Spectroscopic Techniques for Pharmaceutical & Biopharmaceutical Industries
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Lecture 23 - Fluorescence Spectroscopy

Hello students! Welcome back to the lecture. In the last lecture, I have discussed about applications of UV visible spectroscopy and I started discussing about fluorescence spectroscopy.

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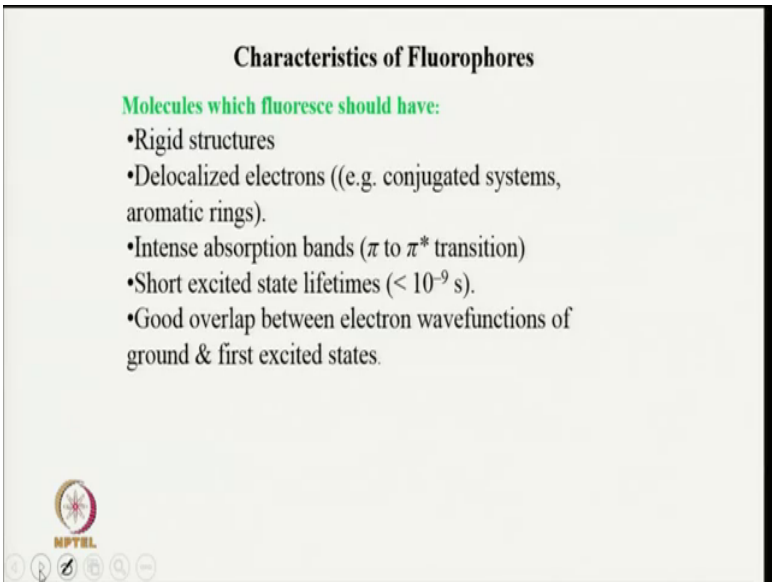

We discussed about few basic things about fluorescence spectroscope, we will again go back and have a recap on those topics and then I will discuss few more concepts which are required to understand the fluorescence spectroscopy. The characteristic of fluorophores are the molecule which process should have that the molecule must have rigid structure.

It must have delocalized electrons. For example, you can have a conjugated system or aromatic ring; they must have intense absorption bands pi to pi star transition. They should have short excited state lifetime less than nanosecond and there should be good overlap between electron wave functions of ground and first excited state. So we start with a molecule which has that characteristic and then we look at their fluorescence spectra.

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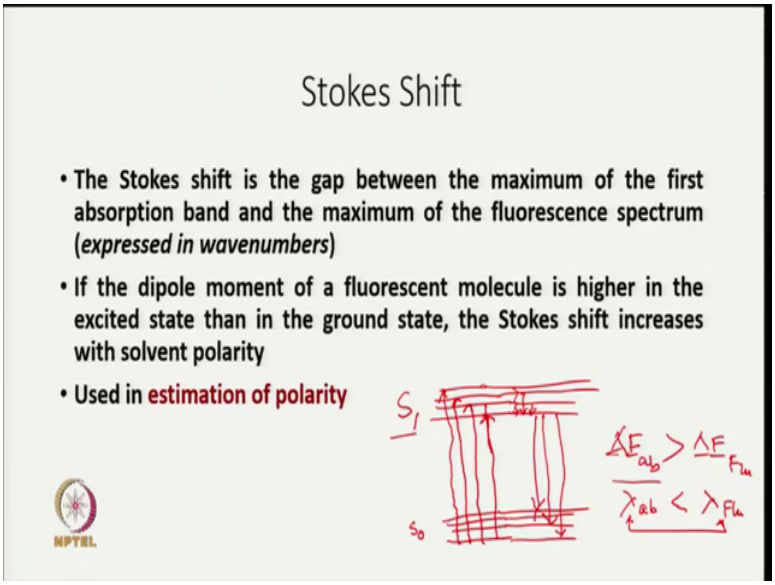

As I discussed in the last lecture one of the very important concept in fluorescence spectroscopy is stock shift, it is basically the gap between maximum of the first absorption band and the maximum of fluorescence spectrum.

So basically what we are looking at is the gap between the two bands, one corresponding to absorption and another corresponding to fluorescence spectrum. Later on I will show you that if dipole moment of a fluorescent molecule is higher in excited state than in ground state, the stock shift increases with solvent polarity and that can be used for estimation of polarity. So, I discussed about what is the reason for the stock shift in the last lecture, just to remind you what happens in fluorescence spectroscopy is when a fluorophore and fluorophore observes the radiation, what happens it goes from S0 ground state to S1 excited state and it can go to, sorry for this, it can go to any of the vibrational levels. It can go to any of the vibration levels, any of the vibrational levels on absorption of light.

But before coming back to ground state all of this will come back to the lowest vibrational level of x1 excited state, x1 excited state and then fluorescence takes place. Then fluorescence takes place. This is the way fluorescence happens. Now you can see that here Delta E of absorption is going to be higher than Delta E of fluorescence because your all fluorophore need to come back to lowest state of lowest vibrational state of S1 excited state. So gap between this is smaller than the gap which is between S naught and S 1 and so if Delta E absorption is higher than Delta E of fluorescence what does that mean is lambda absorption is going to be less than lambda fluorescence. So they are not equal and the difference between these two will give you the value of stock shift.

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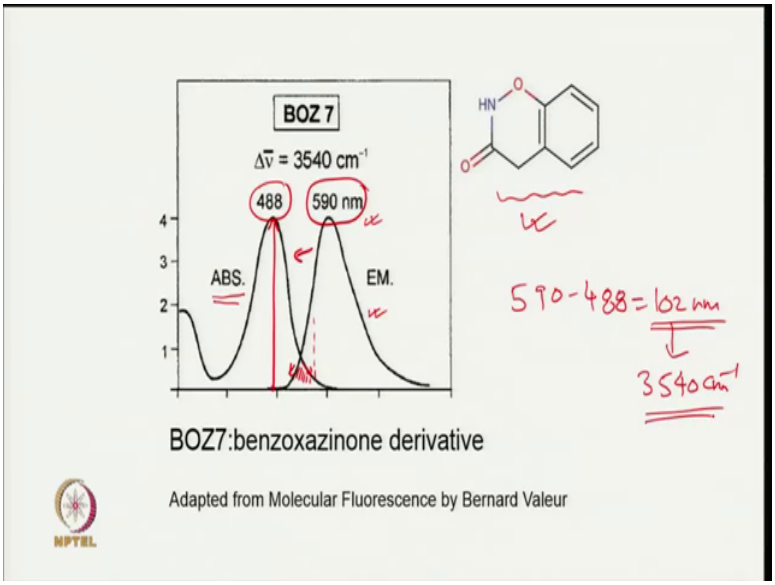

Now let us look at absorption spectrum or excitation spectrum of the molecule, this molecule BOZ 7 and the emission spectrum of the molecule. So this is your absorption spectrum and this one is emission spectrum. This molecule BOZ 7 absorbs at 488 nanometer and it emits at 590 nanometer and the difference is around 590 minus 488 it is 102 nanometer and this is equal to 3540 centimeter inverse in wave number unit and so stock shift is 102 nanometer or if you express in terms of wave number unit it is 3540 centimeter inverse.

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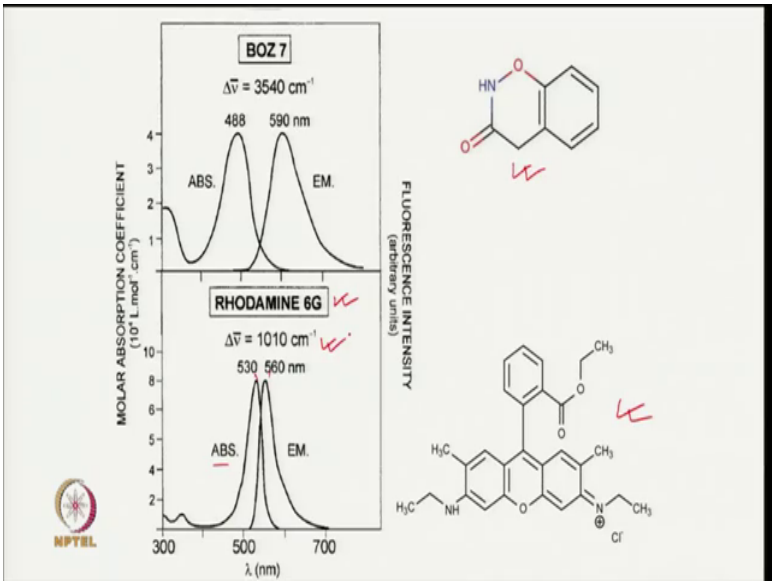

So this is what the molecule which we discussed. Now look at another molecule. Now you can see there are a lot of aromatic ring and conjugated system. So this molecule are typical fluorophore, this molecule is rhodamine 6G, it absorbs at 530 nanometer and emits at 560

nanometer. So your Delta Lambda is 30 nanometer and delta nu bar is 1010 centimeter inverse for rhodamine 6G. So this is stock shift for rhodamine 6G.

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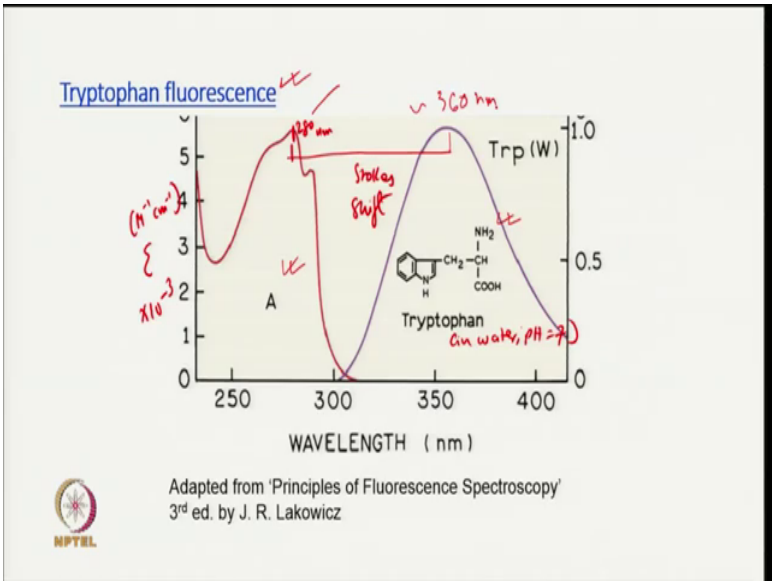

So in protein as I discussed you in the UV visible spectroscopy chapter there are 3 aromatic rings or molecule with side chain as aromatic ring, these amino acids are tryptophan, tyrosine and phenylalanine. So let us look at how does they fluoresce. This is tryptophan fluorescence. Here is your absorption band and here is emission band. So tryptophan absorbs at 280 nanometer and it emits around 360 nanometer and the difference between these two is around 80 nanometer.

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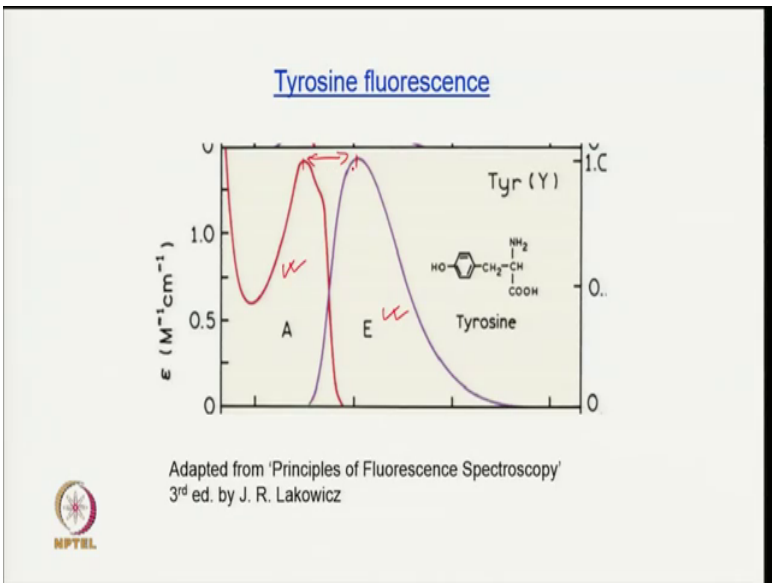

This is the tyrosine fluorescence profile. This is absorption band of tyrosine and this is emission band of tyrosine. Now you can look at that there is a difference between lambda max of absorption and lambda max of emission and this shift is known as your stock shift.

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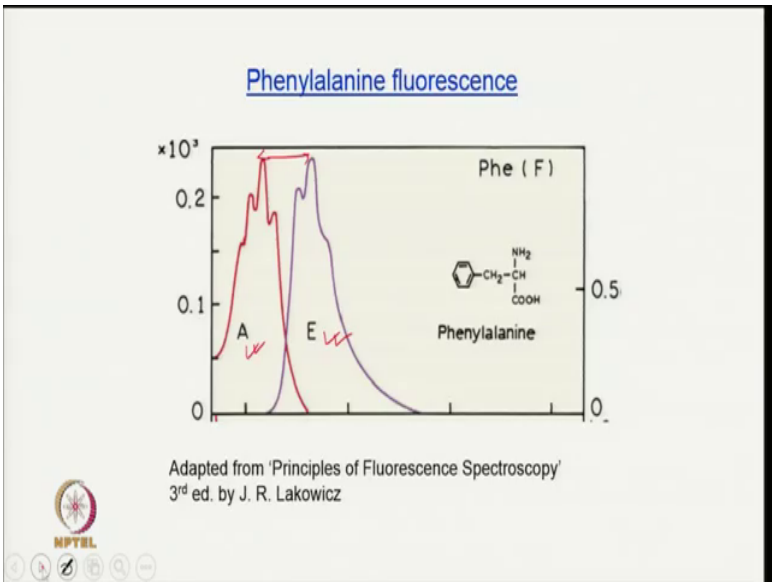

Similarly we can also look at phenylalanine fluorescence and this is your typical absorption band and this is your emission band for phenylalanine and there is again difference here between lambda max of absorption band and lambda max of emission band. And this is your stock shift for phenylalanine. So I discussed about stocks shift, now let us go to quantum yield.

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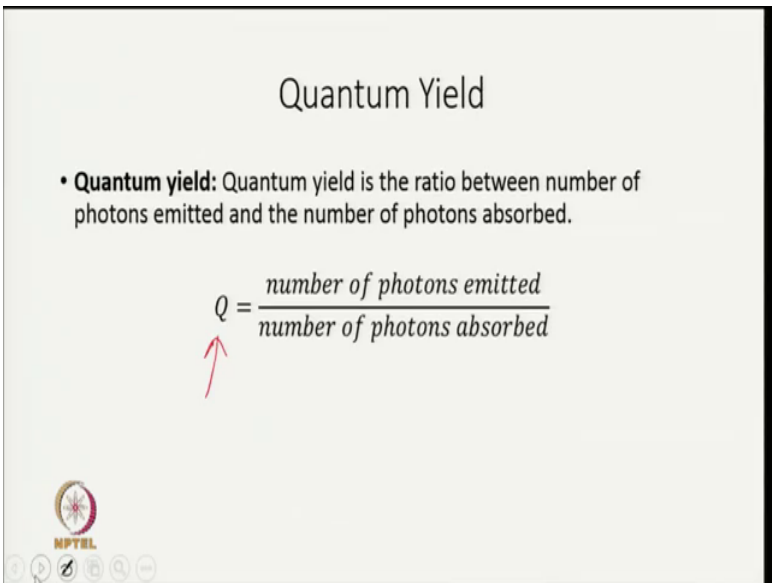

Quantum yield is the ratio between number of photons emitted and number of photons absorbed. So it is a kind of efficiency. It is represented by a symbol Q. So Q is equal to number of photons emitted divided by number of photons absorbed.

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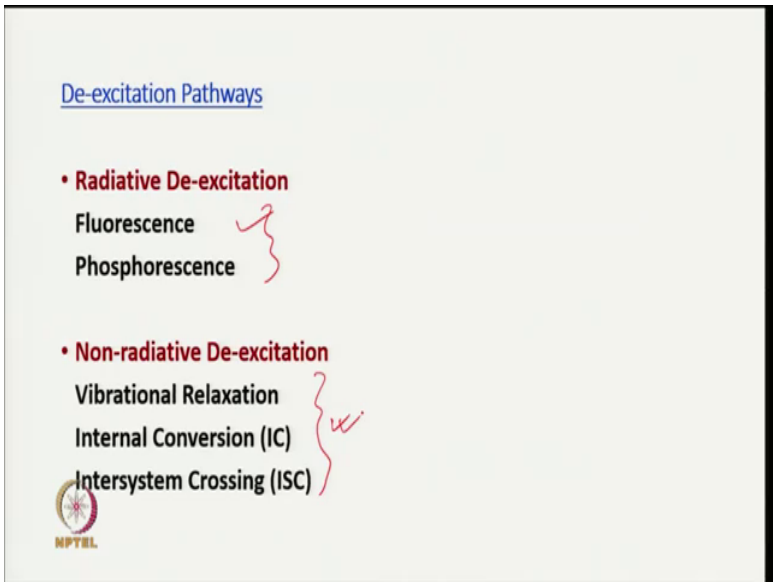

So number of photons emitted is going to be less than number of photon absorbed. This is because of your D excitation pathways which is due to vibrational relaxation, internal conversion and inter-system crossing. I discussed a bit about that in the last chapter, but these are the three non-radiative de-excitation and these are the radiative D excitation which happens with the emission of photons.

So the quantum yield for fluorescence is going to be less than 1 since fluorescence competes with these three processes and your quantum yield is given by number of photons emitted by number of photons absorbed.

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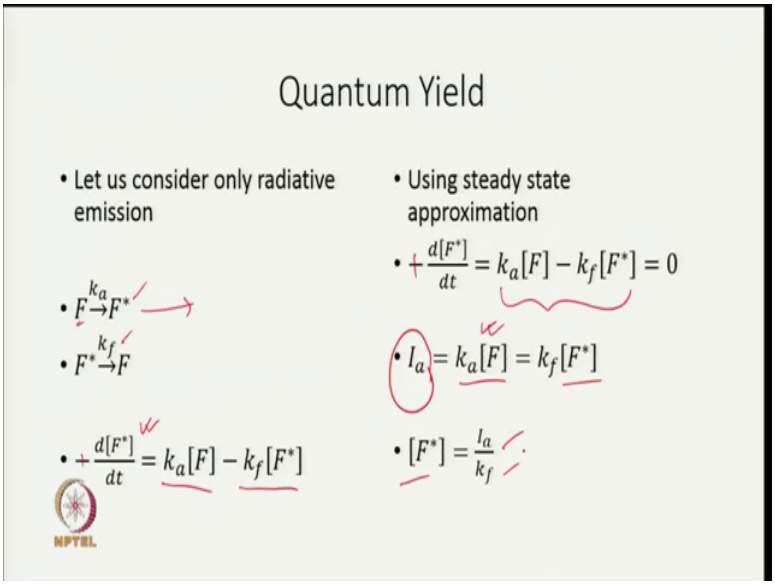

So let us calculate quantum yield and here we will use kinetics. So if suppose if I consider only radiative emission what does that mean only fluorescence is taking place. There is no other way of D excitation. So first step is F is going to F star, it means fluorophore absorbs the radiation and goes to excited state and now excited state comes back to ground state through fluorescence, a rate of fluorescence is given by k f. So as we write kinetic equation we will again write the kinetic equation for the excited state. So this is your rate of decrease of excited state F star. So minus dF star by dt will be given by k a into F minus k f into F star.

So first one has so this is basically plus okay, so this will be plus. So what we are looking at is how F star changes with time and since F star is formed by this reaction, so there will be plus sign here and here there will be minus sign. Since F star is getting used up F star is getting consumed in the second step. Now we know that F star is intermediate and so we can apply steady state approximation because F star is quite unstable, it goes very fast to the ground state and so we can apply a steady state approximation. When we apply a steady state approximation we will make this equal to zero. So dF star by dt is equal to k a into F minus k f into F star, this should be equal to zero. What does that mean is k f is equal to k f multiplied by concentration of F star and this is basically equal to intensity of absorption.

So we generally do not write k into F we simply denote that by I a. So what is F star? F star is simply I a by k f. So now I know what is the amount of excited fluorophore amount of excited fluorophore that is given by I a by k f.

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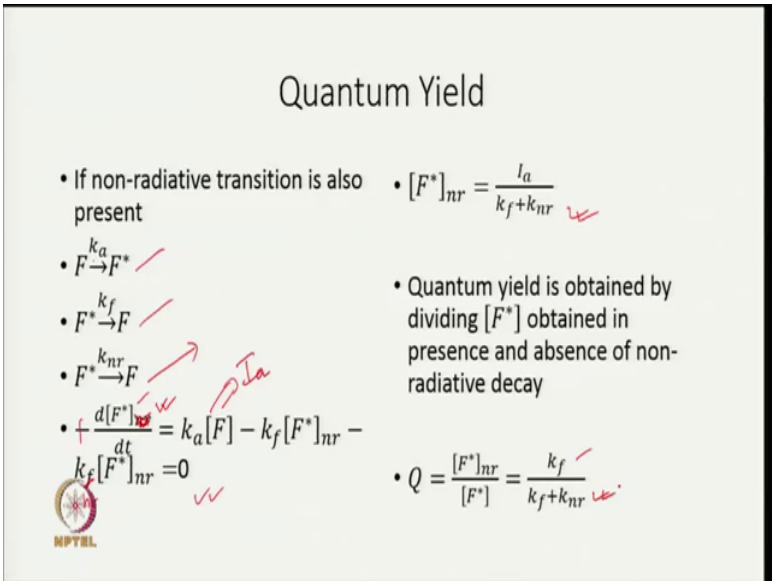

Now let us think of if non-radiative transition is also present, then apart from these two steps the third step is also taking place. So now F star is going to ground a state not only by fluorescence but it is also going by non-radiative process. In that case we can write dF star non-radiative by dt. So in the presence of this is basically your F star in presence of non-radiative process. So we can just write simply - d F star nr by dt is equal to k a into F minus k f, again there will be plus sign here. k f into F star and k f into k nr into F star nr, k nr into F star nr is equal to 0.

So F star nr should be equal to I a by, I a divided by, I a is basically k a F. So this is equal to I a and if I put 0 then F star should be this is I a divided by k f plus k nr and now I can obtain quantum yield. So quantum yield is basically the if you remember it is the amount of photon emitted divided by total amount of photon absorbed. So basically Q is F star in presence of non-radiative process divided by F star and so what you will get is k f divided by k f plus k nr and that will give you the quantum yield.

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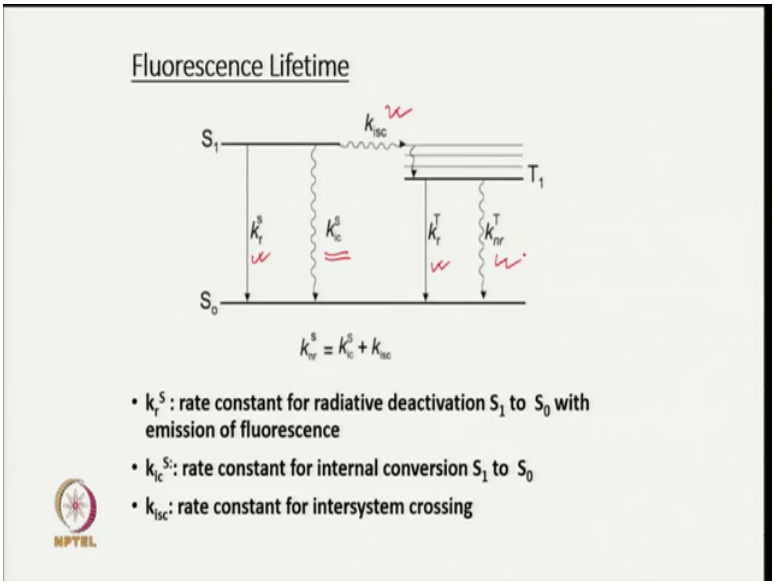

Now let us discuss about another very important concept in the fluorescence which is fluorescence lifetime, so the three processes which we have already discussed, one is fluorescence. Fluorescence is radiative deactivation between S1 and S naught whereas internal conversion is rate constant for non-radiative process from S1 to S naught and this is again a non-radiative process, it is a rate constant for intersystem crossing and this is your fluorescence, the rate constant for phosphorescence and this is the rate constant for non-radiative process from T1 to S naught. So these are the different rate constant which we can think.

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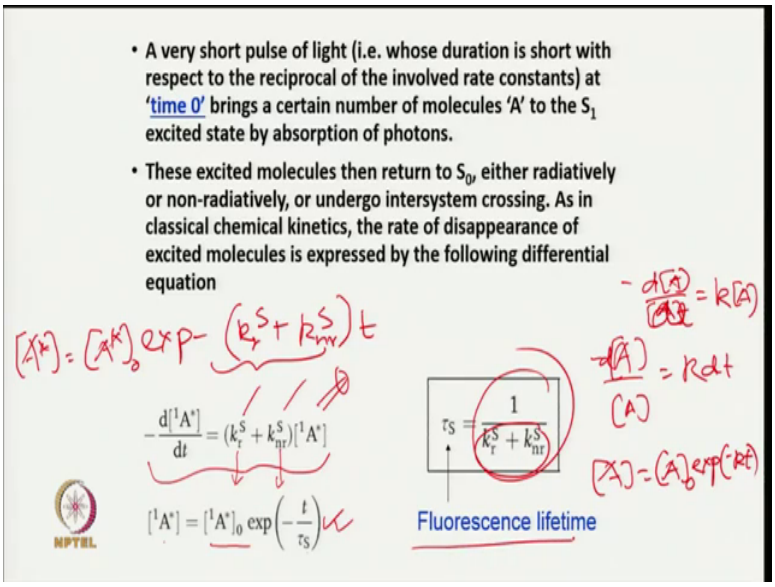

So if suppose we apply a very short pulse of light whose duration is short with respect to reciprocal of the involved rate constant at time 0. So initially we just apply a very short pulse, what will that do is it will bring a certain number of molecule K 2 S 1 excited state by absorption of photon. This excited molecule then returned to S naught. As I discussed previously either radiatively or non-radiatively or can undergo intersystem crossing. So as in chemical kinetics rate of disappearance of excited molecule can be expressed like this: This already I have discussed but now A star is getting decreased by radiative and this is by non-radiative process and these are the rate constant for radiative process.

This is the rate constant for non-radiative process and from that what you will get is A star which is basically the F star which I was discussing earlier, that A is here fluorophore and star means excited state, so A in excited state at a given time will be given by A star naught into exponential minus T or basically divided by tau S where tau S is simply the inverse of this rate constant, inverse of this. So this is simple first order chemical reaction if I have minus dA by A is equal to K or dA by dt is equal to K A, then we write minus dA by A is equal to K dt and the solution of this is A is equal to A naught exponential minus k t.

And so if I saw this what I am going to get is A star is equal to A star naught into exponential minus this K rs plus K nr S into t and what I did is I took tau S is equal to 1 by this whole quantity and so what you can write is your this value will be equal to 1 by tau S. So that is how you get this equation and now you can think of that since this is the tau is inverse of rate constant. So it will have the unit of time and this time is known as lifetime and that is what we mean by fluorescence lifetime.

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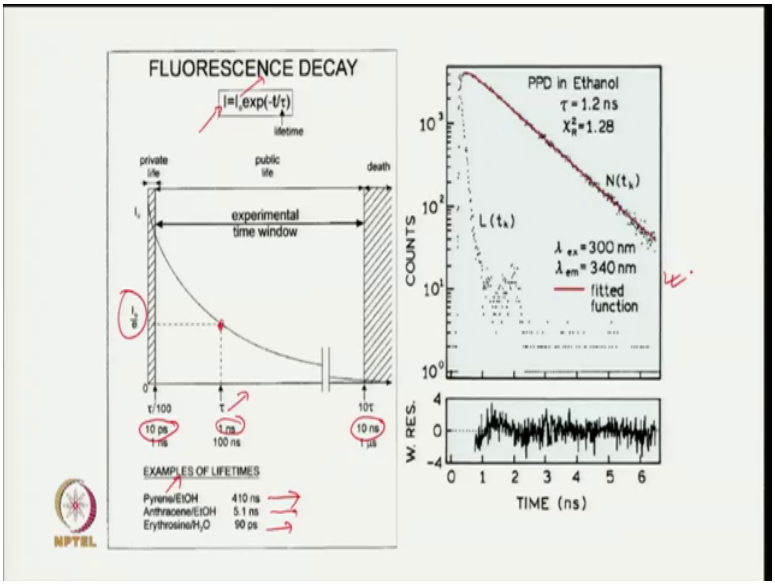

And if I write this equation, here I is fluorescence intensity at time t. I naught is initial fluorescence intensity and this is exponential minus t divided by tau, we expect I to decrease exponentially and that is what you can see here and if I is equal to I naught by E or F is equal to F naught by E then the time will corresponds to tau value and this will give you lifetime. So for example, pyrene in ethanol has lifetime around 410 nanosecond, anthracene in ethanol has lifetime 5.1 nanosecond, Erythrosine H2O gives you 90 picosecond. This graph I will explain when I will discuss the time-resolved fluorescence, so I will leave it here.

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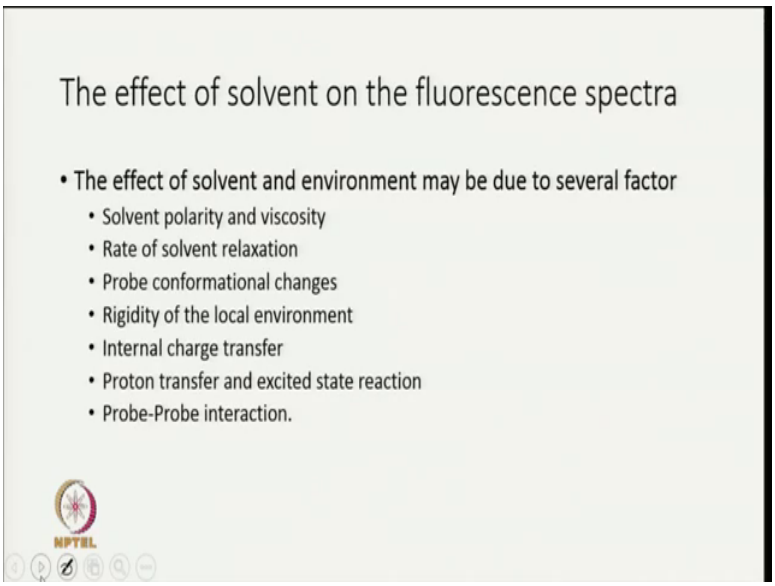

So now we know what is the quantum value and what is the lifetime. Now let us go and look at the effect of solvent on fluorescence spectra. The effect of solvent and environment may be

due to several factors, one is your solvent polarity and viscosity. The second is rate of solvent relaxation. Third is conformational changes due to solvent. Fourth is rigidity of local environment. Fifth is internal charge transfer, then another factor is proton transfer and excited state reaction and last one is probe interaction. So we will discuss few of them but same logic can be applied in every other cases.

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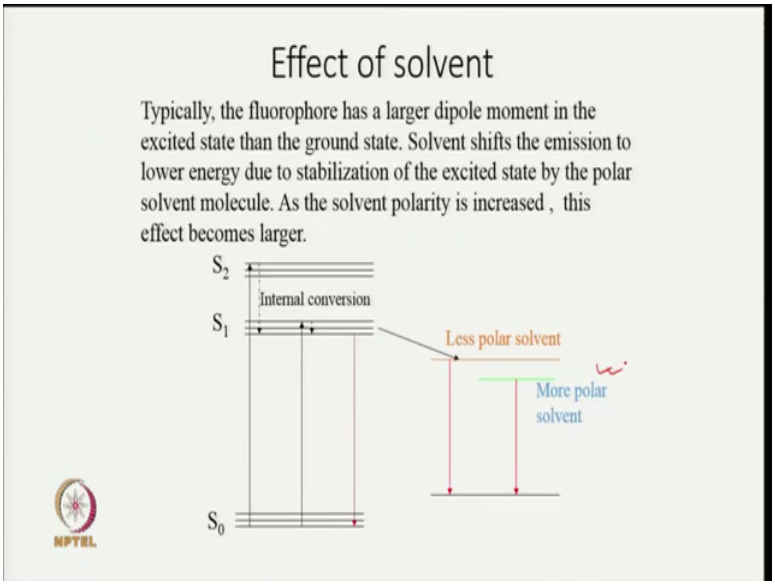

So how does solvent affect the fluorescence intensity or lambda max? Typically the fluorophore has a larger dipole moment in the excited state than in the ground state. So solvent shifts the emission to lower energy due to stabilization of the excited state by the polar solvent molecule. So what it does is what a polar solvent does is it stabilizes both S naught and S 1 but it stabilizes the excited state to a greater extent and what does that mean is that if you have a more polar solvent the energy gap is going to be smaller in comparison to the fluorophore in less polar solvent and when this energy gap is smaller what does that mean is lambda max increases in more polar solvent.

So the more polar solvent generally leads to increase in lambda max, while in less polar solvent the lambda max is smaller.

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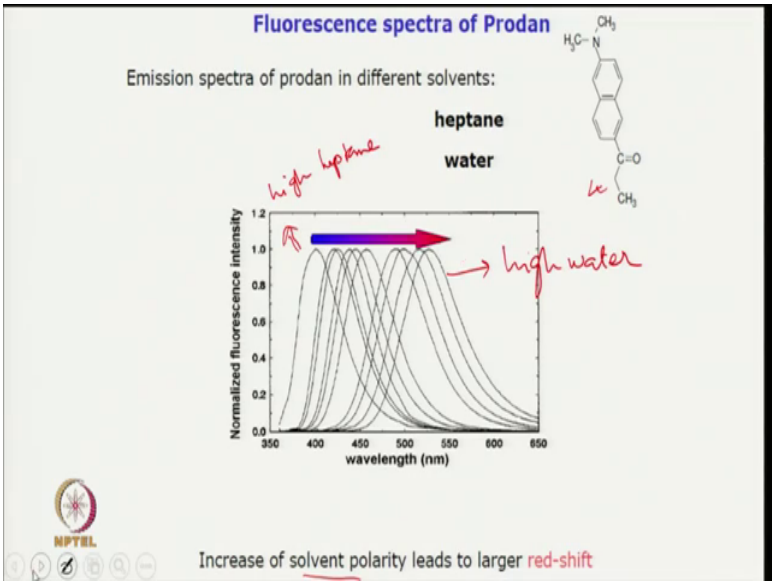

Now I will give you one example, this compound is known as prodan and here is the emission spectra of prodan in heptane water mixture and what you can see is that increase in your solvent polarity, there is increase in lambda max. So this is the fluorescence spectra of prodan in heptane water mixture of different ratio. So when heptane is high then you have more non-polar solvent and when water is high you have polar solvent. So you are going from less polar solvent to more polar solvent. So here we have a high percentage of high heptane and at this place you have a high water content. But all the solvents are mixture of heptane and water and as you go from high heptane to high water polarity increases and once polarity increases lambda max also increases and that leads to red shift.

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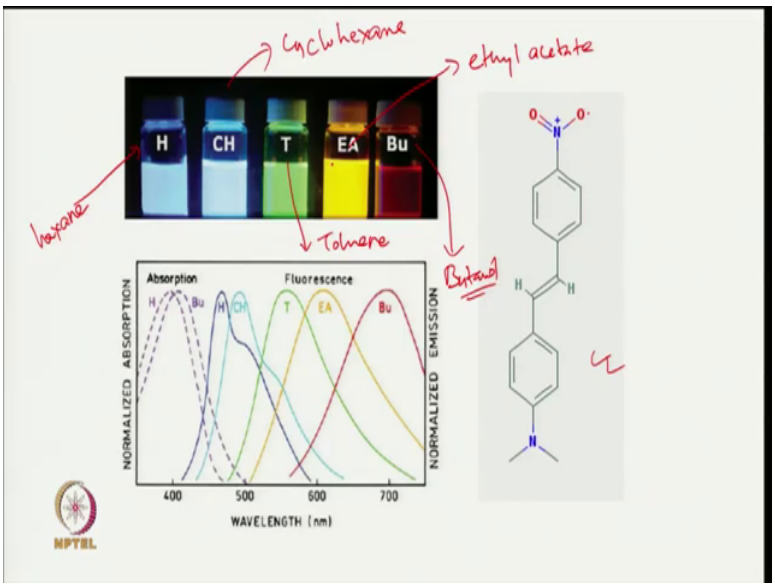

Now look at this dye and what here has been done is the spectra of this dye has been taken in different solvent. For example, H basically tells you about hexane, so now you have taken this dye in hexane. This is cyclohexane, this solvent is toluene, this is ethyl acetate and this is your butane and now you can see that from hexane to cyclohexane to toluene to ethyl acetate to butane, you see there is a shift towards higher wavelength and that basically tells you about how you are going from your high polar to less polar solvent, your lambda max is increasing.

So as the polarity of solvent increases the lambda max of the dye increase. Sorry this view is for butanol. So please correct it, this is not for butane, this is for butanol and that is why this one is more polar compared to ethyl acetate.

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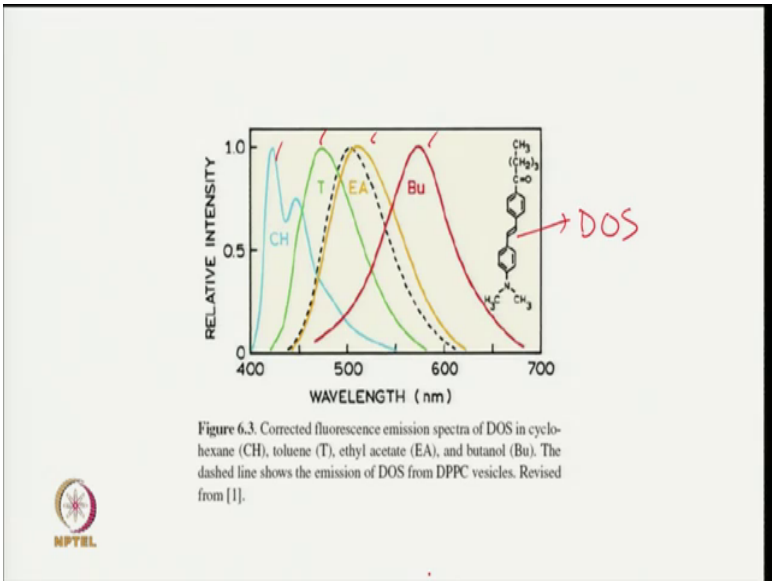

Now this is the spectra of a dye called DOS in again cyclohexane, toluene, ethyl acetate and butanol and you can look at again there is a shift towards higher wavelength and that means that if you have a, if you put a fluorophore in a polar solvent lambda max is going to be higher or there will be Red shift. Now this the effect of polarity on fluorophore polarity of environment of solvent on the fluorophore has many important implications. One of that is if you have a protein and there is a fluorophore the lambda max of fluorophore in a protein will depend on its environment, lambda max of fluorophore in protein will depend on environment. If the environment of the fluorophore is polar then lambda max will be higher. If it is in nonpolar condition then then lamda max is going to be lower.

And that is the reason the effect of or the shift in lambda max is used to probe conformational changes in a protein and as I discussed you that most common fluorophore which we study in protein is your tryptophan.

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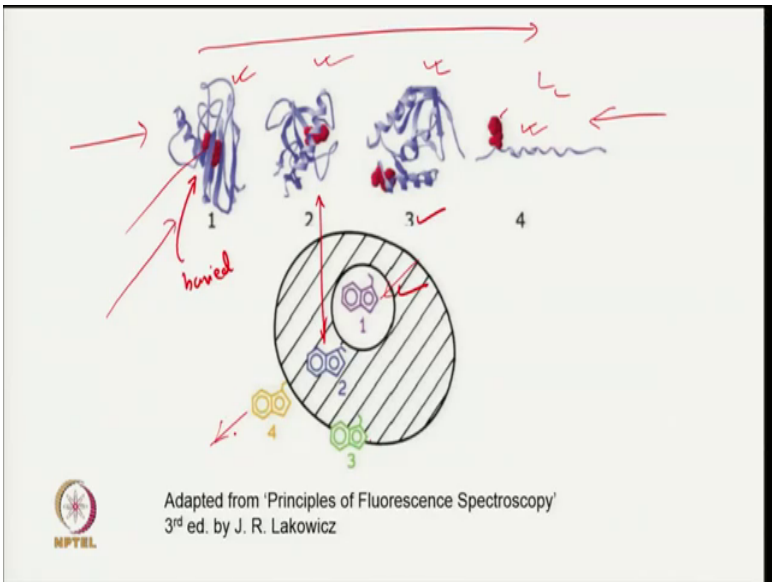

So now look at the different conformation of same protein. So here is a native protein and this one is your unfolded protein and there is a tryptophan here, this is your tryptophan. Now in this conformation tryptophan is buried, in this conformation tryptophan is totally exposed and you have other conditions where the accessibility of tryptophan is different. So as you go from this conformation to this conformation accessibility of protein will increase. Now you can think of this is keen to tryptophan being in a hydrophobic solvent when you talk about conformation 1. So as you can see that in conformation 1 tryptophan is completely buried, that means when tryptophan is buried it is surrounded by hydrophobic environment and so you can think of tryptophan being in a hydrophobic environment.

And when it is in the secondary state you can think of it is here and when it is third stage, third conformation it is at this position and when the it is incompletely exposed tryptophan is completely exposed in protein then it is basically in the water.

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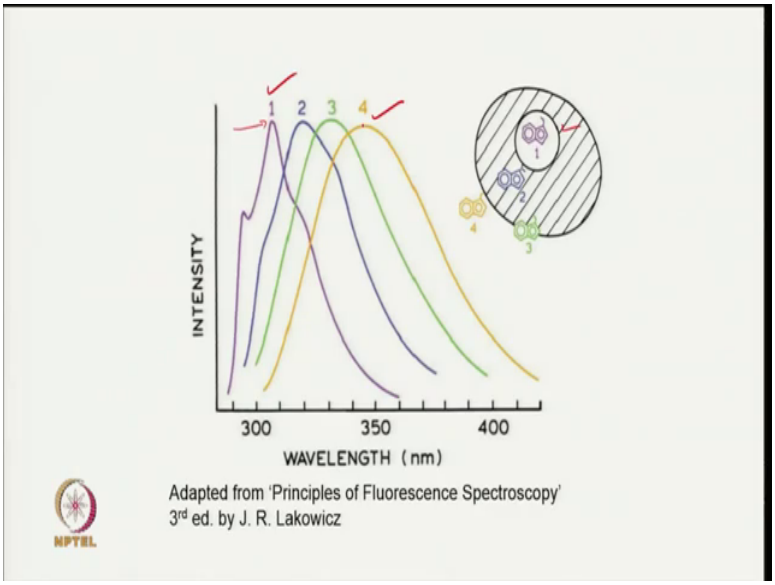

And as expected when fluorophore is in different environment the lambda max will change. If fluorophore is in more polar environment then lambda max will be higher. When fluorophore is in hydrophobic environment lambda max is going to be lower and that is what you can see that when this fluorophore is in very hydrophobic condition then lambda max is the lowest. And when it is in water then lambda max is high and so fluorescence of a fluorophore can be used to know the accessibility of tryptophan in a protein and that will in turn give you idea about the conformational changes taking place in protein.

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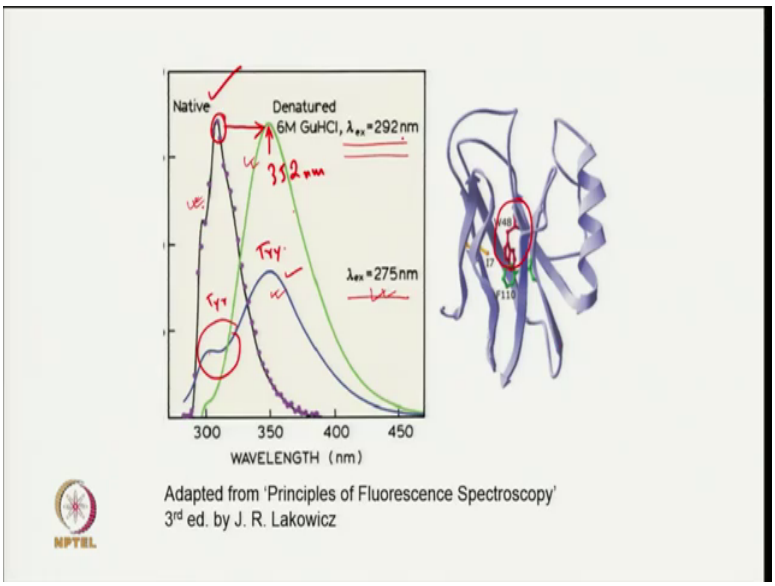

Now look at this protein and here you are looking at tryptophan this is tryptophan, this is tyrosine, this is for native state. So for native state you have this and if the protein is in six

smaller vanadium hydrochloride then you have this curve. When you excite the tryptophan at 292 nanometer then you get this two curve, this is for native, this is for denatured. So denatured means again tryptophan is completely exposed. This tryptophan 48 is completely exposed. You can also excite your protein at 275 nanometer but what it will do is it will excite both phenylalanine and tryptophan and then you see the tryptophan fluorescence will go down and this is for tryptophan and this is for tyrosine. There is one tyrosine also, so this is for tyrosine and so generally in the lab what we do is if we want to look at tryptophan we generally excite it to 92 nanometer.

Fluorescence can also be used to look at the pH of the medium it can be used as a pH indicator since fluorescence spectra of some dyes are sensitive to pH and why it is sensitive? Because there can be equilibrium between protonated form of the dye and deprotonated form of the dye and protonated and deprotonated form of the dye or the fluorophore will differ in a structure and that will result into difference in a spectral properties. So quantum yield for protonated form and deprotonated form can be different. And so just by looking at how the fluorescence changes we can talk about changing your pH value and this is very important particularly when you are trying to look at pH inside cells.

Because generally if we are trying to look at pH we use pH meter but we cannot take pH meter inside the cells or electrode inside the cells. In that case it is easy for us to use a dye and look at the fluorescence, so fluorescence spectroscopy can measure pH inside of the cells and cellular compartment.

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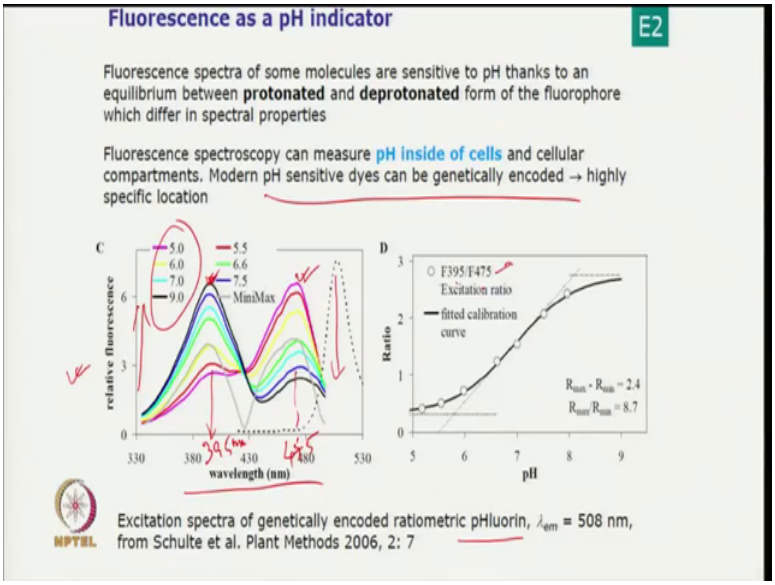

Modern pH sensitive dyes can be genetically encoded and that is why it has highly specific locations. Now here look at the fluorescence with wavelength for a dye which is pH luorin and now you look at what happens when I go from pH 5 to 9. Here you see this is pH 5 and this is pH 9. So if I increase the pH this will increase whereas if you look at here this will decrease and that is because with the change in pH one form of the protein is going to one form of the dye is going to another form of dye. And we can expect that when I go to lower pH protonated form will be in excess and protonated form has your lambda max at, sorry lambda max at this.

This is 450 nanometer and 450 or 460 nanometer whereas at high pH, pH 9 it has around you can look at this is around 390 nanometer. So by using the ratio of fluorescence at 395 nanometer this is around 395 nanometer and this is around the 475 nanometer. So by taking the ratio between fluorescence at 395 nanometer and fluorescence at 475 nanometer we can measure the pH inside the cell.

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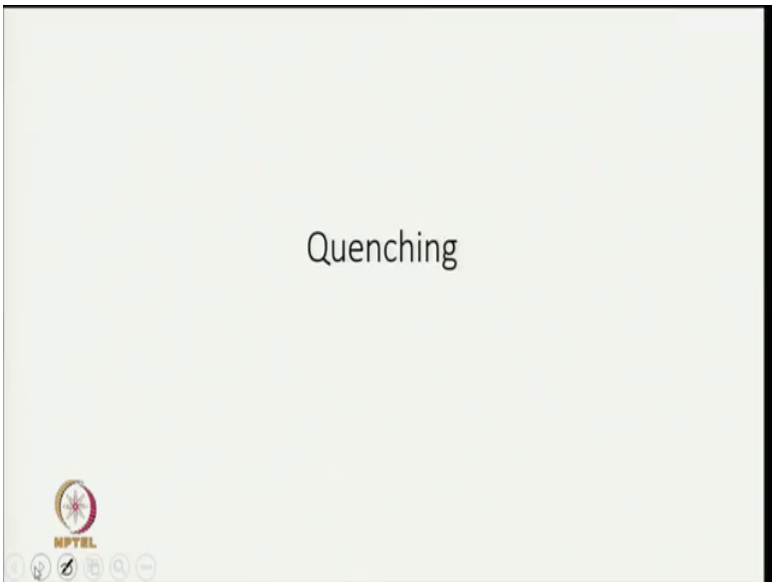

So till now we looked at the effect of solvent. Now we will try to discuss another phenomenon called quenching and this is particularly important when there is a different molecule in the vicinity of a fluorophore.

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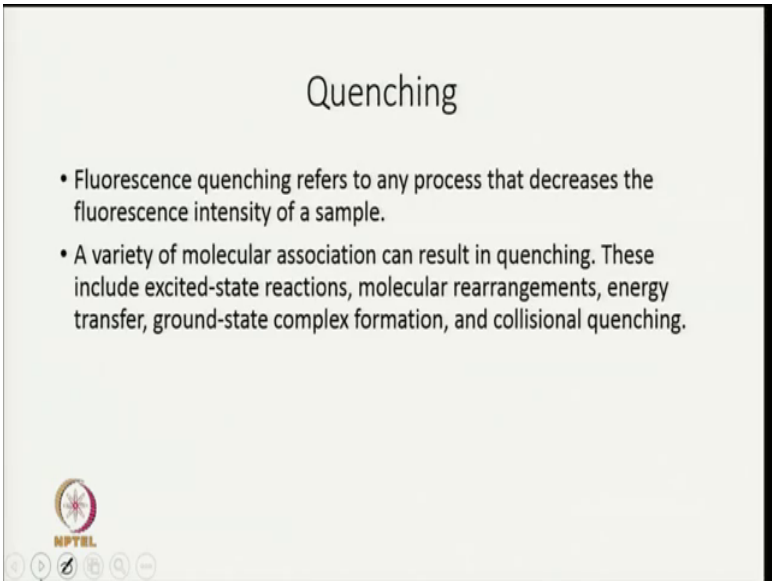

What is quenching? Quenching is any process that decreases the fluorescence intensity of the sample and the variety of molecular association can result into quenching and that is why quenching can be used to study molecular association, the processes include excited state reactions, molecular rearrangements, energy transfer, ground state complex formation and collisional quenching, we will go one by one and we will discuss it.

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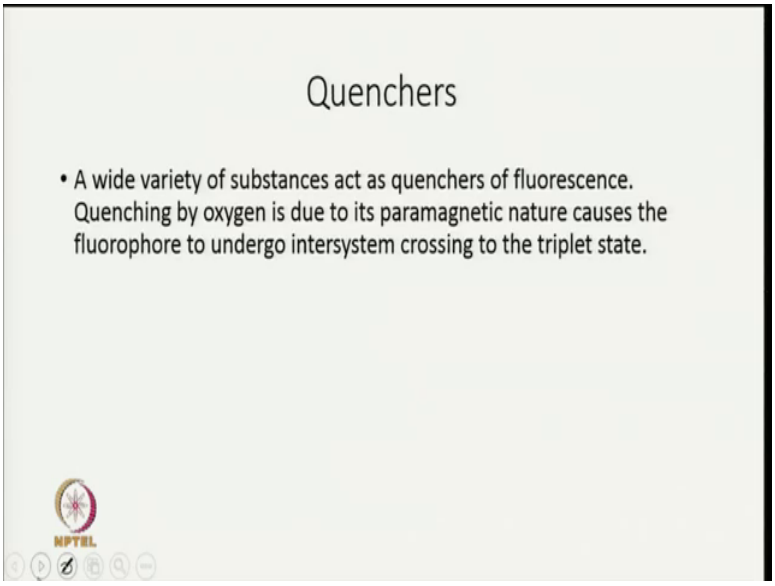

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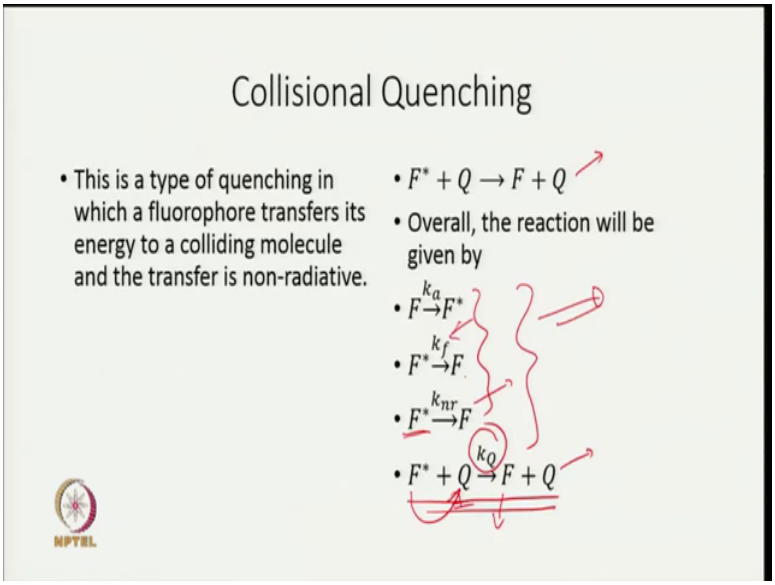

Now we will start with collisional quenching, what is collisional quenching? This is a type of quenching in which fluorophore transfers its energy to a colliding molecule and the transfer is non-radiative. For example, F star plus Q giving you F plus Q, overall the reaction will be given by these four steps. So till now, I have discussed about three steps, now what I am going to do is going to add this quencher and we can look at the reaction between excited state of a fluorophore and a quencher. So this process denotes your quenching.

So what happened? What is happening is excited state of fluorophore is reacting with a quencher to give you this Q plus F. Now F is going to the ground state, F is going to ground state. So basically F star is transferring its energy to Q and this k Q is the rate of quenching, rate of collisional quenching. These are steps I have already discussed, F is going to F star with K. F star will go to ground state by fluorescence, the rate constant is k f, excited fluorophore is going to ground state by non-radiative transition with rate constant equal to k nr and if we have this equation we can easily write how the rate equation looks like.

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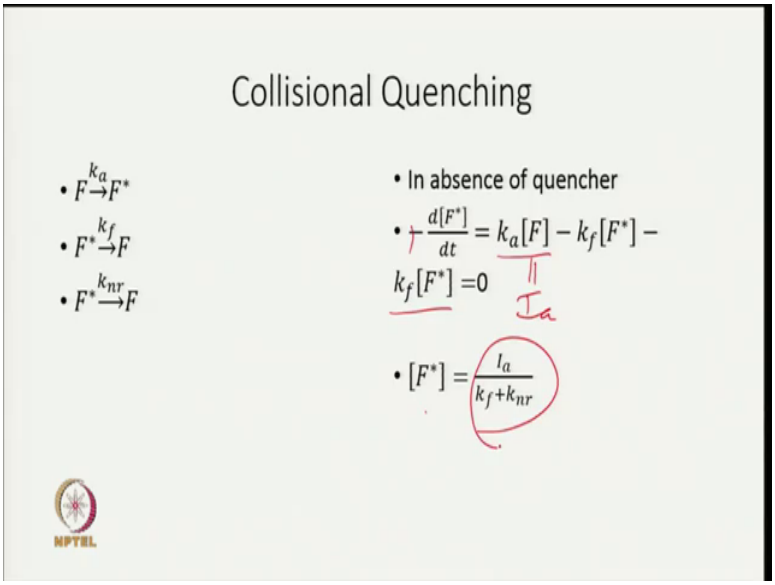

So again plus dF star by dt, if there is no quencher what we will write plus dF star by dt is equal to k into F minus k f into F star minus k f into F star is equal to 0. So F star is equal to k f which is equal to I a. I a divided by k f plus k nr. So this is the way I can get F star.

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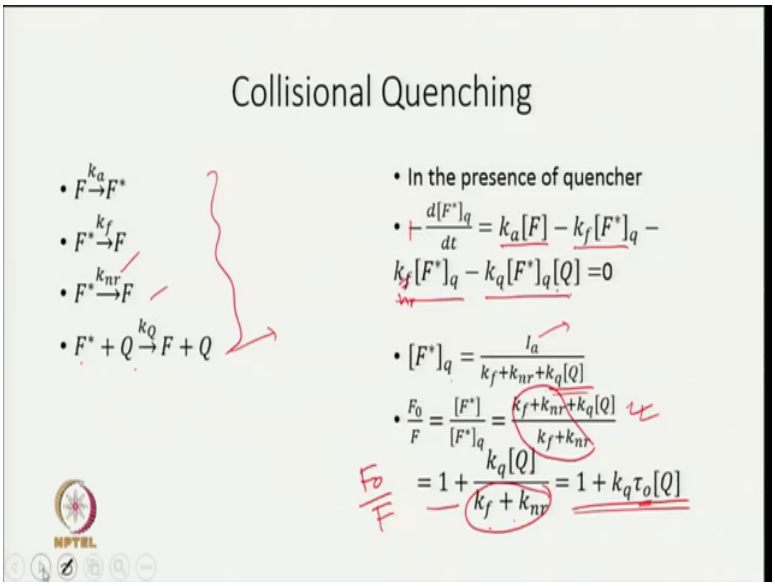

Now let us go and look at what will be the F star when you have a quencher. Now since there are four equations by which F star can be either formed or used up, so there will be four terms here. This is the term from which F star gets formed, this is term for this step, this is the term for the third step and this is the term for the fourth state. So you can see this that q, k q into F star into Q. So plus dF star by dt for the fourth step is minus k q F star into Q. And if I put this equal to 0 which we do for an intermediate then F star in presence of quencher will be

given by I a which is k a f divided by k f plus k nr. So let me put this, this will be k nr, please correct this because here is k nr and plus k q into Q.

So F naught by F will be equal to F star divided by F star quencher and so will be equal to this term which is equal to 1 plus, so this term will be equal to 1 plus k q into Q divided by k f plus k nr and this one divided by this thing is known as lifetime and so we have this equation F naught by F is equal to 1 plus k q tau naught into Q.

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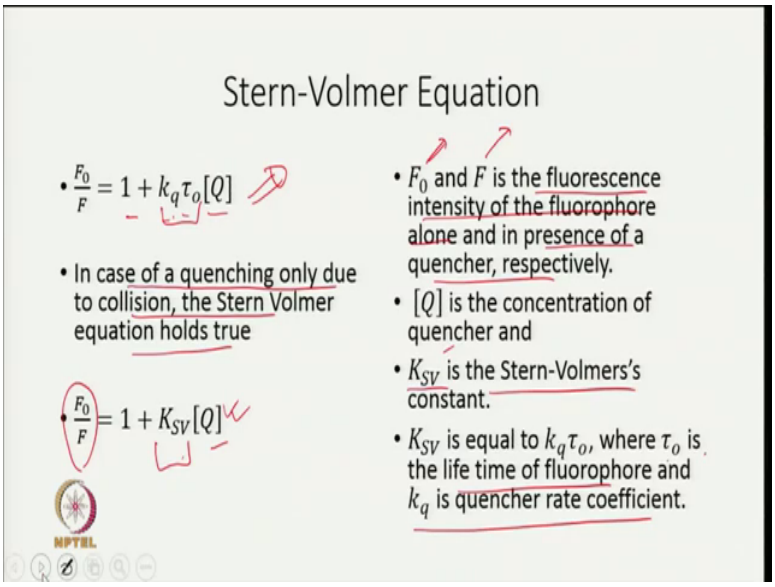

This is well-known Stern-Volmer equation. F naught by F is equal to 1 plus k q into tau naught into Q. In case of quenching only due to collision Sterne-Volmer equation holds true and this k q into tau naught is known as a Stern-Volmer constant and again to explain this in more detail F naught is your fluorescence intensity of fluorophore alone and F is fluorescence intensity in presence of a quencher in presence of a quencher, q is the concentration of the quencher and K sv is called a Stern-Volmer constant where K sv equal to k q into tau naught, tau naught is the lifetime of the fluorophore and k q is the quencher rate coefficient. Quencher rate coefficient, and now if you look at this we can plot F naught by F versus Q, I mean we do that, we expect to get a straight line with slope equal K sv.

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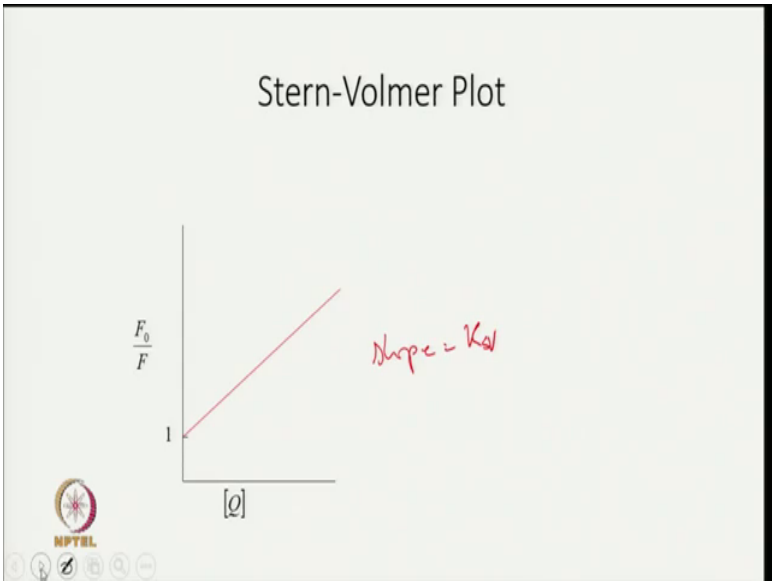

So intercept will be 1 and slope will be equal to K sv and this is well-known Stern-Volmer plot.

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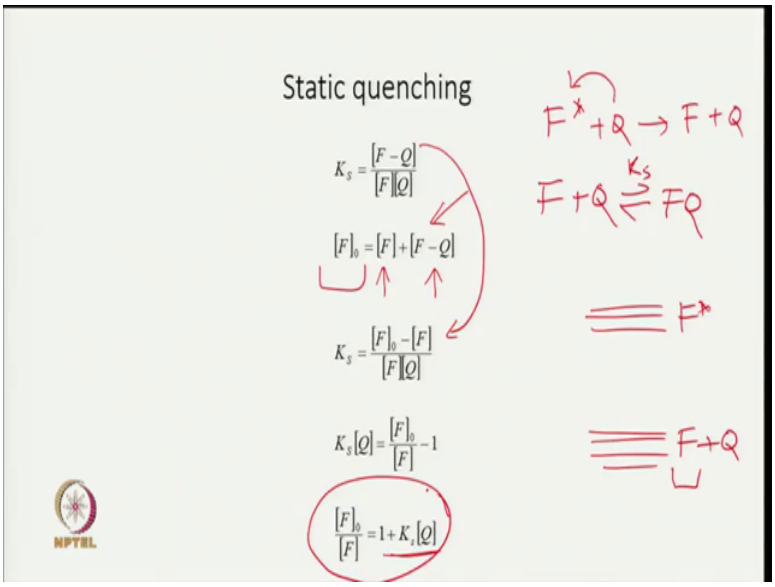

There can be second way of quenching and that is known as static quenching. In a static quenching reaction of quencher does not happen with the excited state, it happens with the ground state and so amount of F decreases and that is why there is a quenching. Now here the reaction is between F and Q to give you FQ and that is different from your dynamic or collisional quenching where reaction takes place between F star plus Q to give you F plus Q to give F plus Q, energy transferred between F and Q.

In the static quenching the excited state is involved, it is the ground state which is involved in complex formation with a quencher and if you take the equilibrium constant as K s, then K s is equal to F Q divided by F into Q and your total fluorophore concentration is in this case will be equal to fluorophore concentration plus complex concentration. So fluorophore in free form plus fluorophore in complex form and so K s will be given by if I just replace this FQ by F naught minus F then you know this will be equal to F Q will be equal to F naught minus F and so we can write K s is equal to F naught minus F divided by F into Q.

And so K s into Q is equal to F naught by F minus 1 and F naught by F is 1 plus K s Q. So we have another equation which is quite similar to Stern-Volmer equation for collisional quenching and when we plot F naught by F versus Q it will be a straight line when in case of a static quenching.

So today I looked at the quenching due to collision and quenching due to complex formation of quencher with the ground state of fluorophore to give a complex. We ended up with the same equation. Now in the next lecture I will discuss about how to differentiate between dynamic quenching and static quenching and what happens when both kind of quenching involved, in that case how we can get K s value or K sv value. K sv value is your Stern-Volmer constant value which we can obtain from F naught by F versus Q. You must keep in mind that K s is association constant. So if I know that the reaction involves static quenching, we can know the binding constant between F and Q using a Stern-Volmer equation. So I will stop here and thank you very much for listening.