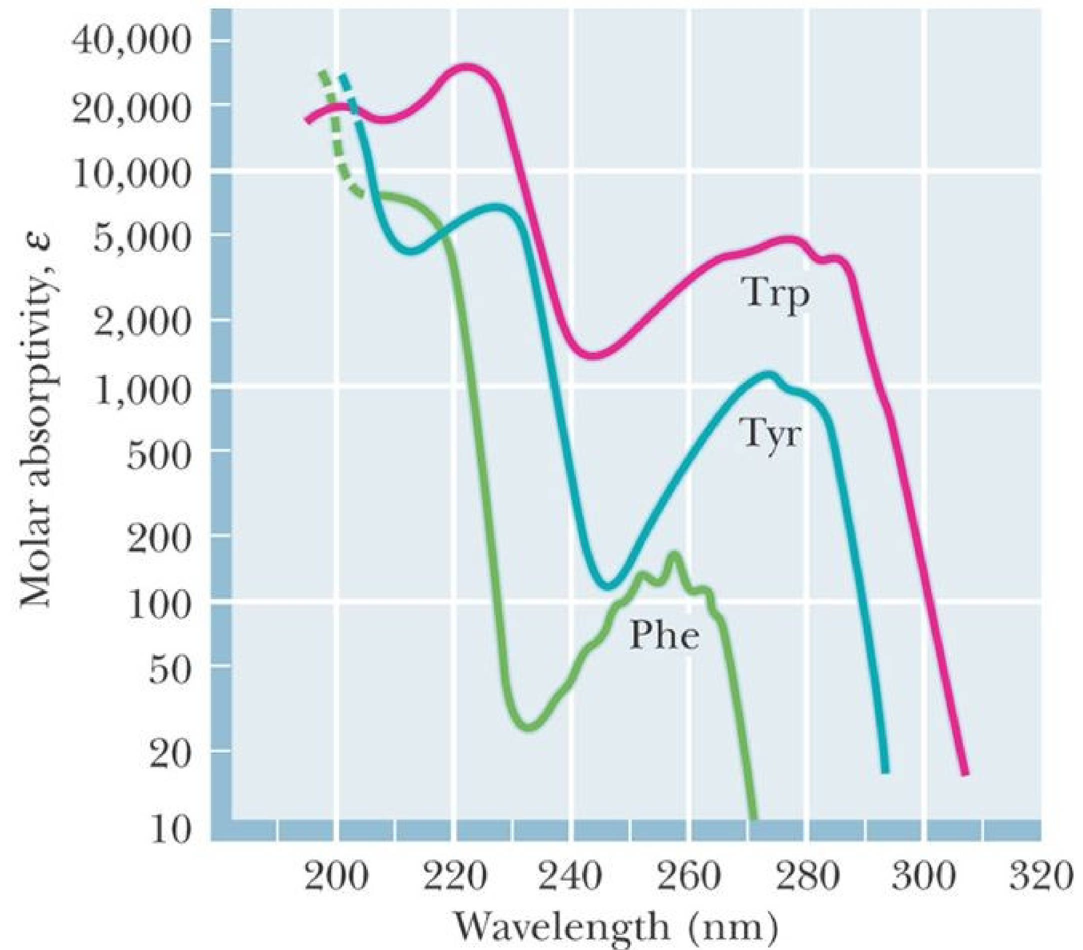
Tyr (Y)



Phe (F)

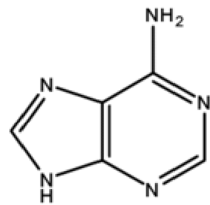
Spectroscopy

The ultraviolet absorption spectra of the aromatic amino acids at pH 6. (*From Wetlaufer, D.B., 1962. Ultraviolet spectra of proteins and amino acids.* Advances in Protein Chemistry **17:***303–390.*)

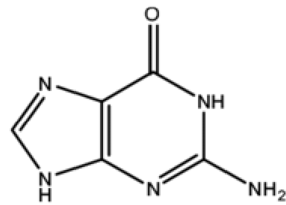


© 2005 Brooks/Cole - Thomson

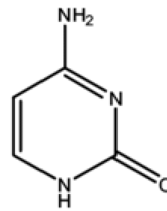
Spectroscopy



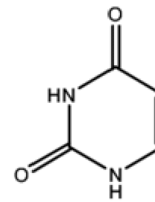
Adenine
A



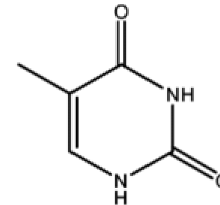
Guanine
G



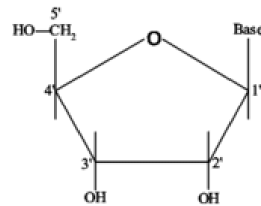
Cytosine
C



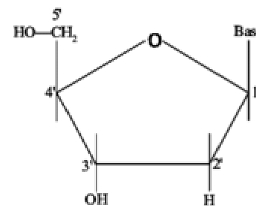
Uracil
U



Thymine
T



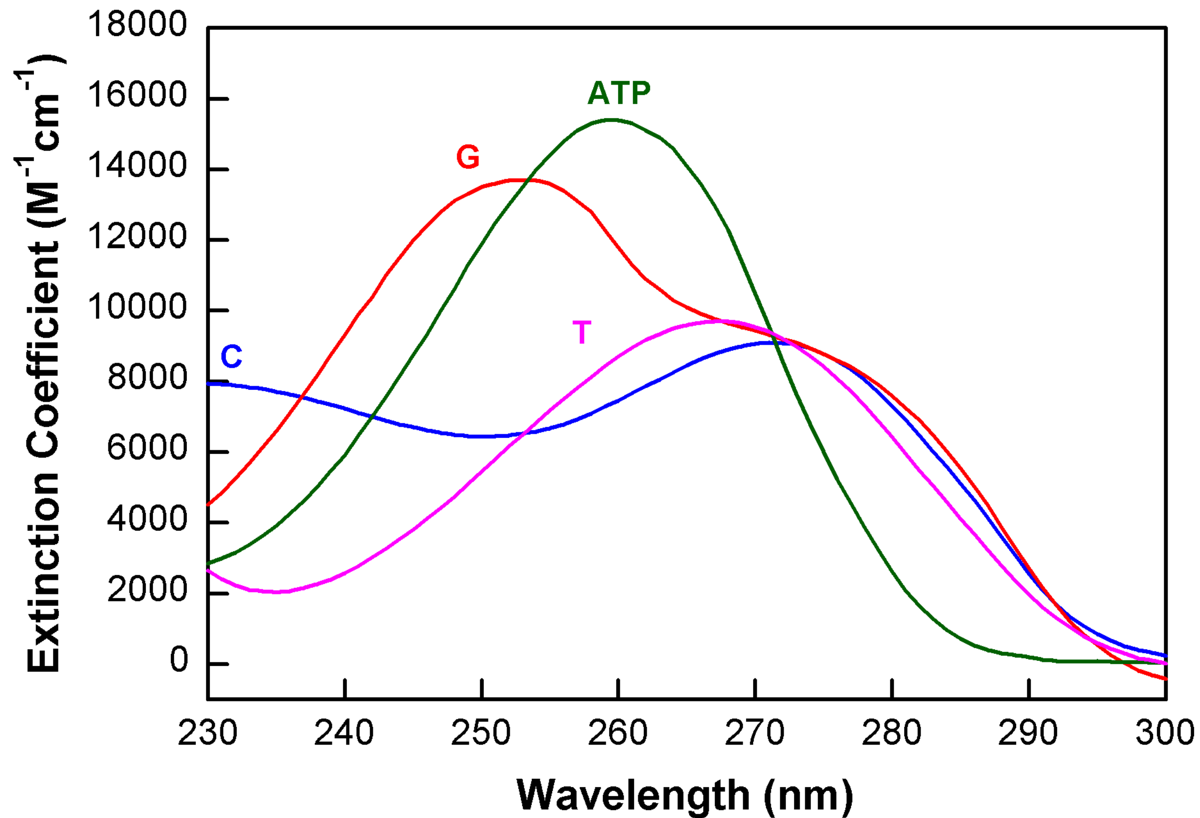
Ribose



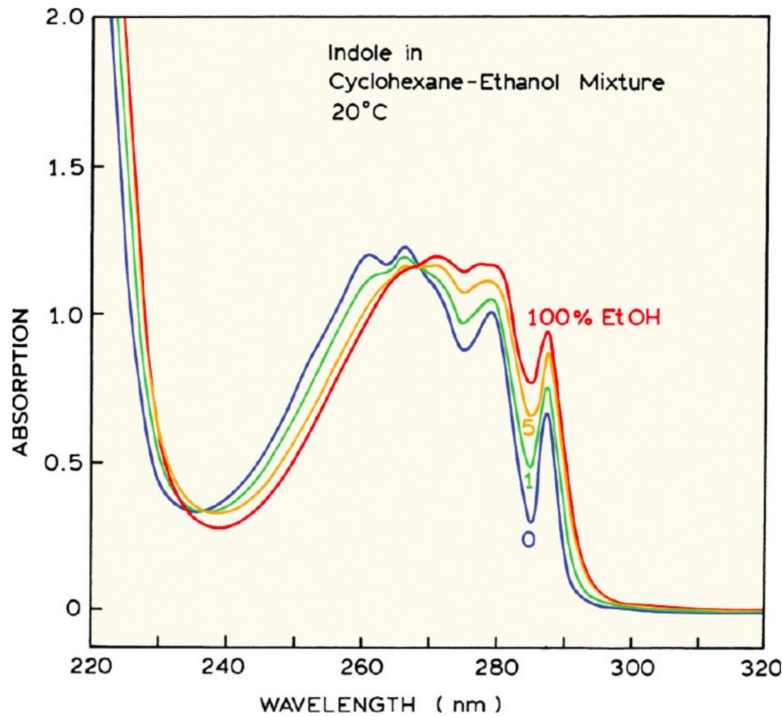
Deoxyribose

Figure 1.1. Structures of nucleic acid constituents

Absorption Spectra of the Nucleic Acids



Spectroscopy



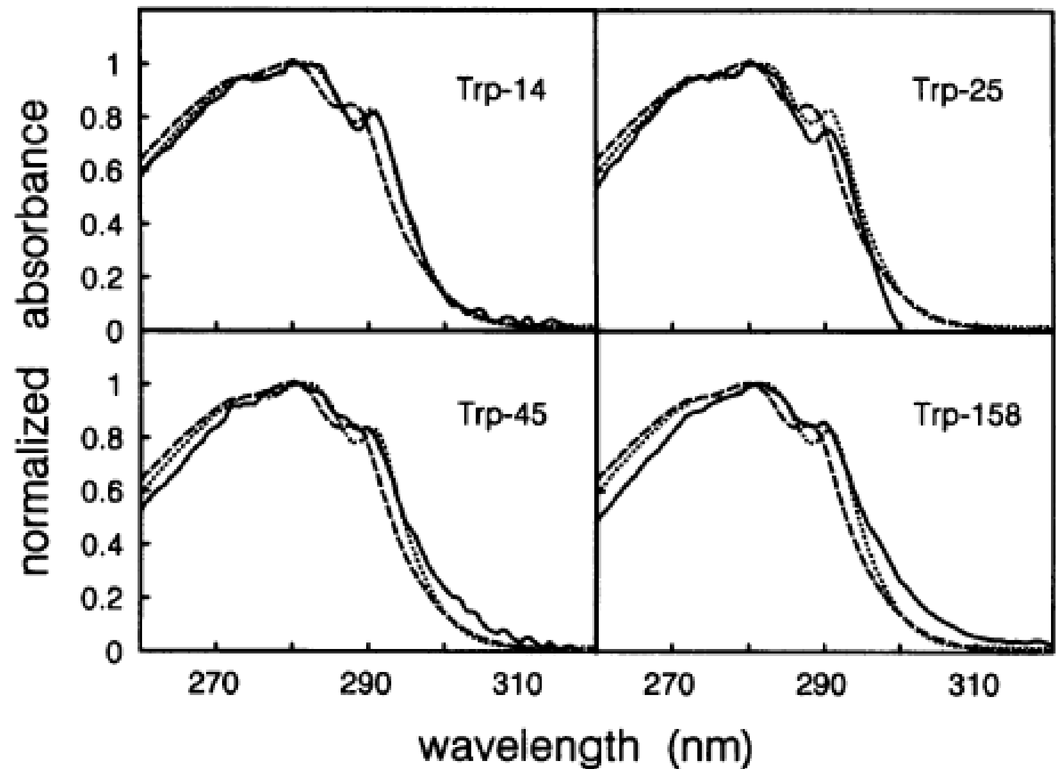
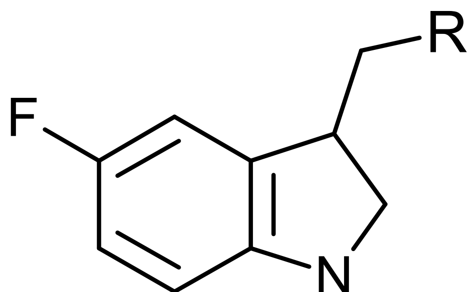


FIGURE 4 Comparison of normalized absorbance spectra of the four Trp in sTF (–) with that of NATA in aqueous buffer (- - -) and in dioxane (····).

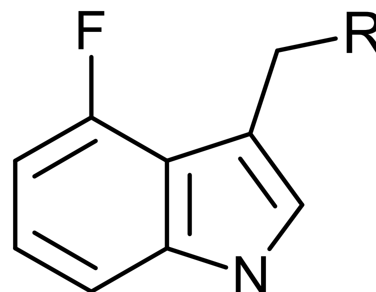
Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

Hasselbacher et, al., Biophys J 60, 20 (1995)

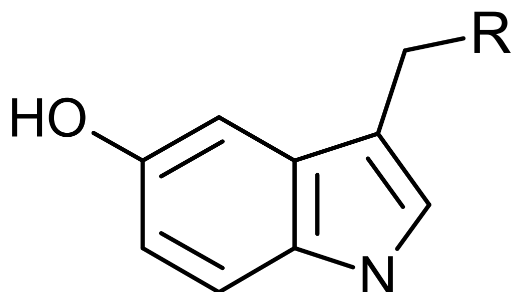
Tryptophan Analogs



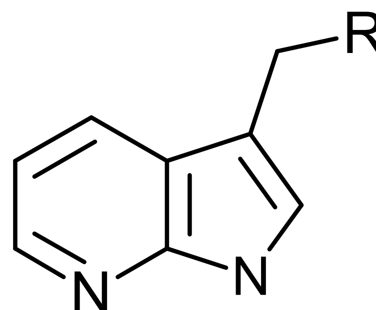
5-fluorotryptophan



4-fluorotryptophan



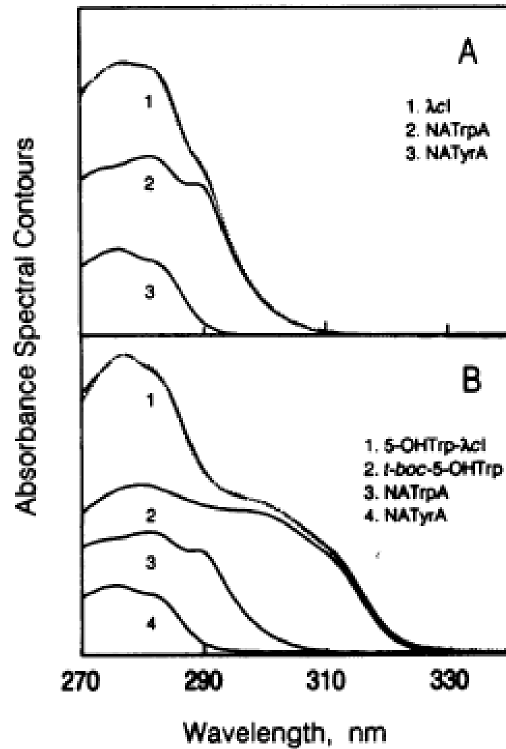
5-hydroxytryptophan



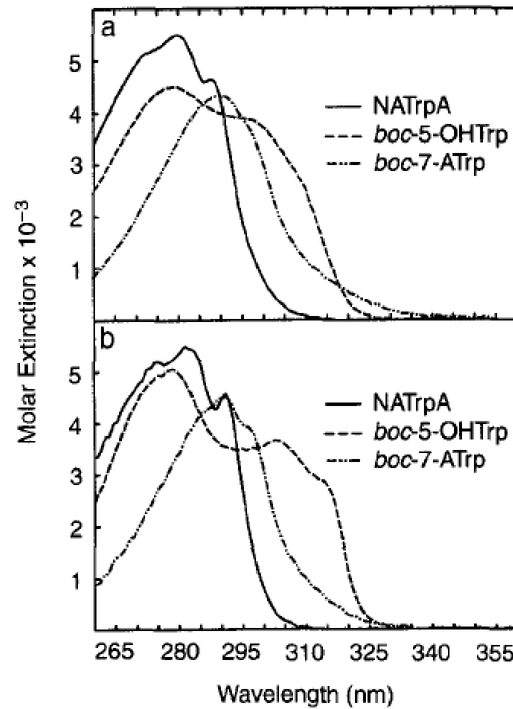
7-azatryptophan

Ross et al.,Topics in Fluorescence Spectroscopy 6, Lakowicz ed. 2000

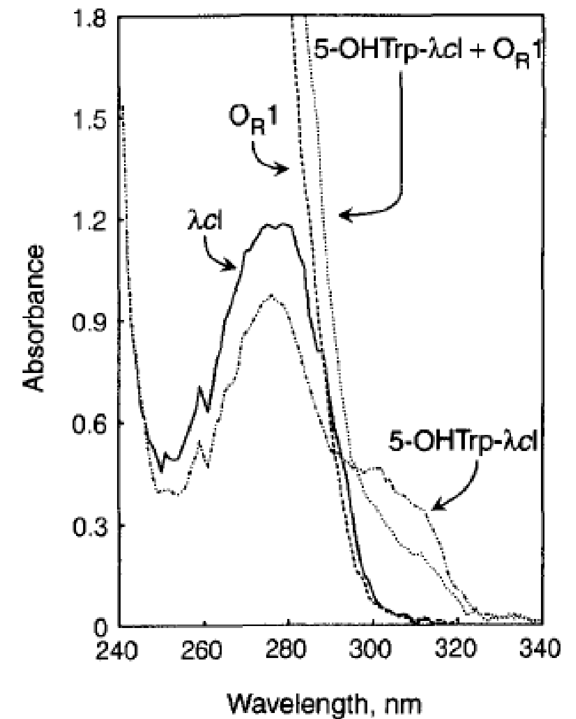
Tryptophan Analogs



Ross et al., Proc Natl Acad Sci USA 89, 12923 (1992)



Ross et al., Meth Enzymol 278, 151 (1998)



Ross et al., Meth Enzymol 278, 151 (1998)

Tryptophan Analogs

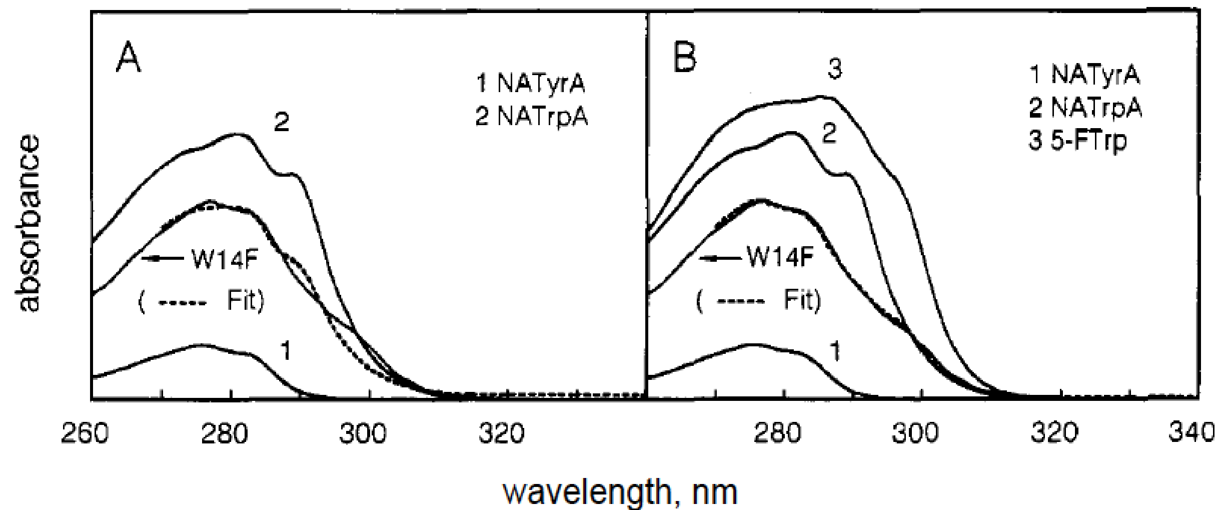
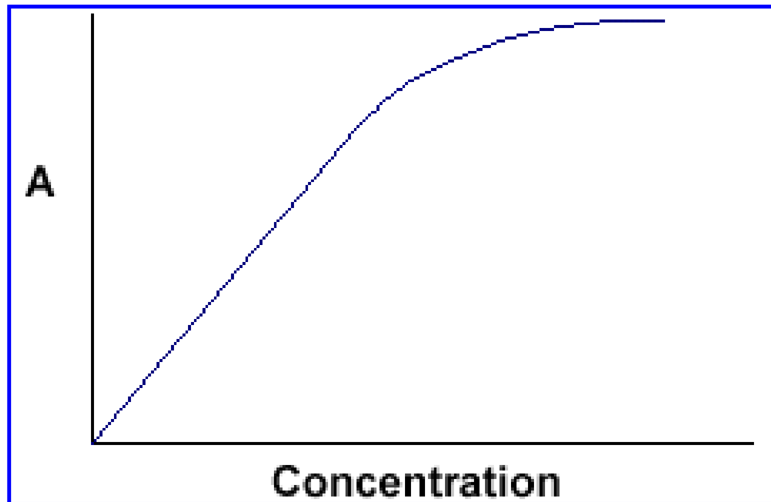


Figure 2.2. LINCS analysis of W14F sTF expressed in the presence of 5-FTrp. Panel A shows the fit from 270 to 340nm (dashed line) of the protein absorbance spectrum (solid line) using the NATyrA and NATrpA basis sets. Panel B shows the corresponding fit (dashed line) when 5-FTrp is included as a third basis set.

Ross et al., Topics in Fluorescence Spectroscopy 6, 17 (2000)

Why is this plot not linear for the entire range?

Relationship between Absorbance and Concentration



At some point the absorbance is so high that not sufficient light passes through to the detector, and linearity is no longer satisfied.

The absorbance at which an instrument becomes non-linear depends on the following factors:

1. Concentration of the analyte
2. Lamp intensity at the measured wavelength
3. Extinction coefficient of the analyte at measured wavelength
4. Sensitivity of detector at the measured wavelength

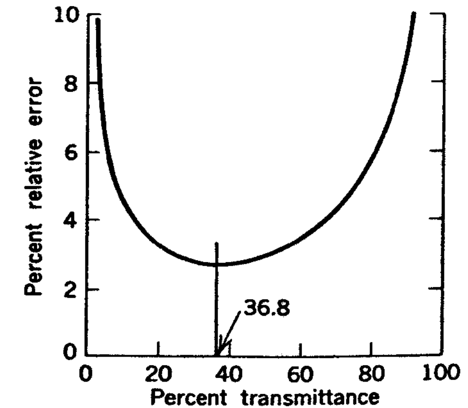
- **For best accuracy, always measure between 0.1 – 1.0 OD**

Spectroscopy

- Concentration

  Beer-Lambert Law (Beer's Law): $A_\lambda = \varepsilon_\lambda c l$

  most accurate range to obtain *c*: between 15 and 65 %T
  which is A between 0.2 and 0.8



- Identification of Chromophore(s) by Spectra

  Are spectra from multiple chromophores in a macromolecule additive?

  $$A(\lambda,\text{sample}) = \alpha A(\lambda,a) + \beta A(\lambda,b) + \gamma A(\lambda,c) + \delta A(\lambda,d) + \ldots..$$

  LINCS: LINear Combination of Spectra

  approximately linear in proteins

  but not linear in DNA or RNA

- Investigate Interacting Systems by Difference Spectra

  Quantification of Binding

  Evaluation of Conformational Changes

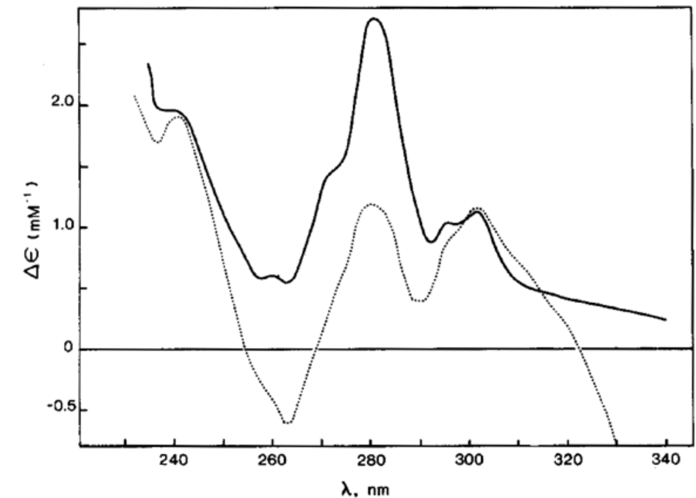


FIG. 2. Absorption difference spectra due to ternary complex formation with liver alcohol dehydrogenase: ——, enzyme·NAD^+·trifluoroethanol complex; · · · ·, enzyme·NADH·isobutyramide complex.

Laws and Shore, J. Biol Chem 254, 2582 (1979)

PRINCIPLES OF FLUORESCENCE SPECTROSCOPY

Sir George Stokes
1819-1903

Born in County Sligo, Ireland, Stokes was the Lucasian Professor of Mathematics at Cambridge from 1849 until his death in 1903.

Some major contributions:

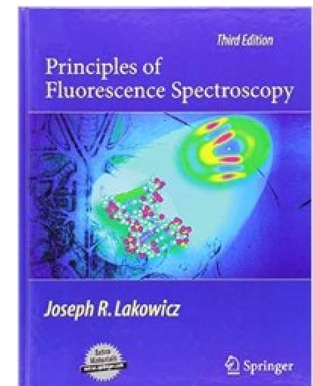
Fluid dynamics (Stokes' Law)

Wave theory of light

Polarization of light

Fluorescence of minerals

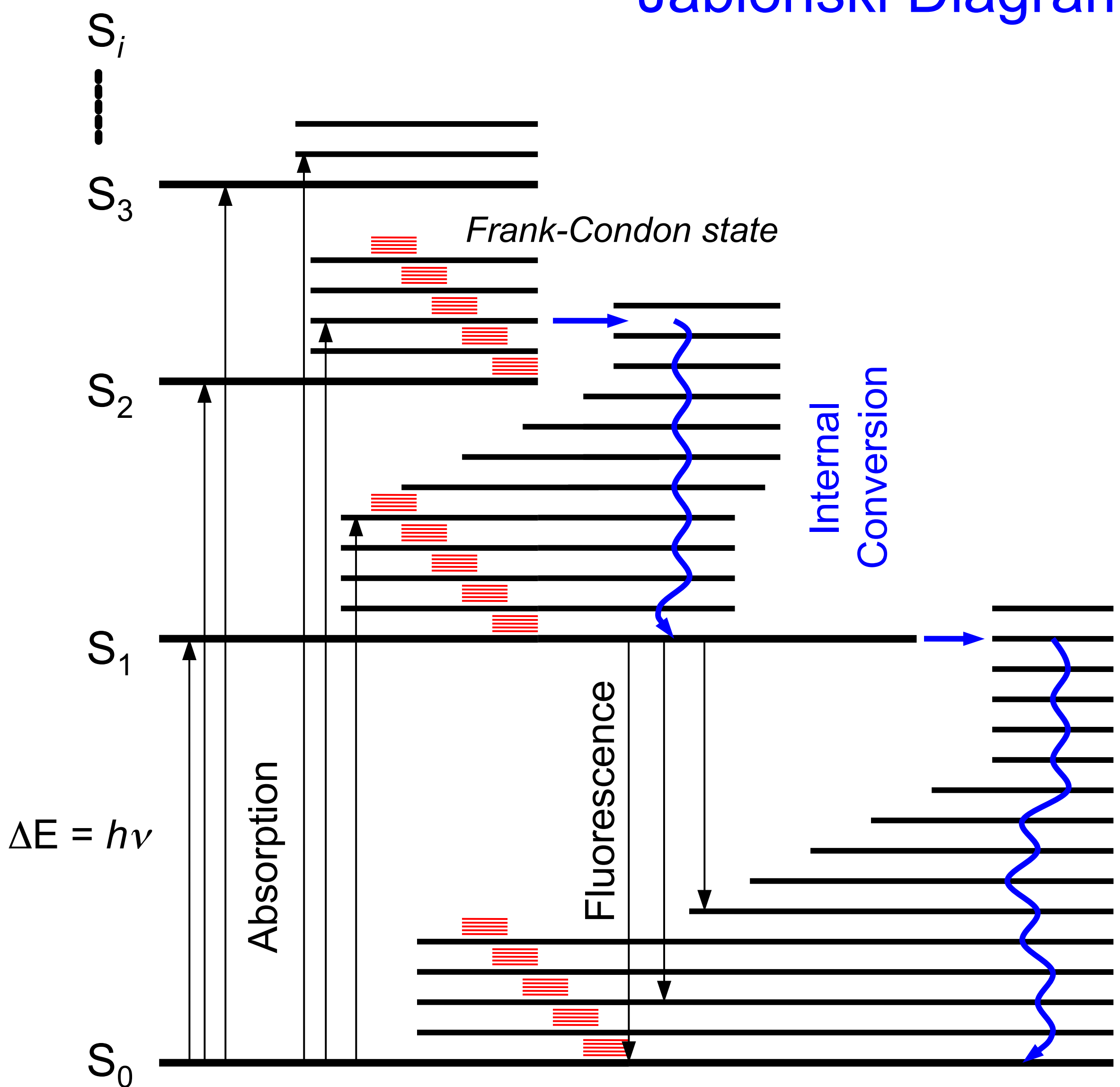
Stokes' line (Raman scatter)



Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

Spectroscopy

Jabłonski Diagram



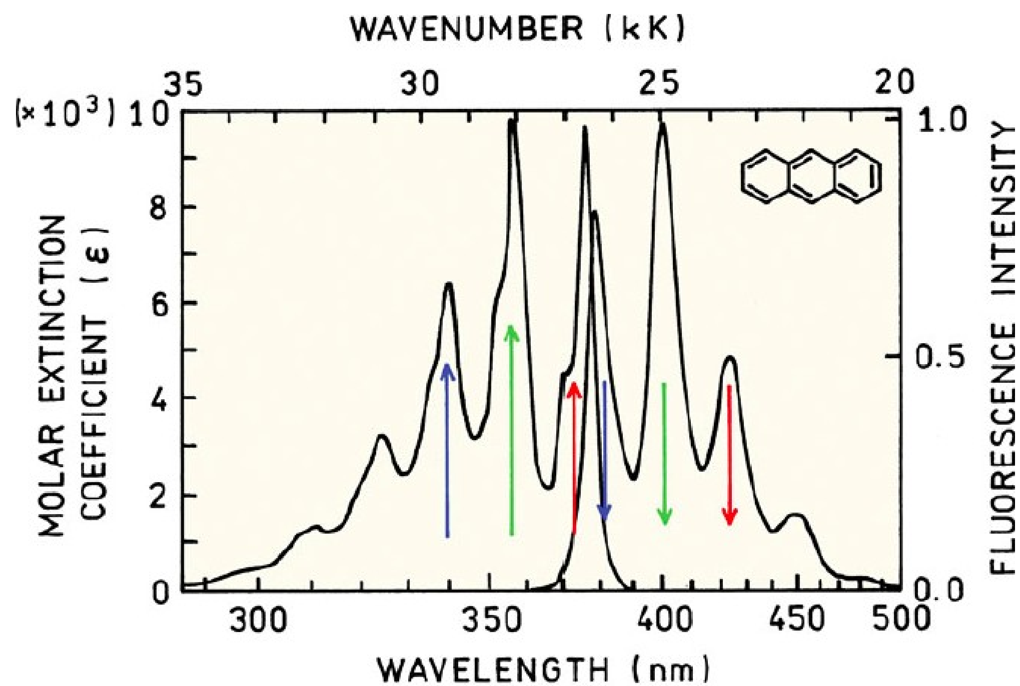
Time Scales

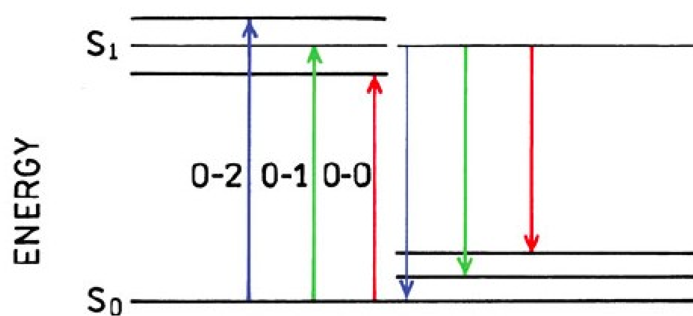
Abs.: ~ 10^{-15} s

IC.: < 10^{-12} s

Fluor: ~ 10^{-9} s

Spectroscopy





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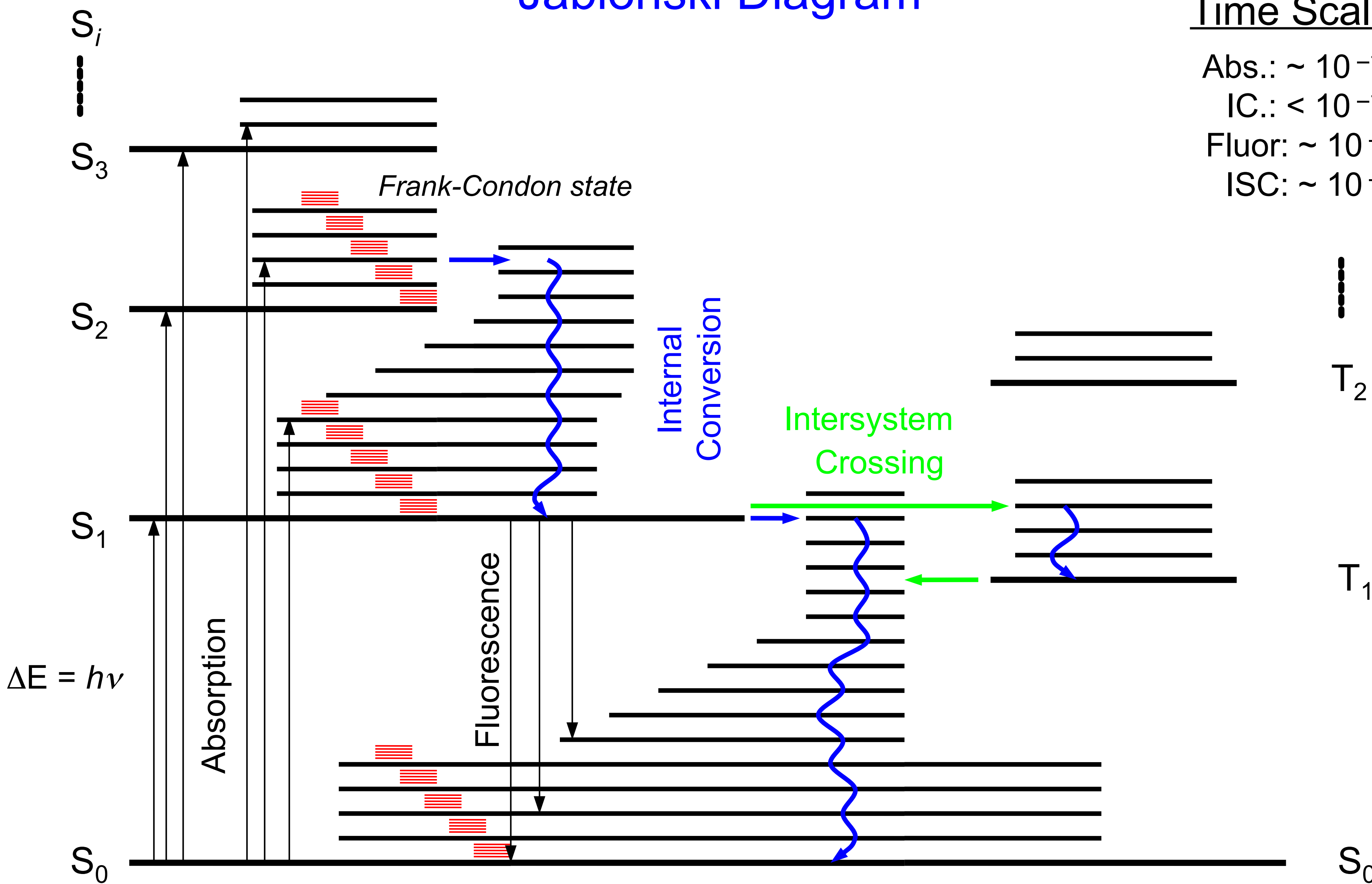
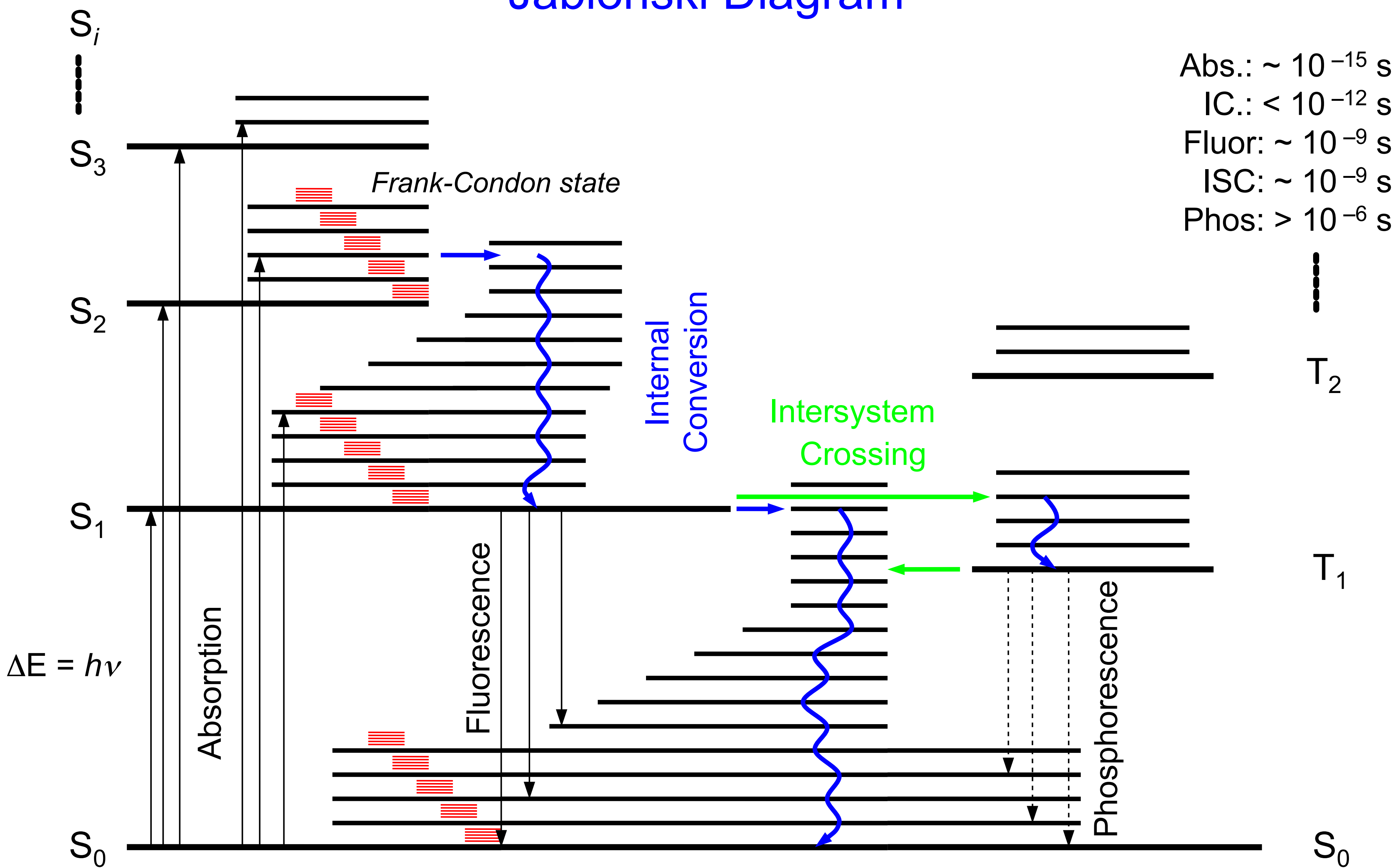
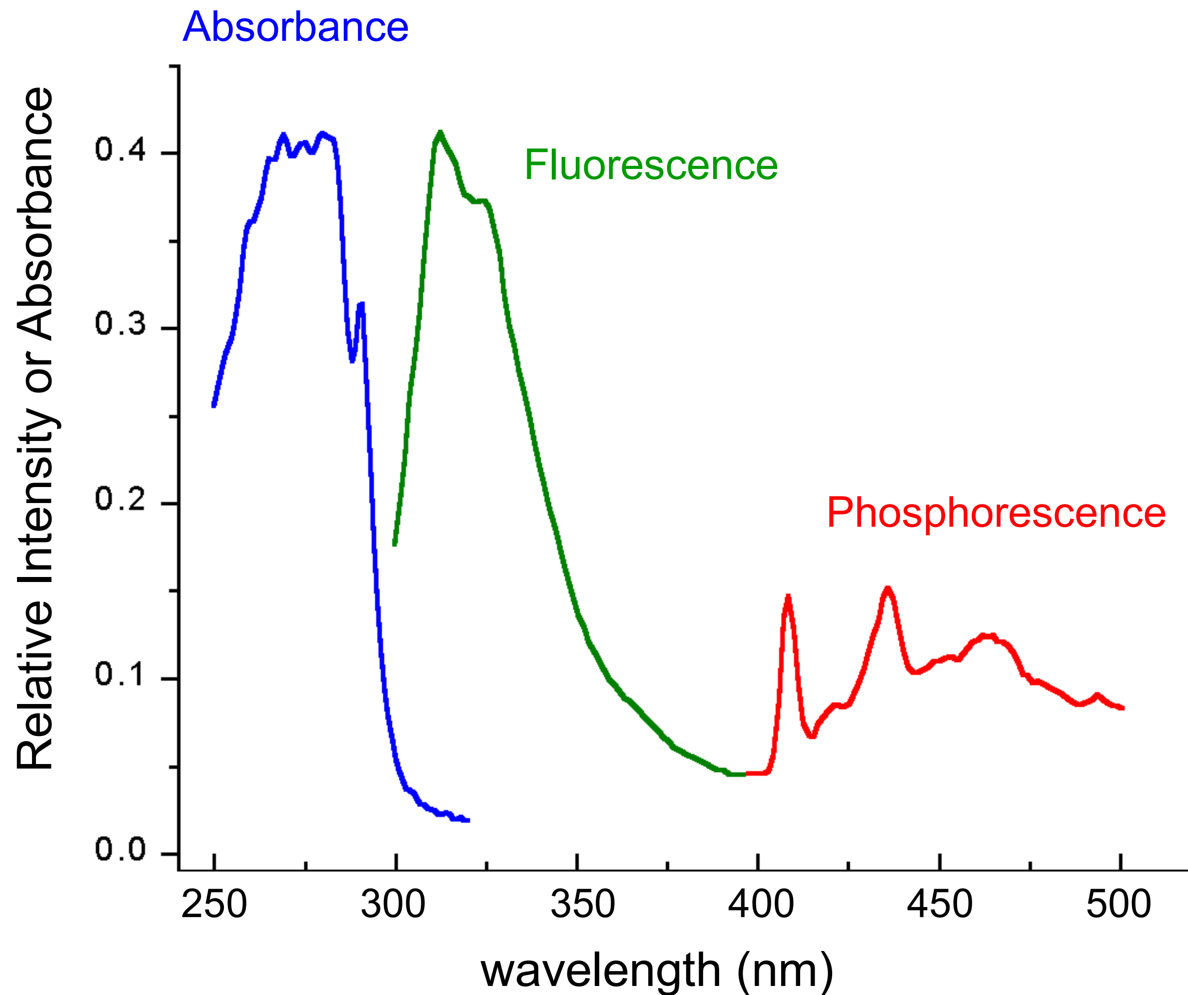


Spectroscopy



Single Trp Residue in Cod Parvalbumin; 77 K



Loss of Energy from Excited State back to Ground State

- Internal Conversion (IC; mainly through vibrational relaxation)
- Quenching: collisions with solvent, solutes, or groups of chromophore
- Intersystem Crossing (ISC)
  phosphorescence from long-lived triplet state
- Förster Resonance Energy Transfer (FRET)
- Emission of a photon
  fluorescence from lower energy than from initial Frank-Condon state; Stoke’s shift
- Excited-State Reactions