Basics of Fluorescence Spectroscopy
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Lecture – 20

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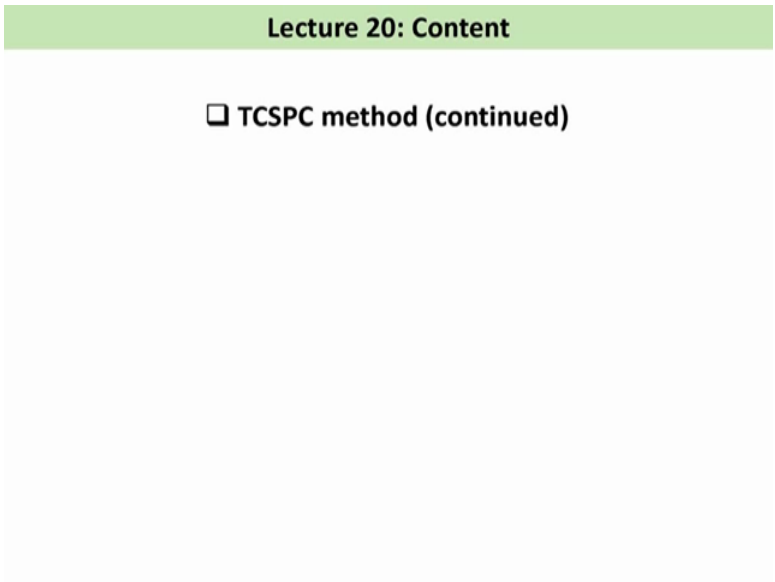

Welcome back to the lecture number 20. Till last class we are discussing about the TCSPC system and I have already told you several parts of this TCSPC system.

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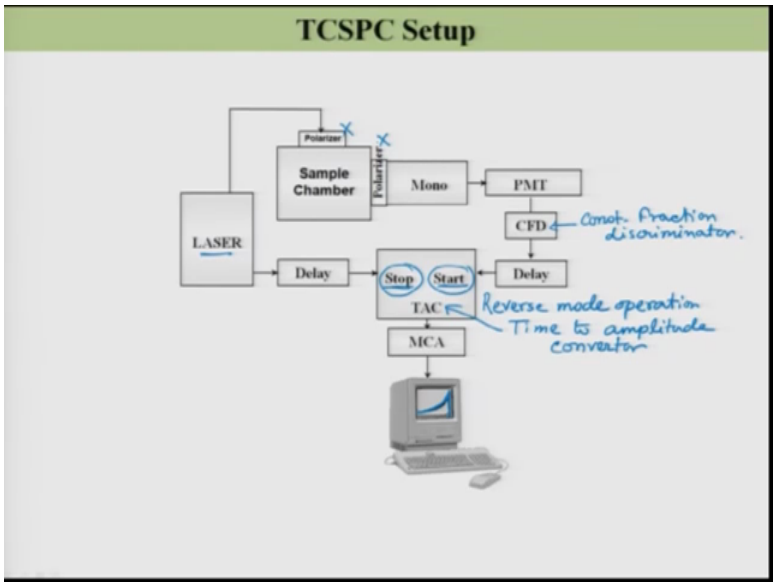

Let us have a quick go through though all these different component of the TCSPC set up. So, as you remember I need a laser source which will produce the past laser light and then this laser light will excite the sample and the emission from the sample will be collected through this monochromator and then this PMT, and when this PMT count will come and start the stopwatch over here then these stopwatch will also be stopped. So, you see here this is the reverse mode operation. So, ultimately the function of this constant fraction discriminator right will remember constant fraction discriminator, the function of this is to reduce the time theta originates from the amplitude theta of the photomultiplier tube and then I also discussed about this TAC which is time to amplitude comment converter.

Basically, what we will going to get is the histogram of this fluorescence intensity, but it looks just in the reverse mode. Like a mirror image of this histogram which is actually is because here my start and stop are different. And you also note that I have not discussed these two things these two work the importance of these two polarizer over here which are going to discuss later.

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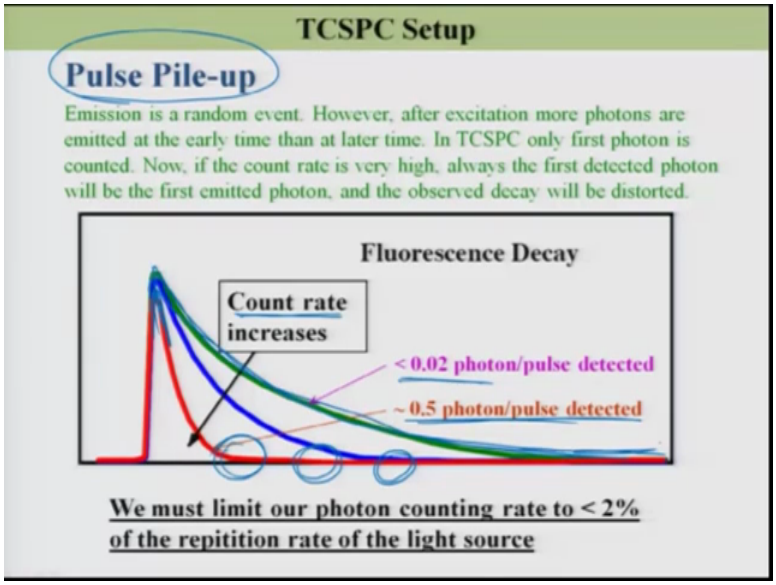

Before moving to this instrument response function, let me tell you that this count rate of this TCSPC system is very important. Why it is important because in TCSPC the first detected photon is the most important photon, it means that for a normal life normal sample where the life time has some value let us say about a nanosecond, then the

photons are emitted throughout the 5 nanosecond time window. If the life time is 1 nanosecond then photons are emitted throughout the 5 nanosecond time window that we have already seen.

Now, consider and I also told you that this lifetime means the average time the molecules paint is at in the excited state, it means that some molecule will come back from the excited state to the ground state at early time, some will come at a later time. So, throughout this 5 nanosecond time window some molecule will come now some molecule will come then and so on and so forth; that means, some molecule will emit is photon early time, some molecule will emits it photon later time.

Now, if I increase my count rate that means, the molecule and; obviously, at a time I am not going to excite only single molecule any many molecules are being excited; because the number of photons in this excitation light pulse are huge. So, once I excite the sample I must get a photon which is emitted at very later time otherwise those photon will never be detected, that is why in TCSPC I already have discussed briefly these things. So, in this TCSPC system the detection rate has to be very very low, otherwise the probability of getting the photon which is emitted at the later time will never be the first detected photon because in TCSPC the first detected photon is only getting detected by the system and you will get a count in the respective time channel right. If you have any question you can ask me right in the photon later.

So, now as I have shown you these problems is known as Pulse Pile up. So, in pulse pile up if the count rate is high that means, 0.5 photon per pulse is detected, here 0 point is over here like. So, then the that the count coming up in the early time is more; that means, you are not getting count at this time, this time, this time so that means, your actual decay is getting distorted.

But if you decrease the count to 0.02 photons per pulse; that means, so the count rate is very high less than 2 percent or so, in that case you can get the decay in that case the decay is the correct decay profile which is actually showing by the molecule. Having said this let me show you the actual measured fluorescence transient or fluorescence lifetime decay of a molecule which is called Anthracene in Cyclohexane.

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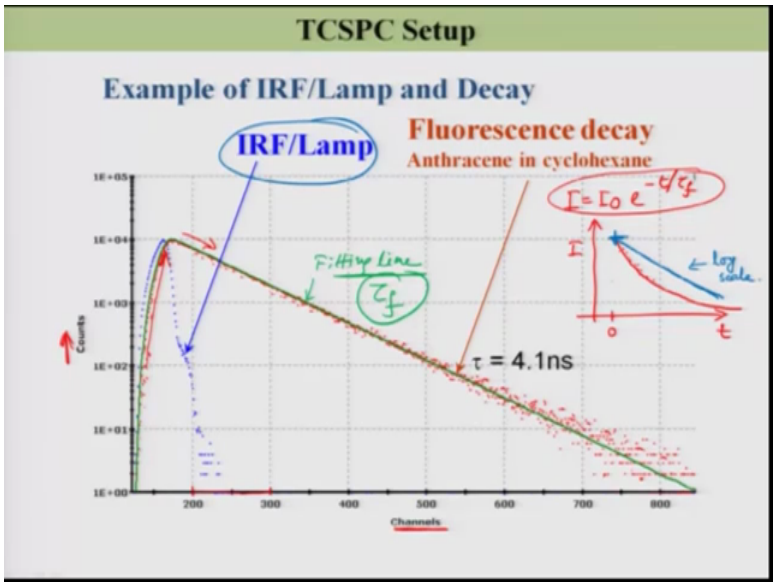

So, as you can see here this is red dots see these red dots over here, these red dots these are the measured intensity as a function of time. You see these red dots these are the measured intensity as a function of time, I told you these fluorescence lifetime the decay of the intensity with time is exponential in nature, but here you see the straight line because these axis is not linear axis this is a log axis. So, that is why it looks like a straight line.

But it that means, in the in the linear axis if this axis is linear then it should be exponential in nature right. So, I have all this measured data point you see when the count is low because this is my channel you see here, here it is written as channels that each channel this is 200 channel this is 300 channel that means, from here to here I have 100 channels at each channels having some value of time right. So, from channel 200 to 201 means some value of time right. It could be 0.005 picoseconds, it could be 0.005 nanosecond it depends on the TAC range; I already mentioned briefly while discussing the TAC.

So, depending on the TAC range; that means, depending on how much time will be required to raise the voltage in the TAC from 0 to 10 volt that you can set from outside. So, depending on that you could have different time value in each channel. So, this is the multi channel analyzer it works that in that way only. So, if you remember when I discussed this fluorescence lifetime, I simply wrote this equation I is equal to I 0 that is

initial intensity, into e to the power minus t by tau f and I said that this tau f is the fluorescence lifetime, and I also said that it means that the average lifetime of the molecule in the excited state.

So, then I also had plotted these things I versus t and I told you that if this is the time of excitation 0 and excitation is usually very very fast in time that means, no time will take for the excitation and if the excitation pulse width is a delta function, then immediately at time t equal to 0 you will going to create some population which will decay exponentially; that means, this decay will be something like this exponential decay let me draw a line like this exponential decay. The same thing if I now plot on the log scale then this will looks like this; that means, at time 0 there is no count and the count is very high at time t equal to 0. So, everything starts from here from this point then it decays in the log scale.

So, it is just started from here at a high value and then it is decaying, but in this case you see the red dots as I showed you these there are some red dots are still even here here here, it looks like that something is rising and then it is decaying. It looks like something is rising from here to here something is rising and then it is decaying right, but from our basic equation like this, I do not see any rise because we simply decay exponential decay. So, now, question is from where that rise part comes. The answer is very simple to get this equation or while explaining this equation I said towards that word is that excitation is very fast and that is true absorption is in the order of 10 to the power minus 15 second that we have already seen.

Another thing what I said is the excitation pulse is a delta pulse that is not correct because I do not have such kind of delta pulse excitation light source, but I have I have the pulse laser output. So, that those pulse laser have some specific pulse width, it could be one nanosecond it could be 100 picoseconds, it could be 100 femtosecond, but it is not 0 because of these I am not going to get just simply all the population at time t equal to 0 is maximum.

So, if the pulse width is something it could be on 1 nanosecond, it could be 1 picosecond then when the tail part of the pulse will interact with the sample then few molecule will going to will go to the excited state, but when the peak of the pulse will interact with the sample then most of the molecule will go to the excited state and when the later tail little

later part of the pulse will interact with the sample then another then again few more molecule will go to the excited state. That is why in this case decay is distorted and to analyze this you see these red dots are like this shape. So, to analyze this that analyzed curve is this green curve as you can see here see this green curve this is my analyzed curve to get the value, this is the fitting line right then I will understand what is the value of tau a from here.

So, to get this I also need these blue one as I have mentioned over here, I also need this blue one that is called the IRF or lamp that is the instrument response function. It is a collective response of the laser how many device we have in TCSPC laser? I have constant fraction discriminator what is the job, it is reducing the time jitter coming from the amplitude jitter of the PMT, then I have the PMT itself where it has some transit time spread which I cannot really I can reduce it, but it cannot be 0. So, taking all this time error component in the TCSPC setup, I will going to get a collective response of the instrument which is called the instruments response function.

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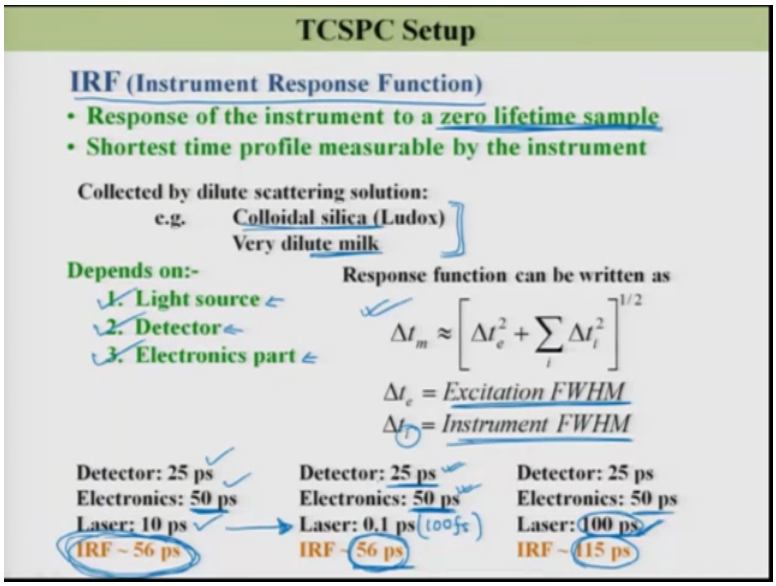

And let me show you that this instrument response function what I have written here is response of the instrument to the 0 lifetime sample right; you will note here is written 0 lifetime sample. What is 0 life time sample? 0 lifetime sample is nothing but the scattering; scattering is it 0 life time right once there is a light there is scattering if light is stopped scattering is stopped.

So, instead of taking some flow raport that some molecules which emits light if I now can take scattered are again, that means that scatter are will going to give me scattered light that could be rally scattering that also could be Raman scattering does not matter because in both the case these scattering is instantaneous process. So, I will going to use a scatter. So, some people use the dilute milk, milk that is also very good scattered. So, dilute milk one can use, one can use a colloidal silica. So, whatever scattered you want to use you can use those are does that scattered light is a 0 lifetime samples in our case.

So, in this case, I will get the response of the instrument is not it. So, if the response of the instrument is only depends on the pulse width of the excited excitation laser source, then if I change the laser source then the IRF of the system will change accordingly, so that we can check how this collective response actually change by changing the different component of this TCSPC system.

So, as I told you that this is instrument response function depends on the light source depends on the detector depends on the electronics part. Here this is my CFT here is my PMT, here is my PMT, here is my CFT, and here is my laser. So, that can be written like such kind of form where this is delta t e is my excitation, FWHM this is the laser part and this I this is the instrument part. So, one can calculate easily that if the detector response transit time spread or response time is 25 picoseconds, electronics part is 50 picosecond, and laser is 10 picosecond.

Then the total IRF will be something like 56 picosecond that obviously, we have to measure we have to measure how by changing the sample with the scatterer and just simply change the wavelength of the emission monochromator a same wavelength of that of my laser light then I can simply monitor it. If you want to monitor the Raman scattering then you can also go to the different wavelength than the excitation light and measure the instruments response function.

Now, what I have done, I have just changed the laser from 10 picoseconds to 0.1 picosecond that means, 100 femtosecond is much better laser, but if you calculate it what will going to see the IRF remains 56 picosecond. That means, it has some effect, but it is that effect is not so prominent here, because it is overruled by these two values. So, if you change the laser from 10 picosecond to 100 picoseconds then you see the effect over here now the IRF will be 115 picosecond.

So, the limit is over here the electronics part in this case the electronics part in this case the laser part because it is the highest value among these 3, so IRF is like the highest value. In this case the highest value among these three is this one. So, the IRF is guided by this value that highest value, in these two three in this case the highest value is this one. So, IRF is like this 56 picosecond, but the problem is that you cannot go below 25 picosecond. So, IRF cannot be less than 25 picosecond in case of TCSPC system.

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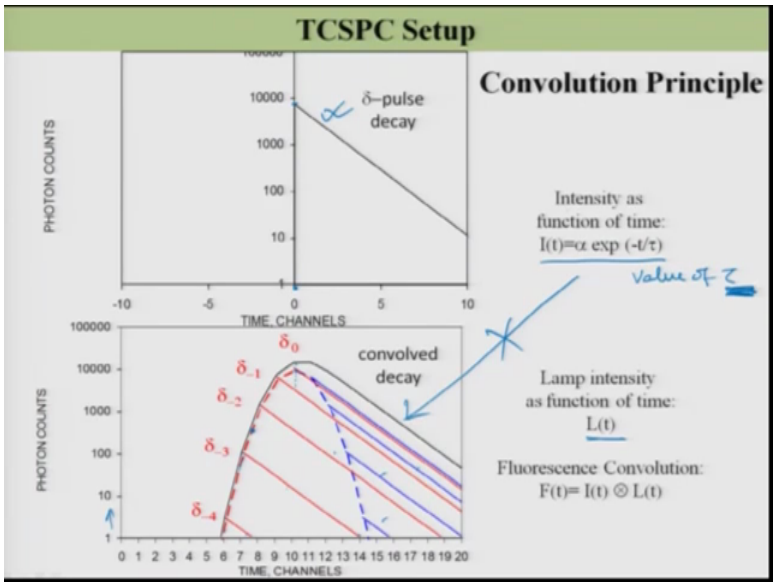

Whatever I said let me also show you in this animation; so, for the delta pulse excitation immediately at time t equal to 0 I got maximum intensity and this is simply decaying like this alright. So, the intensity as a function of time can be written as I t equal to alpha so that initial intensity exponential minus t by tau. But then I said that this cannot be delta it has some pulse width right. So, let me take this L t as the lamp intensity as a function of time which is denoted by these part, and I have taken this mid part as my time 0 right. So, when this part the negative time is interacted with the sample then some molecule will go to the excited state and immediately after going to the excited state, those molecules will start decaying.

So, if when this part of this excitation pulse will interact with the system, those molecule like some other some other molecule will go to the excited state and those molecule immediately after excitation will start decaying and so on and so forth, that what I am trying to show you over here. So, this part is showing a fluorescence decay like this that

time delta minus for this part fluorescence decays like this please note that this is in the log scale here is not linear scale.

So, in this case here all these decays are straight line and here there is a decay that means, the total fluorescence decay will be some this plus this plus this plus this plus this all these things and it will looks like this black curve. So, there is something rising and then there is something decays. But as you know if I have a delta pulse then it should be a simple like this, and then I can fit it and I will get the value of tau, but from here you cannot fit this with such kind of equation these cannot be fitted with such kind of equation impossible.

So, I have to do something to get this value of tau, what I should do that is my question now and we will going to discuss that.

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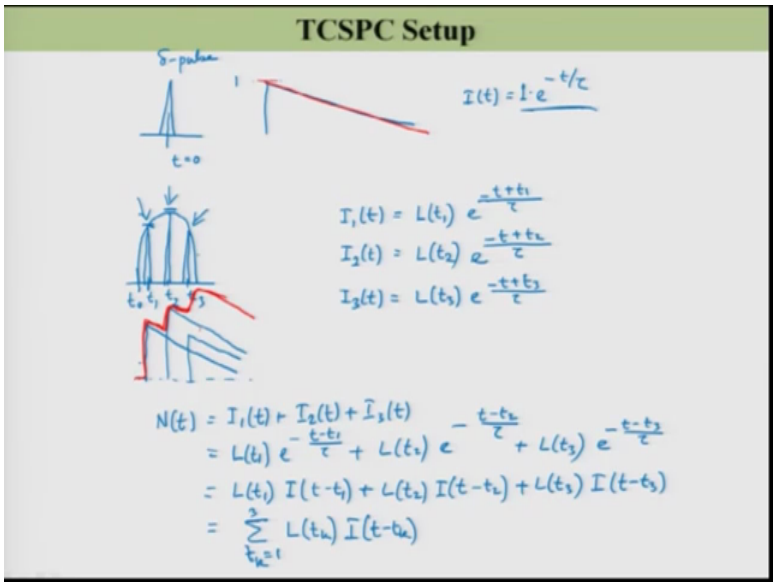

Let me take this as my excitation pulse at time t equal to 0, in this case the lifetime is I t equal to e to the power minus t by tau if I take this as 1. So, this 1 is over here, 1 into e to the minus t by tau, now let me take the broad pulse this is my delta pulse. Now for this broad pulse I can think of that these broad pulse here is my t 0, these broad pulses is consists of three delta pulses that are my choice I decided to think like that, this is my first one, the second one is in the middle and this is my third one.

So, this is actually coming at time t 1, this is at time t 2, this is at time t 3. So, each of these pulse independently I can think of is giving the fluorescence emission and obviously, each of these we will have it is own fluorescence decay. So, the first one will looks like this, the second one will be like this because second one is the higher intensity so, the intensity will be high and the third one will be like this. So, altogether what I will have is like this. So, everything will add together. So, now, the decay looks like this it is no longer a straight line starting from an initial value which was here starting from initial value a straight line it is not distorted.

Let me write the individual equations here, for the first one I can write I 1 t equal to the intensity L t 1; because it has some intensity and the some intensity over here and the fluorescence intensity at time t equal to 0, t equal to t 1 will be proportional to that intensity. So, I simply write L t 1, e to the power minus t plus t 1 divided by tau; you got my point here in this case it is not simply minus t by tau, because some time t 1 is already delayed over here this is now starting not starting from t 0.

So, minus t plus t 1 divided by tau. For the second one I to t is equal to L t 2, because the intensity is little higher over here and the fluorescence intensity will be proportional to the excitation light intensity that we all know, e to the power minus t plus t 2 this is at some other time divided by tau. For I three t similarly I will write t 3 e to the power minus t plus t 3 tau. So, the total fluorescence intensity I can write n t equal to I1 t plus I 2 t plus I 3 t.

So, I can simply write L t 1, e to the power minus t minus t 1 by tau, plus L t 2, e to the power minus t minus t 2 by tau, I just took the negative signs out over here plus L t 3, e to the power minus t minus t 3 by tau good. So, this I can write as L t 1, I t minus t here look here some constant it is (Refer Time: 25:49) t by tau is this t. So, if I have t minus something then here also t minus that time. So, L t 1, I t minus t 1, plus L t 2, I t minus t 2, plus L t 3, I t minus t 3, this I can write as a sum over I or let us write t k is equal to 1 2 3, L t k, I t minus t k. So, this is for the summation for this 3 pulse here is my number 1, number 2 and number 3.

Now, I can consider that I have in finite number of delta pulse which is making this excitation pulse like I can say so.

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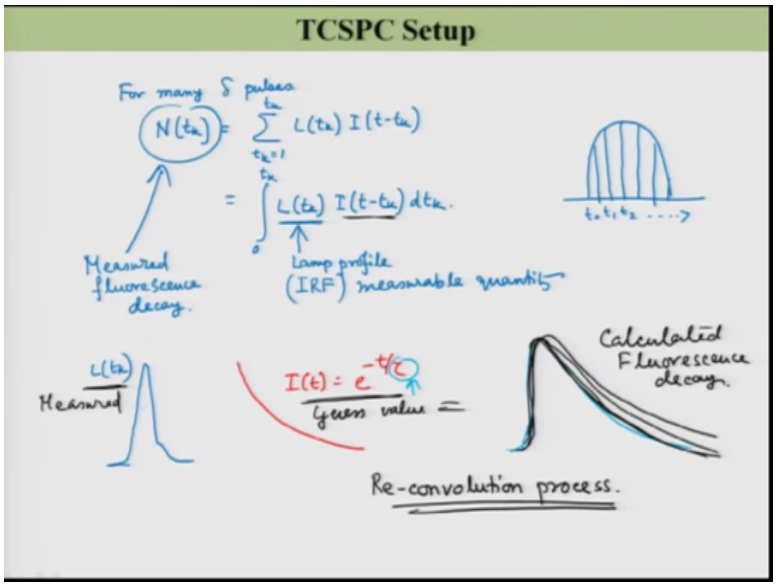

So, what I can write for many delta pulses this in t k can be represented as some of over t k 1 2 t k, L t k 2 I, t minus t k for many many things I can simply integrate integration 0 to t k, L t k, I t minus t k, d t k right. So, this is the effect. So, this is the effect of many many delta pulse in that excitation pulse light. Now these L t k is my lamp profile; that means this is nothing but that what I said here is my one delta pulse here is my another delta pulse another, another, another, another, like that like this is t 0, t 1, t 2 and so on that is what I said. So, this is nothing but instruments response function that is the response of the instrument versus 0 lifetime sample. So, this is IRF I can measure it. So, this is measurable quantity the centigrade this is nothing but your measured fluorescence decay.

So, now if you have this L t k looks something like this, just I am drawing it let us say this is your L t k that you have measured and you have just guessed. So, guessing that the process lifetime of this sample is 4.2 nanosecond. So, for these 4.2 nanoseconds simply this will follow the e to the power minus t by 4.2 nanosecond so the intensity will decay accordingly; so ideal value ideal case. So, in simply you will get such kind of curve. So, this is a true I t which has the form e to the power minus t by tau. So, this (Refer Time: 30:08) and this (Refer Time: 30:09) will give you from this equation this is nothing but this I the form of I this L t is nothing but this. So, these two will give you like such kind of decay this is your calculated decay. What is not measured is over here this is the calculated, this is your measured, this is guess value. So, the measured one and this

calculator and let us say the measure this looks like, this the measured one is like this. So, then after you calculate you compare with the measured one, if they are same now they are not same.

So, you change the value guess value to a different value then again you match with this according to this equation. So, then the different you will come like this still it is different. So, you again change and once and some time and for one value of this tau this will be similar to the measured value that is called the least square fitting method and you will get the lifetime of your sample. So, like this way we are going to measure the lifetime in TCSPC system, and this process is called the iterative Re-convolution technique.

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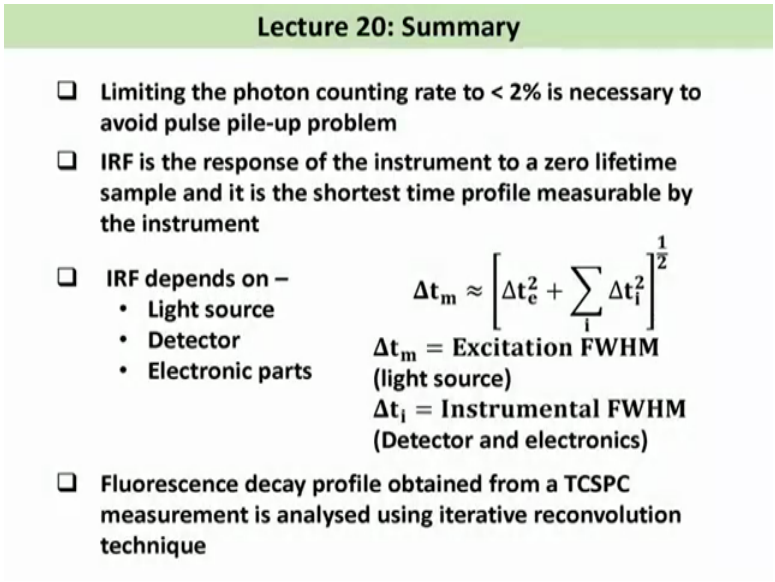

With this let me stop here and we will continue our discussion in the next class.

Thank you very much.