## Practical use of UV Spectroscopy
## Example: Multi-wavelength AUC

**Approach:**

**To exploit the dynamic range of the UV spectrophotometer, measure multiple concentrations of the analyte over a wide wavelength range and globally fit the resulting absorbance traces to an intrinsic absorption spectrum over all wavelengths. Each analyte must be dialyzed into the same buffer, and the spectrophotometer must be blanked with the buffer to get pure analyte spectra.**

**Model the UV spectrum with a linear sum of Gaussians:**

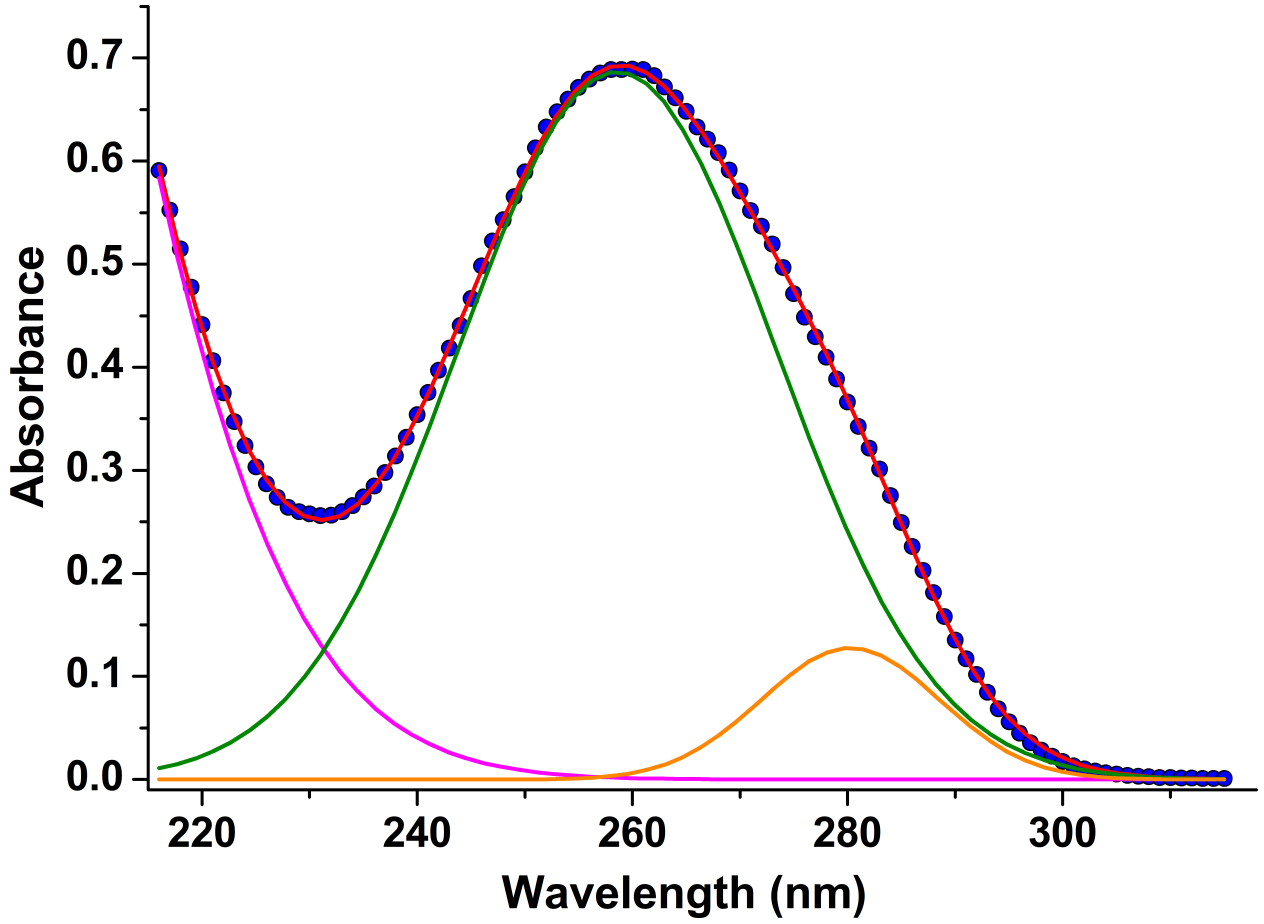
$$\text{Spectrum} = \sum_{i=1}^{n} c_i G_i(\lambda)$$

$$\text{where } G(\lambda) = \frac{1}{\sigma\sqrt{2\pi}} \exp\frac{-1}{2}\left(\frac{\lambda-\mu}{\sigma}\right)^2 \text{ and } \lambda = \text{wavelength},$$

$$\sigma = \text{standard deviation (width), and } \mu \text{ is the mean}$$

## Practical use of UV Spectroscopy

### Example: DNA fitted by three Gaussian terms:



## Practical use of UV Spectroscopy

### Example: Multi-wavelength AUC

**Decompose an unknown mixture of two or more analytes into a linear combination of intrinsic absorption profiles to determine the amplitude (partial concentration) of each analyte in the mixture:**

$$\text{Spectrum ( Mixture )} = \sum_{j=1}^{m} a_j \, Spectrum_j$$

**Minimize with NNLS (Lawson CL, Hanson RJ. Solving Least Squares Problems. Prentice-Hall, Inc. Englewood Cliffs, New Jersey, 1974).**

**DEMO**

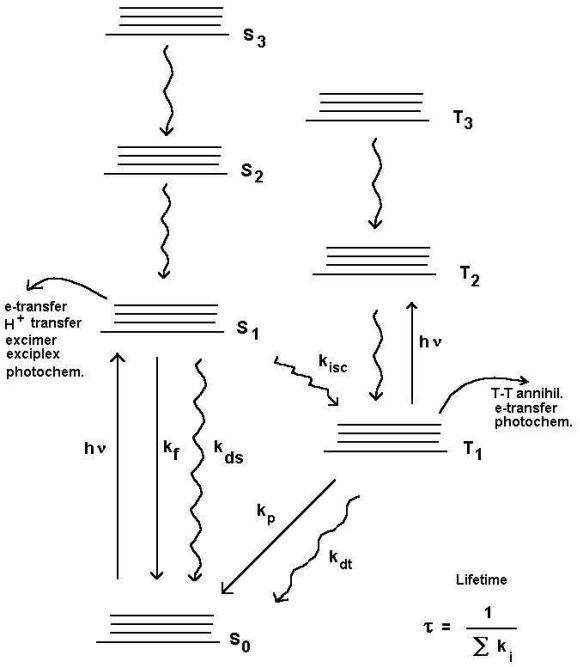
# *Fluorescence Spectroscopy*

## 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)**
- **Fluorescence - Emission of a photon at a lower energy than from initial absorption; Stoke's shift**
- **Excited-State Reactions, bond breakage, formation, rearrangement, proton transfer**

# Fluorescence Spectroscopy

Aleksander Jabłoński



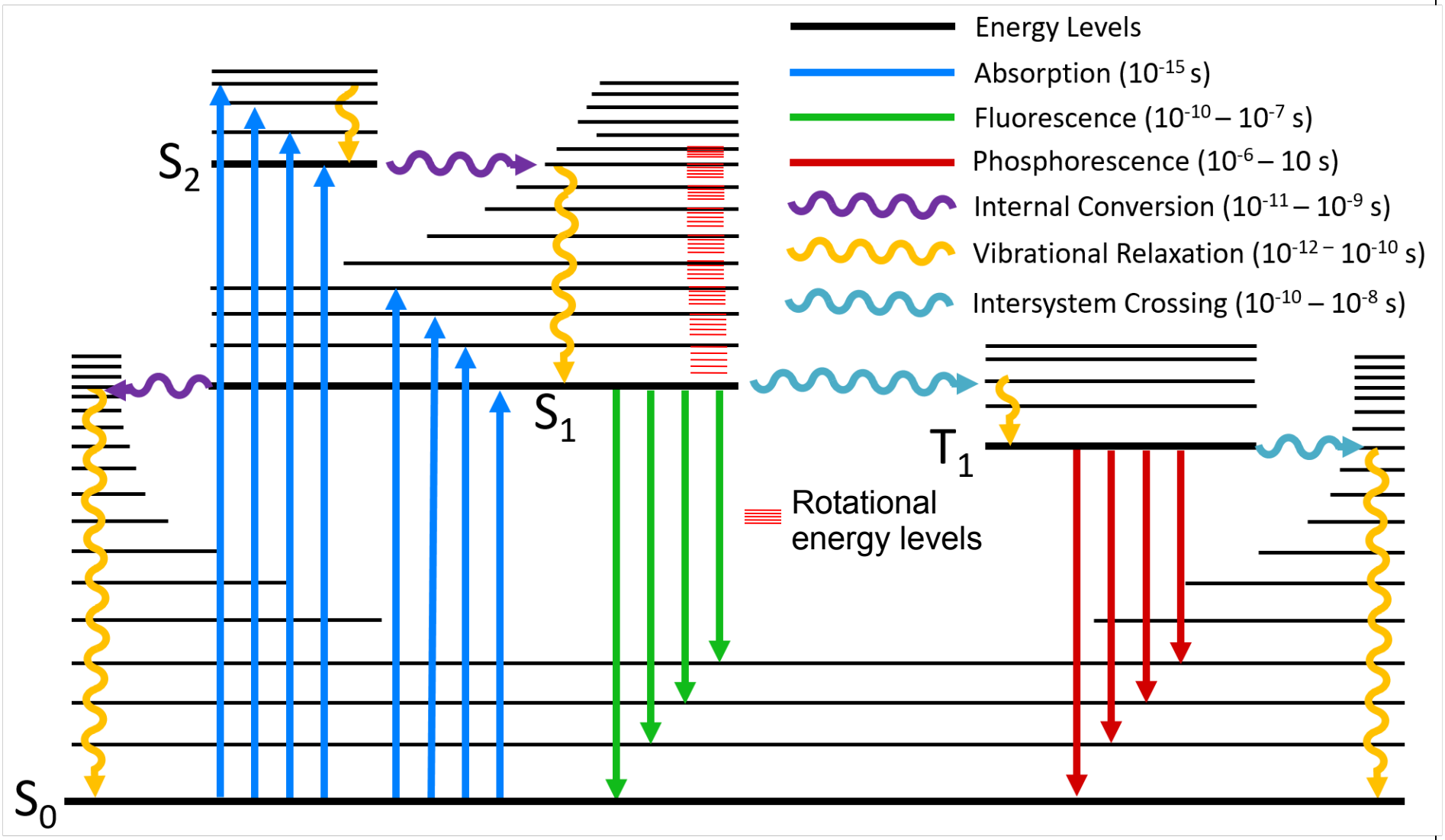

1934 habilitation

"On the influence of intermolecular interactions on the absorption and emission of light"

Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

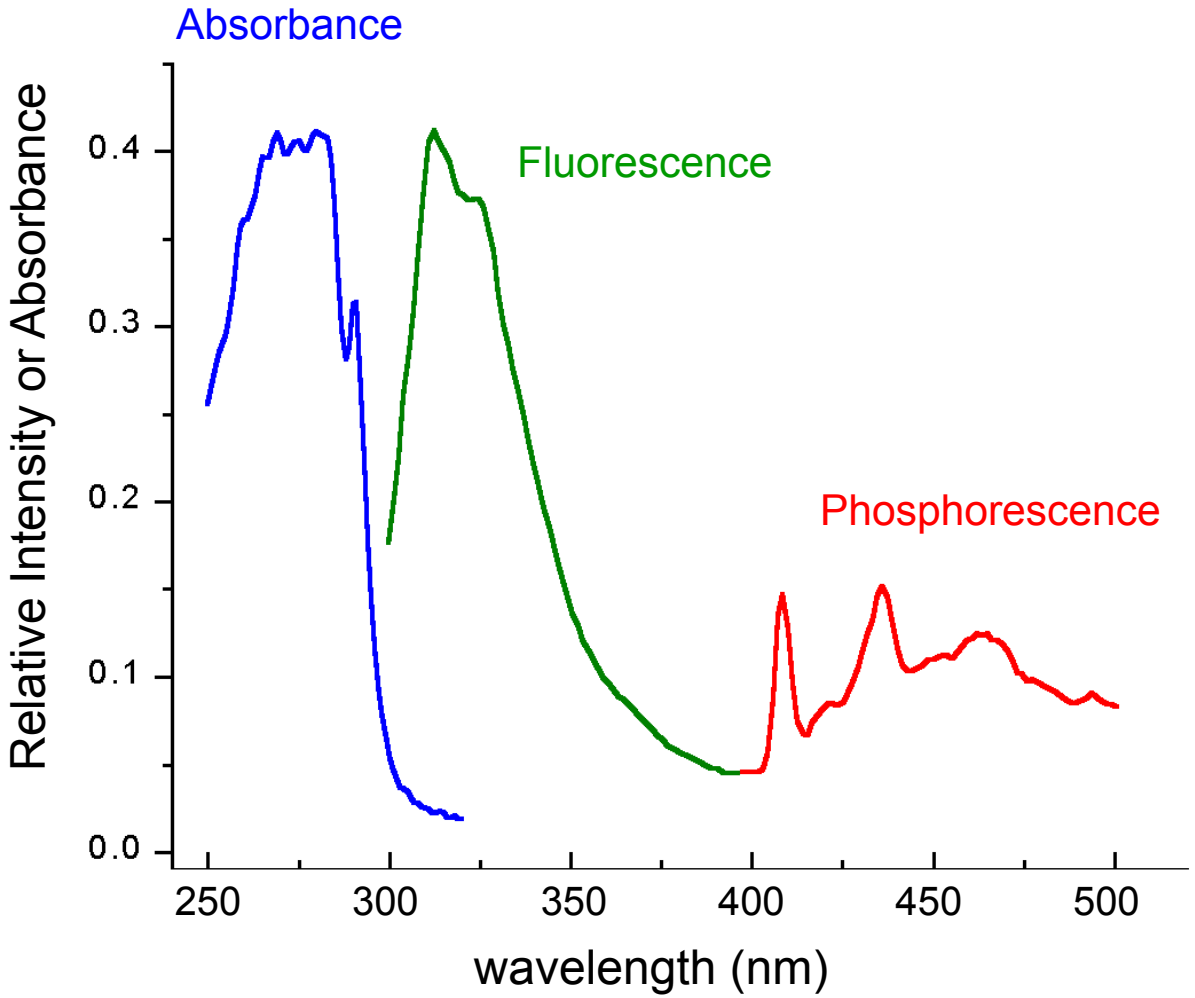
# Fluorescence Spectroscopy

## Jabłonski Diagram



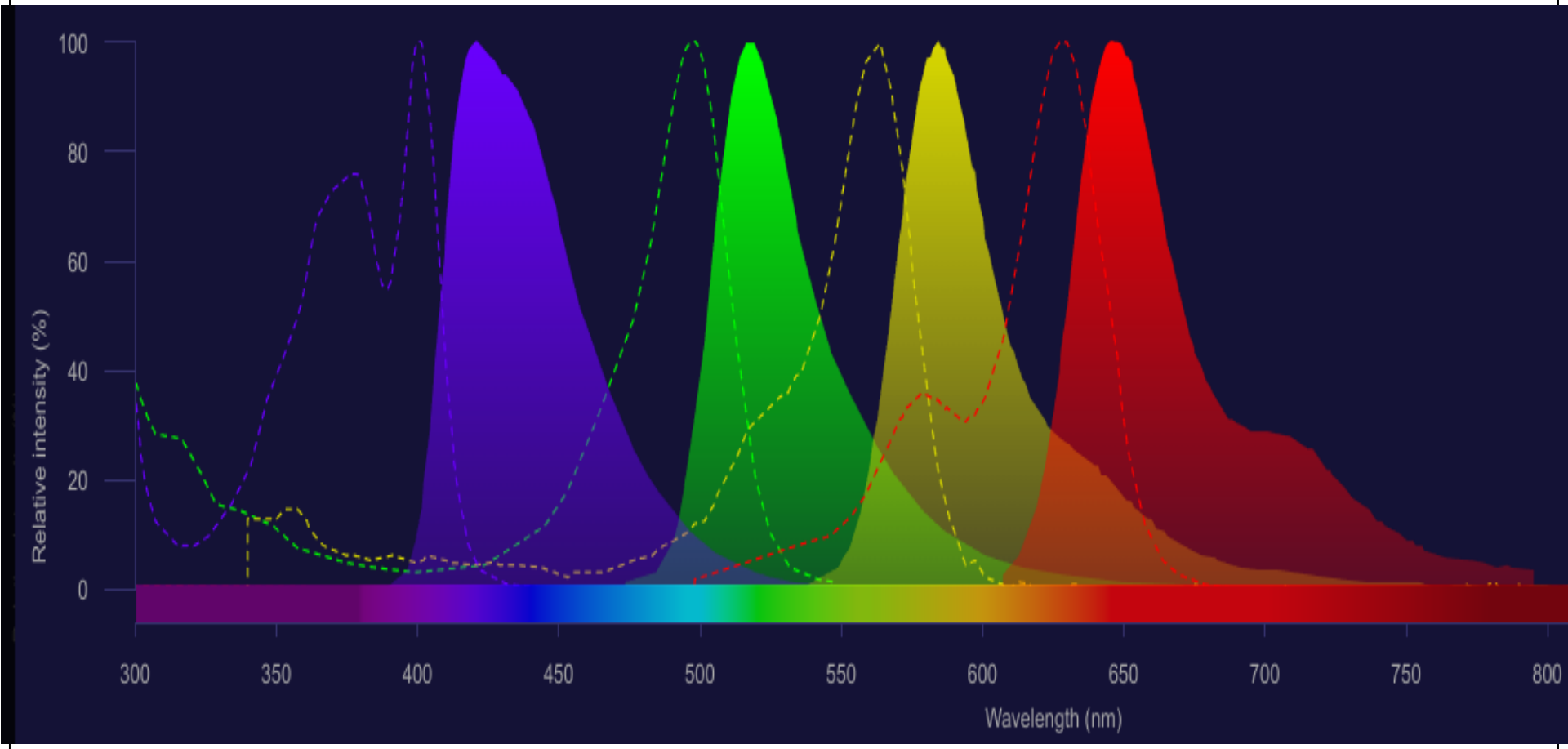
# Fluorescence Spectroscopy

## Single Trp Residue in Cod Parvalbumin; 77 K



# *Fluorescence Spectroscopy*

## Absorbance and emission spectra of various Alexa Fluors 405, 488, 586, 633 nm




https://www.thermofisher.com/order/fluorescence-spectraviewer

# Fluorescence Spectroscopy

## Commercially Available Fluorophores:

### Extrinsic probes

- Alexa dyes

Molecular Probes Handbook:
https://www.thermofisher.com/us/en/home/references/molecular-probes-the-handbook.html

Alexa 488 TFP ester

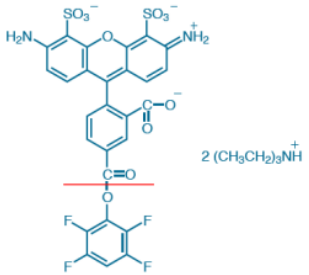
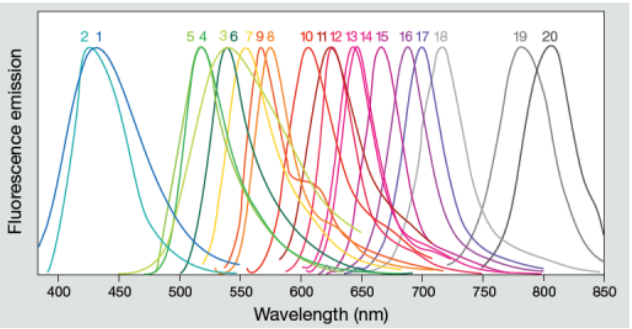



**Table 1.5** Fluorescence quantum yields (QY) and lifetimes (τ) for Alexa Fluor® dyes.

| Alexa Fluor® Dye * | QY † | τ (ns) ‡ |
|---|---|---|
| Alexa Fluor® 488 | 0.92 | 4.1 § |
| Alexa Fluor® 532 | 0.61 | 2.5 |
| Alexa Fluor® 546 | 0.79 | 4.1 |
| Alexa Fluor® 555 | 0.10 | 0.3 |
| Alexa Fluor® 568 | 0.69 | 3.6 § |
| Alexa Fluor® 594 | 0.66 | 3.9 § |
| Alexa Fluor® 647 | 0.33 | 1.0 |
| Alexa Fluor® 660 | 0.37 | 1.2 ** |
| Alexa Fluor® 680 | 0.36 | 1.2 |
| Alexa Fluor® 700 | 0.25 | 1.0 |
| Alexa Fluor® 750 | 0.12 | 0.7 |

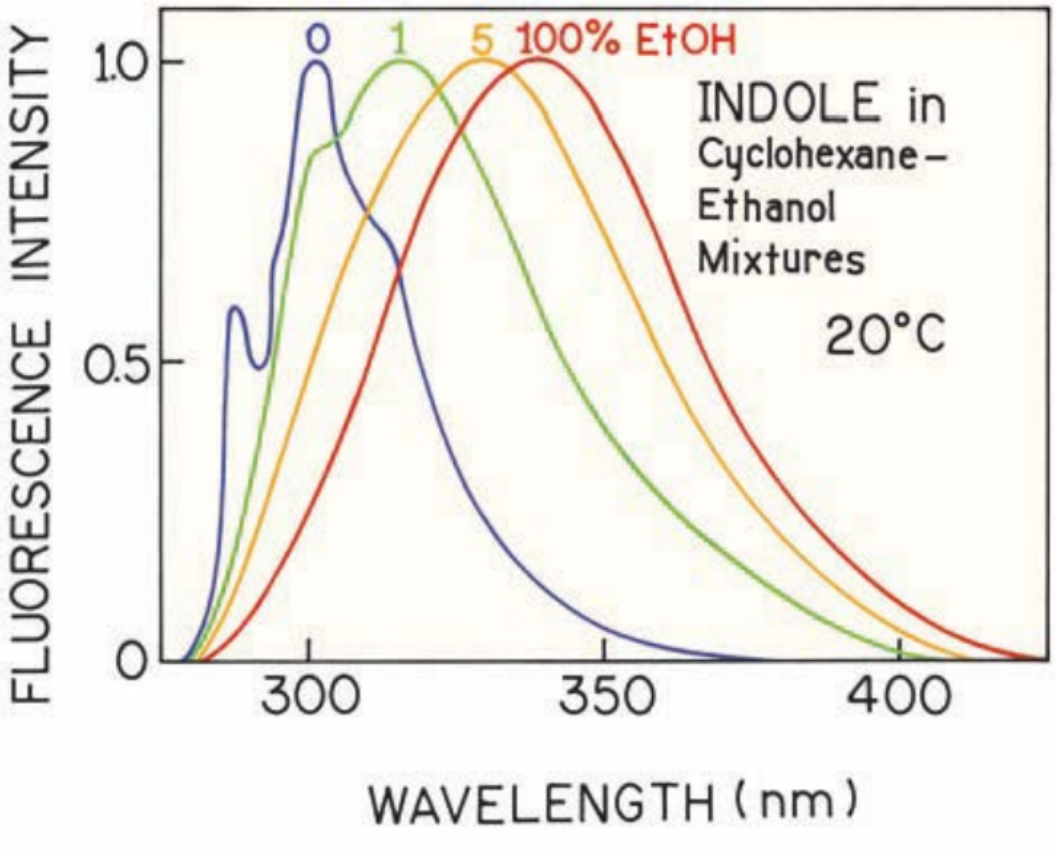
**Table 1** Spectral properties of Molecular Probes® Alexa Fluor® dyes.

| Alexa Fluor® Dye | Absorption Max (nm) * | Emission Max (nm) * | Emission Color † | Extinction Coefficient ‡ |
|---|---|---|---|---|
| Alexa Fluor® 350 | 346 | 442 | Blue | 19,000 |
| Alexa Fluor® 405 | 402 | 421 | Blue | 35,000 |
| Alexa Fluor® 430 | 434 | 539 | Yellow-green | 15,000 |
| Alexa Fluor® 488 | 495 | 519 | Green | 73,000 |
| Alexa Fluor® 514 | 518 | 540 | Green | 80,000 |
| Alexa Fluor® 532 | 531 | 554 | Yellow | 81,000 |
| Alexa Fluor® 546 | 556 | 573 | Orange | 112,000 |
| Alexa Fluor® 555 | 555 | 565 | Orange | 155,000 |
| Alexa Fluor® 568 | 578 | 603 | Red-orange | 88,000 |
| Alexa Fluor® 594 | 590 | 617 | Red | 92,000 |
| Alexa Fluor® 610 | 612 | 628 | Red | 144,000 |
| Alexa Fluor® 633 | 632 | 647 | Far-red | 159,000 |
| Alexa Fluor® 635 | 633 | 647 | Far-red | 140,000 |
| Alexa Fluor® 647 | 650 | 668 | Far-red | 270,000 |
| Alexa Fluor® 660 | 663 | 690 | Near-IR § | 132,000 |
| Alexa Fluor® 680 | 679 | 702 | Near-IR § | 183,000 |
| Alexa Fluor® 700 | 702 | 723 | Near-IR § | 205,000 |
| Alexa Fluor® 750 | 749 | 775 | Near-IR § | 290,000 |
| Alexa Fluor® 790 | 782 | 805 | Near-IR § | 260,000 |



# Fluorescence Spectroscopy

Fluorescent intensity and wavelength can change with solvent:

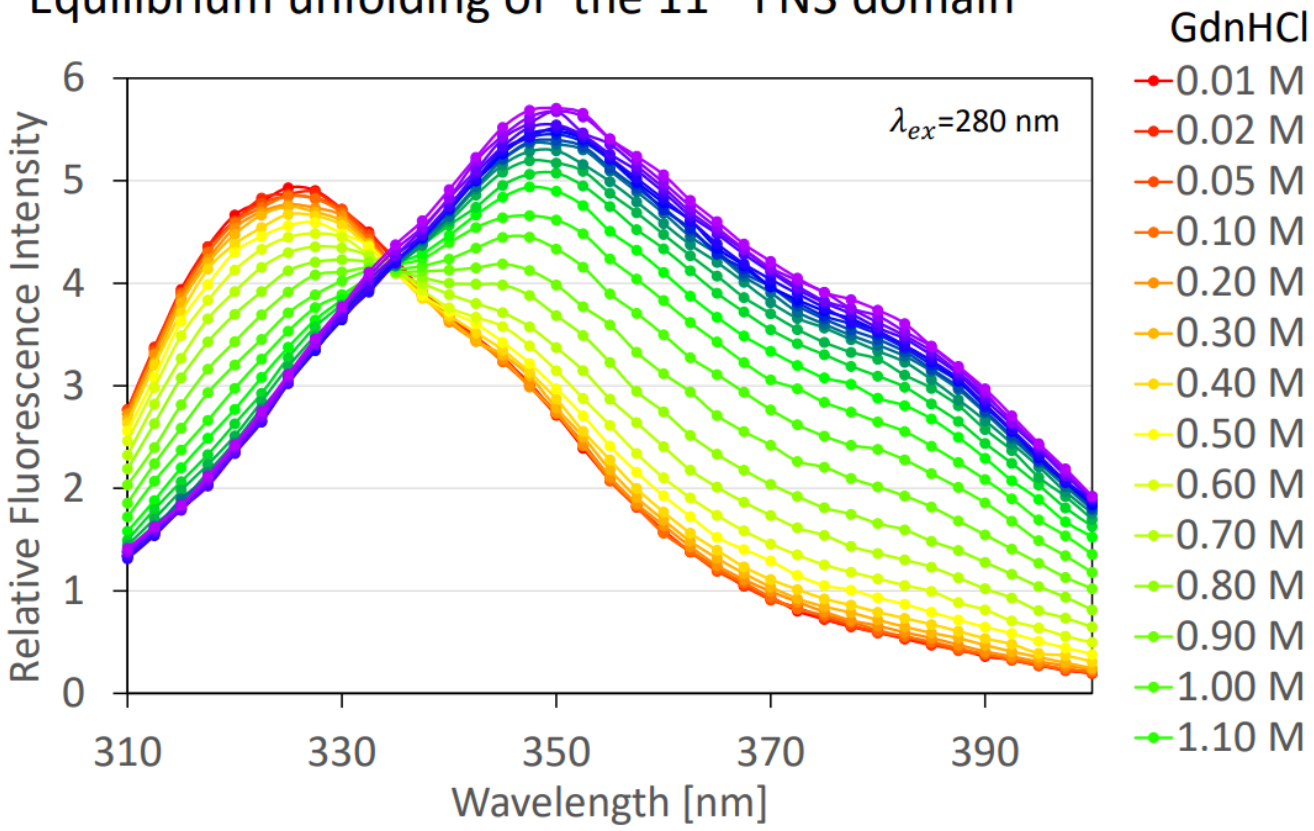



Lakowicz, J.R. Principles of Fluorescence Spectroscopy, 3rd edition, 2006, Springer

# Fluorescence Spectroscopy

## Fluorescent intensity and wavelength can change with solvent:

Application to protein folding:
Equilibrium unfolding of the 11$^{th}$ FN3 domain

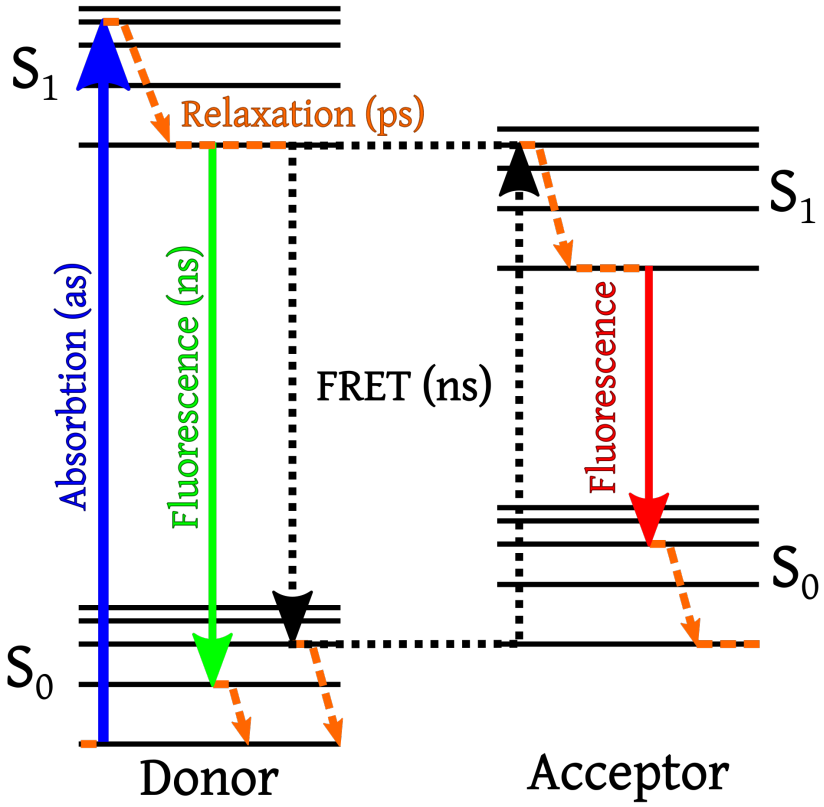



**Credits: Klara Briknarova**

# Förster resonance energy transfer (FRET)

**Förster resonance energy transfer (FRET) is the process of energy transfer between two chromophores, a donor and receptor chromophore using nonradiative dipole–dipole coupling.**

**The donor, which has an electron in the excited state, can transfer a portion of the electron's energy to an acceptor chromophore. This process is on the nanosecond time scale, and does not involve the radiation of a photon. The process requires the two molecules to be close in space, and the efficiency of this energy transfer is inversely proportional to the sixth power of the distance (10-100 Å) between donor and acceptor, making FRET extremely sensitive to small changes in distance.**



# Förster resonance energy transfer (FRET)

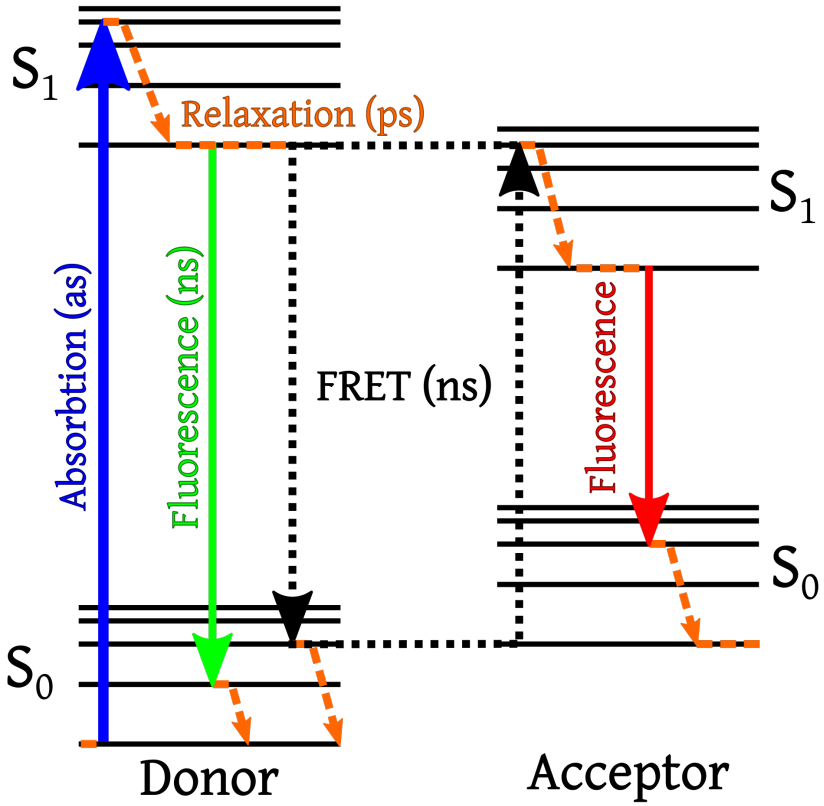
**The absorption spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor, and the efficiency of the transfer depends on the donor-acceptor orientation.**

$$E = 1 - \frac{F_{DA}}{F_D}$$

**Fluorescence intensity of the donor in the presence of acceptor**

**Fluorescence intensity of the donor in the absence of acceptor**

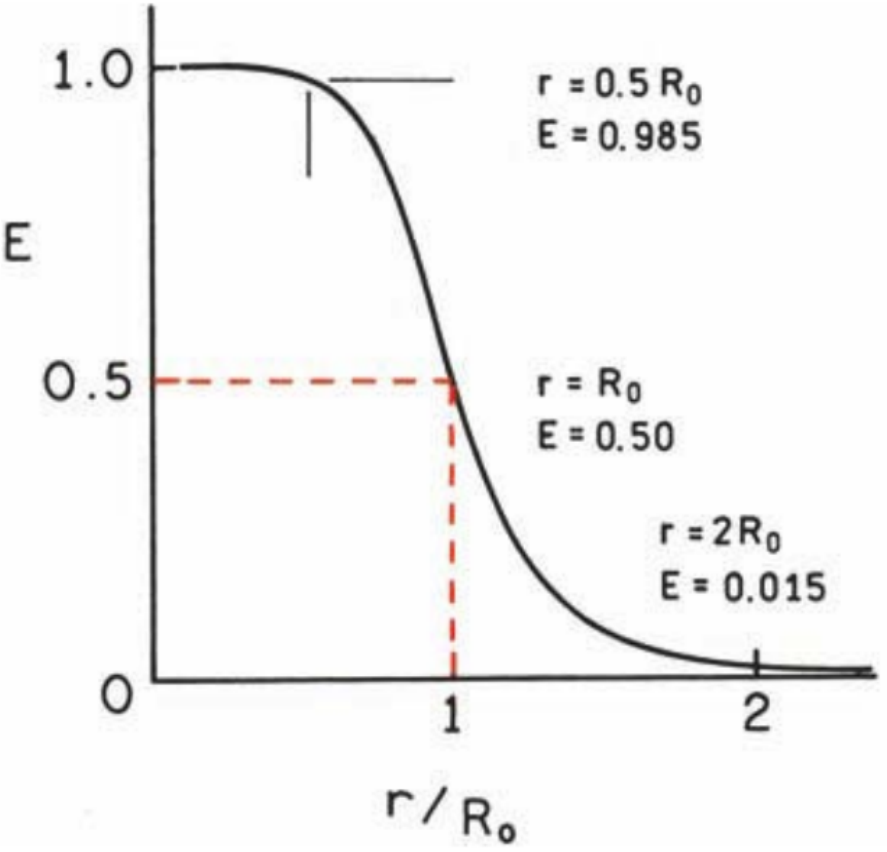
**The efficiency (E) of the transfer is the fraction of photons absorbed by the donor which are transferred to the acceptor.**



# Fluorescence Spectroscopy

Förster distance ($R_0$)

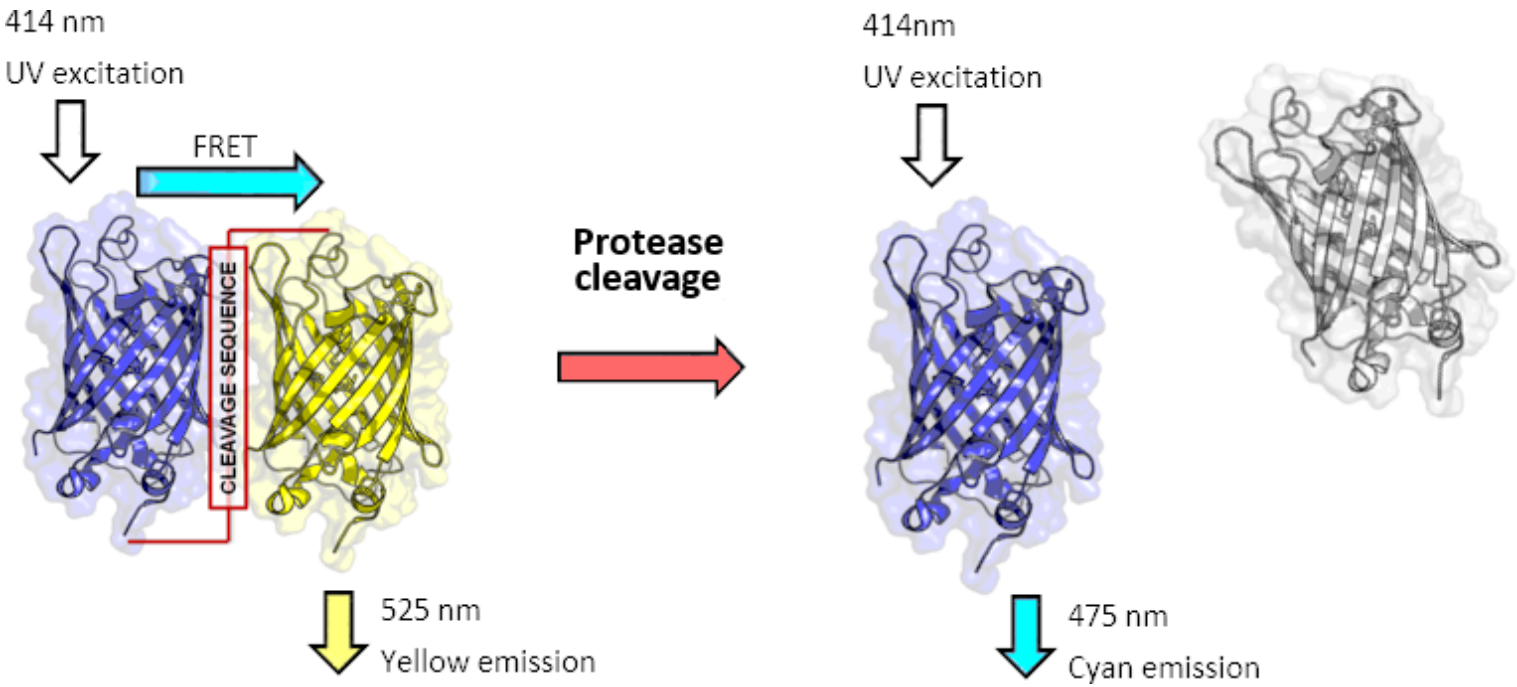
- Distance at which energy transfer is 50% efficient




$$E = \frac{R_0^6}{R_0^6 + r^6}$$

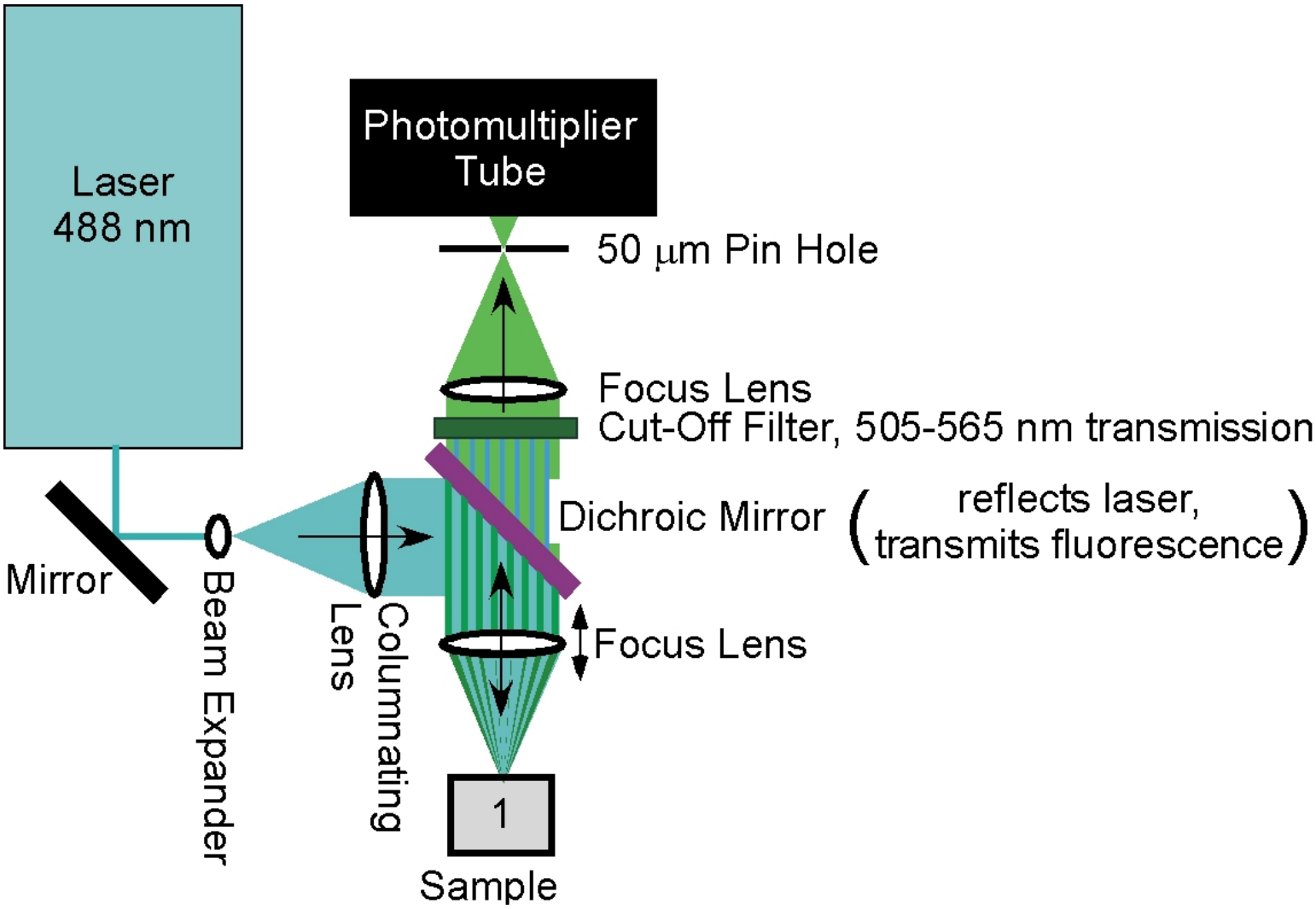
Lakowicz, J.R. Principles of Fluorescence Spectroscopy, 3rd edition, 2006, Springer

# Förster resonance energy transfer (FRET)

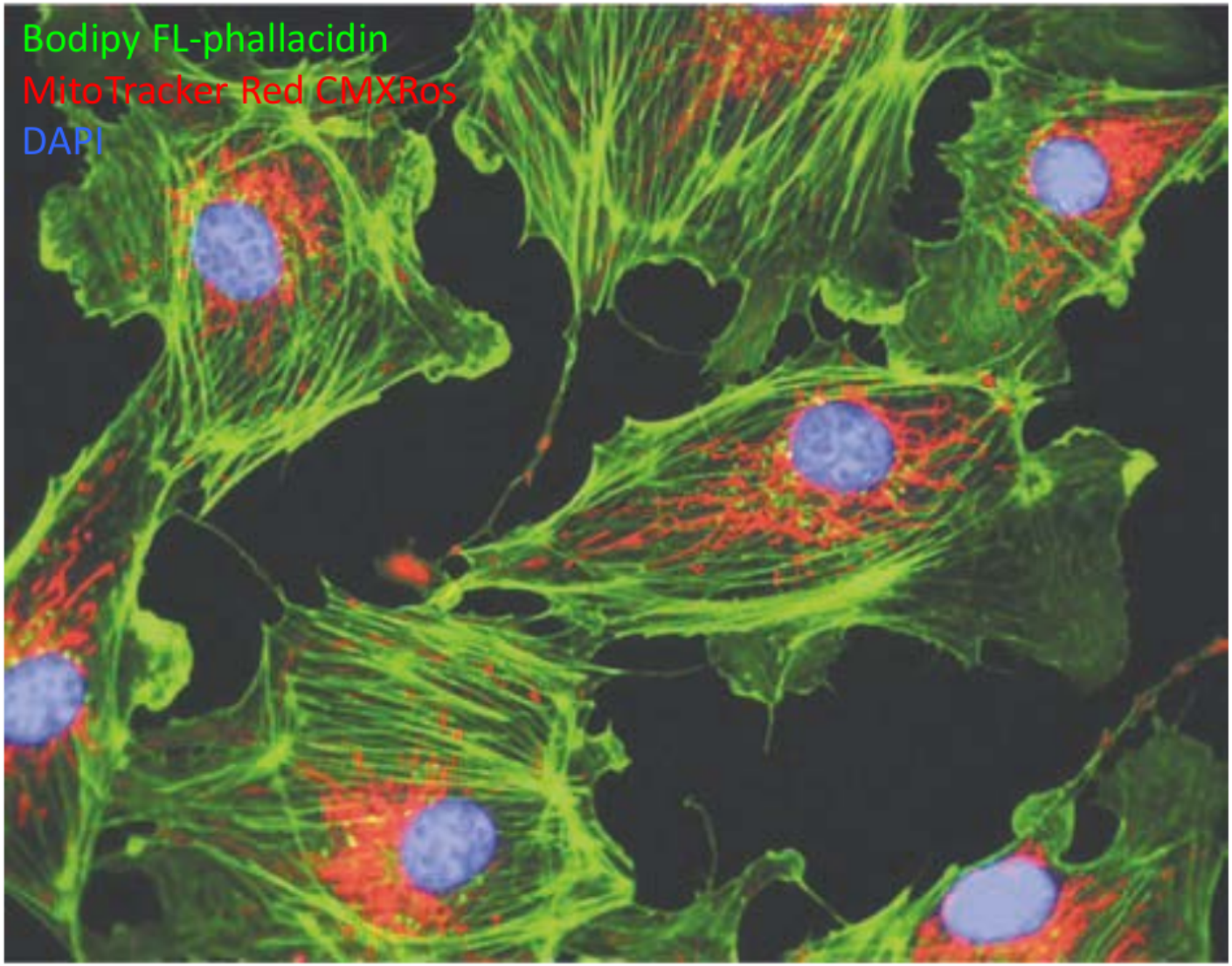



**As seen in the Jablonski diagram, some of the donor photon's energy is dissipated on the picosecond time scale through rotational/vibrational relaxation before energy transfer occurs, which causes the emitted fluorescence to be of longer wavelength/lower energy than the absorbed photon in the donor chromophore.**

# Confocal Microscope Setup

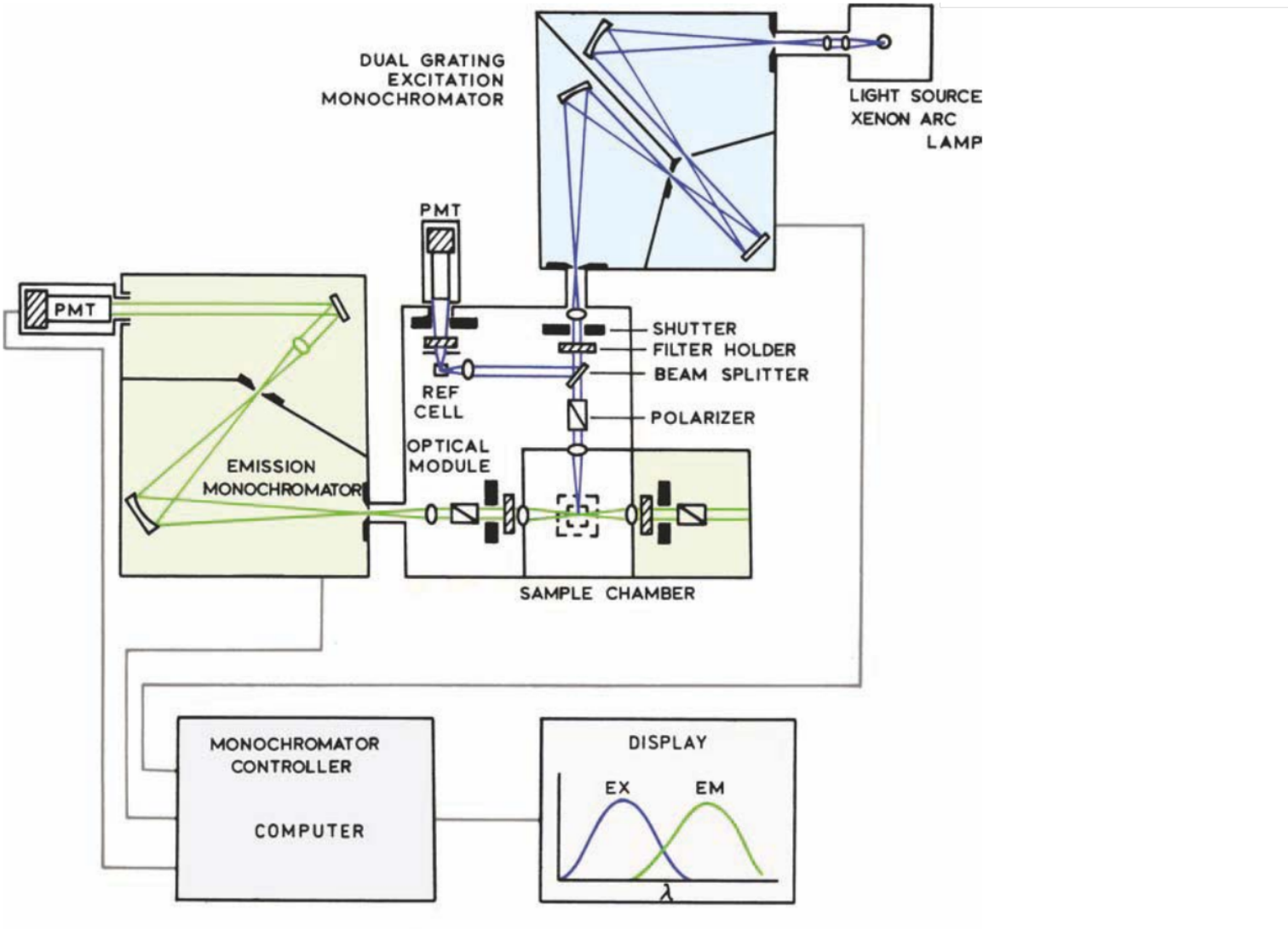


# Confocal Microscopy



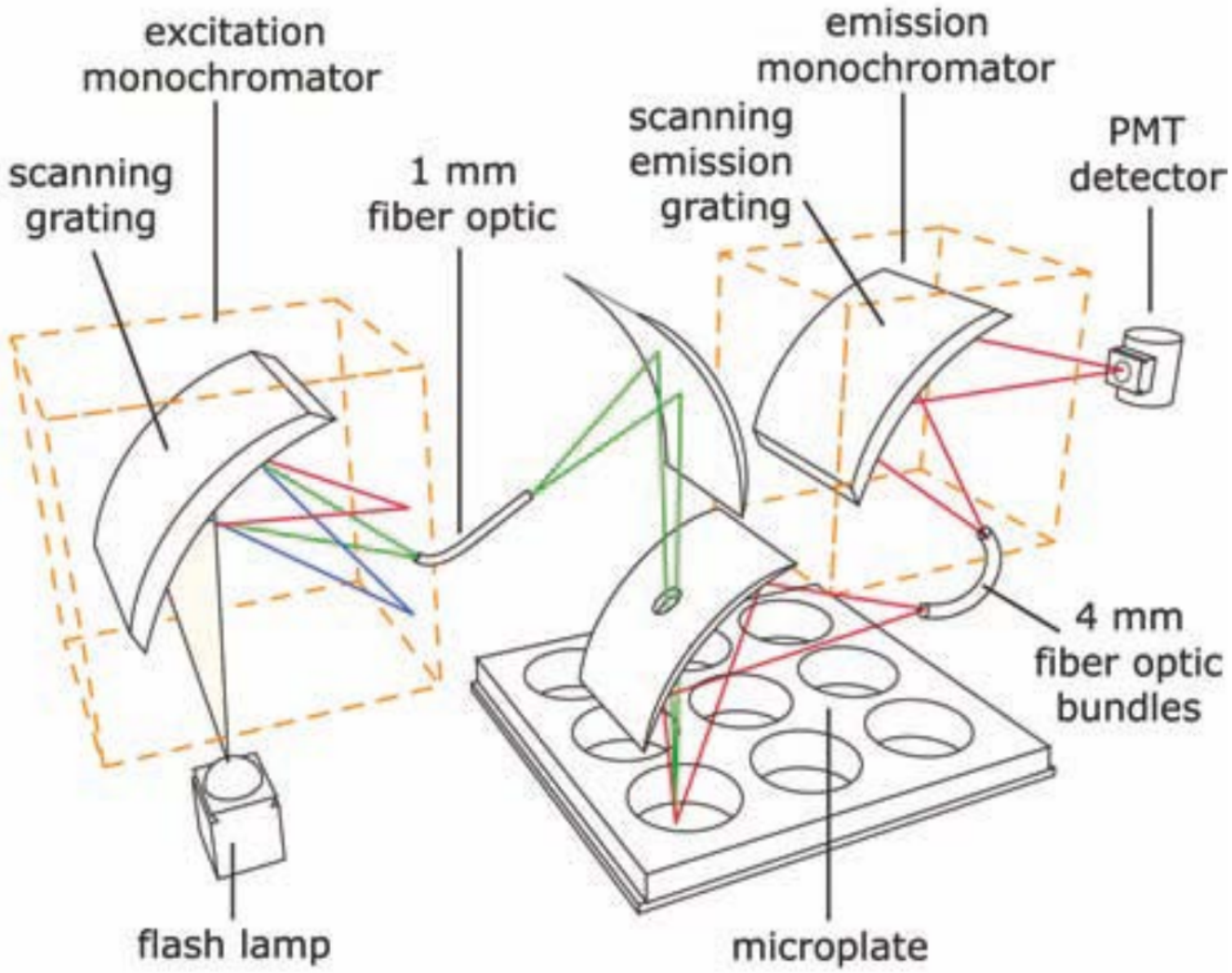

Lakowicz, J.R. Principles of Fluorescence Spectroscopy, 3rd edition, 2006, Springer

# Spectrofluorometer




Lakowicz, J.R. Principles of Fluorescence Spectroscopy, 3rd edition, 2006,Springer

# Plate Reader:




Lakowicz, J.R. Principles of Fluorescence Spectroscopy, 3rd edition, 2006, Springer