Spectroscopic Techniques for Pharmaceutical & Biopharmaceutical Industries
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Lecture 26
Time-resolved Fluorescence Spectroscopy

Hello students, in the last few lectures I have discussed about fluorescence spectroscopy. In the last lecture I was discussing about steady-state fluorescence. In this lecture I will discuss about Time Resolved Fluorescence Spectroscopy.

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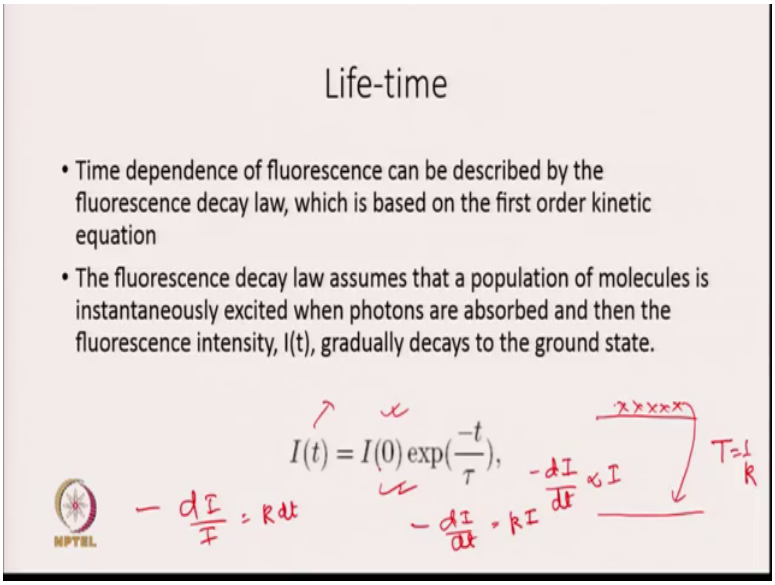

Time dependence of fluorescence can be described by fluorescence decay law which I have already discussed to you when I explain how to get the, how to get the lifetime from the fluorescence decay law which is basically based on the first-order kinetic equation. The fluorescence decay law assume that a population of molecules is instantaneously excited when photons are absorbed and then the fluorescence intensity gradually decays to the ground state.

So, what you are basically looking at that if there is n molecule in the n molecule in the excited state on the absorption of photon, this molecule has gone from the ground state to excited state. So, how the population of this will decay with time? And the kinetic equation which has been used to know the number of excited molecule getting decayed with time is given by dI by dt is proportional to I.

So, what we can write is minus dI by dt is equal to some constant k into I where k is rate constant of the decay so you can simply write dI by I is equal to k dt. And when you integrate

this what you are going to get is I is equal to I naught exponential minus t by Tau where Tau is simply 1 by k, Tau is 1 by k. So here what is assumed that the fluorescence decay follows first-order kinetics, follows first-order kinetics. So we can simply write minus dI by dt is proportional to I when you do that what you get is this equation.

And this is your the intensity at time t which is proportional to the number of molecules, number of fluorophores in the excited state at time t and this is I naught is number of excited state fluorophore when time is equal to 0.

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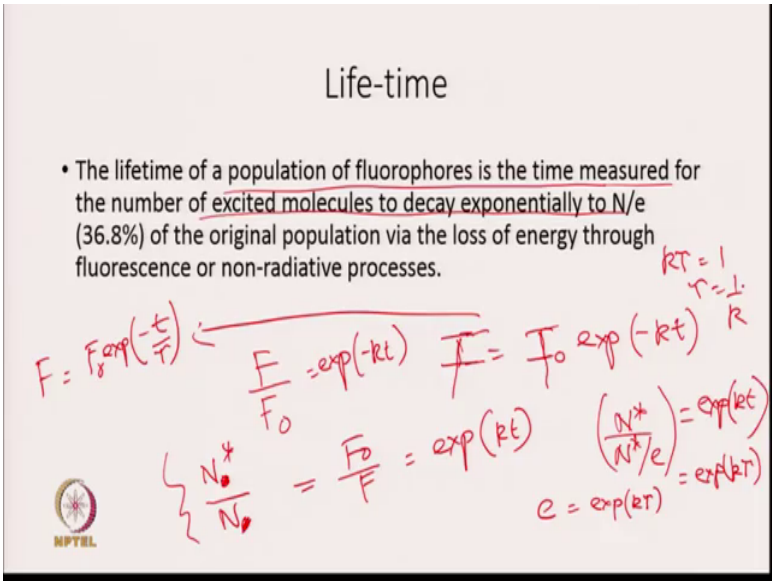

Now, the lifetime of a population of fluorophores is the time measured for the number of excited molecules to decay exponentially to N by e of the original population via the loss of energy through fluorescence or non-radiative processes. So, what I wrote is I is equal to I naught exponential minus k into t this was the equation which we get when we solve the first-order kinetic equation for the fluorescence decay, for the fluorescence decay you can also write F is equal to F naught it is quite same sometime people write fluorescence intensity, fluorescence intensity as F or sometimes they write as I.

So, F by F naught is equal to exponential minus kt and you can also write F naught by F is equal to exponential simply kt and this is nothing but N naught in the excited state by N naught, where N naught is your or simply you can write N star divided by N. So N star is number of excited fluorophore at time T is equal to 0, whereas N is number of fluorophore in excited state at time t is equal to t.

So this N star by N will be equal to exponential kt, so what I am saying is that lifetime of a population of fluorophore is the time measured for number of excited molecules to decay exponentially to N by e of the original population. So, basically you can think of that N star by N star divided by e this is your, this thing and this should be equal to exponential kt and here this t is equal to your t is equal to now in this case this is exponential k and simply Tau.

So time, lifetime is time required for number of excited molecules to decay exponentially to N by e. Now when you solve this you will get e is equal to exponential k Tau and that simply means that k Tau is equal to 1 and so Tau is equal to 1 by k. So sometime this equation is also written like F is equal to F naught exponential minus t divided by Tau since k is equal to 1 by Tau, k is equal to 1 by Tau. So this is the way lifetime is are defined so again lifetime of a fluorophore is the time measured for the number of excited molecule to decay exponentially to N by e of the original population.

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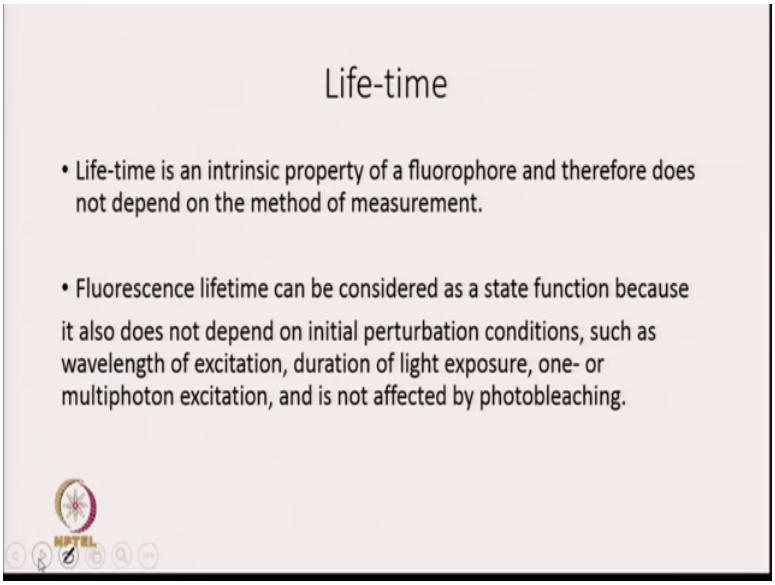

So now there are some very important facts about lifetime which you should know. Life-time is an intrinsic property of a fluorophore and therefore does not depend on the method of measurement so this is very important property and that gives you a tool to use this property in different kind of applications.

The second important thing is fluorescence lifetime can be considered as a state function if you have slight idea of thermodynamics. You know what is the importance of state function, so fluorescence lifetime can be considered as a state function because it also does not depend on initial perturbation conditions such as wavelength of excitation, duration of light exposure,

one or multi-photon excitation and also it is not affected by photobleaching which is not the case if you are dealing with fluorescence intensity and that is why lifetime measurement is much more accurate comparison to in compare to fluorescence intensity measurement.

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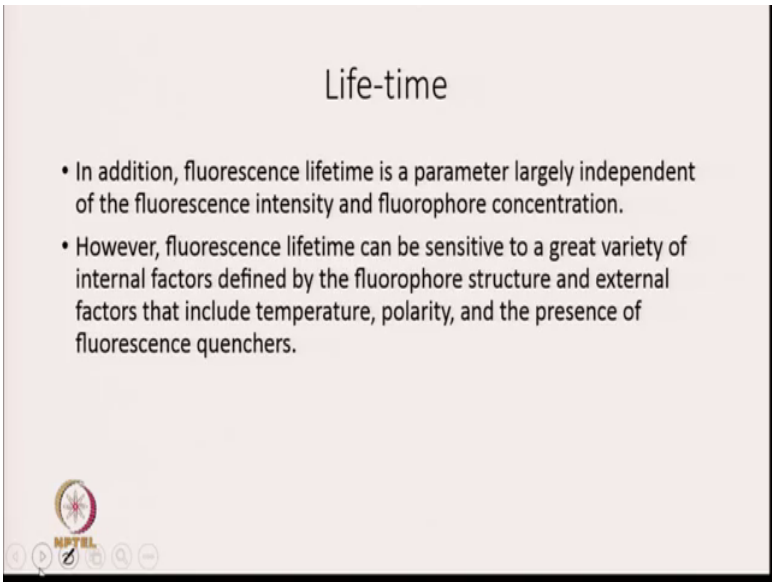

In addition, fluorescence lifetime is a parameter largely independent of the fluorescence intensity and fluorophore concentration so it does not matter. But, concentration of fluorophore you start with lifetime is going to be same, again this is in contrast to fluorescence intensity.

However, fluorescence lifetime can be sensitive to great variety of internal and external factors, internal factors such as fluorophore structure, external factors such that temperature, polarity and presence of fluorescence quenture and that is why if I want to look at the effect of temperature, effect of polarity, effect of conformational change or binding, lifetime can be easily utilized, lifetime, concept of lifetime can be utilized to understand these effects. So it is not dependent on concentration, but it is dependent on internal and external factor.

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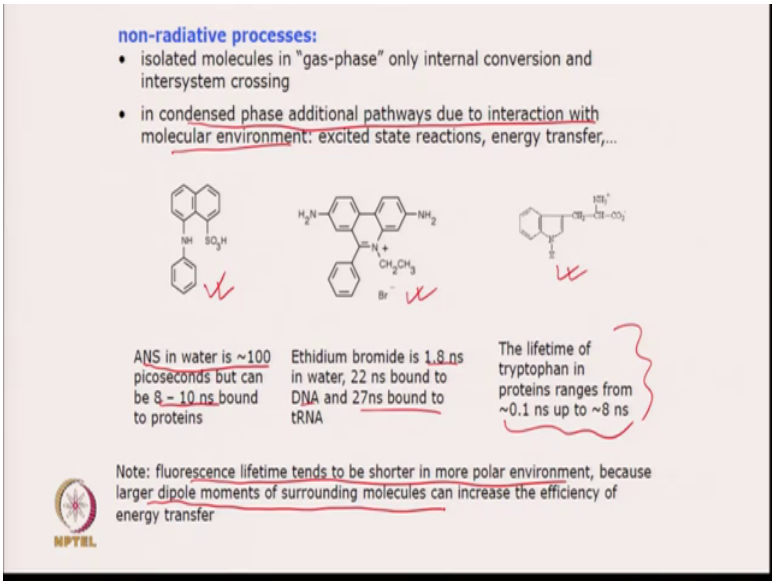

Now here is three examples which I will show, one is this die and this is Ethidium bromide this is ANS and this is your tryptophan these are the three fluorophores. Now, I will show you how its lifetime changes on change in the condition. Here you see ANS in water is 100 picoseconds so lifetime of ANS in water is 100 picoseconds, but when it is bound to protein the lifetime increases to 8 to 10 nanosecond. Ethidium bromide the lifetime is 1.8 nano second in water, but when it is bound to DNA its lifetime is around 22 nanosecond, whereas if it is bound to t NA then its lifetime is 27 nanosecond, it is around 27 nanosecond.

Now, lifetime of tryptophan can also vary and people have observed lifetime of tryptophan in different proteins to range from 0.1 nanosecond up to 8 nanosecond what does that mean is your lifetime is affected by the environment of the fluorophore and this happens since in the condensed phase additional pathways due to interaction with molecular environment happens and so lifetime may change.

So indifferent condition there will be different pathways for, different pathway for energy transfers and so lifetime will be different. Fluorescence lifetime tends to be shorter in more polar environment, because of large dipole moments of surrounding molecules can increase the efficiency of energy transfer these things are very important that in polar solvents lifetime tends to be smaller, in polar solvent or in the polar environment your lifetime is going to be smaller and so lifetime measurement can also give you an idea about the environment of the fluorophore.

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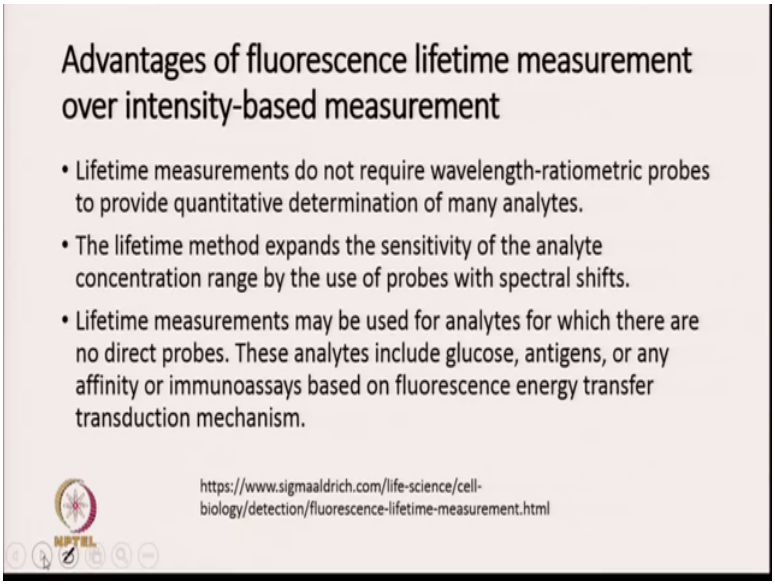

What are the advantage of fluorescence lifetime measurement over intensity-based measurement? One is that lifetime measurements do not require wavelength-ratiometric probes to provide quantitative determination of many analytes. Similarly, lifetime methods expands the sensitivity of the analyte concentration range by the use of probes with spectral shift.

And then lifetime measurement may be used for analyte for which there are no direct probes. These analyte includes glucose, antigens, or any affinity or immune acid based on fluorescence-based energy transfer transduction mechanism. So there are a lot of advantage of time resolved fluorescence over steady-state fluorescence.

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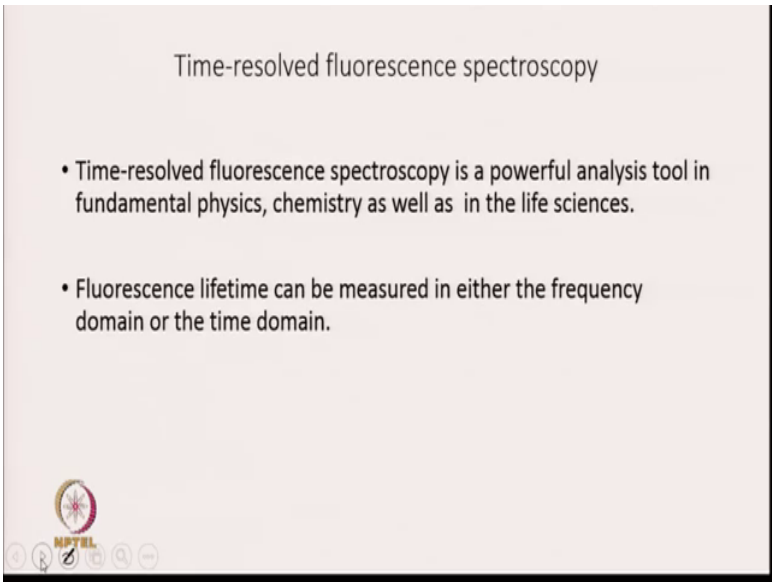

So time resolved fluorescence spectroscopy is a quite powerful analysis tool in the fundamental physics, chemistry as well as in the life sciences. Now, how to measure the lifetime? It can be done in two ways lifetime measurement can be done either in frequency domain or the time domain and I have already discussed about what is the difference between time domain spectroscopy and frequency domain spectroscopy. So, let us think about if I am trying to get lifetime using time domain method what is a problems and what is the solution.

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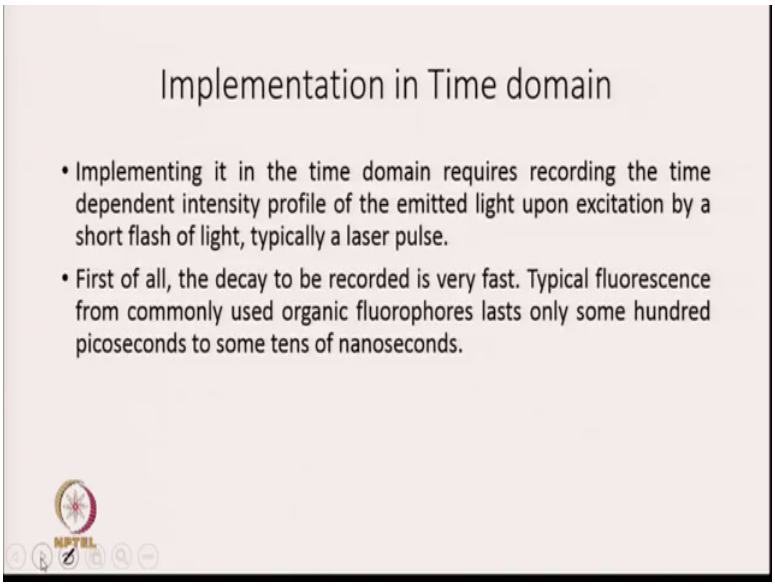

So, first thing is when we are trying to implement this in time domain measurement of lifetime in the time domain what we need generally is that we look at time-dependent intensity profile of the emitted light upon excitation by a short flash of light and typically we use a laser pulse.

First of all the decay to be recorded is very fast, it generally happens from picoseconds to nanosecond range and the typical fluorescence from commonly used organic fluorophore lasts only some 100 picoseconds to some 10 of nanosecond. So the fluorescence lifetime is or the fluorescence from a fluorophore generally lasts from 100 picoseconds to some 10 of nanoseconds.

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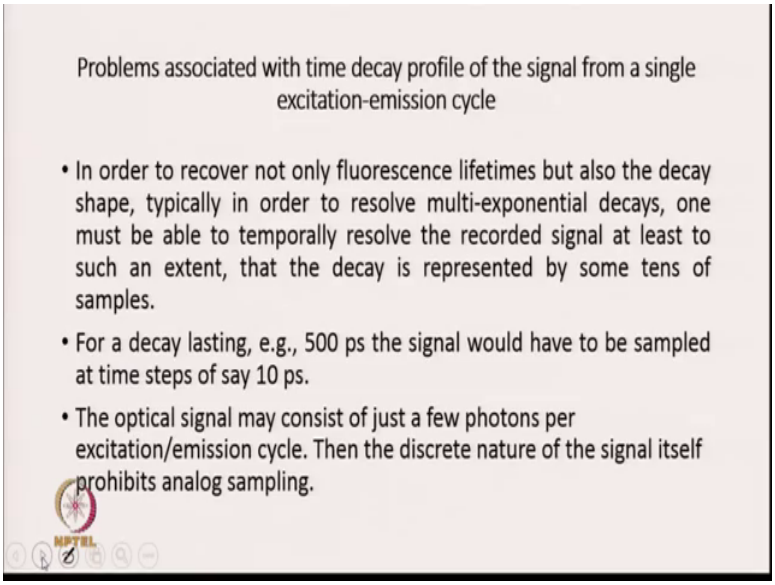

So, what we need to do? If I want the not only to get fluorescence lifetime, but also the decay shape, it is very difficult to get it from a single excitation emission cycle, single excitation emission cycle. So, why we do need the decay shape? Because decay shape if we analyze decay shape, then we can resolve multi exponential decays. So, if there are more than one fluorophore in the system, then your decay will be your multi exponential decay and if we do not have decay shape we cannot resolve multi exponential decay and so one must be able to temporarily resolve the recorded single signal at least to such an extent that the decay is represented by some 10 of the samples so you need some points.

So suppose, we decay started from this point and last to this point, then we need to measure several points in between, but since it is too fast in the one excitation and one emission cycle it is very difficult to get 10 points between you know initial start and final decay. For an example, if the signal is 500 picosecond long. So from the excitation to emission decay zero. Suppose this is 500 picosecond long, then signal would have to be sampled at the time of step say 10 picosecond so that we have almost 50 data.

So between these we need to get several points such that we can know what is the decay shape and then we will be able to resolve multi exponential decay. Optical signal may consist of just a few photons per excitation emission cycle. And so the discrete nature of signal itself prohibits analog sampling so suppose we want to do analog sampling that discrete nature of signal will prohibit the analog sampling.

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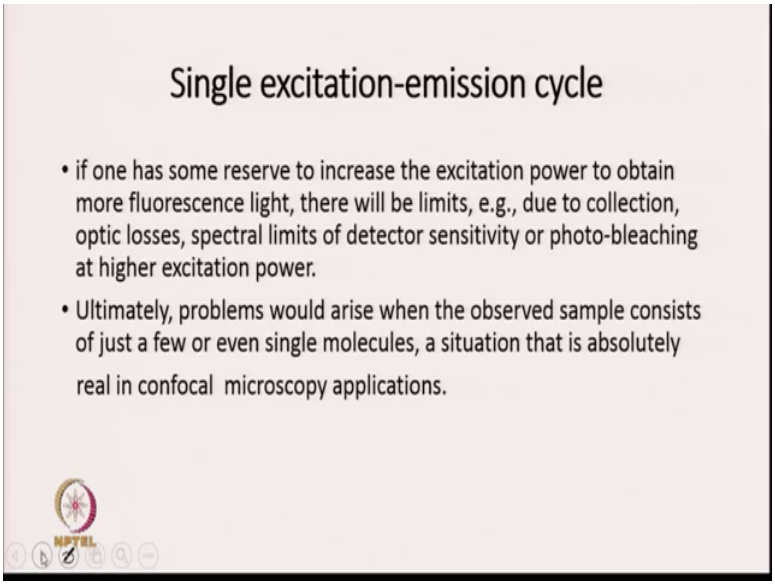

So one way we can think of that okay let us increase the excitation power to obtain more fluorescence light, but there will be limits because of collection, there can be optical losses and then there are spectral limits of detectors sensitivity and if I use higher excitation power then there is a chance of photobleaching. And so these are the problems associated with single excitation emission cycle. So, problem can arise when the observe sample consist of just a few or even single molecule and that is the situation commonly found in confocal microscopy applications.

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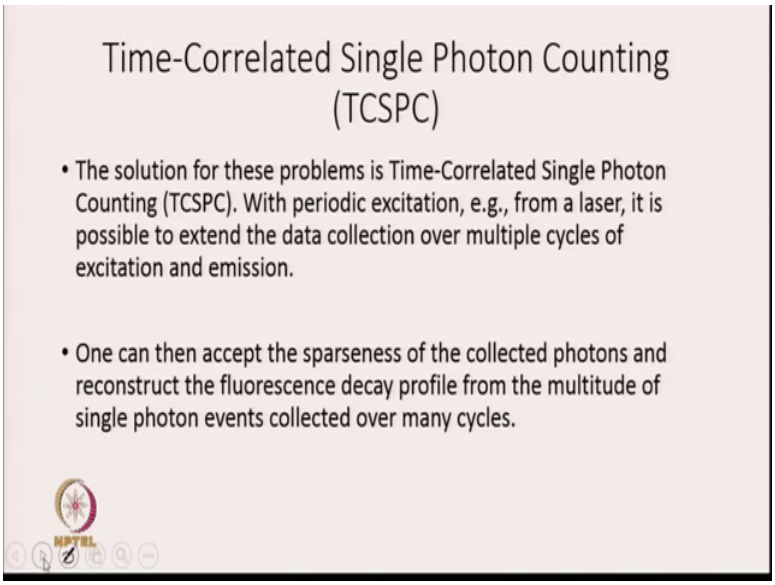

Now, what is the solution? Solution of this is using the method called TCSPC which stands for Time-Correlated Single Photon Counting. So, in this we do not use only one single

excitation emission cycle, here we do periodic excitations from a laser and then we can extend the data collection over multiple cycles of excitation emission. One can then accept the sparseness of collected photons and reconstruct the fluorescence decay profile from the multitude of the single photons event collected over many cycles I will explain this line in the next slide.

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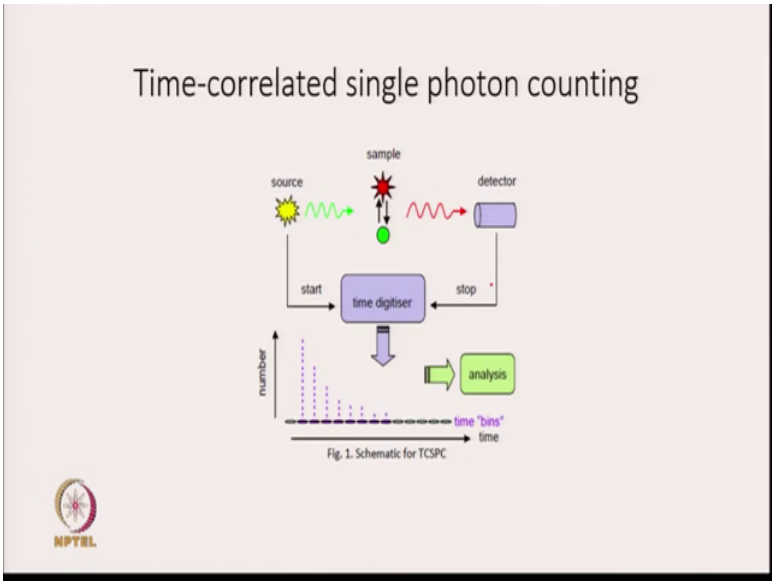

So this is the way we basically doing time correlated single photon counting, what we are doing? This is source of light and here fluorophore from the ground state goes to the excited state and then you are detecting photons, when photons you come across a photon this will stop and time digitizer will tell you the time gap when you receive one of the photon and the number of photons seen versus time which is called lag time is basically plotted in this. Again you see next one then it will be more clear.

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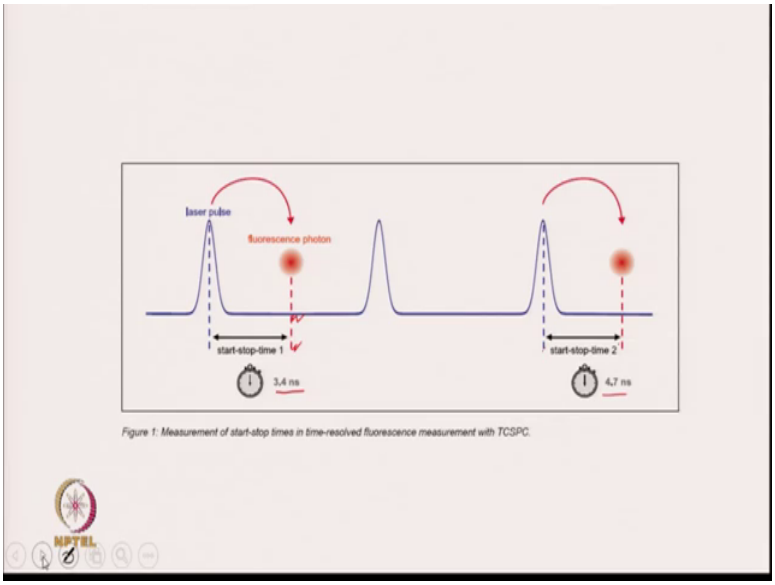

So what we do in this is first we applied laser pulse so we applied laser pulse and then we started looking for photon. Suppose photon comes at this which is around 3.4 nanosecond so between this when you excited and this one when you observe a photon the time gap is 3.4 nanosecond and that is what you are plotting. So one photon at 3.4 nanosecond.

Now look at this, again I excited, but there is no photon in between so within this period I did not see any photon, again I excited so multiple excitation is going on, so again I excited I did see a photon when the time gap between these two is 4.7 nanosecond. So, what we do is we do repeat this cycle.

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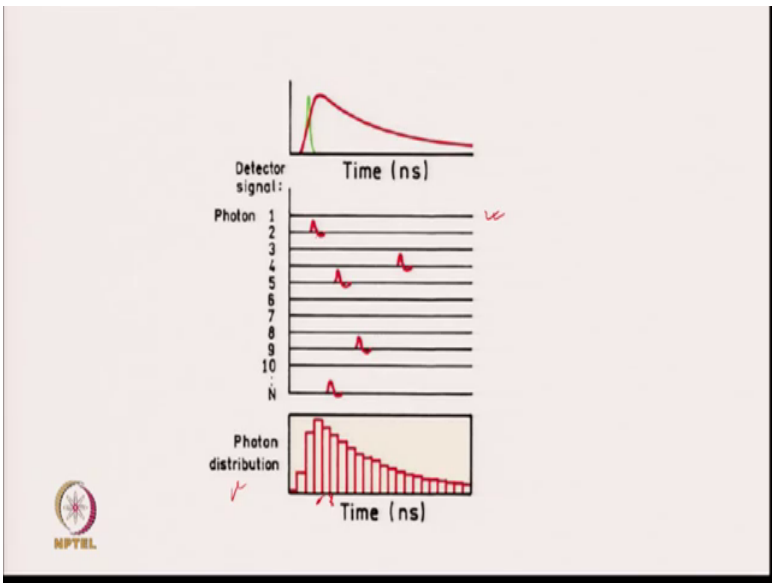

And then here is your you know different cycles and when did you see a photon so what will happen that in first cycle I did not see a photon, in the second cycle I did see here with the time nanosecond in this nanosecond, third I did not see, fourth I did see at this point, five I did see here at this point, in nine I did see at this point and at end I see at this point. Now, what I will do is I will look at photon distribution between small gap of time. So, what you will get is this kind of profile, this kind of profile.

So, initially this intensity increases and basically number of photon increases, then there will be a decay there will be a decay. What does that mean is in this period after this we will see that within this range of nanosecond there is a less number of photons seen. But you have to do this several thousands of time so that you can get a very nice photon distribution curve, photon distribution curve. Now, you have this curve and now you can just fit it and see what is the lifetime.

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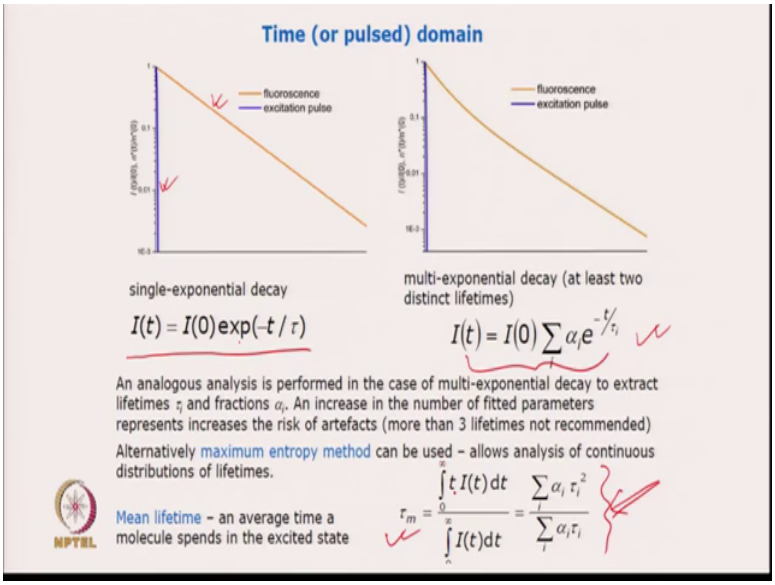

So this is the time domain data, this is the excitation pulse and this is your fluorescence decay. As I discuss with you this is I t will be given by this equation so I t is equal to I naught into exponential minus t divided by Tau. If there is only one fluorophore, only one fluorophore and that one fluorophore has only one lifetime, only one lifetime then you will get this kind of curve.

This is when there is more than one lifetime, there is more than one lifetime and that can be because of suppose if we are discussing protein are maybe two tryptophan which is

undergoing NO decay, undergoing decay and so there will be some from two different tryptophans.

Also it may be because of there is only one tryptophan, but it is changing confirmation between two different conformers. For example, native state with the denatured state. Native state, tryptophan in native state and tryptophan in denatured state and there is a equilibrium between two different conformation of proteins or two different conformation of fluorophore then you can get this kind of data.

So, we can calculate the mean lifetime and mean lifetime is calculated using if you understand, if you remember your quantum what you have studied in BSc courses you will remember how to get the lifetime, it is simply 0 to infinity t multiplied by I t dt divided by 0 to infinity I t dt. So this is the way you calculate your lifetime of a fluorophore.

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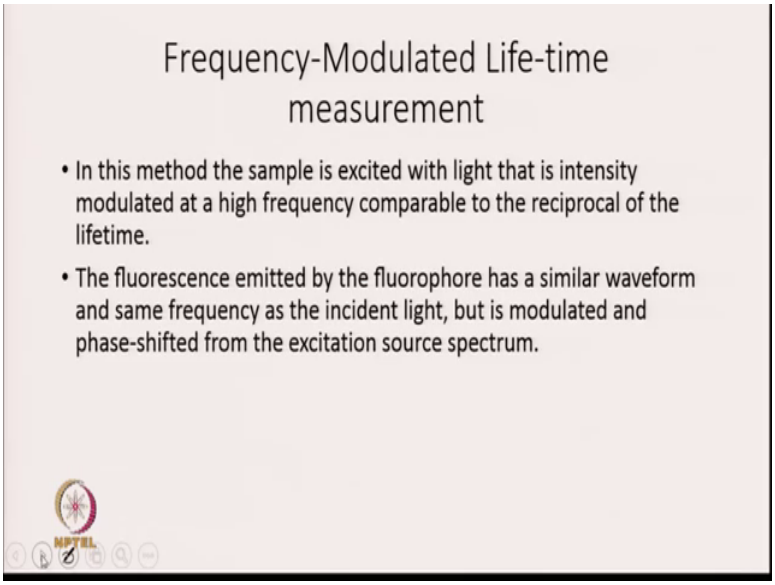

Now, the second way of measurement of lifetime is by using frequency modulated lifetime technique. What you do in this is? The sample is excited with the light that is intensity modulated at a high frequency comparable to the reciprocal of the lifetime, so that is what we do. Then what we will see, you look at the emitted light the fluorescence emitted by the fluorophore is going to have a similar waveform and same frequency as the incident light, but is modulated and phase shifted from the excitation source spectrum.

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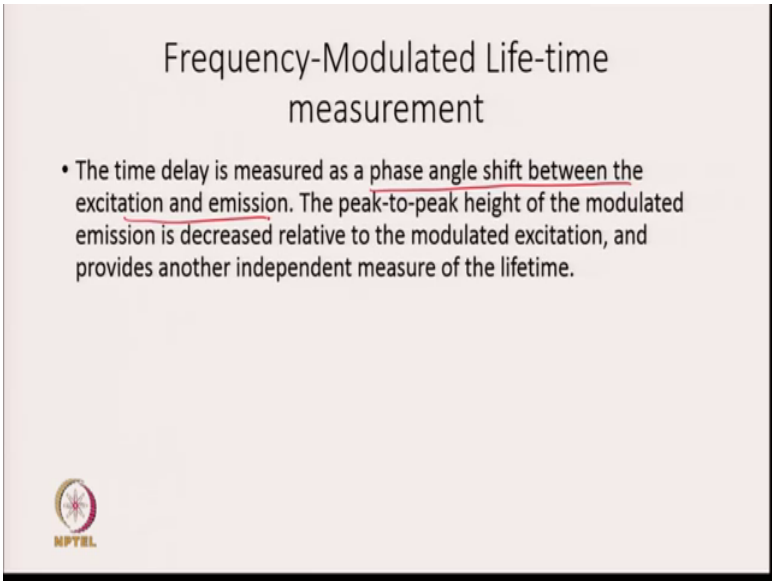

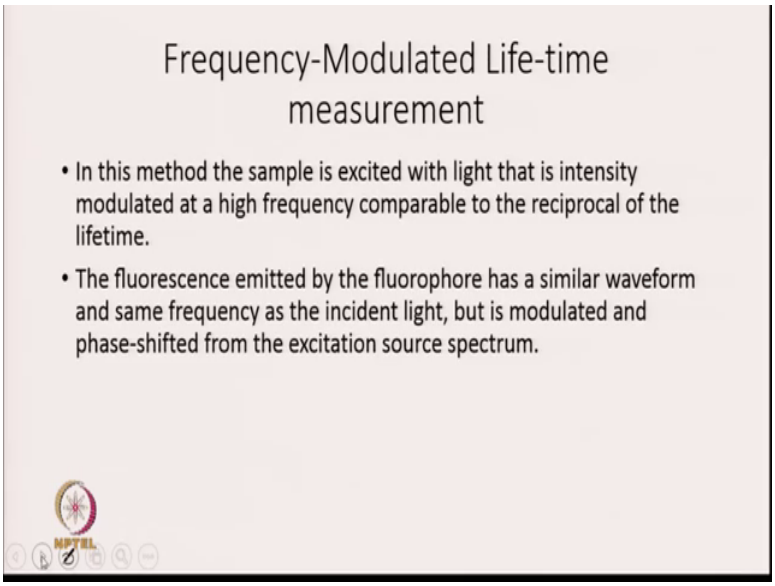

So, what here we are looking at is that time delay is measured as a phase angle shift between excitation and emission. So, what we did? First we excited the fluorophore with the light which is intensity modulated and now we are looking at the fluorescence emitted, fluorescence emitted which has same frequency only thing is it is phase shifted from the excitation source spectrum and is modulated and now we are measuring the difference between phase.

So, if I can measure phase angle shift between excitation and emission, then I can calculate what is the lifetime? Similarly, the peak to peak height of the modulated emission generally decreases, each decrease related to modulated excitation and that also gives you another independent measure of the lifetime so phase angle is related to the lifetime and also the

modulation peak to peak height of the modulated emission is also related to the lifetime. Now let us now understand what is basically happening.

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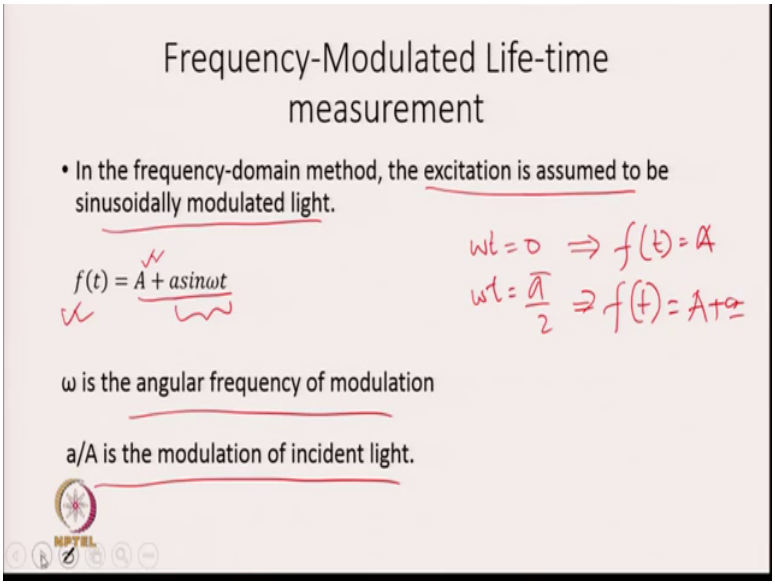

So, as I told you that we are using the intensity modulated light, so the excitation assumed to be sinusoidally modulated light what does that mean is that this is given by A plus a sin omega t, so this is capital A plus small a sin omega t, capital A plus a sin omega t. So, what does that mean is when we have omega t is equal to 0, then we have f t is equal to A because this sin term will be 0 and when omega t is equal to pi by 2 then we have f t is equal to A plus a.

So, when omega t is 0 then f t is equal to A, when omega t is equal to pi by 2 then this value sin omega t becomes 1 and a into sin omega t becomes a. Here omega is the angular frequency of modulation and a by A this ratio small a by capital A is basically known as modulation of incident light.

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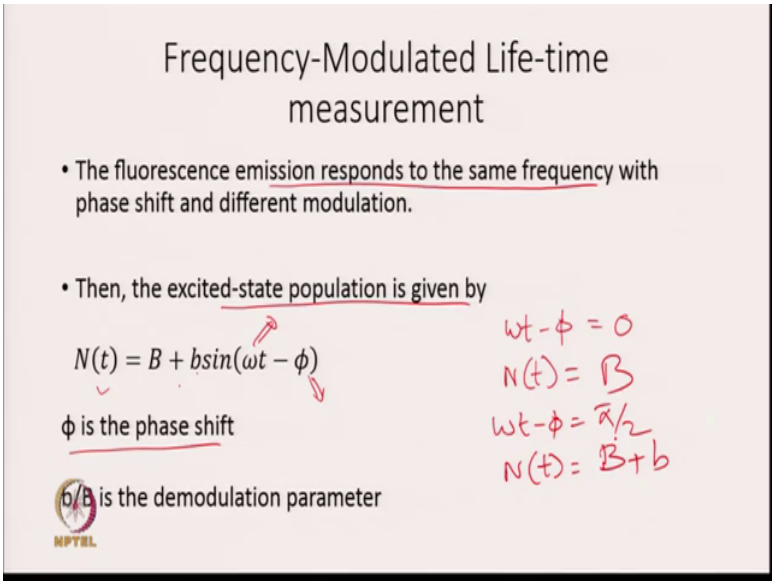

Now, let us talk about the emission. So, fluorescence emission response to same frequency with phase shift and modulation. Then, the excited-state population is given by Nt is equal to B plus small b sin omega t minus phi. Now you can see the frequency is same, what is different? There is a phase shift where phi is your phase shift.

And the modulation is also different and now modulation is b divided by B, now you can think of this is also same kind of curve only thing in now omega t minus phi will be 0, then your Nt will be equal to 0, then Nt will be equal to B and when omega t minus phi is equal to pi by 2, then Nt is equal to capital B plus small b so sin pi by 2 will be equal to 1 and then. So these are two different curves you keep in mind what are equations I have given so this is basically your intensity versus time profile.

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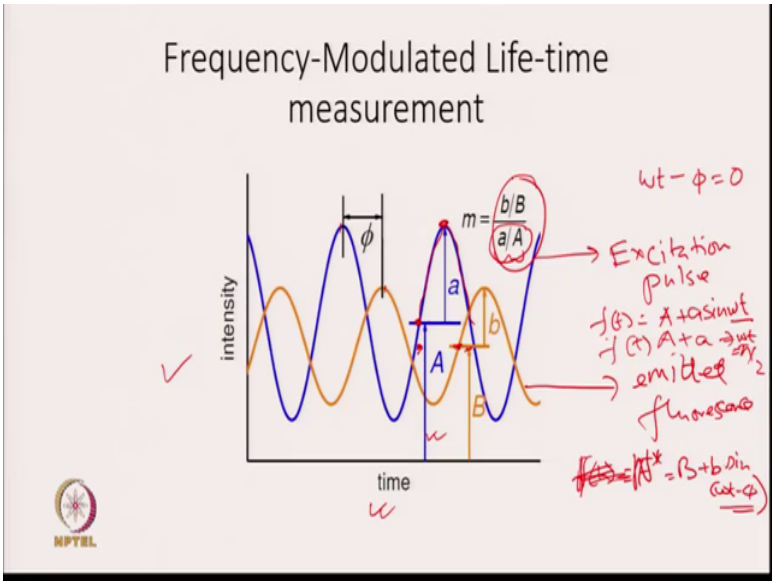

This is your excitation, excitation pulse and intensity modulated excitation pulse and this is your emitted, emitted fluorescence, fluorescence. Now see here if you look at this, this is basically amplitude is 0 here and so you see if you remember what I told that this is given by equation the f t is equal to A plus a sin omega t, am I right? So, if omega t is 0 then f t is equal to A so f t is equal to A. This is your intensity which is equal to A.

Now, and this is remember any wave starts from this point, alright? Waves goes like this and comes back and then go it like this. So this is when our omega t is equal to 0, then you have f t is equal to A. Now, omega t is equal to pi by 2 here, am I right? So omega t is equal to pi by 2 then f t will be A plus a when you have omega t is equal to pi by 2 and that is what you can see here. This is whole intensity is A capital A plus small a, capital A plus small a.

And now, if you remember then what is the modulations small a by A so this is a by A is the modulation for this excited pulse. Now let us look at the emitted, emitted light, here if you look at there is a phase shift, phase shift is this, this is your phase shift phi okay. So, if you go from this point so suppose this point to this point there is a shift of this point to this point, alright? There is shift of phi and so at this point omega t minus phi is going to be 0, omega t minus phi is going to be 0 okay omega t minus phi is going to be 0.

In that case if you remember that in this case your intensity f t is given by or not f t you just write fluorescence or N star basically is given by B plus b sin omega t minus phi, so if this is 0 then this is equal to B and if this is 1 then the whole thing will be capital B plus small b. So, this will tell you about what is the principle behind frequency modulated lifetime

measurement. What we need to do is, we need to measure this phi or we need to measure this B factor because phi and this N value is related to the lifetime. And the way we can do is given in this slide.

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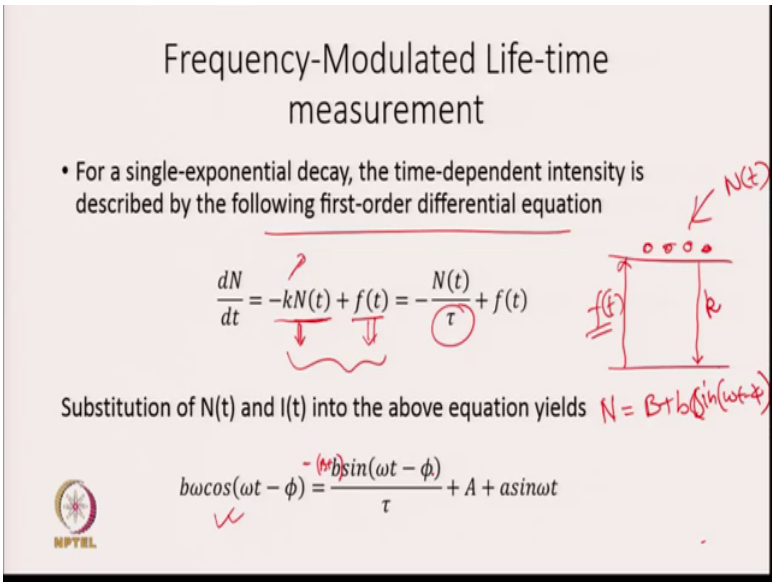

So, if we consider single exponential decay (the time independent) or time dependent intensity will be described by this first-order differential equation dN by dt is equal to k into Nt plus f t. And, if you remember that what we were talking about this is your ft is basically increases the (number of photons) number of fluorophore in the excited state and this is the Nt which is number of fluorophore in excited state.

So, if I am applying pulse ft will increase the population of, population of excited state and the population will decrease because of your fluorescence and the decrease in the population of excited state is your proportional to number of fluorophore in the excited state and so we have minus k into Nt. So this tells you about the decrease in fluorescence with time due to emission and this tells you increase in number of excited state, number of fluorophore in excited state with time, with time due to excitation.

Again this term tells you number your increase in number of, increase in number of excited a number of fluorophore in the excited state and this tells you increase in number of fluorophore in excited state due to excitation and this is your decrease in number of fluorophore in excited state due to emission there are two terms.

We know that this rate constant is equal to 1 by lifetime and that is what we have written here so k is equal to 1 by Tau. So this equation dN by dt can be written as equal to minus Nt

divided by Tau plus ft. Now, if you remember N is given by B plus b sin omega t minus phi. So dN by dt will be equal to b omega cos omega t minus phi. Now minus k Nt, Nt is here I have missed this so minus B plus b sin omega t minus phi by Tau plus ft is equal to A plus a sin omega t, a sin omega t.

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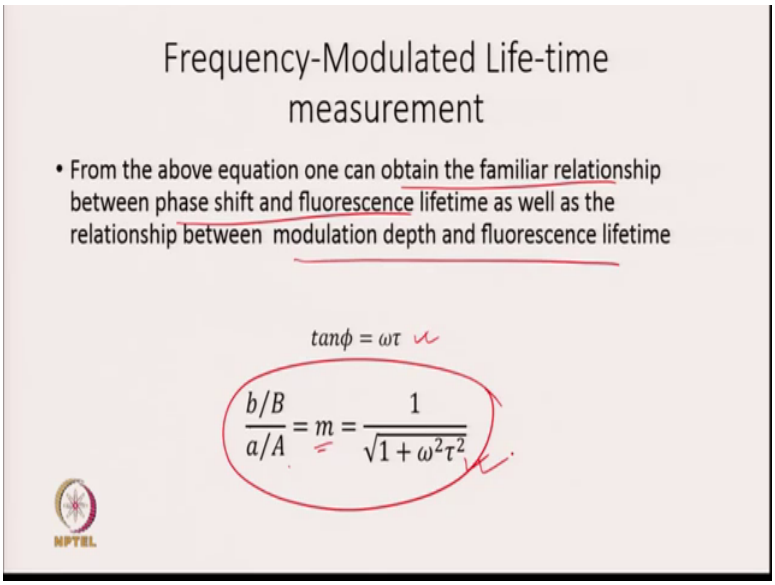

And from the above equation one can obtain the familiar relationship between phase shift and fluorescence lifetime as well as relationship between modulation depth and fluorescence lifetime, so tan phi is equal to omega t and this we can get from this equation if we solve this equation we can get the value of tan phi, tan phi gives is equal to omega into Tau, Tau is your lifetime and b divided by B and divided by a by A will be equal to m and that will also give you Tau. You can get the value of lifetime from the phase shift or you can get the value of lifetime from the m value, from the m value.

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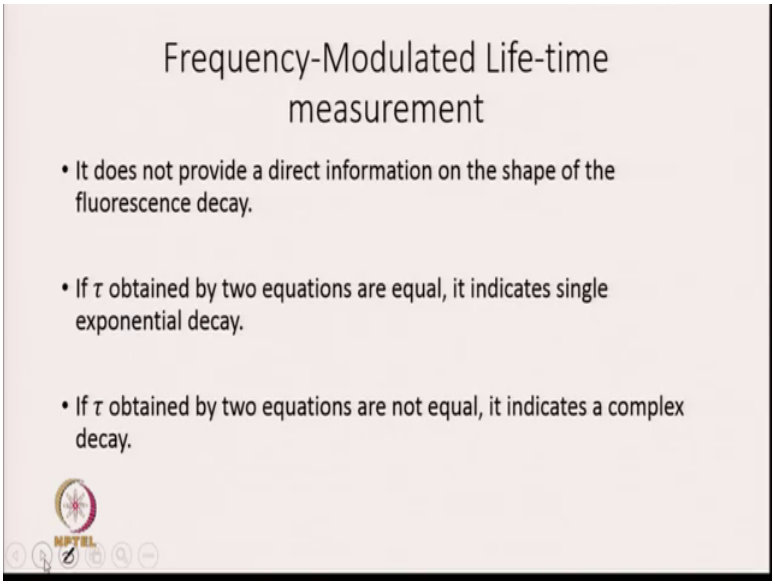

The problem with this method is it does not provide a direct information on the shape of the fluorescence decay. I told you there are two ways in which you can get the value of Tau, one is through the phase shift and another is through the what is called demodulation parameter, demodulation parameter. If the Tau obtained by two equations are equal then it indicates there is a single exponential decay, but if it is not equal then it means there is a complex decay and we need to use different equations.

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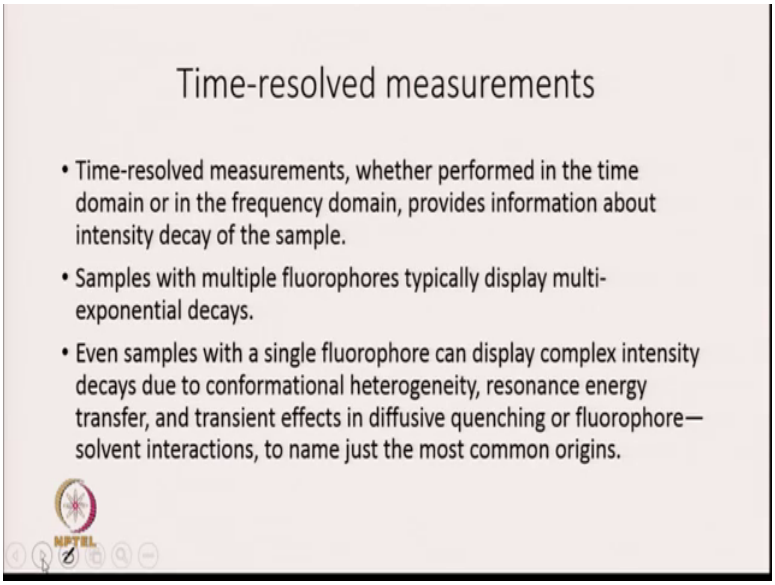

Time resolved measurements, whether performed in time domain or in the frequency domain, provides information about intensity decay of the sample, intensity decay of the sample. Samples with multiple fluorescence typically display multi exponential decay. Even samples

with a single fluorescence can display complex intensity decay due to conformational heterogeneity.

So it is not true that if there is a single fluorescence and it will always show single exponential decay, it may show a complex intensity decay because of conformational heterogeneity. So fluorophore are in different conformation in the same sample. There can be resonance energy transfer and the transient effect in diffusive quenching or fluorophore solvent interaction, so there are some of the origins why a single fluorophore can display complex intensity decay.

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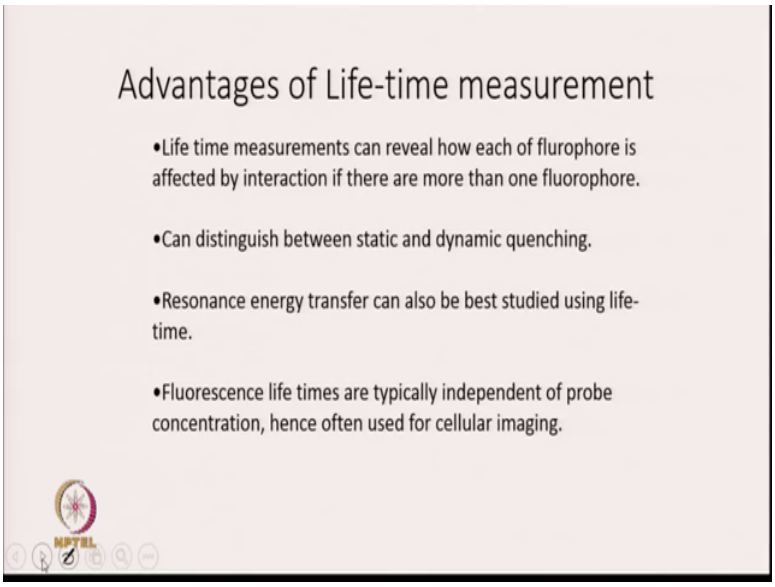

Advantage of lifetime measurement is lifetime measurement can tell you how each of the fluorophore is affected by interaction if there are more than one fluorophore which is not possible when we are trying to study the study, study the fluorescence by steady-state fluorescence measurement. It can also distinguish between static and dynamic quenching.

In static quenching in a static quenching fluorophore does not, fluorophore in excited state does not interact with the quencher, quencher interacts with fluorophore in the ground states. So in the static quenching there is no change in lifetime in presence or in absence of a quencher.

Whereas, in dynamic quenching quencher is associating with your quencher is associating with fluorophore in the excited state and hence and hence the lifetime changes when you have a dynamic quenching. The third important thing is resonance energy transfer can also be best studied using lifetime measurement. Fluorescence lifetime are typically independent of

probe concentration and so they are often used for cellular imaging, they are often used for cellular imaging.

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Now, what is the importance of lifetime measurement? It can tell you about the changes in the environment. For example, if there is a change in viscosity, there is a change in pH, there is change in polarity and change in solvation because these factors affects the lifetime, these factors affects the lifetime. And so we can tell about changes in this physical parameters by measuring the lifetime.

We can also talk about size and shape of the molecules, size and shape of the molecule. We can talk about molecular interaction because lifetime measurement can give you idea about static or dynamic quenching and similarly for FRET measurement we can apply lifetime measurement. It also gives you inter and intramolecular distances. Again this can be done by measuring transfer efficiency using lifetime measurement and then we can also see the kinetic and dynamic rates, we can get the resolution of molecular mixtures using lifetime measurement.

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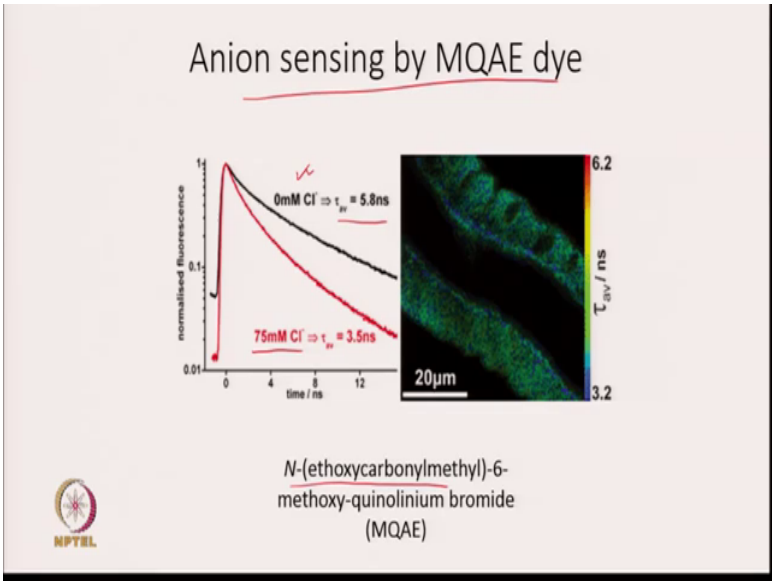

I will give you some applications of the lifetime measurement and this is your anion sensing by MQAE dye. MQAE is basically N-ethoxycarbonylmethyl-6-methoxy-quinolinium bromide dye. It is quite sensitive to chloride ion and here I can show you that in presence of 0 mili molar chloride ion the lifetime is 5.8 nanosecond, whereas in 75 mili molar chloride ion it is 3.5 nanosecond. So, if you increase the concentration lifetime of your MQAE dye decreases, MQAE dye decreases and that is how we can detect chloride ion and also we can talk about concentration of the chloride ion in this case.

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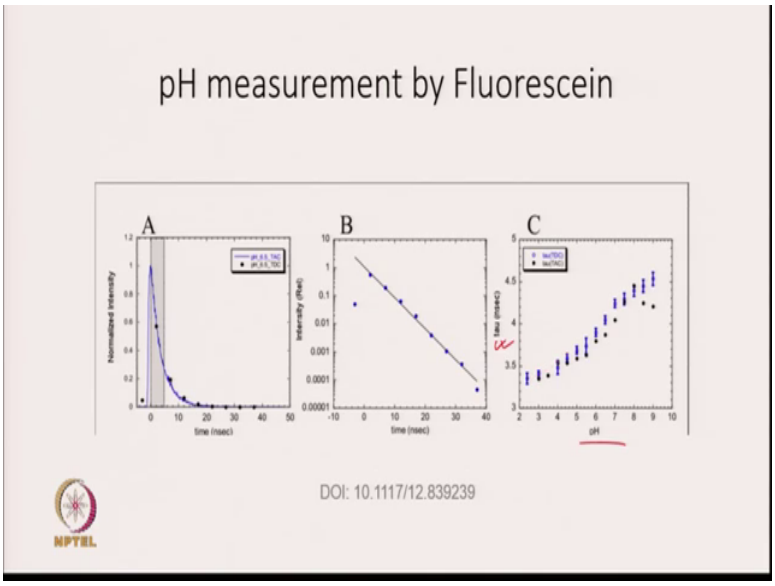

There is another application where we see, we are trying to measure pH by a fluorophore call fluorescein and fluorescein lifetime is also dependent on pH and this is your Tau versus pH

for fluorescence and you can see, see that it is increasing on increasing the pH, increasing the pH. So, what we did here simply we are exciting fluorescein and looking at the decay and from the decay you get the Tau which is in nanosecond.

And now you are plotting at the various pH and what has been seen that pH can be sensed fluorescein if we use lifetime measurement, if we can make lifetime measurement. And the lifetime is going to be different, lifetime of this fluorophore is going to be different in different pH.

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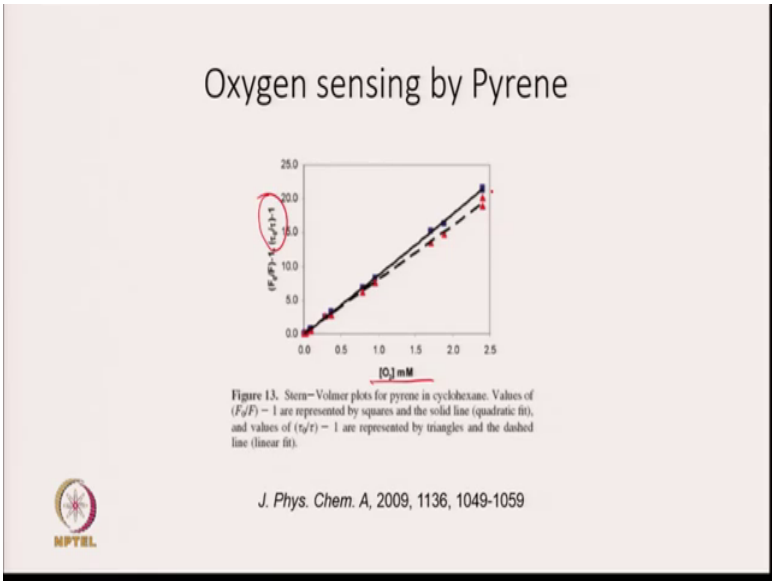

Now another application is your oxygen sensing by pyrene, pyrene is another fluorophore which senses oxygen which is used for sensing oxygen and this is your Stren-Volmer plot again you can see that we can look at the lifetime measurement versus oxygen and you can see that this is basically increasing, so again here you are looking at the quenching by oxygen. Here we are looking at quenching by, quenching of fluorescence of pyrene, fluorescence of pyrene in the presence of oxygen, so oxygen is acting as a quencher. And Stren-Volmer plot is given here and that can give you the value of ksv.

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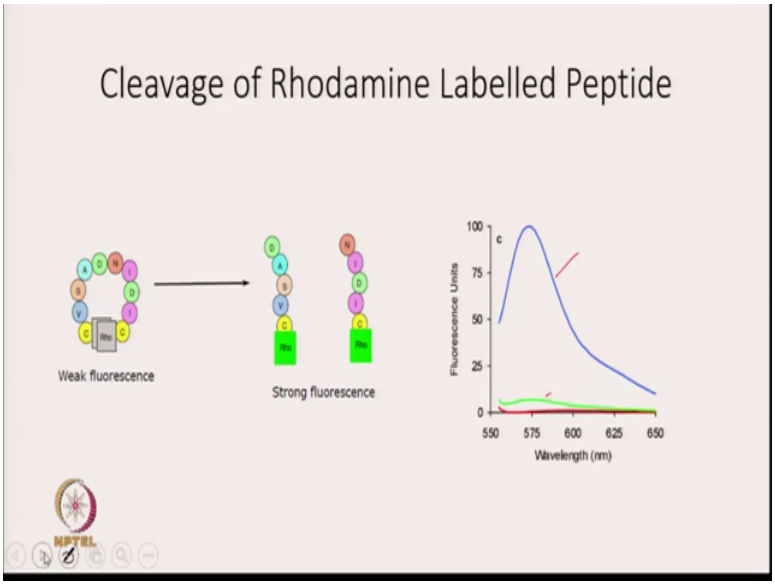

Now this is one example where labelled peptide, the labelled peptide is studied by your time resolved fluorescence. So this peptide is labelled with Rhodamine and this is the peptide and this is the Rhodamine. It has weak fluorescence when it is not cleaved, but if I cleave it then there is a strong fluorescence which is shown here so this is strong fluorescence when it is cleave and this is weak fluorescence when it is not cleaved.

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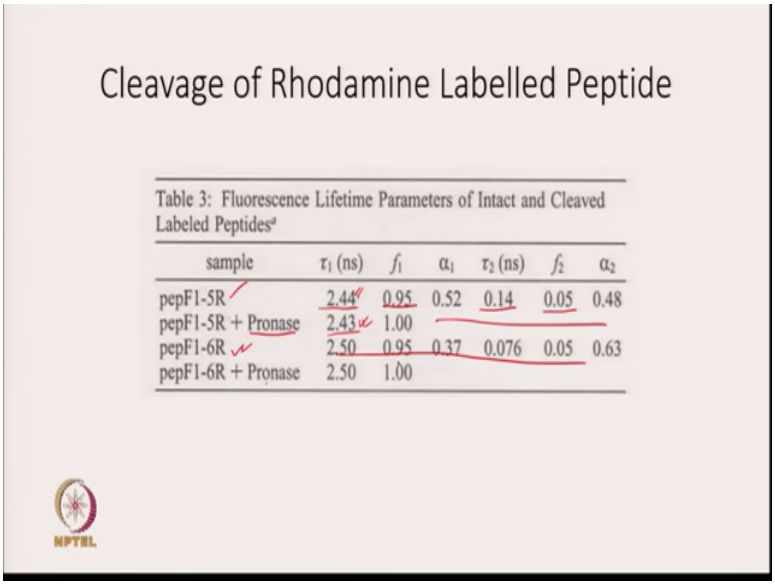

Now, if I look at the fluorescence if I just look at fluorescence intensity what we are going to see that it is cleave or it is not cleave so this is the Rhodamine labelled peptide. It has two lifetime. It is basically bi-exponential its fluorescence decay is basically bi-exponential. So

one lifetime is 2.44 nanosecond and other lifetime a 0.14 nanosecond, 0.14 nanosecond the percentage or F1 is basically 0.95 here F2 is 0.05.

So, fluorophore most of the fluorophore decays with this lifetime only 5 percent of fluorophore decay by this lifetime. What does it tells you that the peptide when it is not cleaved has two conformations, it is going into two conformation, it has two conformation. One conformation contributes 95 percent and other conformation contributes 5 percent.

This kind of information you cannot get from steady-state fluorescence measurement or intensity measurement. This can only be obtained by lifetime measurement so this lifetime measurement tells you that 95 percent of the fluorophore is in one conformation with the lifetime 2.44 nanosecond and the 5 percent are in the conformation which have lifetime 0.14 nanosecond.

Now when you cleave this with this enzyme Pronase what you are going to get is only one conformation you see it can be fitted only by single exponential and all the fluorophore is now decaying with the 2.43 nanosecond lifetime which is quite close to this one. And so what kind of information we got that the uncleaved peptide is an equilibrium between two conformation, but when I cut through pronase it gives you only one conformation with the lifetime 2.43.

This is the data same data but labeling with 6 Rhodamine and when you do that again you see same kind of conformation and when the information you get that again peptide is basically intact peptide is basically a mixture of two different conformation when we cut it then again we are getting only one conformation with single lifetime.

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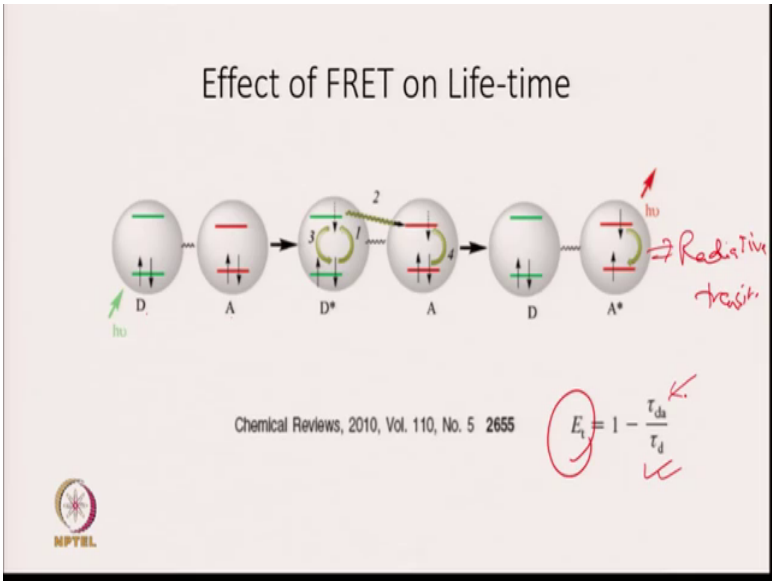

Now what we are going to see is the effect of FRET on the lifetime, effect of FRET on the lifetime. So, this is important if I want to study the protein ligand interaction or protein-protein interaction using lifetime measurement. So, now you see that this is your donor, this is acceptor when you put light then what will happen it goes too excited state it comes back and there is a transfer non-radiative transfer, non-radiative transfer to acceptor what it does?

It excites one of the fluorophore to the excited state and then what happens that this is same acceptor there is emission of the photon due to fluorophore coming from the excited state to ground state and this being radiative transition, this being radiative transition, transition. Now here transfer efficiency can also be given in terms of lifetime and transfer efficiency is equal to 1 minus lifetime for donor in presence of acceptor and this is your lifetime of donor.

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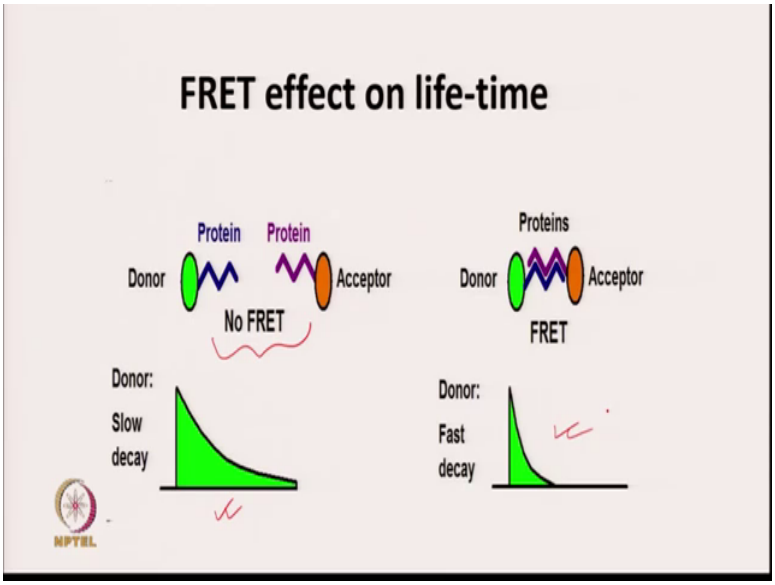

So, what is the FRET effect on lifetime? So, if there is no FRET then donor undergoes slow decay, when there is a FRET donor undergoes fast decay, donor undergoes fast decay. So just by looking at the lifetime you can know, you can know what is the your, you can know whether interaction between two proteins is taking place or not? And thus, that gives you a very useful application.

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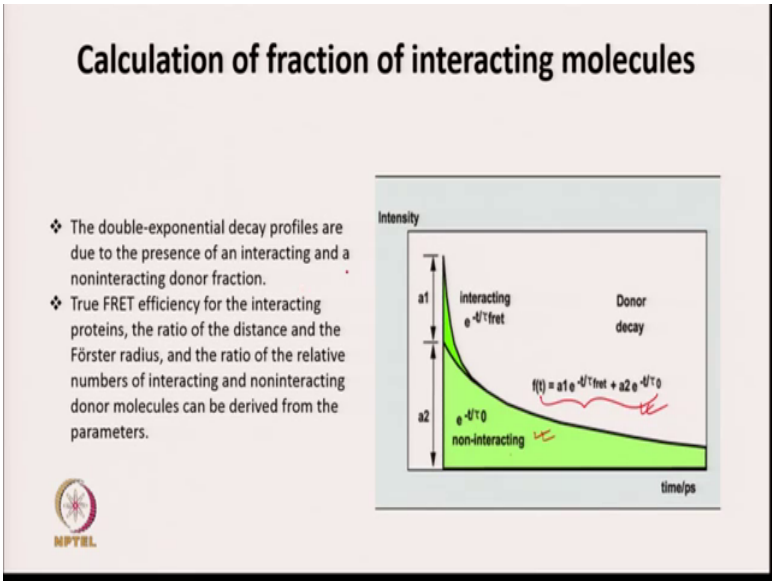

You can even calculate fraction of interacting molecule. So, suppose there is a donor when it is not interacting then this is your decay profile and you can see this is slow decay, slow decay, but if they are interacting then this donor goes a fast decay, fast decay. So the double

exponential decay profile are due to presence of an interacting and a non-interacting donor fraction.

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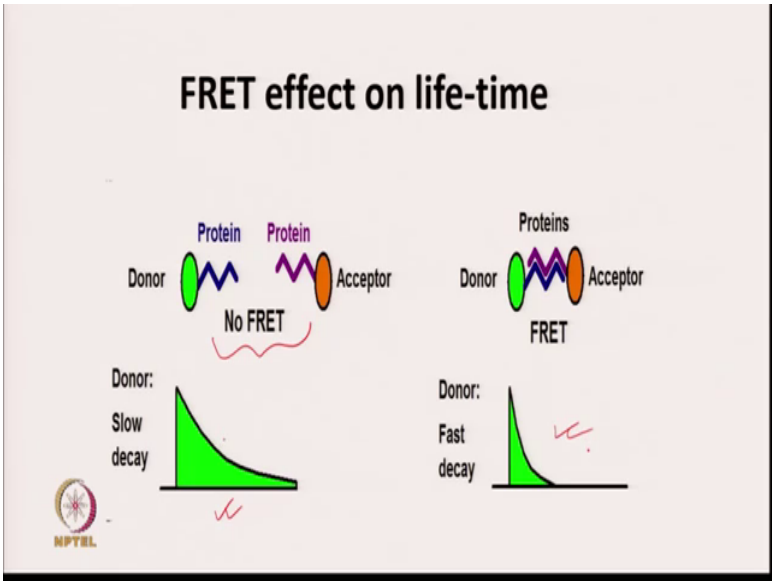

So, if you see donor which is interacting at this profile, donor which is not interacting at this profile, so if there is a mixture of interacting fluorophore and non-interacting then you get this kind of curve which will be bi-exponential and just by you know analyzing this and taking the ratio of A1 and A2 we can know what is the fraction of interacting molecule.

So true FRET efficiency for the interacting proteins, the ratio of the distance and the Forster radius, and the ratio of relative numbers of interacting and non-interacting donor molecule can be derived from this parameters.

So, I think I am now done with the lifetime measurements. So, today we have discussed about the lifetime measurements. What are the different methods to get the lifetime and lifetime as very important application, we saw how lifetime can be use for various applications, I also discuss about the combination of FRET or getting information from FRET using lifetime measurement, lifetime measurement and that can be utilized to understand the interaction between two different molecule. We can also do microscopy using these techniques, but I will discuss microscopy in the next lecture.

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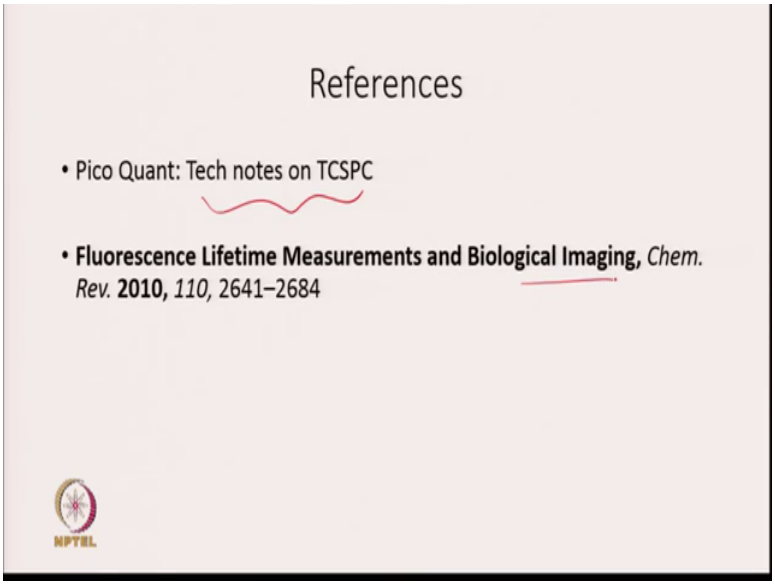

The two books and notes which I referred making this slides are your tech notes on TCSPC from Pico Quant and the fluorescence lifetime measurement and biological imaging, this is a review an chemical review and this is your detail about this paper. Thank you very much for listening, see you.