

**Structural Biology**  
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**Lecture – 33**  
**Fluorescence Spectroscopy and Green Fluorescence Protein (GFP)**

Hi everyone, welcome again to the course of structural biology. We are continuing to structural Biology techniques. We have already discussed about high resolution techniques like x-ray crystallography NMR. Currently we are talking about low resolution techniques majorly on the category of Spectroscopy. Today I am going to discuss fluorescence.

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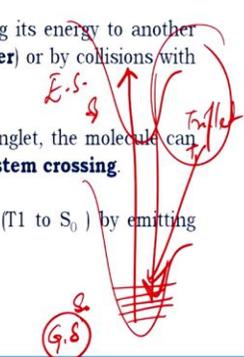
**Introduction:**

When a molecule absorbs light, an electron is promoted to a higher excited state (generally a singlet state, but may also be a triplet state). The excited state can get depopulated in several way

The molecule can lose its energy non-radiatively by giving its energy to another absorbing species in its immediate vicinity (**energy transfer**) or by collisions with other species in the medium

If an excited state triplet overlaps with the excited state singlet, the molecule can cross over into this triplet state. This is known as **inter system crossing**.

If the molecule then returns to the ground state singlet ( $T_1$  to  $S_0$ ) by emitting light, the process is known as **phosphorescence**

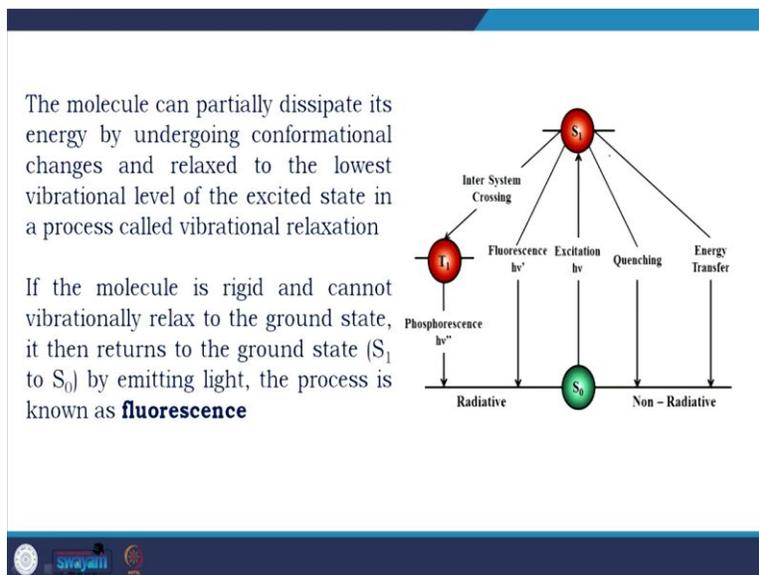


So to introduce fluorescence, you know when a molecule absorbs light, a lot of electronic phenomena are used to take place. And electron after absorbing light is promoted to the higher excited state. It generally should be singlet state but there are exceptions. It could also be a triplet state. The excited state can get depopulated in several ways.

You have a low energy stable state which is mostly populated and higher energy excited state which is less populated. So when a molecule absorbs energy and becomes excited, it goes to the higher populated state, but coming back would be in several ways giving rise to several spectroscopy. The excited state can lose its energy nonradiatively by giving its energy to another absorbing species in its immediate vicinity, which is called as energy transfer or by collision with other species in the medium. If an excited state triplet overlaps with the excited state singlet the

molecule can cross over into this triplet's state known as intersystem crossing. If the molecules return to the ground state singlet, so it goes from  $S_0$  to  $S_1$ , and then go to  $T_1$  then come to  $S_0$  then this phenomenon is called phosphorescent.

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So if you see, here is  $S_0$  goes to  $S_1$  by being excited. Now in several ways it is coming, through quenching, through energy transfer, they are called non radiative transfer. If it comes from  $S_1$  to  $S_0$ , then this is called fluoresce. If it goes to  $T_1$  by intersystem crossing and then come back, these are radiative transfer and this is called phosphorescence. So the molecule can partially dissipate its energy by undergoing conformational changes and relaxed to the lowest vibrational level of the excited state in a process called vibrational relaxation. If the molecule is rigid and cannot vibrationally relaxed the ground state it then returns to the ground state ( $S_1$  to  $S_0$ ) by emitting light, these process is called fluoresce which we are going to study in this module.

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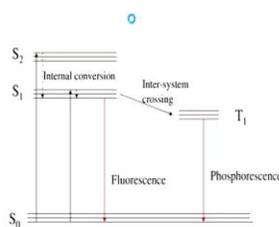
## Jablonski diagram:

**Jablonski diagram**, named after the Polish physicist Aleksander Jabłoński illustrates the electronic states of a molecule and the transitions between them

The states are arranged vertically by energy and grouped horizontally by spin multiplicity

The transitions are indicated by straight arrows

The electronic states are further divided by vibrational states



Jablonski diagram. It is named after the polish physicist Alexander Jablonski, it illustrates the electronic state of the molecule and the transition between them. The states are arranged vertically by energy and grouped horizontally by spin multiplicity. The transitions are indicated by arrows. The electronic states are further divided by vibrational states.

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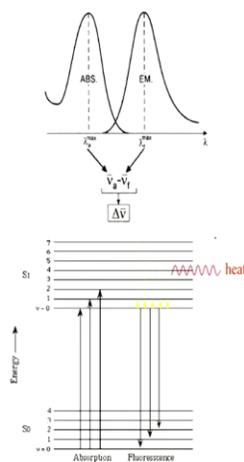
## Stokes Shift:

The Stokes shift is the gap between the maximum of the first absorption band and the maximum of the fluorescence spectrum

The energy of emission is typically less than that of absorption

Fluorescence typically occurs at lower energies or longer wavelength

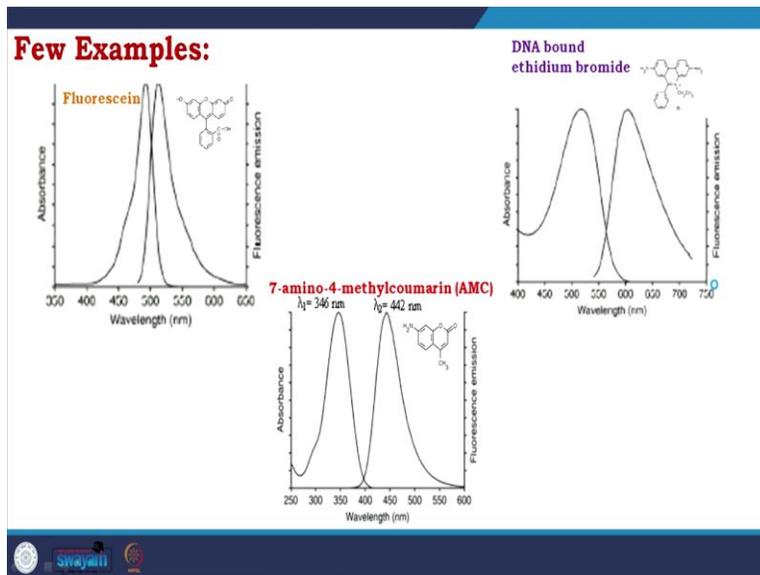
This is due to loss of vibrational energy in the excited state as heat by collision with solvent



The stoke shift is the gap between the maximum of the first absorption band and the maximum of the fluorescence spectrum. The energy of emission is typically less than that of absorption because if you remember, from the ground electronic state to the excited state the molecule goes, then it dissipate the energy and come back that is fluorescence.

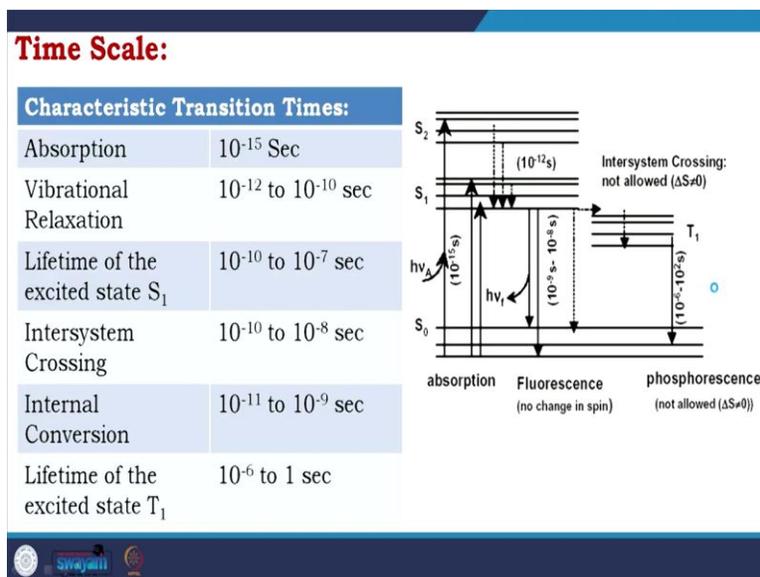
So there is the dissipation of energy in any form and that is why the emission energies typically less. Fluorescence typically occurs at lower energies for longer wavelength. This is due to loss of vibrational energy in the excited state as heat, by collision with solvent.

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These are some of the examples, this is fluorescein and you see the absorption and fluorescence emission, this is DNA bound ethidium bromide, when ethidium bromide present alone in the solution, it does not show any fluorescence, but when it bound to DNA, that time it is showing fluorescence. 7 amino 4 methylcoumarin (AMC) you again see the profile.

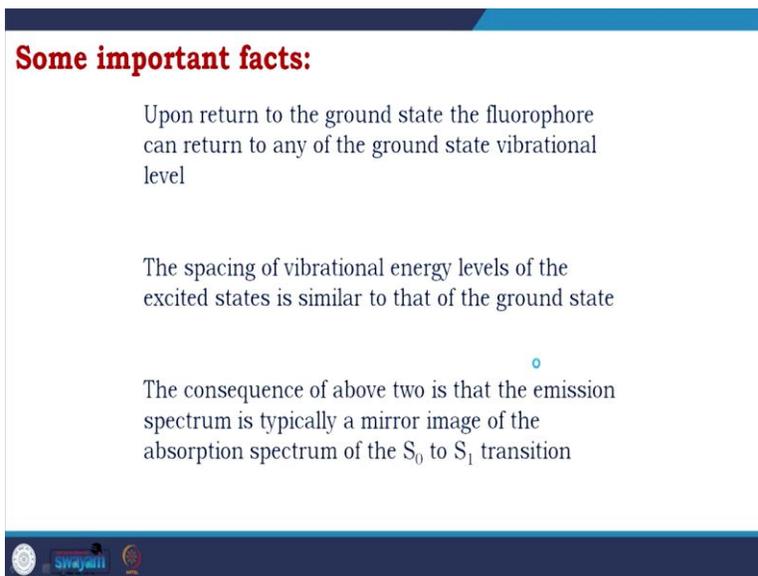
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So what are the timescale of those conversions for absorption? The time scale is  $10^{-15}$  second for absorption, for vibrational relaxation  $10^{-12}$  to  $10^{-10}$  second. For lifetime of the excited state at S1 is  $10^{-10}$  to  $10^{-7}$  second, for intersystem crossing  $10^{-10}$  to  $10^{-8}$ , internal conversion  $10^{-11}$  to  $10^{-9}$ , and lifetime of the excited state T1  $10^{-6}$  to 1 second.

So if you see, here are the transition happens, and timelines are given. Among them the lifetime of the excited state S1  $10^{-10}$  to  $10^{-7}$  second, this is fluorescence. The lifetime of the excited state T1 is actually defining phosphorescence.

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**Some important facts:**

- Upon return to the ground state the fluorophore can return to any of the ground state vibrational level
- The spacing of vibrational energy levels of the excited states is similar to that of the ground state
- The consequence of above two is that the emission spectrum is typically a mirror image of the absorption spectrum of the  $S_0$  to  $S_1$  transition

Some important facts, upon return to the ground state the fluorophore can return to any of the ground state vibrational level. The spacing of vibrational energy level of the excited state is similar to that of the ground state. The consequence of above two is that, the emission spectrum is typically a mirror image of the absorption spectrum of the  $S_0$  to  $S_1$  transition.

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## Quantum Yield:

$$\text{Quantum Yield} = \Phi_f$$

$\Phi_f$  = number of fluorescence quanta emitted divided by number of quanta absorbed to a singlet excited state

**Quantum yield is the ratio of photons emitted to photons absorbed by the system:**

$$\text{Quantum Yield} = \frac{\text{Number of photons emitted}}{\text{Number of photons absorbed}}$$

Quantum yield: Quantum yield is very important, it is the number of fluorescence quanta emitted divided by the number of quanta observed to a singlet excited state. Alternatively it could be also told, Quantum yield is the ratio of photons emitted to photon absorption in the system. So number of photons emitted by a number of photons absorbed. This ratio is called Quantum yield in the later session we will talk about Quantum yield and its application. The molecule with a higher Quantum yield can use as a probe.

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## Fluorescence life times and quantum yield:

Quantum yield is the ratio of the number of photons emitted to the number absorbed

The lifetime of the excited state is defined by the average time the molecule spends in the excited state prior to the return to the ground state

$$Q = \frac{\Gamma}{\Gamma + k_{nr}} \quad \tau = \frac{1}{\Gamma + k_{nr}}$$

$\Gamma$  = the emissive rate of fluorophore.

$k_{nr}$  = rate of non-radiative decay

Fluorescence life time and Quantum yield: Quantum yield is the ratio of the number of photons emitted to number absorbed. The lifetime of the excited state is defined by the average time the molecules spend in the excited state prior to the return of the ground state.

$$Q = \Gamma / (\Gamma + k_{nr})$$

Here Q is used for quantum

$$\tau = 1 / (\Gamma + k_{nr})$$

Where  $\Gamma$  is the emissive rate of the fluorophore. It is characteristic of any fluorophore and  $k_{nr}$  is the rate of non-radiative decay.

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## Fluorescence life times and quantum yield:

The lifetime of the fluorophore in the absence of radiative process is called the intrinsic or natural life time

Fluorescence lifetimes are near 10 ns

Scintillators have large  $\Gamma$  value. Hence they have large Q and lifetime

The fluorescence emission of aromatic substances containing nitro group are generally weak due to large  $k_{nr}$  value

$$\tau_n = \frac{1}{\Gamma}$$

Fluorescence lifetime and Quantum yield: The lifetime of fluorophore in the absence of radiative process is called intrinsic or natural life time. Fluorescence life times are near 10 nanosecond. Earlier scintillators have large  $\tau$  (tau) value hence they have large Quantum yield (Q) and lifetime. The fluorescence emission of aromatic substances containing nitro group are generally weak due to their large  $k_{nr}$  value.

$$\tau = 1 / \Gamma$$

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**Quantum Yield would be determined with respect to standard:**

Quantum Yield [Q.Y.] Standards	Q.Y. [%]	Conditions for Q.Y. Measurement	Excitation [nm]
Cy3	4	PBS	540
Cy5	27	PBS	620
Cresyl Violet	53	Methanol	580
Fluorescein	95	0.1 M NaOH, 22°C	496
POPOP	97	Cyclohexane	300
Quinine Sulfate	58	0.1 M H <sub>2</sub> SO <sub>4</sub> , 22°C	350
Rhodamine 101	100	Ethanol	450
Rhodamine 6G	95	Water	488
Rhodamine B	31	Water	514
Tryptophan	13	Water, 20°C	280
L-Tyrosine	14	Water	275

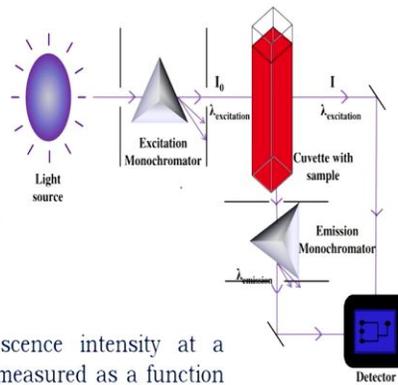
Quantum yield have to be determined with respect to some standard. So here there are some standard Cy 3 with Quantum yield 4, and Quantum yield is characteristic in a certain buffer. So this Quantum yield of Cy 3 is measured in the PBS buffer. cy5 quantum yields is 27 and it measured in PBS also. Cresyl Violet quantum yield is 53, solvent is methanol, fluorescein quantum yield is 95 in 0.1 M NaOH at 22°C.

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## Instrumental Setup:

**Emission spectrum:** Excitation wavelength is kept constant and fluorescence intensity measured as function of wavelength, i.e. spectrum of emitted light is determined

**Excitation spectrum:** Fluorescence intensity at a particular fixed wavelength is measured as a function of the excitation wavelength



This is the internal setup of the instrumentation. You have the light source, then you have the excitation Monochromator, and the initial light ray energy will go, and hit the sample, then you get the intensity of the fluorophore which is coming, and this will come through the emission monochromator and it would be detected by a particular fluorescence detector.

Excitation spectrum: It is the fluorescence intensity at a particular fixed wavelength is measured as a function of the excitation wavelength.

Emission spectrum: Excitation wavelength is kept constant and fluorescence intensity measured as a function of wavelength, that is spectrum of the emitted light is determined.

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## The effect of solvent on the fluorescence spectra:

The effect of solvent and environment may be due to several factors:

Solvent polarity and viscosity

Rate of solvent relaxation

Probe conformational changes

Rigidity of the local environment

Internal charge transfer

Proton transfer and excited state reaction Probe-Probe interaction

The effect of solvent of the fluorescence spectra: Effect of solvent and environment may be due to several factors like solvent polarity and viscosity, rate of the solvent relaxation, probe conformational changes, rigidity of the local environment, internal charge transfer and proton transfer in excited state reaction, probe-probe interaction.

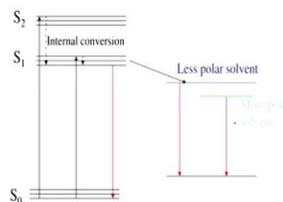
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## Effect of solvent:

Typically, the fluorophore has a larger dipole moment in the excited state than the ground state

Solvent shifts the emission to lower energy due to stabilization of the excited state by the polar solvent molecule

As the solvent polarity is increased, this effect becomes larger

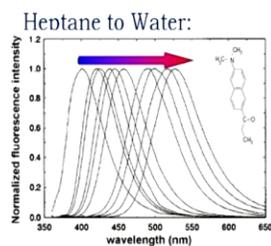


So typically the fluorophore has a larger dipole moment in the excited state than the ground state. Solvents shift the emission to lower energy due to stabilization of the excited state by the polar solvent molecules. So with less polar and more polar you will see different effect. As the solvent polarity is increased these effects become larger.

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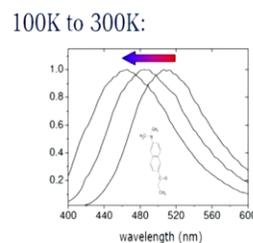
## Fluorescence Spectra of Prodan:

### Effect of Solvent:



Emission spectra in different solvent  
Increase in solvent polarity leads to red shift

### Effect of Temperature:



Decrease in temperature means increase in viscosity which ultimately increase the fluorescence contribution of non-relaxed state resulting blue shift

If you see here; fluorescence spectra is taken for prodan, where solvents are used from heptane to water, from nonpolar to polar, and you see several different spectra obtained. So emission spectra is collected from prodan from different solvent, increase in solvent polarity leads to red shift. Effect of temperature 100K to 300K: Three points are taken and there is clear shifting of the spectrum. So, decrease in temperature means increase in viscosity because when you increase the temperature the dynamicity would be enhance.

So decrease in the temperature is decrease in the dynamism which means increasing the viscosity which ultimately increased the fluorescence contribution of non relaxed state resulting blue shift. So I talk about red shift. I talked about blue shift. What is red shift and what is blue shift?

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## Red and Blue shift:

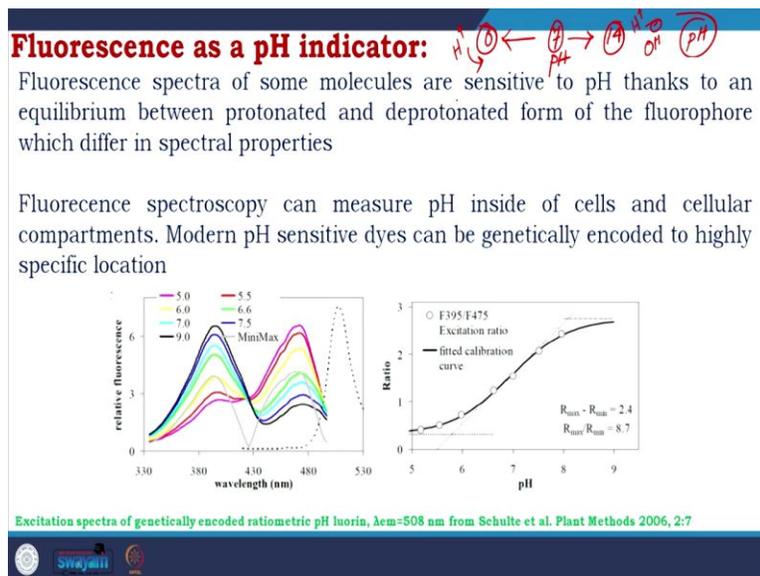
A spectral **shift** towards higher wavelengths (i.e. lower energy and lower frequency) is called a **red-shift** or a bathochromic **shift**

A spectral **shift** towards lower wavelengths (i.e. higher energy and higher frequency) is called a **blue-shift** or hypsochromic **shift**.

A spectral shift towards higher wavelength that is lower energy and lower frequency is called a red shift or a bathochromic shift. So, from lower wavelength to higher wavelength happened the shift of the emission spectra with the change of the solvent when the polarity of the solvent is enhanced? Whereas, when temperature is increased the opposite effect was observed from the higher energy to lower energy the emission spectra sifted so that is called Blue shift.

Spectral shift towards lower wavelength, which is higher energy and higher frequency is called a blue shift or hypsochromic shift.

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Fluorescence would be used as a pH indicator. Fluorescence spectra of some molecules are sensitive to pH, thanks to equilibrium between the protonated and deprotonated form of the fluorophore which differ in the spectral property. Fluorescence spectroscopy can measured pH inside of cells and cellular compartments. Modern pH sensitive device can be genetically encoded to highly specific location. If you see here, relative fluorescence is measured with the change of pH start with 5, 5.5, 6, 6.6, 7, 7.5, 9 and when you do that, you see the changes which is plotted to get a correlation. That is why fluorescence here working as a pH indicator.

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## Quenching:

Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample

A variety of molecular association can result in quenching

These include excited state reactions, molecular rearrangements, energy transfer, groundstate complex formation, and collisional quenching

Coming to quenching: Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. A variety of molecular association can result in quenching, include excited state reactions, molecular rearrangement, energy transfer ground state complex formation and collisional quenching.

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## Quencher:

A wide variety of substances act as quenchers of fluorescence

Quenching by oxygen is due to its paramagnetic nature causes the fluorophore to undergo intersystem crossing to the triplet state

Who are quenchers? A variety of substances would act as quenchers of fluorescence. Quenching by oxygen is due to paramagnetic nature causes the fluorophore to undergo intersystem crossing to the triplet state.

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Quenching can occur during the excited state lifetime

for example collisional quenching, energy transfer, charge transfer reactions, photochemistry

or they may occur due to formation of complexes in the ground state

We focus on the two quenching processes usually encountered

- (1) collisional (dynamic) quenching
- (2) static (complex formation) quenching

Quenching can occur during the excited state lifetime. For example collisional quenching, energy transfer, charge transfer reactions, photochemical reactions, or they could occur due to formation of complexes in the ground state. So when the molecule goes in the excited state quencher could go, and could form complex in the ground state. We will focus on two type of quenching one is collisional or dynamic quenching, another one is static quenching. So in collisional they keep interacting. In static they form complexes.

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**Collisional or dynamic Quenching:**

Collisional quenching occurs when the excited fluorophore experiences contact with an atom or molecule that can facilitate non radiative transitions to the ground state

Common quenchers include Oxygen, Iodide and acrylamide

Excited-state molecule returns to ground state via emission of a photon

Excited-state molecule collides with quencher molecule and returns to ground state non-radiatively

Collision with quencher

Collisional or Dynamic Quenching: Collisional quenching occurs when the excited fluorophore experiences contact with an atom or molecule that can facilitate non radiative transition to the

ground state. Some common quenchers include oxygen, iodide ion and acrylamide. So when you see a molecule, it gets excited and gives radiative transfer.

So excited state molecule return to ground state by a emission of a photon (it goes to the excited state give back a photon and come back). The same molecule interacts with the quencher come back to the ground state but could not provide a photon. Excited state molecule collides with quencher molecule and return to ground state non-radiatively. So no fluorescence or less fluorescence is observed.

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**Collisional Quenching: Stern-Volmer Plot**

In the simplest case of collisional quenching, the following relation, called the Stern-Volmer equation, holds:

$$F_0/F = 1 + K_{sv}[Q]$$

Where  $F_0$  and  $F$  are the fluorescence intensities observed in the absence and presence, respectively, of quencher,  $[Q]$  is the quencher concentration and  $K_{sv}$  is the Stern-Volmer quenching constant

Thus, a plot of  $F_0/F$  versus  $[Q]$  should yield a straight line with a slope equal to  $K_{sv}$ . Such a plot, known as a Stern-Volmer plot, is shown below for the case of fluorescein quenched by iodide ion (I<sup>-</sup>)

Concentration of I <sup>-</sup> (M)	F <sub>0</sub> /F
0.00	1.00
0.01	1.05
0.02	1.10
0.03	1.15
0.04	1.20
0.05	1.30
0.06	1.40
0.07	1.55

Now, you could quantitatively measure the effect of collisional or dynamic quenching. In the simplest case of collisional quenching the relation is called Stern-Volmer equation. So Stern-Volmer is

$$F_0 / F = 1 + K_{sv}[Q]$$

Where  $F_0$  and  $F$  are the fluorescence intensity observed in the absence and presence of the quencher,  $Q$  is the quencher concentration and  $K_{sv}$  is the Stern-Volmer quenching constant.

What is the importance of the equation? If you plot  $F_0 / F$  versus  $Q$  concentration, it should yield a straight line with slope. So, the slope is  $K_{sv}$ , this plot as I told known as Stern-Volmer plot, is helping you getting the factors. So suppose you want to see the effect of a quencher. The effect of quencher is characteristic of this Stern-Volmer constant. So you could measure that from this plot.

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### Stern-Volmer Equation:

$K_{SV} = k_q \tau_0$  where  $k_q$  is the bimolecular quenching rate constant (proportional to the sum of the diffusion coefficients for fluorophore and quencher) and  $\tau_0$  is the excited state lifetime in the absence of quencher

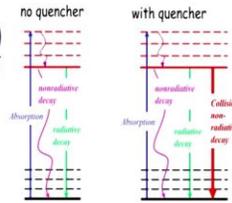
Stern-Volmer equation:  $F_0/F = 1 + K_{SV}[Q]$

Now with  $K_{SV} = k_q \tau_0$

For purely collisional quenching, also known as dynamic quenching:  $F_0/F = \tau_0 / \tau$

$$\tau_0 / \tau = 1 + k_q \tau_0 [Q]$$

In the fluorescein/iodide system (previous slide),  $\tau_0 = 4\text{ns}$  and  $k_q \sim 2 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$



So  $K_{sv} = k_q \tau_0$

$k_q$  is the bimolecular quenching rate constant which is proportional to the sum of the diffusion coefficient for fluorophore and quencher and  $\tau_0$  is the excited state lifetime in the absence of quencher.

Stern-Volmer was

$$F_0 / F = 1 + K_{SV}[Q]$$

Now when

$K_{sv} = k_q \tau_0$  for purely collisional quenching which is as I told also known as dynamic quenching

$$F_0 / F = \tau_0 / \tau$$

$$\tau_0 / \tau = 1 + k_q \tau_0 [Q]$$

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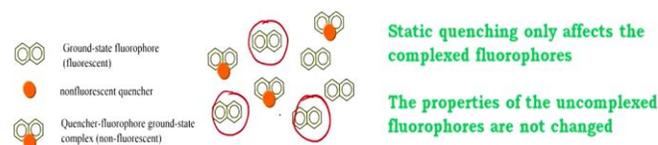
## Static Quenching:

In some cases, the fluorophore can form a stable complex with another molecule

If this ground-state is non-fluorescent then we say that the fluorophore has been statically quenched

In such a case, the dependence of the fluorescence as a function of the quencher concentration follows the relation:  $F_0/F = 1 + K_a[Q]$

Where  $K_a$  is the association constant of the complex. Such cases of quenching via complex formation were first described by Gregorio Weber



Coming to Static Quenching: In some cases the fluorophore can form stable complex with another molecule. So up to now we are talking about dynamic interaction. Now, we are talking about stable complex and when they could form stable complex this is static quenching. If this ground state is non fluorescent, then we say that the fluorophore has been statically quenched. In such a case the dependence of the fluorescence as a function of the quencher concentration follows the relation

$$F_0 / F = 1 + K_a[Q]$$

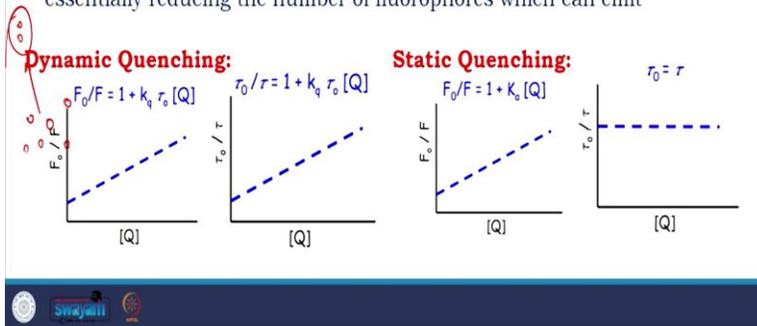
The Ksv Stern-Volmer is now replaced by  $K_a$ , where  $K_a$  is the association constant of the complex. Such cases of quenching the complex formation were first described by Gregorio Weber. So if you see, the ground state fluorophore is there, it is not bound to the non fluorescent quencher, but the now there is bound and form the ground state complex. So the static quenching only affects the complex fluorophore it would not affect the fluorophore which are open.

While in dynamic it could interact with any of them. The properties of the uncomplexed fluorophore are not changed. They show property of a normal fluorophore.

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## Quenching and Lifetime of Fluorophores:

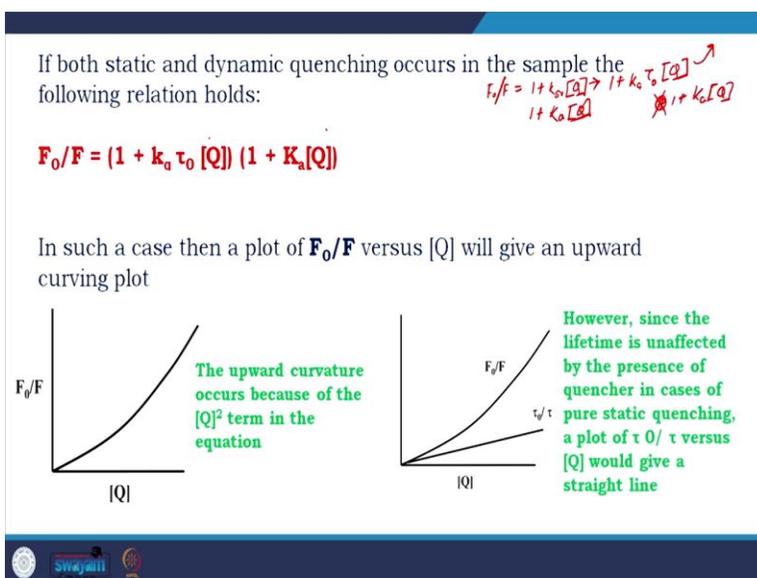
Static quenching will not reduce the lifetime of the sample since those fluorophores which are not complexed and hence are able to emit after excitation will have normal excited state properties. The fluorescence of the sample is reduced since the quencher is essentially reducing the number of fluorophores which can emit.



Quenching and Lifetime of Fluorophore: Static quenching will not reduce the lifetime of the sample since those fluorophore which are not complex working like a normal one and hence are able to emit after excitation. Will have normal excited state properties. The fluorescence of the sample is reduced since the quencher is essentially reducing the number of fluorophore which can emit.

So, if you see in dynamic quenching this plot of  $F_0 / F$  and this is the plot of  $\tau_0 / \tau$  is mostly same. Whereas in case of static quenching this is the  $F_0 / F$ , whereas  $\tau_0 / \tau$ , it straight line because here  $\tau_0$  is equal  $\tau$ .

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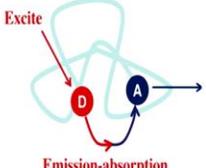
So in both Static and dynamic quenching, sometime both the things occur together, both the static and dynamic quenching together. So now they have

$$F_0 / F = (1 + k_q \tau_0 [Q]) (1 + K_a [Q])$$

In such a case a plot  $F_0 / F$  versus  $Q$  will give an upward curve in plot rather than straight one. The upward curvature occurs because of the  $Q$  square term in the equation. Now when you put  $\tau_0 / \tau$  vs  $[Q]$ , you still get a straight line, since the lifetime is unaffected by the presence of the quencher in case of static quenching.

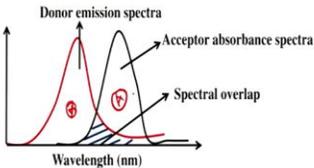
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**Förster's Resonance Energy Transfer (FRET):**



Fluorescence emission from the donor (D) is absorbed by the acceptor (A)

The emission spectrum of donor and the absorption spectrum of the acceptor must have a spectral overlap



FRET is a non-radiative process. The FRET efficiency is dependent on the distance between the donor and acceptor.

Coming to Förster's Resonance Energy Transfer or FRET: What is the concept of FRET? If you have fluorophore A, you excite, so you have an excitation wavelength, you get an emission. Interestingly, emission wavelength actually comes in the range of the excitation wavelength of a molecule B instead of getting the emission of A and B you get a very big resonating emission peak, and this relation is known as FRET or Förster Resonance Energy Transfer. So fluorescence emission from donor is absorbed by the acceptor. As I told it is emitting it gives energy to the A. Emission spectrum and absorption spectrum of acceptor must have a spectral overlap. That is the relation of two molecules being into FRET relation.

They are overlapping, the emission spectrum of the donor is overlapping with the absorption spectra or excitation spectra of acceptor. FRET is a nonradiative process. The FRET efficiency is dependent on the distance between the donor and acceptor.

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## Milestones in the Theory of Resonance Energy Transfer:

**1918** J. Perrin proposed the mechanism of resonance energy transfer

**1922** G. Cario and J. Franck demonstrate that excitation of a mixture of mercury and thallium atomic vapors with 254nm (the mercury resonance line) also displayed thallium (sensitized) emission at 535nm

**1924** E. Gaviola and P. Pringsham observed that an increase in the concentration of fluorescein in viscous solvent was accompanied by a progressive depolarization of the emission

**1928** H. Kallmann and F. London developed the quantum theory of resonance energy transfer between various atoms in the gas phase. The dipole-dipole interaction and the parameter  $R_0$  are used for the first time

**1932** F. Perrin published a quantum mechanical theory of energy transfer between molecules of the same species in solution. Qualitative discussion of the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor

**1946-1949** T. Förster developed the first quantitative theory of molecular resonance energy transfer

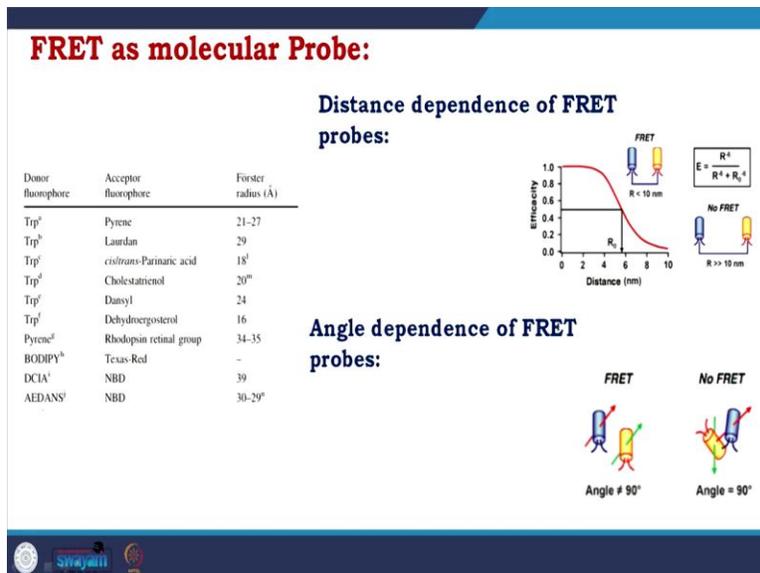
FRET is a very interesting concept and a lot of background researches are developed. In 1918 J. Perrin proposed mechanism of resonance energy transfer, by the way, if I just want to give your memory a little bit refresher. If you remember the nuclear overhauser effect here also tracking 2 hydrogen if their interaction or not. So you could think this as a similar effect if 2 probes comes close to each other and there in FRET relation instead of getting small emissions you get a resonating emission.

In 1922 Cario and Franck demonstrated the excitation of a mixture of Mercury and thallium atomic vapour's with 254 nanometer the mercury is resonance line also displayed thallium emission at 535 nanometer. In 1924 E. Gaviola and, P. Pringsham observed that an increase in the concentration of fluorescence in viscous solvent was accompanied by a progressive depolarization of the emission.

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And finally 1946 to 49, T. Forster in whose name today, we know developed the first quantitative theory of molecular resonance energy transfer.

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So without going into much depth I just want to say that this relation FRET is used globally as a molecular probe. The condition is the donor and acceptor would be in a distance where FRET relation should be valid. Here you see a lot of molecules like Pyrene, Laurdan, Cistrans Parinaric acid, Cholestatrienol, Dansyl they all are actually having FRET relation with tryptophan. There are other pairs like Pyrene with Rhodopsin retinal group, BODIPY with Texas red, DCIA with NBD and AEDANS with NBD, all you see there is a Forster radius in angstrom. FRET is distant dependent, R is the distance and when you see the efficacy, the efficacy reduced with distance.

More interestingly, if you see that in some of the pair, people found that change in the angle, when the angle comes at 90 degree, there is no FRET. So we know that FRET is distance dependence, distance dependence of the FRET is known but new research is also coming with the angle dependence of FRET.

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## Fluorophores in Biology:

Intrinsic fluorophores in proteins include Trp, Tyr & Phe

Trp & Tyr give stronger spectra than Phe, & Tyr is frequently quenched as a result of proton transfer in the excited state

The bases of DNA nucleotides & of some co-factors (e.g. NAD) are also intrinsic fluorophores, although they produce weak spectra

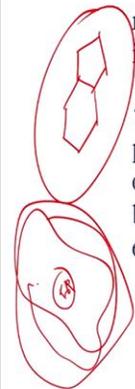
As I told fluorophore in Biology: Intrinsic fluorophores in protein include tryptophan, tyrosin, and Phenylalanine. Tryptophan and tyrosine gives stronger spectra than phenylalanine and tyrosine is frequently quenched as a result of proton transfer in the excited state.

The bases of DNA nucleotides and of some cofactor (like NAD) are also intrinsic fluorophores, although they produce weak spectra's.

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## Tryptophan:

**Tryptophan**, the dominant intrinsic fluorophore. A protein may possess just one or a few Trp residues, which facilitates interpretation of the spectral data.



**Tryptophan** is very sensitive to its local environment. It is possible to see changes in emission spectra in response to conformational changes, subunit association, substrate binding, denaturation, and anything that affects the local environment surrounding the indole ring.

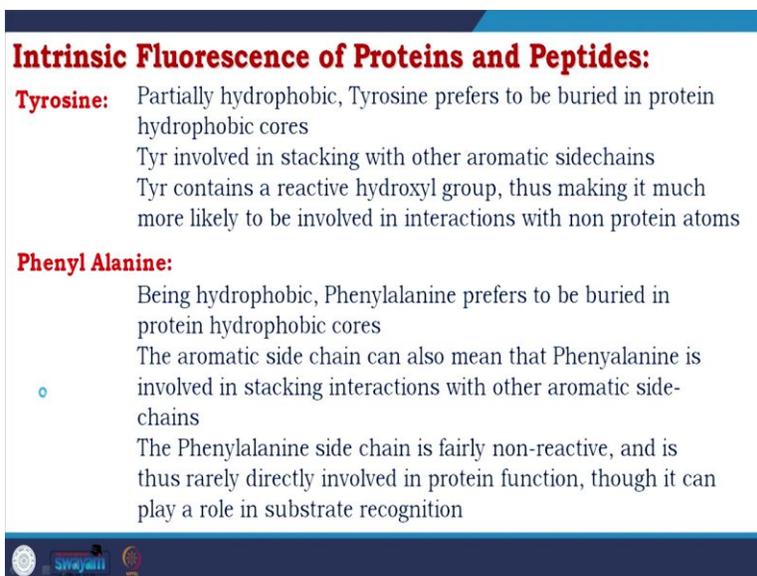
Trp appears to be uniquely sensitive to collisional quenching, either by externally added quenchers, or by nearby groups in the protein.

So as I told tryptophan is very important when it is concern to the fluorophore study of fluorescence of protein. Tryptophan, the dominant intrinsic fluorophore a protein may poses just one of the few tryptophan residues which facilitate interpretation of the spectral data. Tryptophan is very sensitive to its local environment. It is possible to see changes in emission spectra in

response to conformational changes, subunit association, substrate binding, denaturation and anything that affect the local environment surrounding the indole ring. The indole ring has the reason of the fluorescence's but there is something else. What is something else? See tryptophan is quite a big molecule. This is the tryptophan moiety. Now why tryptophan is so important? Suppose this tryptophan is present inside the hydrophobic core of the protein. Now when you add some heat or change the pH or add some denaturing agent, for most of the soluble protein you will see the possible opening, now when the protein start opening tryptophan would be more exposed to water. So there would be continuous shifting. And if you are lucky enough to have one tryptophan you could even calculate quantitative rates. It is also possible for ligand binding and anything which change the confirmation of the protein.

Tryptophan appears to be uniquely sensitive to collisional quenching either by external added quencher or by nearby groups in the protein.

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**Intrinsic Fluorescence of Proteins and Peptides:**

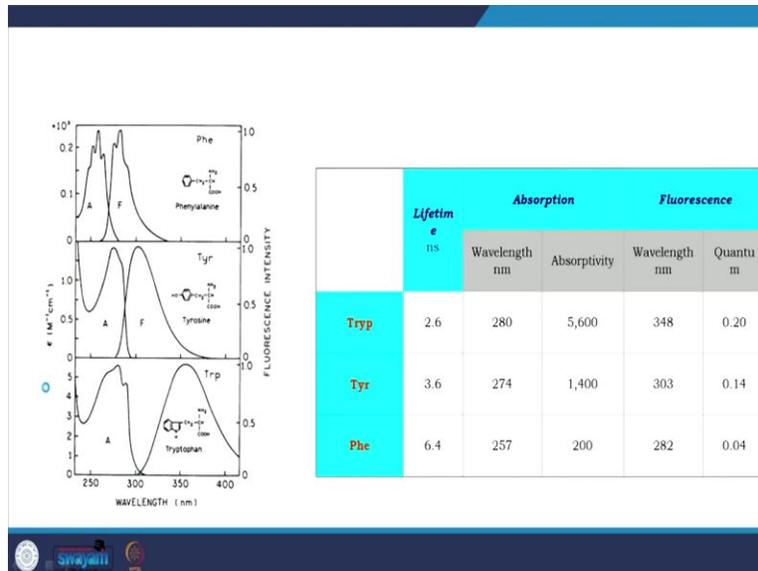
**Tyrosine:** Partially hydrophobic, Tyrosine prefers to be buried in protein hydrophobic cores  
Tyr involved in stacking with other aromatic sidechains  
Tyr contains a reactive hydroxyl group, thus making it much more likely to be involved in interactions with non protein atoms

**Phenyl Alanine:**  
Being hydrophobic, Phenylalanine prefers to be buried in protein hydrophobic cores  
The aromatic side chain can also mean that Phenylalanine is involved in stacking interactions with other aromatic side-chains  
The Phenylalanine side chain is fairly non-reactive, and is thus rarely directly involved in protein function, though it can play a role in substrate recognition

Tyrosine is partially hydrophobic, Tyrosine prefers to be buried in protein hydrophobic course. Tyrosine involved in stacking with other aromatic side chain, Tyrosine content reactive hydroxyl group, thus making it much more likely to involve in interaction with non protein atoms. Phenyl Alanine; it is hydrophobic. Phenyl Alanine prefers to be buried in protein hydrophobic cores. The aromatic side chain can also mean that phenyl Alanine is involved in stacking interactions with other aromatic amino acid side chains. The Phenyl Alanine side chain is fairly non reactive

and is thus rarely directly involved in protein function, though it can play a role in substrate recognition.

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Here is the data of tryptophan, this is tyrosine, and this is Phenyl Alanine. If you see all of them have fair lifetime 2.6 nanosecond 3.6 ns and 6.4 ns, absorption wavelength 280 nm, 274nm, 257 nm, but absorptivity 5600 for tryptophan 1400 for tyrosine and 200 for phenyl alanine. Also, the quantum yield is 0.24 for tryptophan 0.144 for tyrosine and only 0.04 for phenyl alanine.

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**General amino acids in Fluorescence Spectroscopy:**

In general Hydrophobic amino acids can be involved in binding/recognition of hydrophobic ligands such as lipids, those interaction could be studied using FS

Aromatic residues can also be involved in interactions with non-protein ligands that themselves contain aromatic groups via stacking interactions which could be trapped in FS

A common role for Tyr (Thr) within intracellular proteins is phosphorylation could trap through differential FS

Protein kinases frequently attach phosphates to Tyrosines in order to facilitate the signal transduction process

Protein kinases are highly specific (i.e. Tyrosine kinases generally do not work on Serines/Threonines and vice versa)

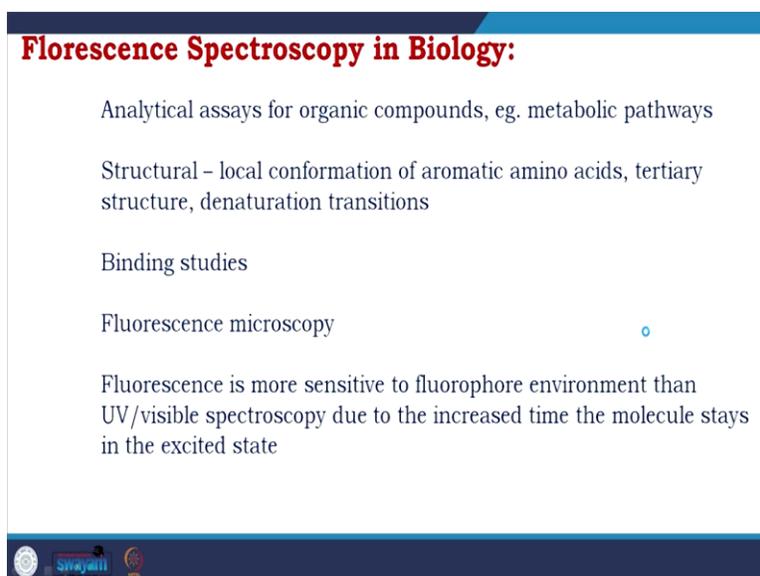
Folding study could be performed using FS targeting Trp and Tyr as internal and ANS as external fluorophores

*serines → water*

So if you look at other amino acids. In general hydrophobic amino acid can be involved in binding/recognition of hydrophobic ligand such as lipids, and those interactions would be studied

during fluorescence spectra. Aromatic residues can also be involved in interaction with non protein ligand that themselves contain aromatic group via stacking interactions which would again be studied using fluorescence Spectroscopy. A common role of tyrosine within intracellular protein is phosphorylation that could trap through differential fluorescence spectrum. Protein kinases frequently attached phosphates to tyrosines in order to facilitate the signal transduction process. Protein kinases are the highly specific (tyrosine kinase is generally do not work on serine and threonine and vice versa). Folding study could be performed using fluorescence Spectroscopy targeting tryptophan mainly but also tyrosine as internal and ANS as external fluorophore.

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**Fluorescence Spectroscopy in Biology:**

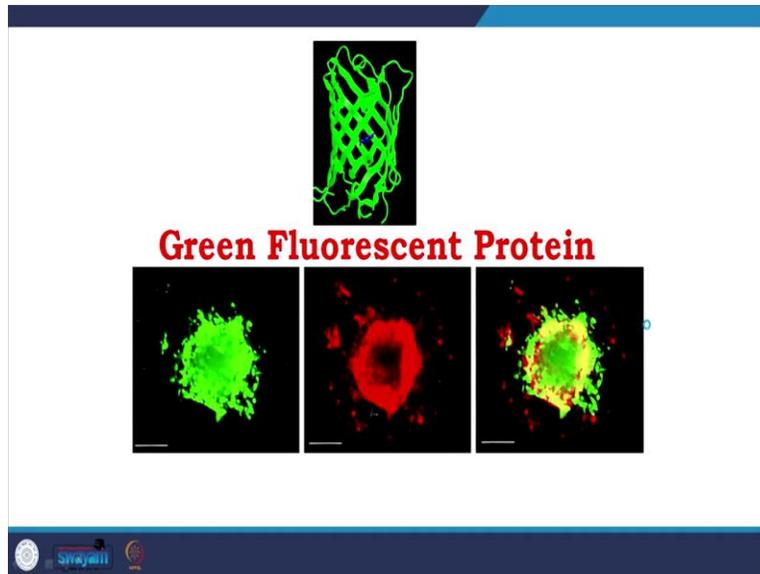
- Analytical assays for organic compounds, eg. metabolic pathways
- Structural - local conformation of aromatic amino acids, tertiary structure, denaturation transitions
- Binding studies
- Fluorescence microscopy

Fluorescence is more sensitive to fluorophore environment than UV/visible spectroscopy due to the increased time the molecule stays in the excited state

Fluorescence spectroscopy in Biology: Analytical access for organic compounds to get the metabolic Pathways. Structural: local conformation of aromatic amino acid, tertiary structure, denaturing transitions. Binding studies, fluorescence microscopy is very popular. Fluorescence is more sensitive to fluorophore environment than UV visible spectroscopy due to increased time the molecules stay in the excited state.

And when I was talking about fluorescence microscopy it remind me with the one of the best innovation of this century which has changed the way we look at Biology it is called green fluorescence protein.

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So what is green fluorescent protein? The protein which existed for more than 160 million years in one of the species? And when you said GFP green fluorescent protein, you should immediately remember the name of Osamu Shimomura.

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### History of GFP: Osamu Shimomura



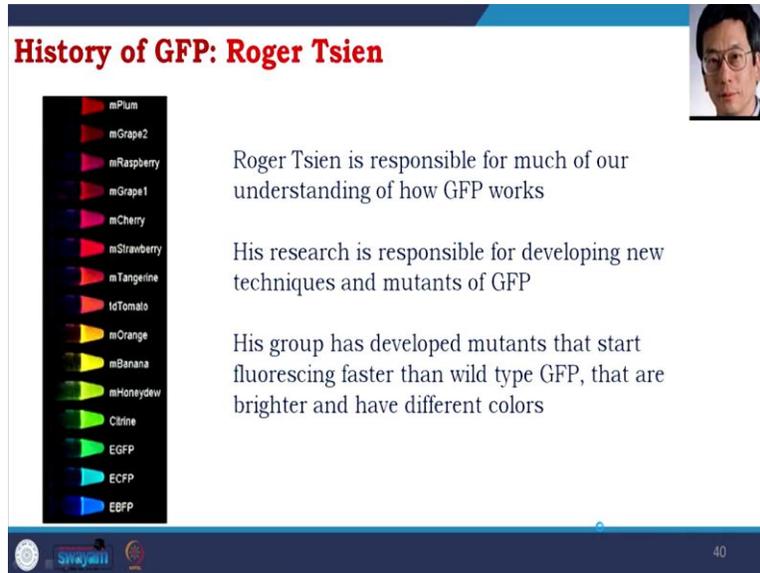
Osamu Shimomura was the first person to isolate GFP and to find out which part of GFP was responsible for its fluorescence  
 His meticulous research laid the solid foundations on which the GFP revolution was built  
 This jellyfish produces green bioluminescence from small photoorgans located on its umbrella  
 When the rings of twenty to thirty jellyfish are squeezed through a rayon gauze, a faintly luminescent liquid called squeezeate is obtained  
 Shimomura went to Friday Harbor, Washington, to collect this squeezeate and to extract from it the substance responsible for its luminescence  
 Shimomura estimates that he collected over a million *Aequorea* specimens, cut off the rings, and produced squeezeate to conduct his research



Who is the father of this protein. So Osamu Shimomura was the first person to isolate green fluorescent protein and to find out which part of the green fluorescent protein was responsible for its fluorescence. His meticulous research let the solid foundation on which GFP revolution was built. This jellyfish produces green bioluminescence from small photoorgans located in its umbrella. So if you see this is the umbrella of the Jelly fish and this small white things are the photoorgans. And when the Rings of 20 to 30 Jellyfish are squeezed through rayon gauze, a

faintly luminescent liquid called squeezeate is obtained. This is the squeezeate this green thing. Shimomura went to Friday Harbour in Washington to collect this squeezeate and to extract from its substance responsible for its luminescence. Shimomura estimates by its own that he collected over a million of Aequorea specimens, cut of the ring and produce squeezeate to conduct his research.

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**History of GFP: Roger Tsien**

Roger Tsien is responsible for much of our understanding of how GFP works

His research is responsible for developing new techniques and mutants of GFP

His group has developed mutants that start fluorescing faster than wild type GFP, that are brighter and have different colors

- mPlum
- mGrape2
- mRaspberry
- mGrape1
- mCherry
- mStrawberry
- mTangerine
- IdTomato
- mOrange
- mBanana
- mHoneydew
- Citrine
- EGFP
- ECFP
- EBFP

40

The person who have changed this, who have converted the green fluorescent protein to enhance Green fluorescence protein and then there are series in blue fluorescent protein, enhance fluorescence protein Roger Tsien is responsible for much of our understanding of how this GFP works. His research is responsible for developing new techniques and mutants of GFP. I will try to talk about this little bit. This is like volume of work. His group has developed mutant that start fluorescing faster then wild type GFP, brighter and different colours.

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## History of GFP:

**Martin Chalfie's** graduate student, Ghia Euskirchen, successfully incorporating the GFP gene into E. coli and they published their results in the February 11, 1994 issue of Science

**Douglas Prasher** was the first person to realize the potential of GFP as a tracer molecule. In 1987, he got the idea that sparked the GFP revolution. He thought that GFP from a jellyfish could be used to report when a protein was being made in a cell

Major breakthrough in GFP applications came when **Sergey Lukyanov** found some GFP-like proteins in corals. His findings (DsRed) resulted in the discovery of many new GFP-like proteins in non-bioluminescent and sometimes even non-fluorescent marine organisms

In 2008 **Osamu Shimomura, Martin Chalfie** and **Roger Tsien** were awarded the **Nobel Prize** in Chemistry for "the discovery and development of the green fluorescent protein, GFP"



Martin Chalfie another landmark another Legend in GFP, his graduate student Ghia Euskirchen successfully incorporates GFP into the E coli. This is the first time they have expressed this gene in other organism. They published their result in Science. Douglas Prasher was not at the initial activities, but he was the first person to realize the potential of GFP as tracer molecule and in 1987 he got the idea that sparked the GFP revolution.

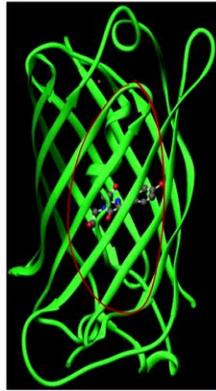
He thought that GFP from Jellyfish could be used to report when a protein was being made in a Cell. Major breakthrough in GFP application came when Sergey Lukyanov found some GFP like protein in Corel. His finding DsRed resulted in the discovery of many new GFP like protein in non bioluminescent and sometimes even non fluorescent marine organisms.

In 2008 Osamu Shimomura, Martin Chalfie and Roger Tsien were awarded the Nobel Prize in Chemistry for discovery and development of the green fluorescent protein.

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## Structure of GFP:

Composed of 238 amino acids



“Paint in a can”

Each monomer composed of a central alpha-helix surrounded by an eleven stranded cylinder of anti-parallel b-sheets

Cylinder has a diameter of about 30Ang and is about 40Ang long

Fluorophore located on central helix

This protein is composed of 238 amino acids. It is called Paint in a can because as I explain already and I am going to talk few words about that. It could have come in any and every colour. So the overall structure as you see is the Beta sheet, but inside that if you could look here, you could have get that there is a Alpha helix and the fluorophore is attached.

The amino acids are attached to the Alpha helix. Each monomer is dimers remember I am showing about some AMS they monomers but initially it was a dimer. Each monomer composed of a central alpha-helix surrounded by an 11 standard cylinder of anti parallel beta sheet. Cylinder has a diameter of about 30 angstrom and is about 40 angstrom long. Fluorophore located on the central helix.

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**Chromophore:** Ser65-Tyr66-Gly67

Deprotonated phenolate of Tyr66 is cause of fluorescence

Forster Cycle (1949-Theodor Forster)

Proton transfer to His148

So what is the Chromophore? It is a protein. So how Chromophore comes; this is the fluorophore. You look at this the resemblance of amino acids I am showing it. This is the crystal structure where you see the actual core active side where the mutations are going on. So the core is actually coming from an Amino acid, 3 amino acids in serine 65, tyrosine 66 and glycine 67. Deprotonated phenolate of tyrosine 66 is cause of fluorescence.

I am going to show you. It is the Forster cycle and proton transfer to Histidine 148. How we are looking at? These are the three serine 65, tyrosine 66 and glycine 67. So, initial oxidation happened and auto catalysing cyclization taking place. Then you get this thing here the final fluorophore content series of conjugated double bonds. So here I am tell you one story when Seema Buddha was working he had a doubt that who is doing this categorization? Who is developing this fluorophore?

Because he was able to identify the fluorophore but looking at that he understood that there is a series of reaction happened. So he had idea that there is other enzyme present in that Jellyfish who is taking care of the cyclization reaction but it is a autocatalytic reaction no enzymes is required and that was understood when it was first expressed in E coli. It was very fine very well. And if you look at this Rigid Beta Barrel structure, they are very good to express very easy to express protein.

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**pGlo: GFP plasmid**

pGlo is a plasmid

- Circular DNA
- Can be transformed into bacteria
- Independently replicating

pGlo has Ampicillin Resistance

GFP on the plasmid is inducible by arabinose  
The GENE is NOT fluorescent, the PROTEIN is fluorescent  
GFP is under the control of a tightly regulated system on the plasmid  
GFP will only be turned on when arabinose is present

Biotechnology Explorer™  
pGLO® Bacterial Transformation Kit  
Catalog Number 164-00032-001  
explains.bio-rad.com

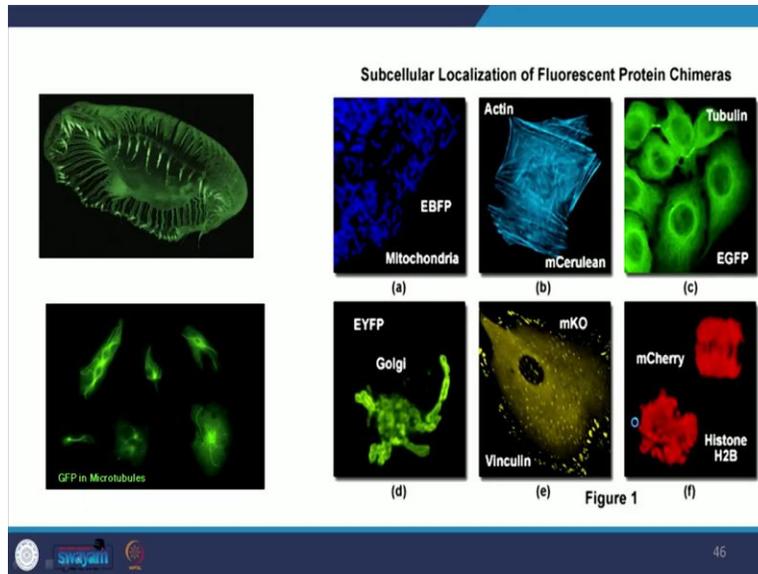
Transformation with a plasmid  
DNA plasmid

Bacterial chromosome  
Uptake of plasmid  
Stable transformation

And the revolution was father when there is a commercial plasmid pGLo. The pGLo is a plasmid with circular DNA can be transformed bacteria and independently replicating. So you have the plasmid. You put your gene you put it into bacteria. You put it into other organism and use, track that with the expression of the green fluorescent protein. It is ampicillin resistance, so you could mark it. Here the GFP on the plasmid is inducible by arabinose, which means that the gene is not fluorescent the Protein is fluorescent.

You could produce the protein by regulating arabinose. So the GFP is under the control of a tightly regulated system on the plasmid. And the GFP will only be turned on when arabinose is present. WE have change the way we look at Biology it has revolutionize. Now we have started with inflorescence. We have so many fluoroscences compound in our hand giving a so many options. I would say it is one of the best innovations to further proceedings of the biological research.

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So with that I would like to finish in this class I have tried to discuss the important point of fluorescence. How the electronic transition happened, how singlet ground state is excited state and returning is giving by releasing of energy giving of fluorescence. On the other hand the transition from singlet to triplet and coming back is giving you phosphorescence but what about the shift energy shift which is stock shift.

And then you go for you see that the fluorescence how it is quenched, how the quenching is affecting static quenching, dynamic quenching, Stern-Volmer plot and then FRET, how FRET is used as a probe and what are the dependence of the fluorescence and associated things and last but not the least the green fluorescent protein which has changed the way we are looking at biology today. Again I request all of you to listen to the classes.

I request all of you to send us the questions will try our best to provide you answer as much as possible. Please keep listening keep asking questions. Thank you.