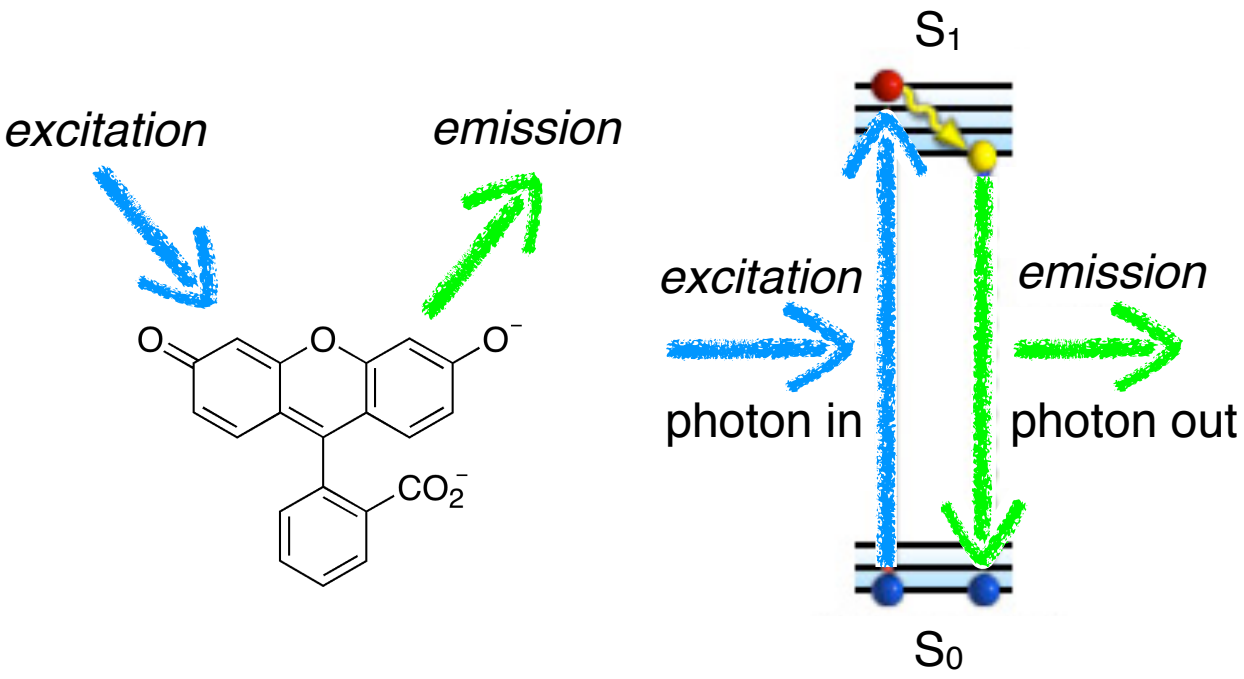
# Concept review: Fluorescence

Some definitions:

- Chromophore. The structural feature of a molecule responsible for the absorption of UV or visible light.
- Fluorophore. A chromophore that remits an absorbed photon at a longer wavelength.
- Extinction coefficient (ε). The absorbance (=-log($I_{out}/I_{in}$) of light of a particular wavelength by 1 cm of a 1 M solution of a chromophore (units of $M^{-1}cm^{-1}$).
- Quantum Yield. The fraction of absorbed photons that are reemitted by a fluorophore.

# Fluorescence

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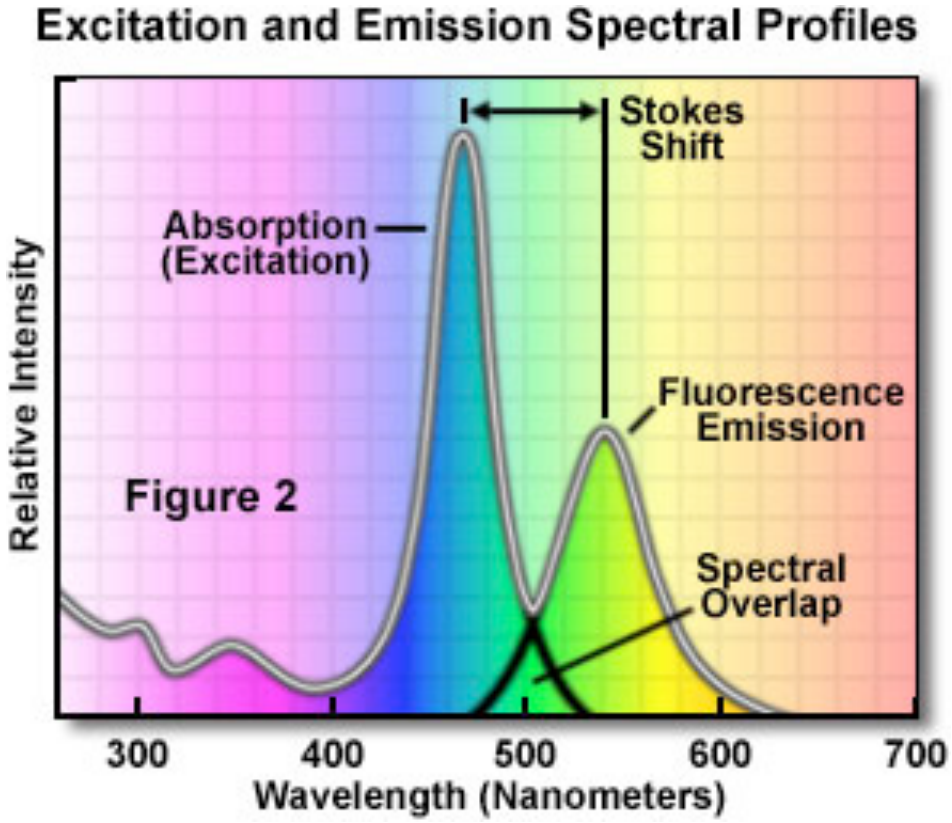
The process responsible for the fluorescence of fluorescent probes and other fluorophores is illustrated by a Jablonski diagram.

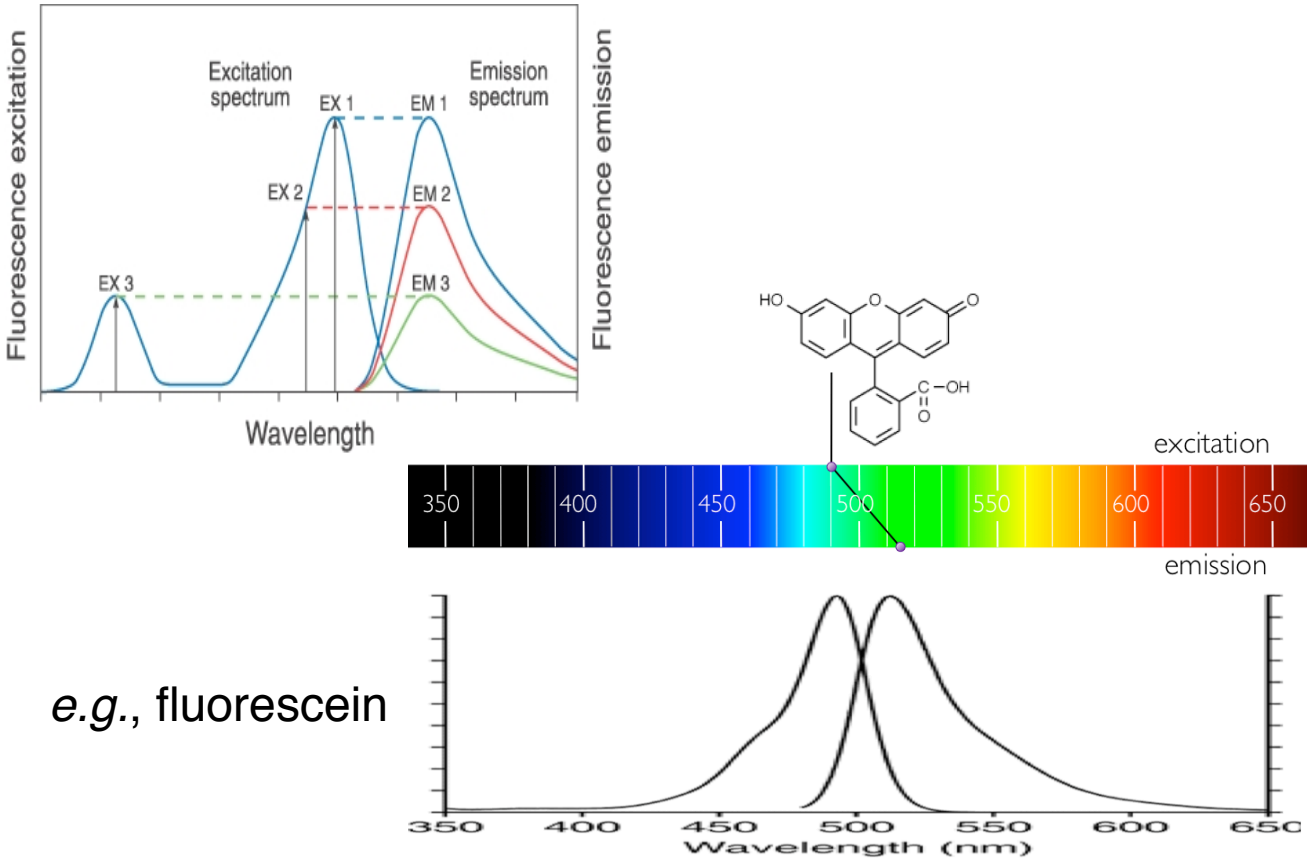
- **Absorption**. A photon is absorbed by the fluorophore, creating an excited electronic singlet state ($S_1$). This process distinguishes fluorescence from chemi- or bioluminescence, in which the excited state is populated by a chemical reaction. The initial excitation may result in the molecule in a higher energy vibrational state.
- **Excited-State**. The excited state exists for a finite time (typically 1–10 nanoseconds). During this time, the fluorophore undergoes conformational changes and the energy of $S_1$ is partially dissipated by the vibrational relaxation. Fluorescence emission originates from the lowest energy vibrational state of $S_1$. Processes such as collisional quenching, fluorescence resonance energy transfer (FRET) and intersystem crossing may depopulate $S_1$.
- **Fluorescence Emission**. A photon of energy is emitted, returning the fluorophore to its ground state $S_0$. Due to energy dissipation during the excited-state lifetime, the energy of this photon is lower, and therefore of longer wavelength, than the excitation photon. The difference in energy or wavelength represented by the absorbed and emitted photon is called the Stokes shift.

# Fluorescence Spectra




- The entire fluorescence process is cyclical. Unless the fluorophore is irreversibly destroyed in the excited state (an important phenomenon known as photobleaching), the same fluorophore can be repeatedly excited and detected. The fact that a single fluorophore can generate many thousands of detectable photons is fundamental to the high sensitivity of fluorescence detection techniques.
- http://micro.magnet.fsu.edu/primer/lightandcolor/fluorescencehome.html
- http://probes.invitrogen.com/handbook/sections/0001.html

# Fluorescence spectra




*e.g.*, fluorescein

Image source: http://www.probes.com/handbook/sections/0001.html

- For polyatomic molecules in solution, the discrete electronic transitions of the previous slide are replaced by rather broad energy spectra called the fluorescence excitation spectrum and fluorescence emission spectrum, respectively. The bandwidths of these spectra are parameters of particular importance for applications in which two or more different fluorophores are simultaneously detected.
- With few exceptions, the fluorescence excitation spectrum of a single fluorophore species in dilute solution is identical to its absorption spectrum.
- Generally speaking, the fluorescence emission spectrum is independent of the excitation wavelength , due to the partial dissipation of excitation energy during the excited-state lifetime. This is known as Kasha's rule after Michael Kasha. The emission intensity is proportional to the amplitude of the fluorescence excitation spectrum at the excitation wavelength. Simply put, the greater the number of molecules that absorb a photon, the greater the number of molecules that will emit a photon as fluorescence.

# Lifetimes of excited state processes

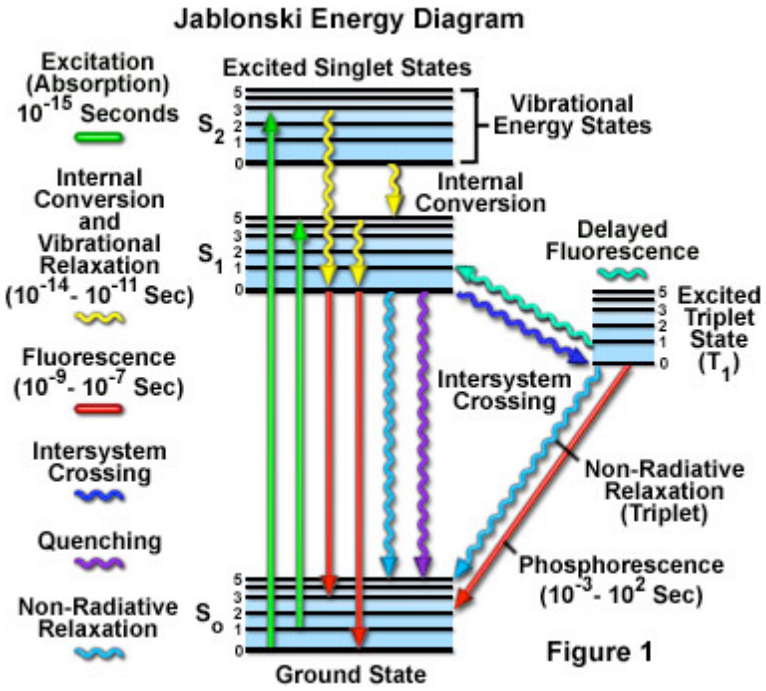
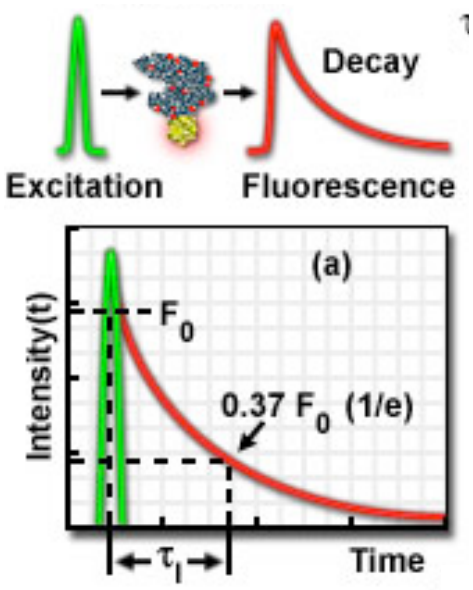
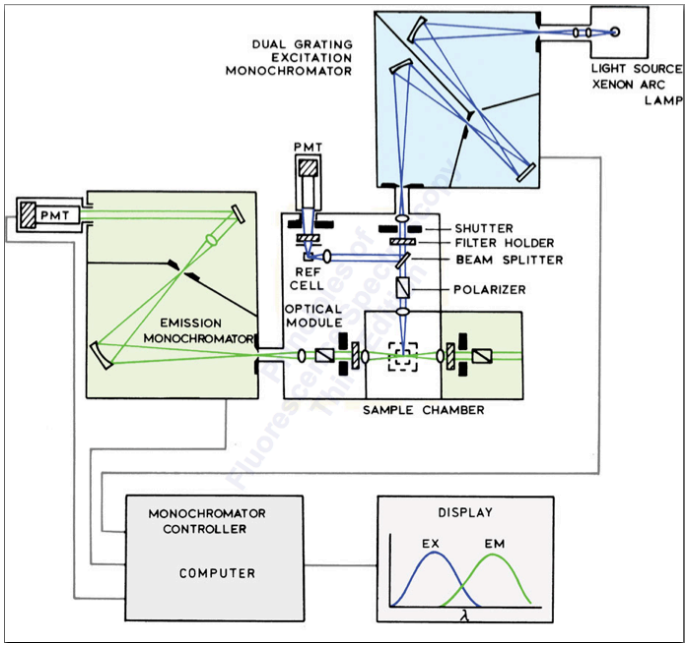



Figure 1

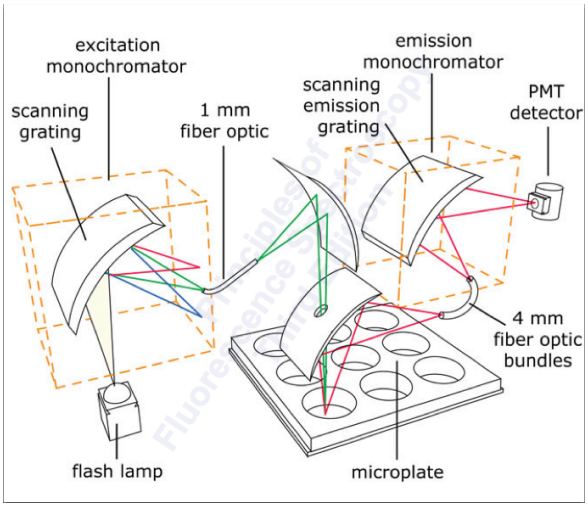



- A key feature of fluorescence is that the molecule spends a measurable amount of time in the singlet excited state. This time is typically in the range of 1-10 ns.
- A number of different things can happen to molecule while it is in the excited state. Fluorescence is, of course, one thing that can happen to the molecule. Other ways of depopulating the excited state include non-radiative relaxation (essentially an internal conversion from $S_1$ to $S_0$) or quenching or intersystem crossing to a triplet state.
- If a triplet state is formed it can emit a photon through the process of phosphorescence or it can non-radiatively relax.

# Instrumentation for detecting fluorescence



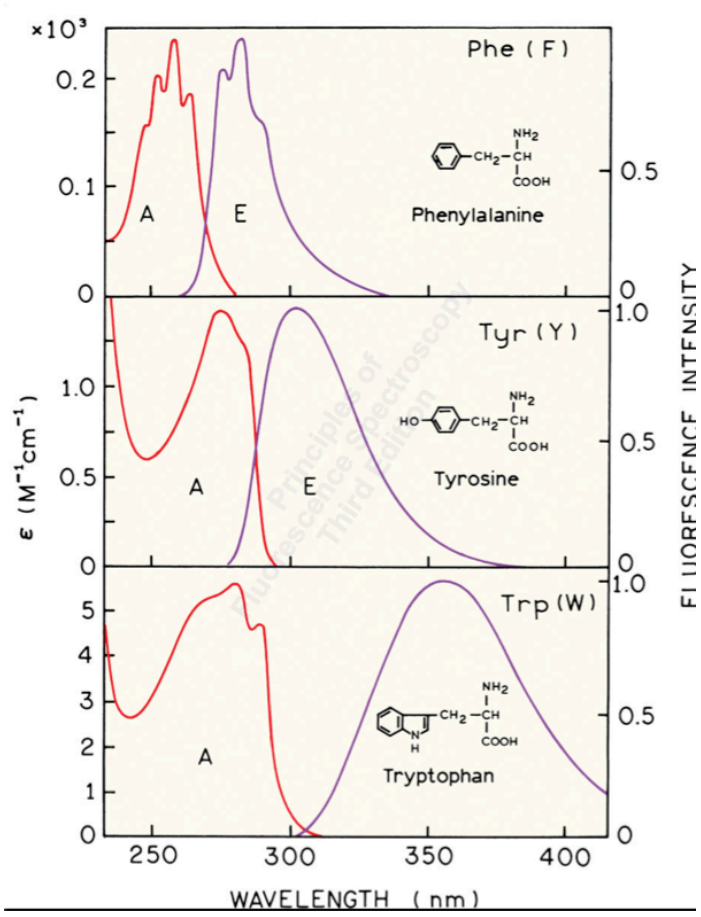

A spectrofluorometer

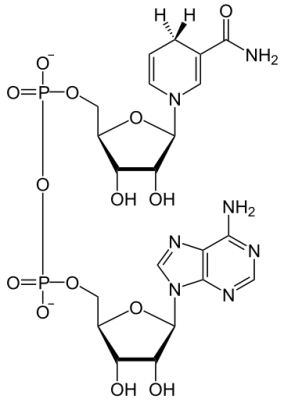



A monochromator-based platereader

- Spectrofluorometers are the most common instrument for measuring of fluorescence. Essentially, they are instruments that are similar to UV-vis spectrophotometers in design except that the emission detector is positioned at a 90 degree angle from the direction of the excitation source.
- It is also quite common to detect fluorescence using a platereader type device. For this type of device, the cuvette is replaced with a microplate with perhaps 96 or 384 (or more) wells on it. Both excitation and collection of emission occur from the same direction. Most instruments could measure from either the top or the bottom (assuming that the bottom of the plate is clear plastic or glass).
- The third common class of instruments for detection of fluorescence are confocal or widefield fluorescence microscopes.

# Intrinsic fluorophores for bioanalytical applications

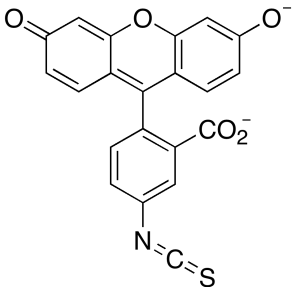


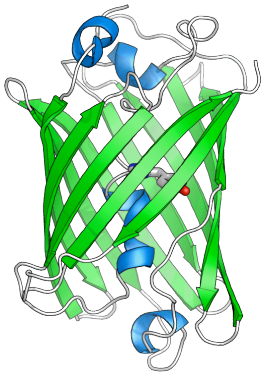



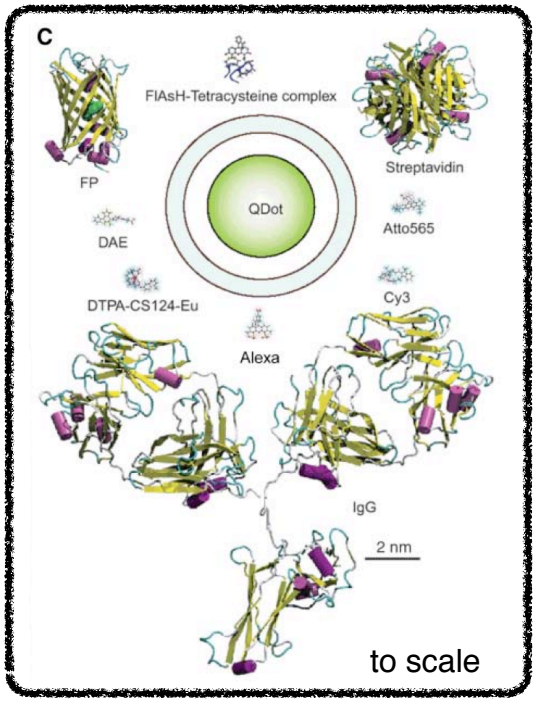
NADH (absorbs ~340 nm, emits at ~460 nm)

- The most useful fluorophores for bioanalytical purposes can be divided into the categories of intrinsic and extrinsic fluorophores.
- **Intrinsic fluorophores** are naturally occuring in the sample to be measured. These would the fluorescent amino acids as well as other common cofactors, such as NADH, that are inherently fluorescent.
- Source: Lakowicz, Principles of Fluorescence Spectroscopy, 3rd Edition. NADH structure from Wikipedia.

# Extrinsic fluorophores for fluorescence applications





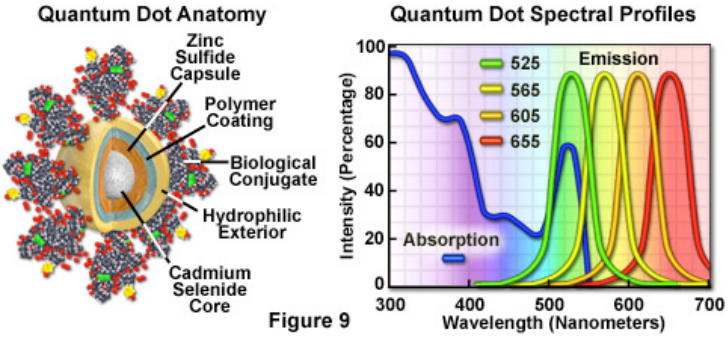




Figure 9

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Giepmans et al. 2006 *Science*. **312**: 217-224.

- **Extrinsic fluorophores** are fluorescent molecules that have been added to the sample by the experimenter. These would include synthetic dyes such as fluorescein, fluorescent proteins, and quantum dots.
- It is fair to say that all of these different fluorophores are associated with specific advantages and disadvantages. The choice of which type of fluorophore to use for a particular experiment requires one to consider all of these factors. Here is a brief overview:
  - Intrinsic fluorophores have the advantage of being naturally present. The disadvantages are that they are relatively dim and high energy.
  - Synthetic dyes come in a wide variety of colors and brightnesses. The disadvantage is that they must be covalently attached to the biomolecules to be detected, and this presents additional challenges.
  - Fluorescent proteins come in a smaller selection of colors than synthetic dyes, but can be attached to proteins using molecular biology.
  - Quantum dots come in a wide variety of colors and are very bright. The primary disadvantage is that they are large and do not allow for monovalent attachment to the biomolecule of interest.