Absorption

- Basic quantum mechanics requires that molecules absorb energy as quanta (photons) based upon a criteria specific for each molecular structure
- Absorption of a photon raises the molecule from ground state to an excited state
- Total energy is the sum of all components (electronic, vibrational, rotational, translations, spin orientation energies) (vibrational energies are quite small)
- The structure of the molecule dictates the likely-hood of absorption of energy to raise the energy state to an excited one

Lifetime

- Absorption associated with electronic transitions (electrons changing states) occurs in about 1 femtosecond (10^{-15} s)
- The lifetime of a molecule depends on how the molecule disposes of the extra energy – but many fluorophores have lifetimes around 5 ns
- Because of the uncertainty principle, the more rapidly the energy is changing, the less precisely we can define the energy
- So, long-lifetime-excited-states have narrow absorption peaks, and short-lifetime-excited-states have broad absorption peaks

Extinction

- Using Beer's law (Beer-Lambert law) for light travelling through a curvette thickness **d** cm containing **n** molecules/cm^3

  $\ln (I_o/I) = \sigma n d$

  where I_o and I are the light entering and leaving and σ is the molecular property called the absorption cross section

- Now we can state that

  $\ln (I_o/I) = \alpha n d$ where C is the concentration and a is the absorption coefficient which reflects the capacity of the absorbing substance to absorb light

- If there are n (molecules/cm^3 ; d in cm, σ must be in cm^2 so if α is in cm^2/mol, C must be in mol/cm^3 do $C=a/10^3$

- giving

  $\log10 (I_o/I) = \varepsilon \alpha d = A$

  where A is the absorbance or optical density

  and ε is the decadic molar exctinction coeficient in $dm^3mol^{-1}cm^{-1}$

4th Ed. Shapiro p 111

Absorbance

- O.D. units or absorbance is expressed in logarithmic terms so they are additive.
- e.g. an object of O.D. of 1.0 absorbs 90% of the light. Another object of O.D. 1.0 placed in the path of the 10% of the light 10% of this light or 1% of the original light is transmitted by the second object
- It is possible to express the absorbance of a mixture of substances at a particular wavelength as the sum of the absorbances of the components
- You can calculate the cross sectional area of a molecule to determine how efficient it will absorb photons. The extinction coefficient indicates this value

4th Ed. Shapiro p 111

Parameters

- Extinction Coefficient
  - ε refers to a single wavelength (usually the absorption maximum) the cross sectional area of a molecule determines how efficient it will absorb photons
- Quantum Yield
  - Q_f is a measure of the integrated photon emission over the fluorophore spectral band
- At sub-saturation excitation rates, fluorescence intensity is proportional to the product of ε and Q_f

Fluorescence

- **Quantum Yield**

$$Q = \frac{\text{photons emitted}}{\text{photons absorbed}} = \frac{k_r}{k_r + k_{nr}}$$

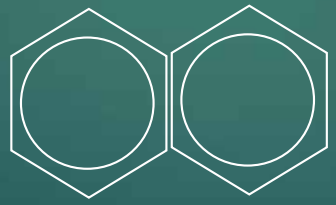
- **Fluorescence Lifetime (τ)**

  - is the time delay between the absorbance and the emission

$$\tau = \frac{1}{k_r + k_{nr}}$$

Fluorescence

- Excitation Spectrum
  - Intensity of emission as a function of exciting wavelength (this is the absorbance component)
- Chromophores are components of molecules which absorb light
- They are frequently aromatic rings

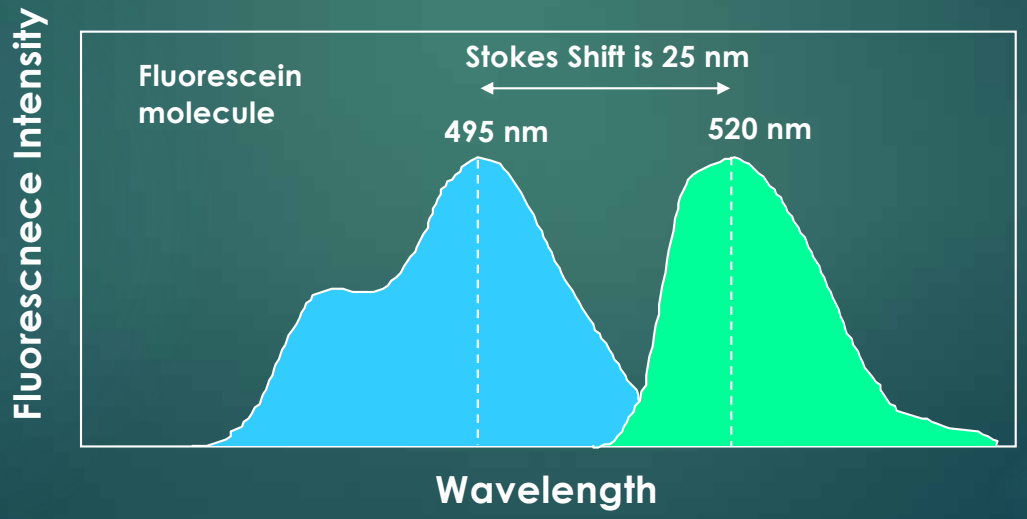


Fluorescence

- The wavelength of absorption is related to the size of the chromophores
- Smaller chromophores, higher energy (shorter wavelength)

Fluorescence

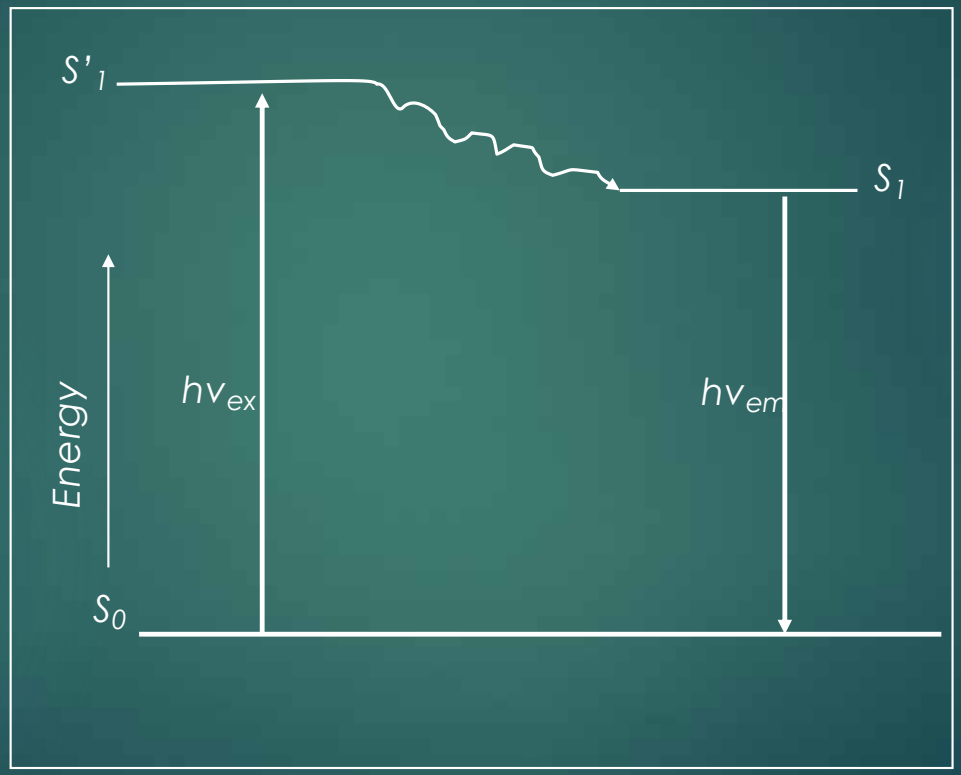
- Stokes Shift
  - is the energy difference between the lowest energy peak of absorbance and the highest energy of emission



Properties of Fluorescent Molecules

- *Large extinction coefficient at the region of excitation*
- *High quantum yield*
- *Optimal excitation wavelength*
- *Photostability*
- *Excited-state lifetime*
- *Minimal perturbation by probe*

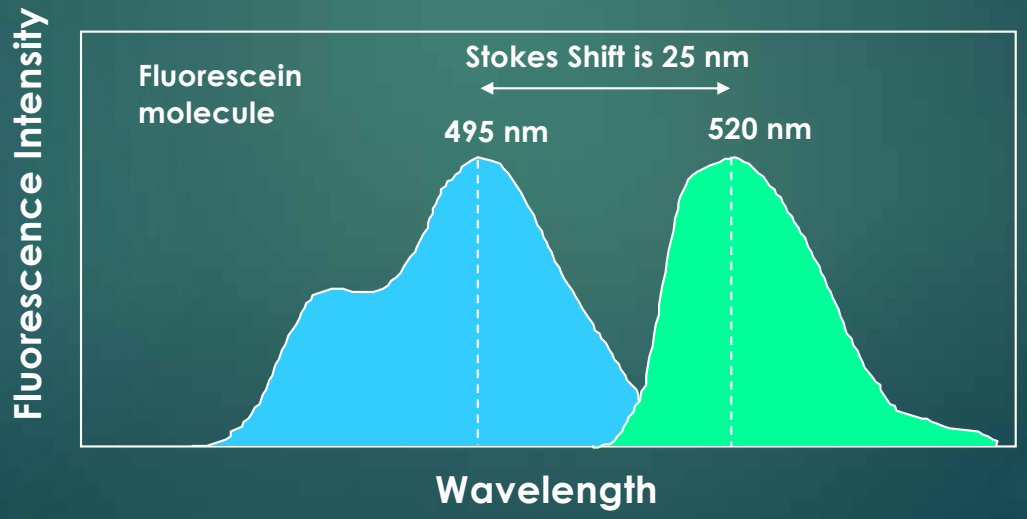
Simplified *Jablonski Diagram*

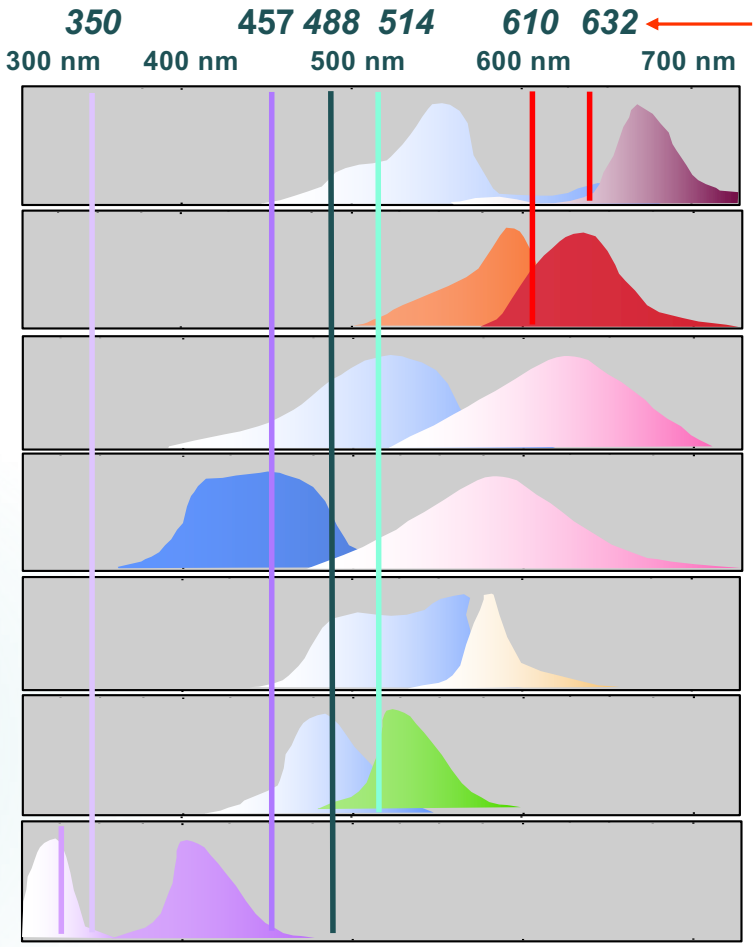


Fluorescence

Stokes Shift

- is the energy difference between the lowest energy peak of absorbance and the highest energy of emission





Common Laser Lines

PE-TR Conj.

Texas Red

PI

Ethidium

PE

FITC

cis-Parinaric acid

Quenching, Bleaching & Saturation

- Quenching is when excited molecules relax to ground states via nonradiative pathways avoiding fluorescence emission (vibration, collision, intersystem crossing)
- Molecular oxygen quenches by increasing the probability of intersystem crossing
- Polar solvents such as water generally quench fluorescence by orienting around the exited state dipoles

4th Ed. Shapiro p 115

Photobleaching

- Defined as the irreversible destruction of an excited fluorophore
- Photobleaching is not a big problem for flow cytometry as long as the time window for excitation is very short (a few hundred microsconds)

Photobleaching example from microscopy

- **FITC** - at 4.4×10^{23} photons cm^{-2} sec^{-1} **FITC** bleaches with a quantum efficiency Q_b of 3×10^{-5}
- Therefore **FITC** would be bleaching with a rate constant of 4.2×10^{3} sec^{-1} so 37% of the molecules would remain after 240 μsec of irradiation.
- In a single plane, 16 scans would cause 6-50% bleaching

Excitation - Emission Peaks

Fluorophore	EX_{peak}	EM_{peak}	% Max Excitation at 488	568	647 nm
FITC	496	518	87	0	0
Bodipy	503	511	58	1	1
Tetra-M-Rho	554	576	10	61	0
L-Rhodamine	572	590	5	92	0
Texas Red	592	610	3	45	1
CY5	649	666	1	11	98

Note: You will not be able to see CY5 fluorescence under the regular fluorescent microscope because the wavelength is too high.

Excitation Saturation

- The rate of emission is dependent upon the time the molecule remains within the excitation state (the excited state lifetime τ_f)
- Optical saturation occurs when the rate of excitation exceeds the reciprocal of τ_f
- In a scanned image of 512 x 768 pixels (400,000 pixels) if scanned in 1 second requires a dwell time per pixel of 2×10^{-6} sec.
- Molecules that remain in the excitation beam for extended periods have higher probability of interstate crossings and thus phosphorescence
- Usually, increasing dye concentration can be the most effective means of increasing signal when energy is not the limiting factor (i.e. laser based confocal systems)

Phosphorescence

- Following absorption, molecules can relax via a non-radiative transition to the T_1 rather than the S_1 state - this is called an intersystem crossing
- While it is forbidden it does happen and has a low probability and takes a longer time - the energy dissipated is called phosphorescence
- Phosphorescence has a longer lifetime than fluorescence (milliseconds rather than femtoseconds
- Phosphorescence generally occurs at longer wavelengths than fluorescence because the energy difference between S_0 and T_1 is lower

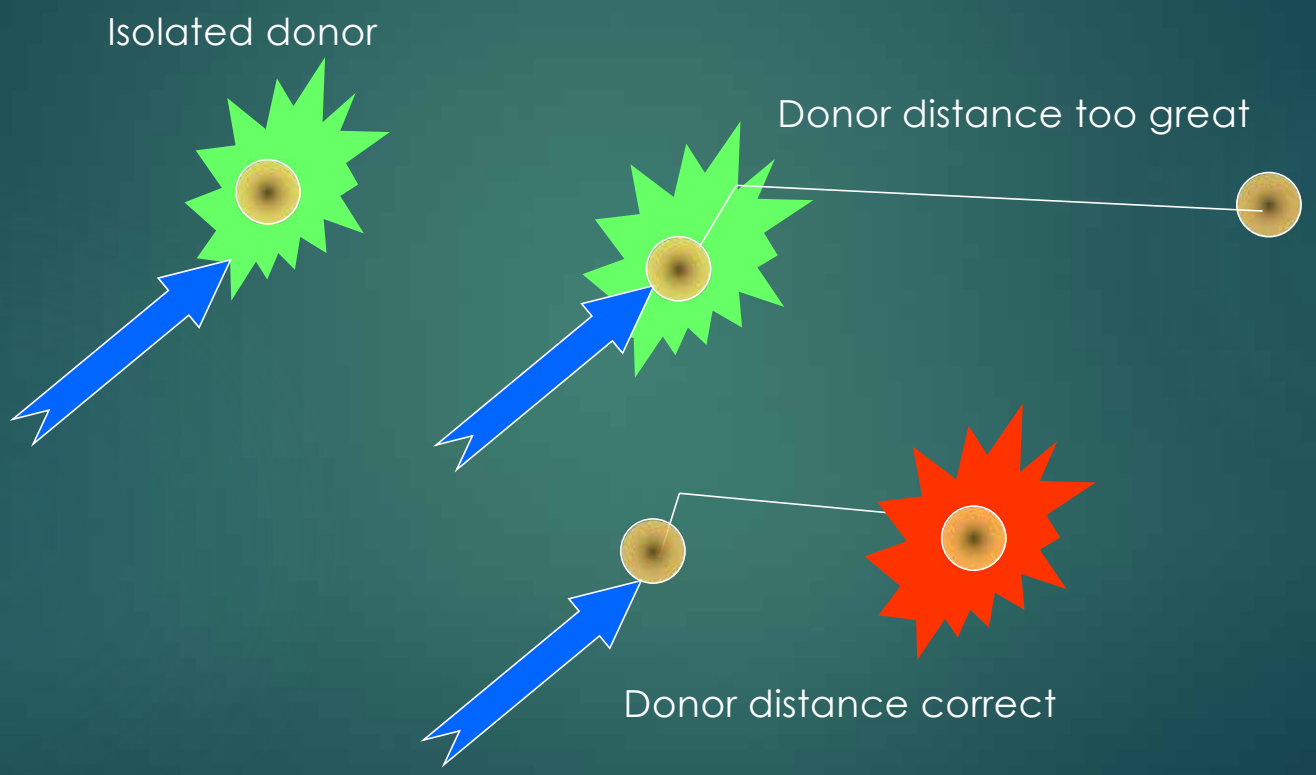
4th Ed. Shapiro p 113

Resonance Energy Transfer

- Resonance energy transfer can occur when the donor and acceptor molecules are less than 100 Å of one another (preferable 20-50 Å)
- Energy transfer is non-radiative which means the donor is not emitting a photon which is absorbed by the acceptor
- Fluorescence RET (FRET) can be used to spectrally shift the fluorescence emission of a molecular combination.

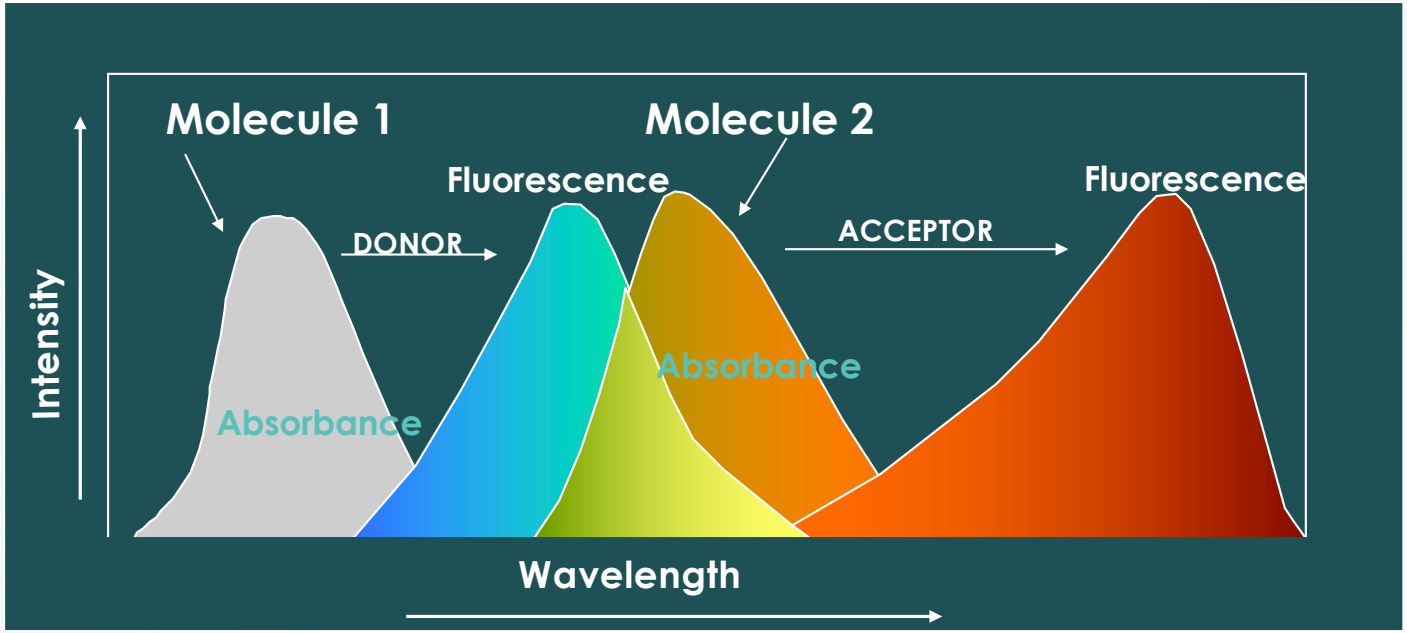
4th Ed. Shapiro p 115

FRET properties



Fluorescence

Resonance Energy Transfer

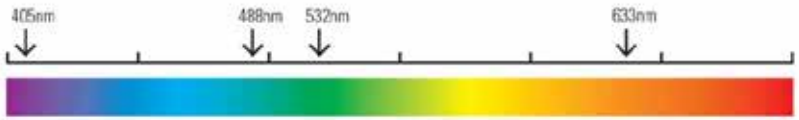


Raman Scatter

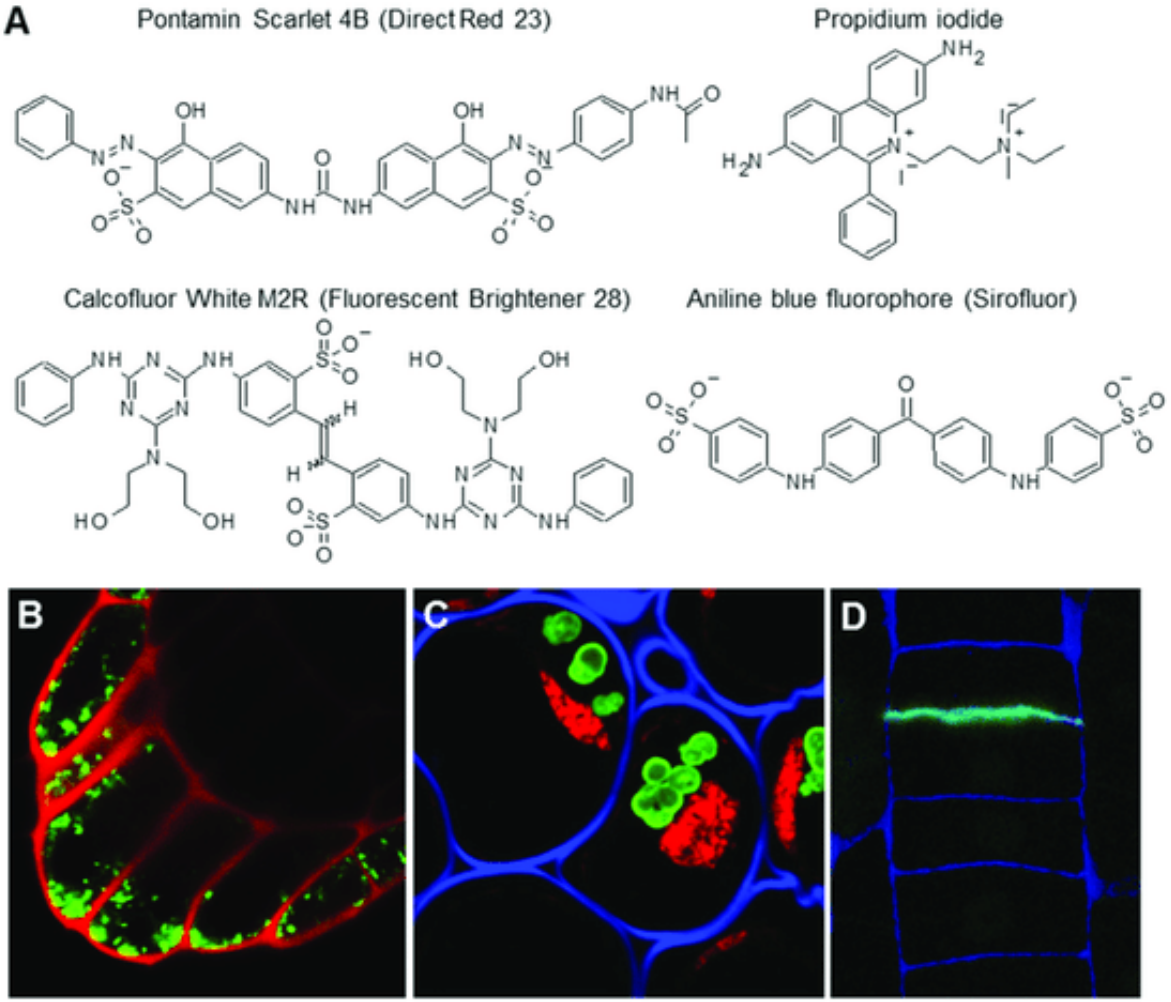
- A molecule may undergo a vibrational transition (not an electronic shift) at exactly the same time as scattering occurs
- This results in a photon emission of a photon differing in energy from the energy of the incident photon by the amount of the above energy - this is Raman scattering.
- The dominant effect in flow cytometry is the stretch of the **O-H** bonds of water. At **488 nm excitation** this would give emission at **575-595 nm**

4th Ed. Shapiro p 118

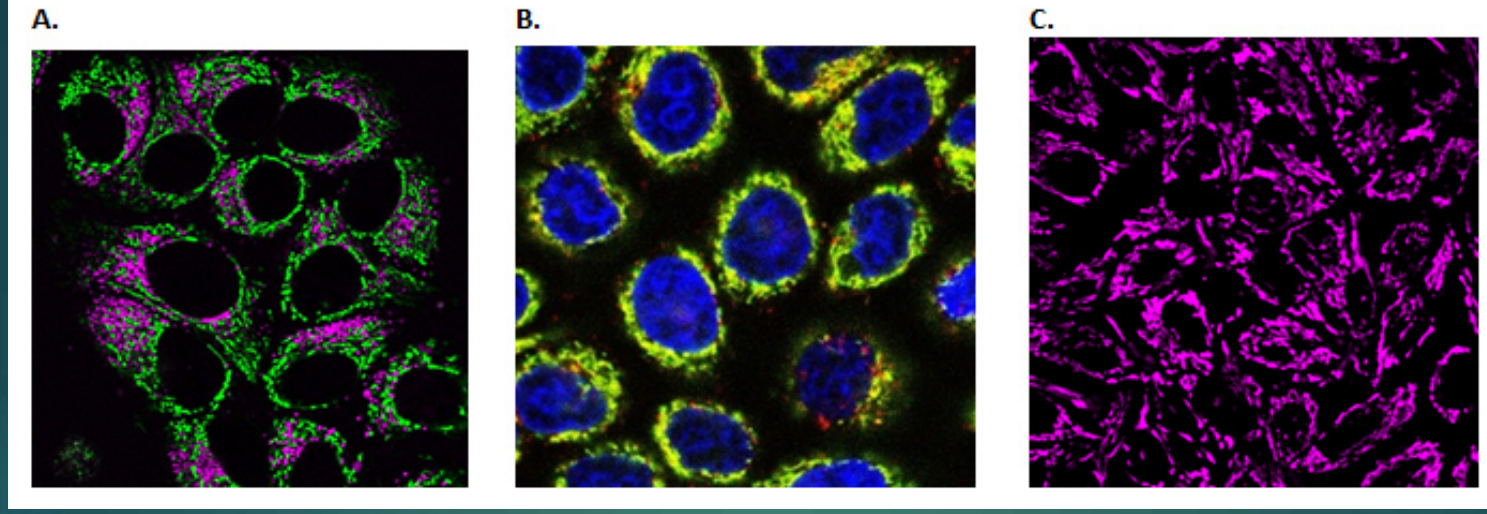
Visible Spectrum



Catalog Code	Description	MW	Excitation (nm)	Emission (nm)	Purpose
890	Certified Blank™				reference
897	Acridine Orange	265	500	525	DNA/RNA
886	Alexa Fluor® 488	643	499	519	conjugate
887	Alexa Fluor® 647	1300	652	668	conjugate
901	Allophycocyanine (APC)	104k	650	660	conjugate
914	APC-Cy™7	104k	650	767	conjugate
898	Chlorophyll (*a* + *b*)	8014 (a) 907 (b)	430,453	642,662	plant pigment
895	Cy™5	792	649	668	conjugate
906	DAPI	277	350	470	DNA (A-T)
913	Far-Out Red	-	475,590	663	reference
891	Fluorescein	389	495	519	conjugate
894	Hoechst 33342	616	346	375,390	dsDNA
916	Pacific Blue™	339	410	455	conjugate
899	PE (R-Phycoerythrin)	240k	480, 565	578	conjugate
908	PE-Cy™5	240k	480,565,650	670	conjugate
889	PE-Cy™7	240k	480	767	conjugate
909	PE-TR	240k	480,565,650	670	conjugate
892	Propidium Iodide	668	536	617	DNA intercalator
905	T.M. Rhodamine (TRITC, TAMRA)	430	557	575	conjugate
893	Texas Red® (Sulforhodamine)	625	589	615	conjugate
915	Violet Laser (Glacial Blue)	-	360	450	reference

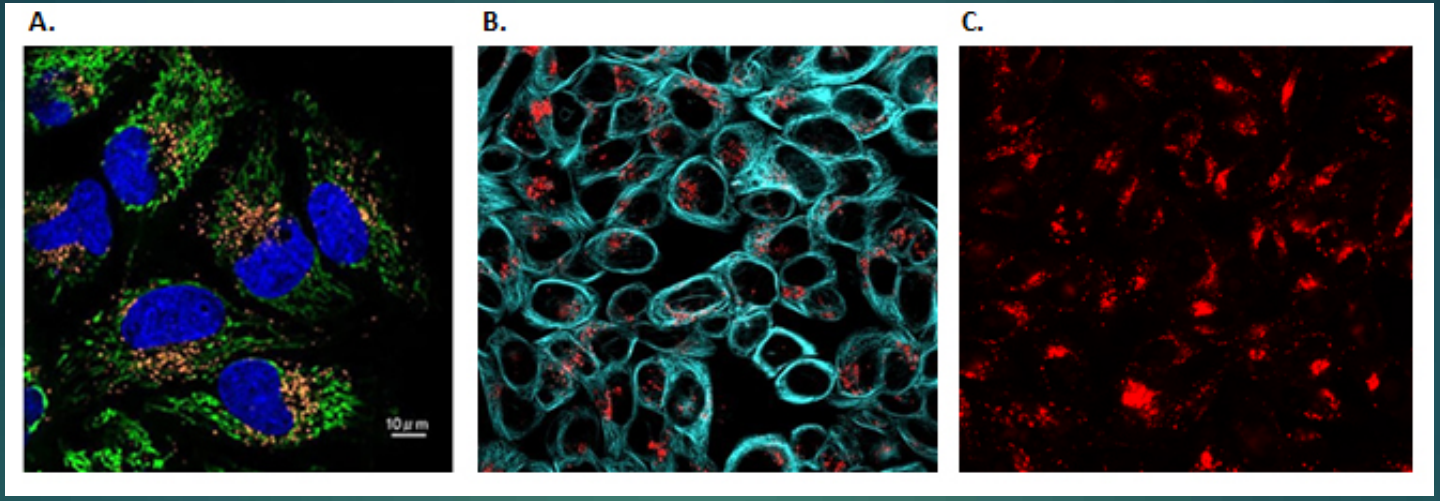

A
Pontamin Scarlet 4B (Direct Red 23)
Propidium iodide
Calcofluor White M2R (Fluorescent Brightener 28)
Aniline blue fluorophore (Sirofluor)
B
C
D

Live Cell Fluorophores



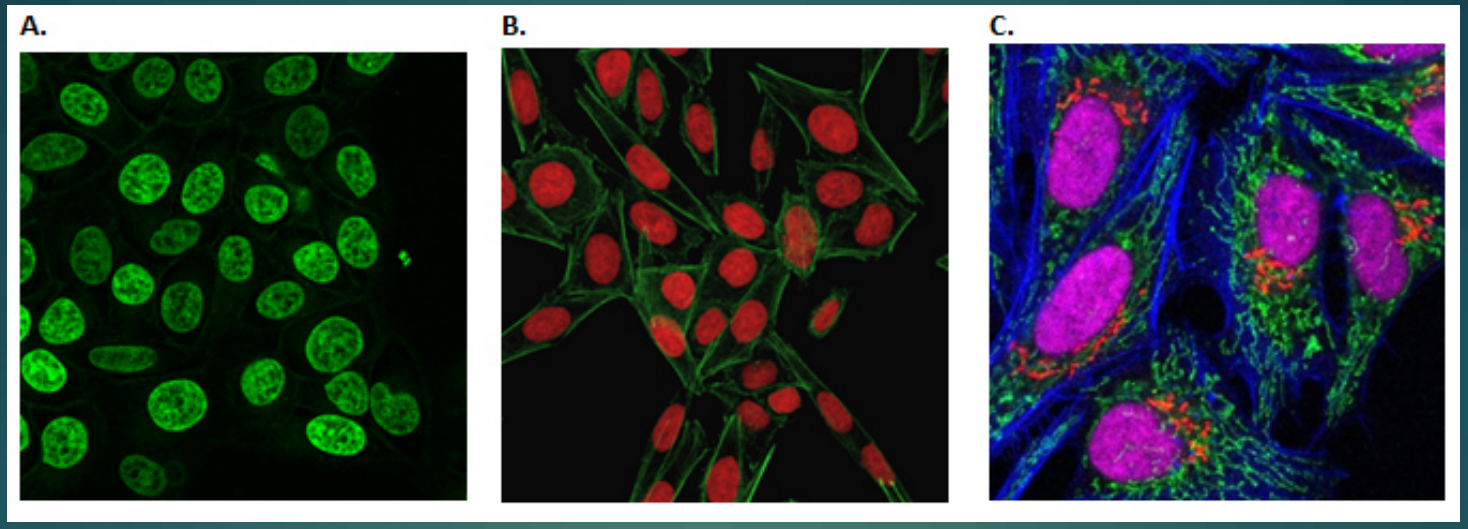
Product Description	Excitation (nm)	Emission (nm)	No.
BioTracker 405 Blue Mitochondria Dye	398	440	SCT135
BioTracker 488 Green Mitochondria Dye	490	523	SCT136
BioTracker 633 Red Mitochondria Dye	622	648	SCT137
BioTracker ATP-Red Live Cell Dye	510	570	SCT045
JC-1 Mitochondrial Dye	485	527/590	T4069

Live Cell Fluorophores



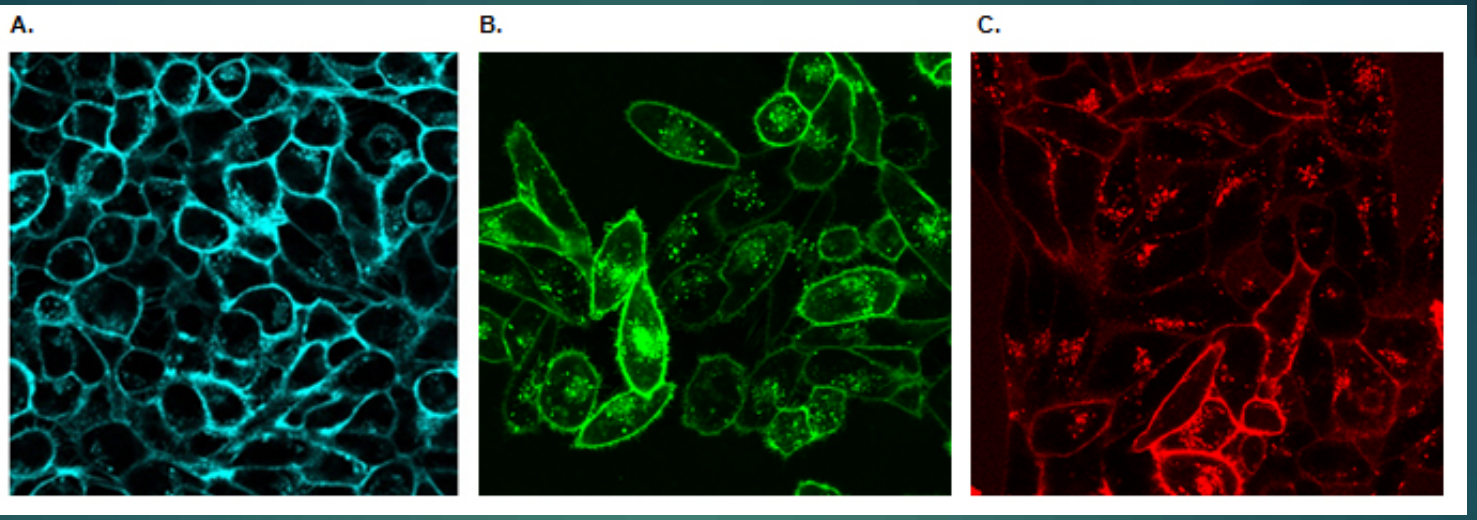
Product Description	Excitation (nm)	Emission (nm)	No.
BioTracker 555 UV-Excitation Red Lysosome Dye	554	583	SCT140
BioTracker 560 Orange Lysosome Dye	535	560	SCT019
BioTracker 540 Red Lysosome Dye	541	634	SCT141
BioTracker NIR633 Lysosome Dye	634	659	SCT138
BioTracker LYSO-TP Live Cell Dye	375	500	SCT044

Live Cell Fluorophores



Product Description	Excitation (nm)	Emission (nm)	Product No.
Hoechst DNA Stain Solution	350	461	94403
BioTracker 488 Green Nuclear Dye	500	515	SCT120
BioTracker 650 Red Nuclear Dye	650	675	SCT119
BioTracker NIR694 Nuclear Dye (water-based)	662	694	SCT117
BioTracker NIR694 Nuclear Dye (in DMSO)	662	694	SCT118

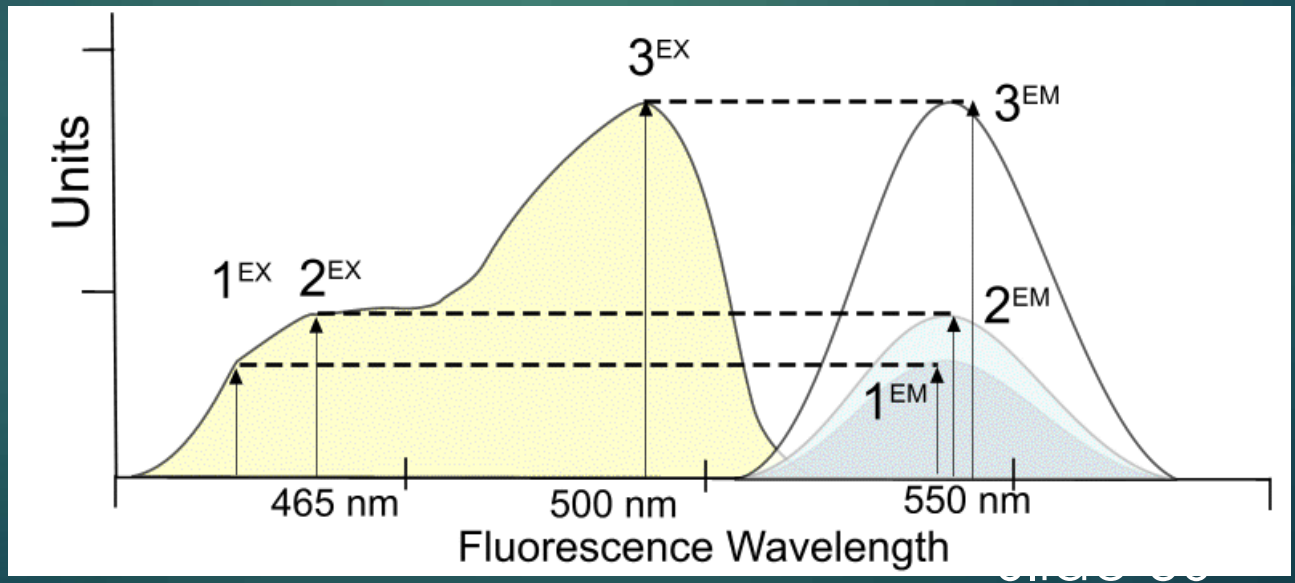
Live Cell Fluorophores



Product Description	Excitation (nm)	Emission (nm)	No.
BioTracker 400 Blue Cytoplasmic Membrane Dye	366	441	SCT109
BioTracker 490 Green Cytoplasmic Membrane Dye	484	501	SCT106
BioTracker 555 Orange Cytoplasmic Membrane Dye	549	565	SCT107
BioTracker 655 Red Cytoplasmic Membrane Dye	644	665	SCT108
BioTracker NIR680 Cytoplasmic Membrane Dye	683	724	SCT112
BioTracker NIR750 Cytoplasmic Membrane Dye	748	780	SCT113
BioTracker NIR770 Cytoplasmic Membrane Dye	767	806	SCT114
BioTracker NIR790 Cytoplasmic Membrane Dye	786	820	SCT115

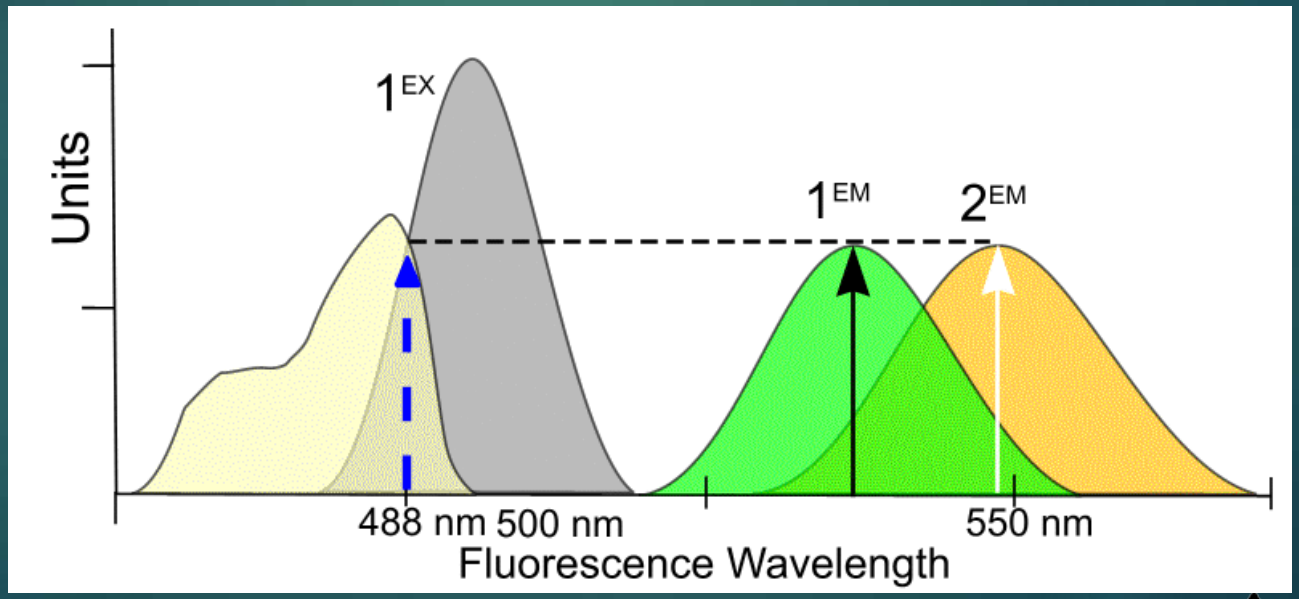
Fluorescence

- The longer the wavelength the lower the energy
- The shorter the wavelength the higher the energy
  - eg. UV light from sun - this causes the sunburn, not the red visible light
- The spectrum is independent of precise excitation line but the intensity of emission is not



Mixing fluorochromes

When there are two molecules with different absorption spectra, it is important to consider where a fixed wavelength excitation should be placed. It is possible to increase or decrease the sensitivity of one molecule or another.



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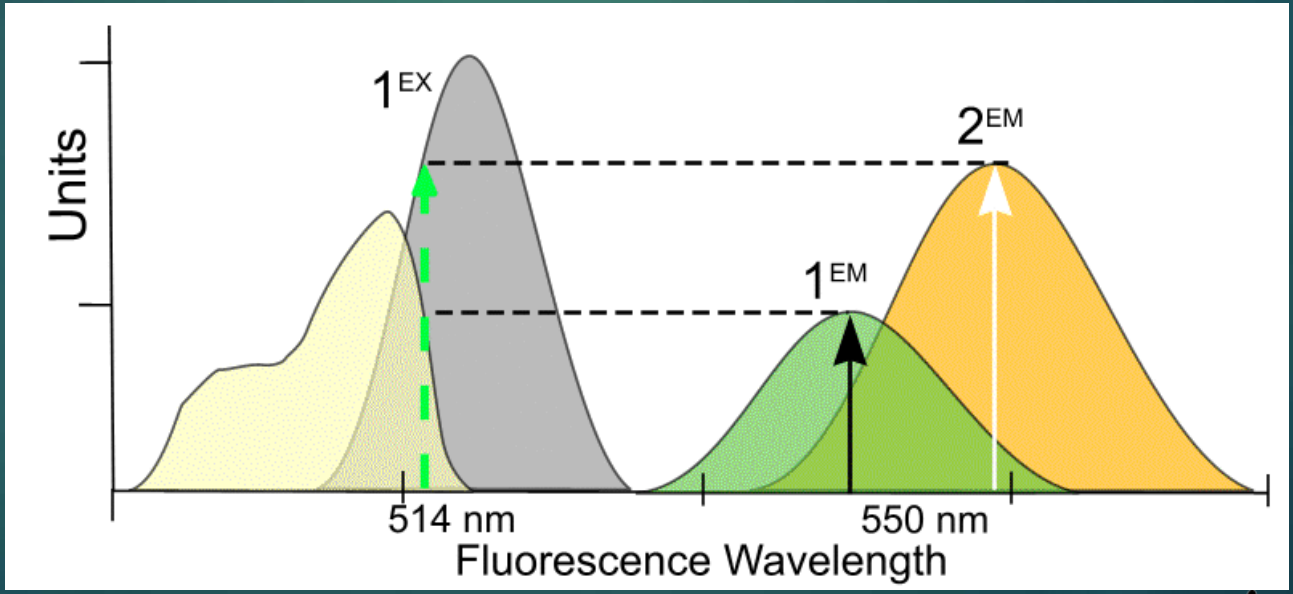

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