F. ELECTRONIC SPECTROSCOPY IN BIOCHEMISTRY

UV/Vis Spectroscopy

The most familiar technique in biophysical chemistry is UV/Vis spectroscopy. It reports on electronic transitions taking place in chromophores that occur naturally in the protein chain. The energy of these transitions is in the ultraviolet and visible region of the electromagnetic spectrum and it makes them amenable to study with relatively inexpensive instrumentation.

Electronic transitions

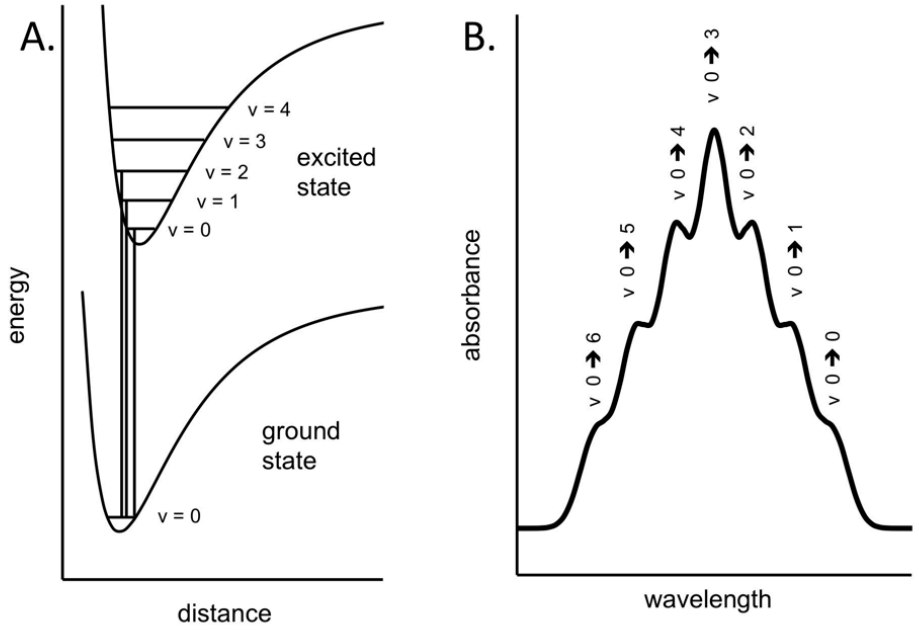


Figure F.1 A. Excitation from the ground state to the excited state of a molecule. Note that the energy "levels" are not level. The x-axis relates to molecular geometry, and the energy of the ground state varies as you apply more vibrational energy and distort geometry. Likewise, in the excited state, multiple vibrational energy levels (labeled ν=0, 1, 2…) mean that the excited state has no fixed energy either. (B) The absorption spectrum reflects the uncertainty of the transition. Starting the ν=0 vibrational state of the ground state, you can make the transition to any number of vibrational states in the excited state. That leads to a number of different wavelengths of light that can promote the transition. Hence the broad absorption band.

UV/Vis spectroscopy monitors the excitation of a molecule from a ground electronic state to an excited electronic state. A photon of an appropriate energy is absorbed and an electron is advanced from a low lying orbital to one higher in energy. In molecules the transition is from one molecular orbital (MO) to another – usually from the highest occupied MO (HOMO) to the lowest unoccupied MO (LUMO). Although only a single electron is changing orbitals, the redistribution of electron density of the molecule with the electron in the ground state to a molecule with an electron in a higher energy orbital changes the energy of the molecule as a whole. Thus, instead of talking about the energy of an individual electron, we talk about the energy of the molecule. It is the *molecule* that advances from the ground state to the excited state upon absorption of a photon. The absorption event is a quantum phenomenon, but unlike narrow atomic absorption bands, one obtains a broad absorption band. That is because the vibrational energy levels for a molecule are superimposed on the electronic energy levels. In OChem, you used low-energy infrared light to excite a molecule *vibrationally* but not *electronically*. In electronic spectroscopy, such as UV/Vis, you can't help but excite a molecule both electronically and vibrationally. Although a pair of electronic energy levels may be separated by a fixed gap, one has the possibility of exciting a molecule to a variety of different vibrational levels as well. The breadth of distribution in vibrational energy levels is seen in the breadth of the absorption band (Figure F.1).

Application of UV/Vis Spectroscopy to Proteins

There are several naturally occurring chromophores in proteins: the peptide bond, the aromatic rings of Phe, Tyr and Trp (and His to a lesser degree), and the disulfide bond in cystine linkages. The most common use of these chromophores is in determining the presence and/or quantity of protein present. In that respect, Tyr and Trp are the most important contributors to absorbance in the ultraviolet spectrum (Table F.1). With absorption maxima at 275 nm and 280 nm respectively, they lead to the common practice of using absorption of light at 280 nm to determine the quantity of protein present. UV/Vis spectroscopy plays additional roles when natural or artificial chromophores are present, but we won't get to that here. UV/Vis is not typically used for structural information, though occasionally there is information in the position of λ_{max} that can be valuable (see fluorescence spectroscopy below).

Table F.1. Spectroscopic properties of the aromatic amino acids.[a]

Amino Acid	**UV Absorbance**		**Fluorescence Emission**	
	□ max	**□ ($M^{-1}cm^{-1}$)**	**□ max**	**Quant. Yld.**
Phe	257 nm	197	282	0.04
Tyr	275 nm	1420	303	0.21
Trp	280 nm	5600	348	0.20

Fluorescence spectroscopy

Fluorescence

In UV/Vis spectroscopy, one monitors absorption of a photon. In most instances, the absorption of the energy provided by the photon heats the sample an no light escapes. For some select molecules, however, re-emission of a photon follows the absorption event. Fluorescence and phosphorescence are both examples of this phenomenon and together they are classified as luminescent phenomena. We will restrict our conversation to fluorescence, which is the most commonly used technique in

biophysical chemistry. In fluorescence, electronic excitation is accompanied by vibrational excitation as we saw in Figure F.1. If the lifetime of the excited state is long enough, the molecule may "relax" to the *ground* vibrational state of the *excited* electronic state. This is a small change in energy relative to the difference in electronic energy states and can be achieved simply by transferring vibrational energy through collision with another molecule. Some of the energy of the incident photon has been lost as heat, but not all of it. If, now, the remaining energy is lost as a photon in what is called *radiative emission*, a photon of longer wavelength that the incident photon is emitted and can be observed. That is fluorescence. A common example is in Day-Glo pigments. They excited in the near UV (light just below 400 nm in wavelength) and emit in the visible (at wavelengths greater than 400 nm). This effect has been particularly entrancing to 8th graders for as long as I can remember.
Here is a step-by-step description, with simple diagrams to show how fluorescence works.

• A **fluorophore** is a molecule that exhibits fluorescence. In the absence of visible light, the molecule resides in the ground electronic state (S_0; S means the molecule is a singlet – it has all paired electrons) and the ground vibrational state. The green dot is intended to show that position.

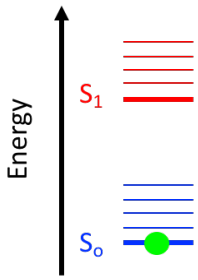


Figure F.2. Fluorophore in the ground state.

• A short wavelength of light irradiates the fluorophore and **excites** the molecule an excited vibrational state within the excited electronic state (S_1).

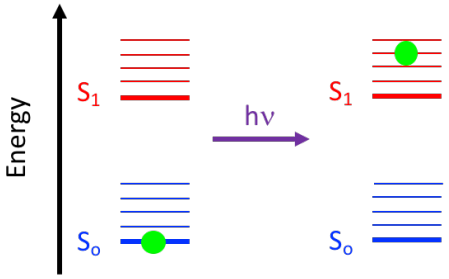


Figure F.3. Fluorophore is excited by a short wavelength photon (purple, because that's a high energy visible photon).

• The molecule undergoes vibrational relaxation, through molecular collision or some other non-radiative process so that it is in a lower energy vibrational state than following initial excitation.

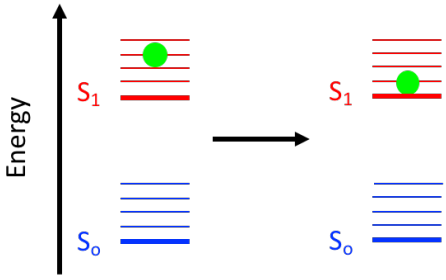


Figure F.4. Vibrational relaxation of an excited fluorophore.

• The vibrationally relaxed but electronically excited fluorophore can now return to the ground state via **emission** of a long wavelength photon (orange, because that's a longer wavelength color that purple – I am trying to be consistent).

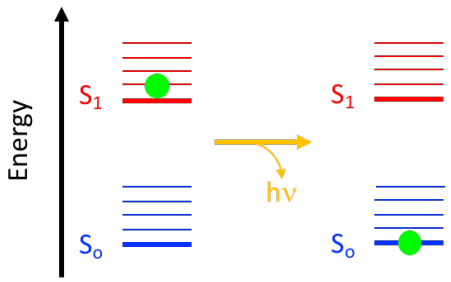


Figure F.5. Now the molecule drops from the lower energy vibrational state of S_1 back to the ground state. A longer wavelength photon is emitted.

Biochemistry uses a relatively limited range of core molecular structures as the basis for fluorophores. The thing they share in common is planar, aromatic structure. The general explanation is that the rigidity of the aromatic ring system prevents the molecules from decaying to the ground electronic state through loss of energy via vibrations.

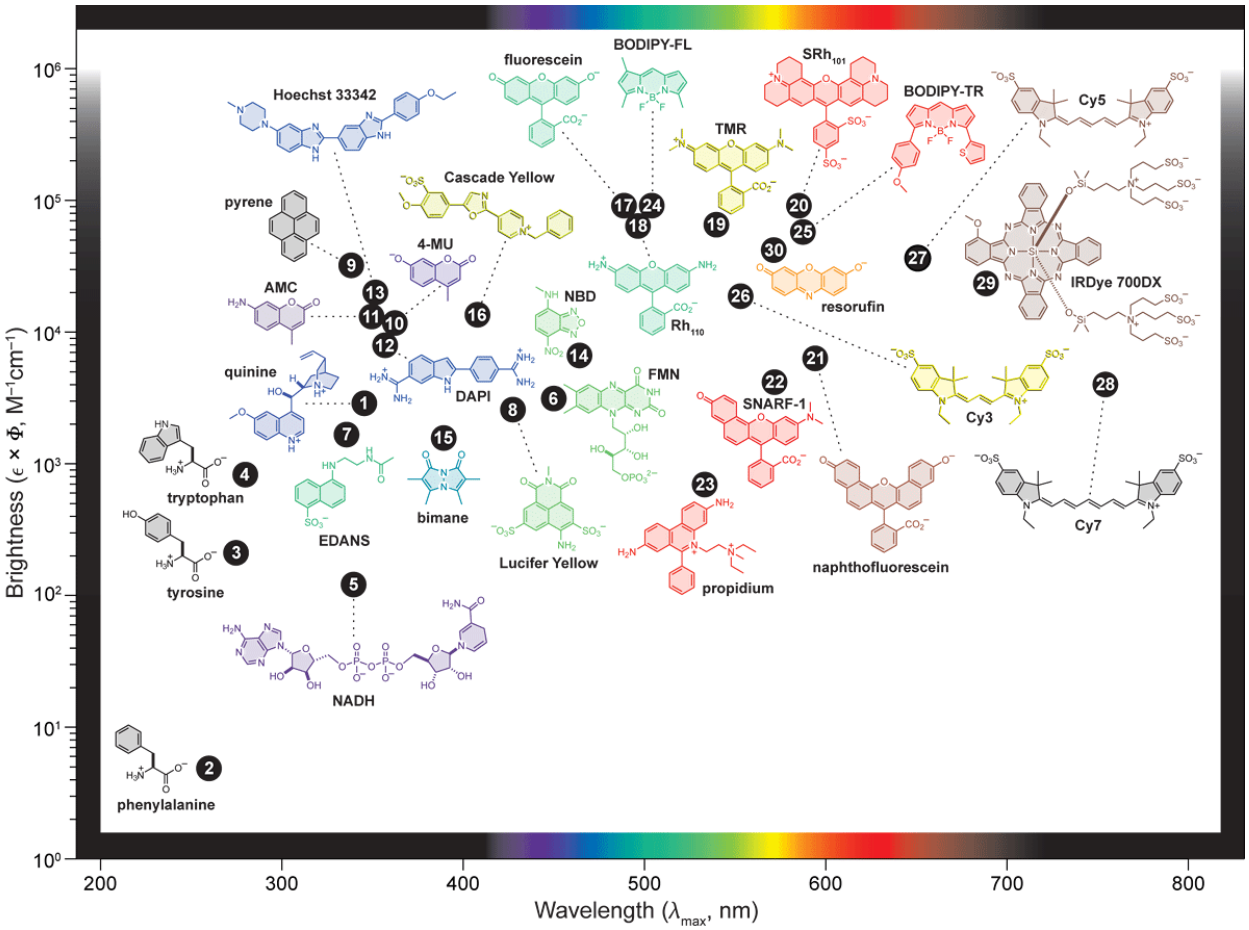


Figure F.6. Some dyes used in biological fluorescence spectroscopy. They are arranged by the wavelength of emission on the x-axis and their intensity of fluorescence on the y-axis (https://www.hhmi.org/research/tailoring-fluorescent-molecules-biological-applications).

Fluorescence Spectroscopy in Proteins

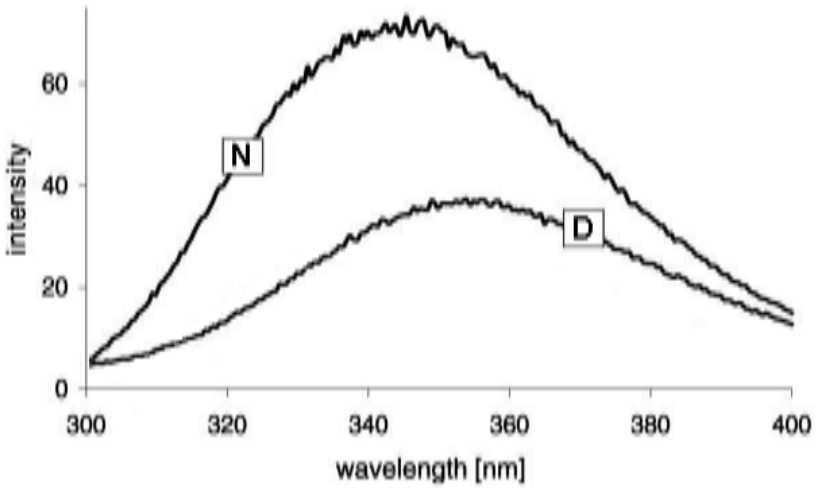
Fluorescence can be an exceptionally useful tool in studying protein structure, because the local chemical environment can lead to significant changes in the intensity of fluorescence, as well as smaller changes in the spectrum of the emitted light. As luck would have it, both Tyr and Trp are fluorescent species, and it is possible to do fluorescent spectroscopy on virtually any protein, though the enhanced fluorescence of Trp makes it a particularly valuable tag.

Intrinsic fluorescence spectroscopy (that is – using Trp and Tyr as fluorophores) is most commonly used to investigate changes in tertiary structure within proteins. As non-polar residues, Trp and Tyr typically occupy positions within or near the hydrophobic core. If the tertiary structure of the protein is disrupted, the residues become exposed to polar solution. As a result there will be **changes** in the fluorescence spectrum. Note the emphasis on the word "changes". It is unnecessary in most instances to understand the exact cause of the change, so long as you can be confident that the change in the fluorescence signal accompanies a change in tertiary structure.

Two key elements of the fluorescence spectrum are typically monitored for changes (Figure F.7). The λ_{max} of fluorescence emission is often affected by environment. The ground states of Trp and Tyr are non-polar, but their excited states are polar. In a non-polar environment, the gap between ground and excited state is typically maximized because you are creating a polar species in an environment that is poorly suited to stabilize it. Thus, there is a "blue shift" in fluorescence – the emission spectrum has a shorter λ_{max} for buried Trp and Tyr residues than for those exposed to solution. The difference is typically only 5-10 nm, but it can be a useful signal.

The other chief impact of structural changes on fluorescence is intensity. When Trp or Tyr residues exposed to solution, they fluoresce less efficiently than those buried from solution. This is due to "collisional **quenching**". While the mechanism is not entirely clear, there are a number of dissolved species in water (most notably O_2) that can collide with fluorophores in the excited state and relax them to the ground state without fluorescent emission. When a Trp residue becomes buried in the interior of a protein (say during protein folding), the fluorescence of the protein increases. Fluorescence is a useful probe of the folded structure of proteins.

Figure F.7. Typical fluorescence emission spectra of the native (N) and denatured (D) forms of a protein. The sample was excited at 280 nm. Note that the λ_{max} for the emission of the native form is at a shorter wavelength by roughly 10 nm and is more intense.



Förster Resonance Energy Transfer (FRET)

Monitoring the distance between biological objects – molecules or even cells – is an important goal in much of biology and biochemistry. Fluorescence is an important tool for that process. Förster resonance energy transfer (FRET) allows one to observe the close approach of two objects if they are labeled with two different fluorophores. One will be labeled with a **donor** that is excited at a short wavelength and the other is an **acceptor** that is excited at a longer wavelength, that overlaps with the wavelength of emitted light from the **donor** (Figure F.7).

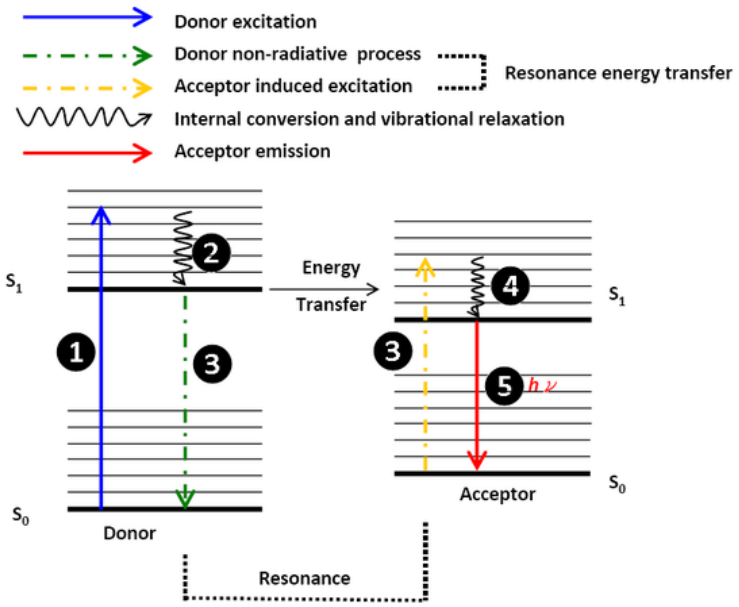


Figure F.7. Donor excitation (**step 1**) is accomplished with short wavelength light. The donor then relaxes (**step 2**) and by non-radiative transfer of a "medium" amount of energy through space to the acceptor, which is excited (**step 3**). The donor is now in the ground state, but the acceptor is in the excited state. The acceptor then relaxes vibrationally (**step 4**) and subsequently emits a long wavelength photon (**step 5**). FRET can be observed either as the loss of emission from the donor, which is no longer emitting medium wavelength photons, or as the gain of emission from the acceptor, which is remarkable, since you are not exciting it with medium wavelength light! (From chem.libretexts.org).

It is important to note that there is a distance dependence for that transfer of energy. For each dye you might use as a fluorophore, there is a characteristic Förster distance (R_o) and the efficiency of energy transfer (E) is determined as shown in equation F.1, where r is the distance between the donor and acceptor, which means that if r is significantly greater than R_o, no transfer will be observed. . R_o is often in the range of 10-50 Å, which proves to be useful in measuring the close approach of biological macromolecules

$$E = \frac{1}{1+(r/R_o)^6} \qquad \text{Eq. F.1}$$