UNCC Biotechnology and Bioinformatics Camp

Dr. Jennifer Weller

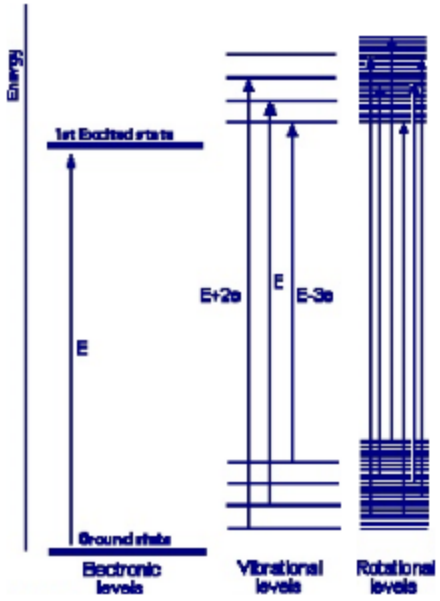
Summer 2010

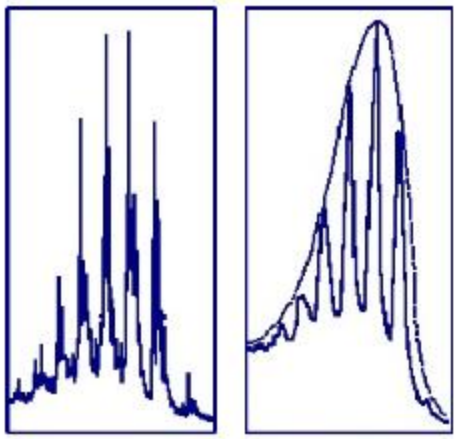
Contents

- Spectrophotometry
- Fluorescence Spectrophotometry
- Spectrophotometry Platforms

Energy states in Molecules

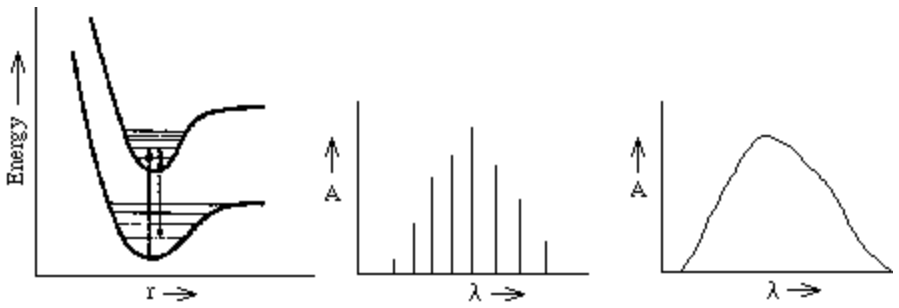
- Molecules have different states characterized by differences in energy of electron orbitals.
- Light has energy, characterized by the frequency or wavelength. (e = hν)
- Electrons can jump between orbitals if the energy provided matches the energy difference in the orbitals, changing the state of the molecule.
  - This energy can be released by electron decay to the first orbital, with photon release, or by transfer to another molecule.
  - For proteins and nucleic acids, photons in the UV and near-UV-visible range have the right energy to be absorbed.
  - Because of the many vibrational energy levels in complex biomolecules there are many absorption peaks of similar but not identical energy – when summed they give a broad peak.

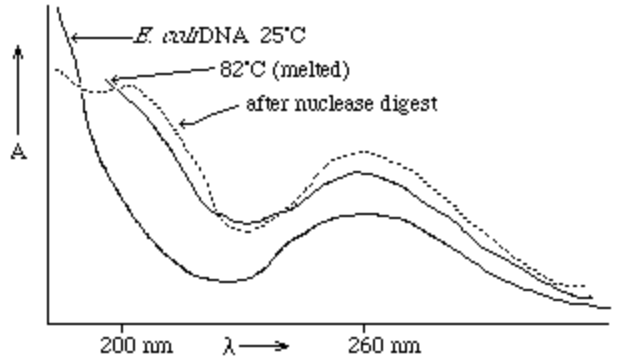




Absorbance spectrum

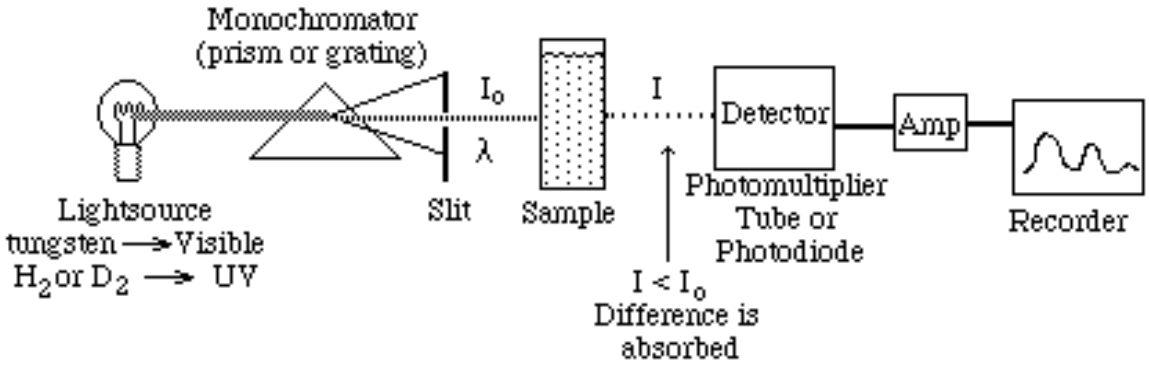
- For light passing through a solution of identical molecules (randomly oriented), the rate at which photons are absorbed is a function of the distance (l) through the sample holder (a cuvette), proportional to the concentration, at a rate having a constant called epsilon (ε).





Spectrophotometry

- Light can be absorbed, transmitted, scattered, reflected, or excite fluorescence.
- A spectrophotometer is an instrument with settings to select light of a desired wavelength from a source, passing only that wavelength through a sample; it has detectors to measure the amount of that a wavelength that reaches the detector.

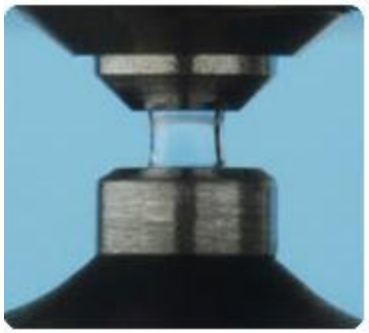


Types of Experiments

- Determine what wavelengths of light a sample absorbs and transmits
  - An absorbance spectrum
- Determine how much of a molecule is in a solution through which light of a certain wavelength is passing.
  - Use Beer's Law to calculate the concentration
    - $A_\lambda = \varepsilon c l$

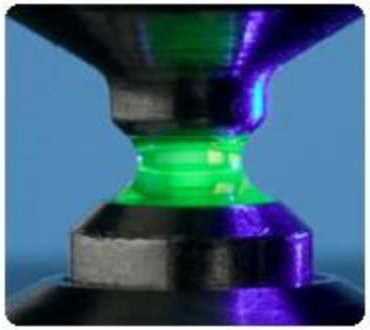
Simulations using spectrophotometers

- A virtual lab showing concepts important to spectrophotometry, including taking an absorbance spectrum, or light-responsive fingerprint of a molecule across a group of wavelengths: http://www.chm.davidson.edu/vce/spectrophotometry/
- Although we will be using different platforms, there is an animation of common experimental steps here: http://ncbionetwork.org/spectrophotometer/
- A video (in two parts) of a biochemistry lab measuring Riboflavin http://carrollslab.blogspot.com/2008/08/biochem-lab-spectrophotometry-of.html



Increasing Detection Sensitivity and Specificity for NAs

- Specificity enhancement
  - Use dyes that
    - interact specifically with single or double-stranded nucleic acid
    - specifically with either DNA or RNA
- Sensitivity enhancement
  - Use dyes with a high quantum yield.
    - Minimize the NA contribution at the emitted wavelength.

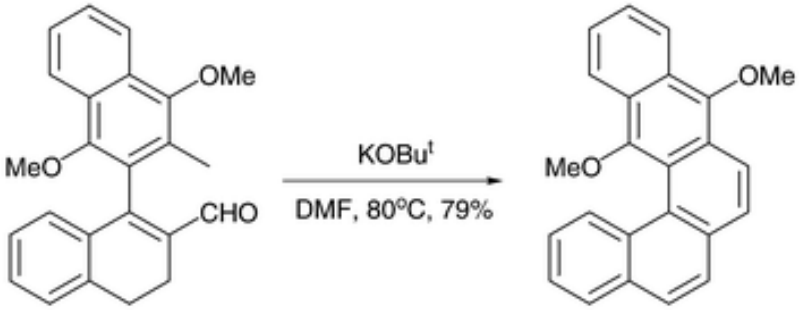


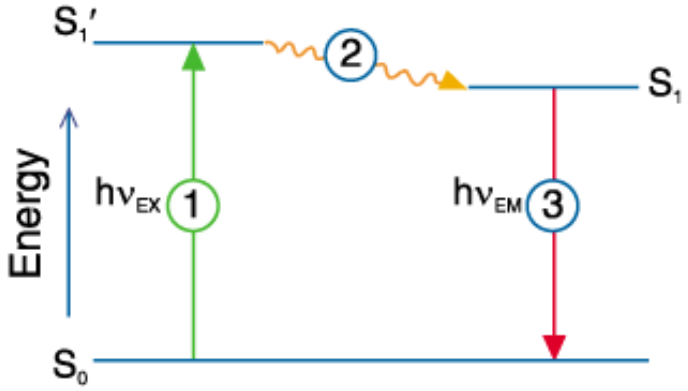
Fluorometry

- Spectrophotometry is a direct assay – a characteristic of the molecules is measured directly.
  - Not always sufficiently sensitive.
- A fluorometer allows indirect assays
  - Associate the MOI with a dye, excite the dye and detect its emitted photons.
  - Ideal: high affinity and binding constant, photons emitted only when bound to MOI, simple binding mechanism and binding ratio.
- For very small amounts of DNA a fluorometer gives a more sensitive reading of the concentration.
  - Fluorometers measure fluorescence in relative rather than absolute units.
    - Thus, after zeroing with a “blank”, always begin an assay by calibrating the instrument to display the known concentration of a standard solution.

Fluorescence

- Fluorescence is the result of a 3-stage process occurring in molecules with certain characteristics
  - Polyaromatic hydrocarbons
  - Heterocyclic compounds

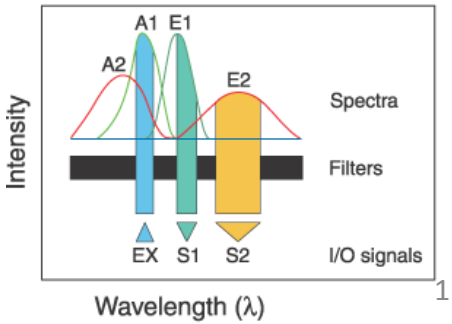




Jablonski diagram

3 stages of Fluorescence

- Stage 1: excitation, where a photon (energy $h\nu_{EX}$) is supplied (from incident light or an energy source)
  - The fluorophore absorbs the photon, an electron is raised to a singlet state (S_1')
- Stage 2: S_1' is short-lived (1-10 nsec), the electron decays to S_1, dissipating energy.
  - The fluorophore undergoes conformation changes and interacts with the environment – a photon may be released or the energy may be lost.
- From the relaxed singlet state S_1 the electron drops back to S_0, the stable ground state, releasing the energy as a photon
  - The energy of the photon is less, so the wavelength of emitted light is longer. The difference between the excited and emitted wavelengths is called the Stokes shift

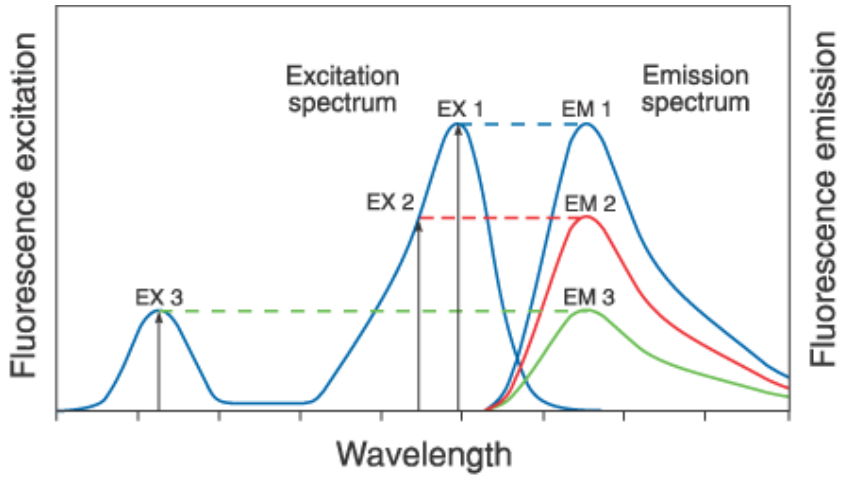


Instrumentation/Detection

- Instruments require
  - Excitation energy source
  - Responsive molecule (dye = fluorophore)
  - Filters to discriminate the different wavelengths of light
  - A detector of the emitted photons with a recorder for output values
- Fluorescent intensity also obeys the Beer-Lambert law, $I = \varepsilon l c$
  - Factors that must be considered include:
    - The quantum yield (QY) of the fluorophore
    - The intensity of the excitation source
    - The collection efficiency of the detector
- The emission intensity is linearly proportional to the amount of dye when
  - The concentration is low (dilute solution; the absorbance should be <0.05)
  - Since the value is detector-dependent, calibration standards are needed.

Fluorescence Spectra

- The excitation, decay and emission process occurs in repeating cycles → the sensitivity of the method.
- For complex molecules in solution you get vibrational states affecting the process, so you get broad bands of both excited and emitted light.
  - The excitation band (for a single molecule in dilute solution) is identical to the absorption spectrum
  - The breadth of the bands is important when choosing multiple dyes



Peak characteristics

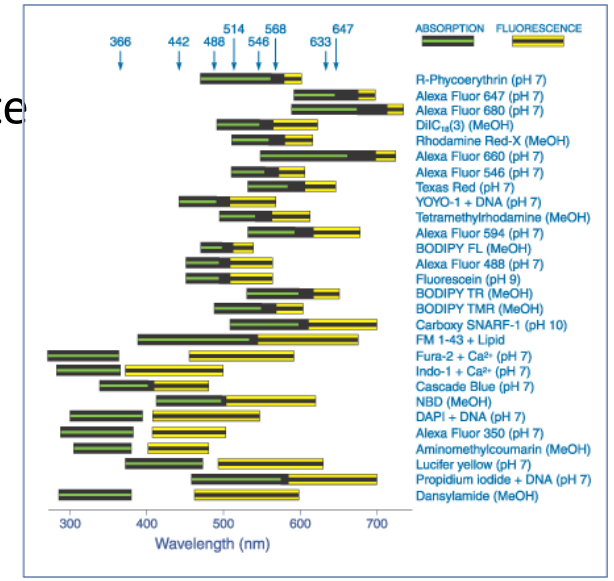
- If the solvent/temperature/concentration stays the same
  - the position and shape of the emission spectrum is independent of the excitation spectrum
  - The intensity (amplitude) of the emission is proportional to the amplitude of the excitation intensity at the maximum.

Fluorescence Detection Sensitivity

- Background sources
  - Autofluorescence from the sample (eg the bases in DNA)
  - Unbound or non-specifically bound probe
- Loss of signal can photobleaching (destruction of the molecule)
    - Starts at the triplet excited state: inter-system crossing occurs from S_1
- The fluorescent quantum yield: the ratio of the number of photons emitted in Stage 3 to the number effectively absorbed in Stage 1.

Selecting Fluorophores

- The instrument and attachment chemistry will constrain the choice of dyes. Measures to consider include
  - Fluorescent output: the efficiency of absorption and emission and the cycling rate
  - These measures are valid for defined conditions (solvent, temperature and wavelength)
    - The molar extinction coefficient of absorption
    - The quantum yield
  - The I_{fl} (per molecule) α [ε*QY]
    - Ranges are 5000-200,000 $cm^{-1}M^{-1}$ for ε
    - 0.05-1.0 $A_{h\nu}$ for QY



Rules for Fluorescent NA quantification

- Be sure that you are in the *linear detection range* for the instrument
  - Perform a series of dilutions, with replication, to make sure you have a reliable value.
  - the effective range for Absorbance is usually 0.05 – 1.0
  - You can prepare a standard curve to validate instrument function, sensitivity and range where the Beer-Lambert law is obeyed.
- Place a cuvette in the light beam that has only the solvent and adjust the output to zero
- Replace the solution with the sample, place in the light beam and record the intensity – most instruments subtract the incident from transmitted light so you actually get the change

Measurement Platforms

- Nanodrop platforms
  - http://www.nanodrop.com/?gclid=CIHDvsT1rp8CFaM45QodrWi60w
  - There is a scientific video on Jove, showing the use of the Nanodrop for protein measurements here http://www.jove.com/index/Details.stp?ID=1610
  - There is a Technology Overview posted on the Readings/Equipment section of the lab Web page.
- BeckmanCoulter DU 800 platform: the manual is provided under the Readings section of the Lab Web site, under Equipment.
- Molecular Devices SpectraMax Plus 384 UV-Vis absorbance plate reader: there are two Webinars produced by this supplier, linked off the Readings/Equipment section that are very informative – the Assay one presents good information about DNA/RNA detection.

Steps in Use

1. Turn on the software and the light source
2. Wipe the detection surface with a Kimwipe
   that has a little 80% ethanol on it.
3. Pipette 1.5ul of your sample on the pedestal
   (base).
   Make sure there is not an air bubble.
4. Close the arm (this forms a liquid column)
   The pedestal moves automatically to
   optimize the pathlength
5. A measurement is taken and reported on the
   screen – note the value in your lab
   book.
6. Open the arm and either pipette off the
   solution or simply blot it away with a
   Kimwipe.
7. Repeat at least once for each sample.
   If measurements are very difference,
   repeat until you get a consistent
   reading.