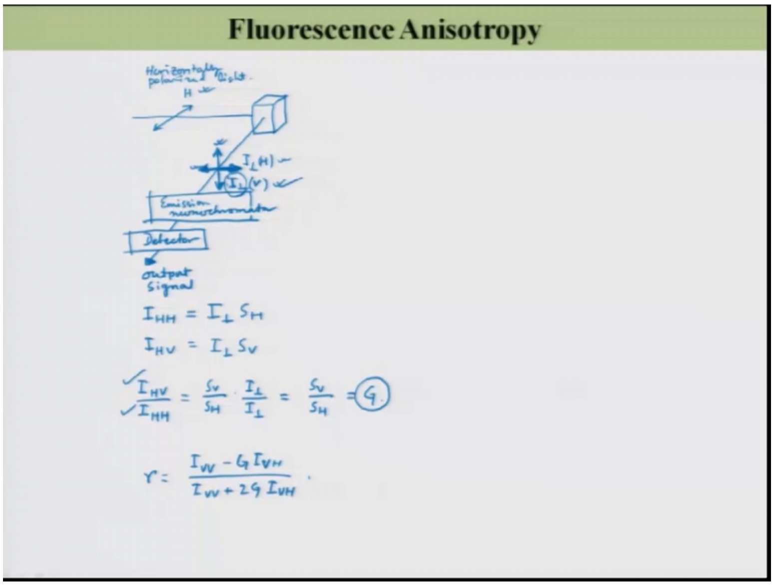
Basics of Fluorescence Spectroscopy

Prof. Pratik Sen
Department of Chemistry
Indian Institute of Technology, Kanpur

Lecture – 28

So, welcome to the lecture number 28. So, we are discussing about this fluorescence anisotropy and as you can see here in our previous discussion.

(Refer Slide Time: 00:24)



Fluorescence Anisotropy

$$I_{HH} = I_\perp S_H$$

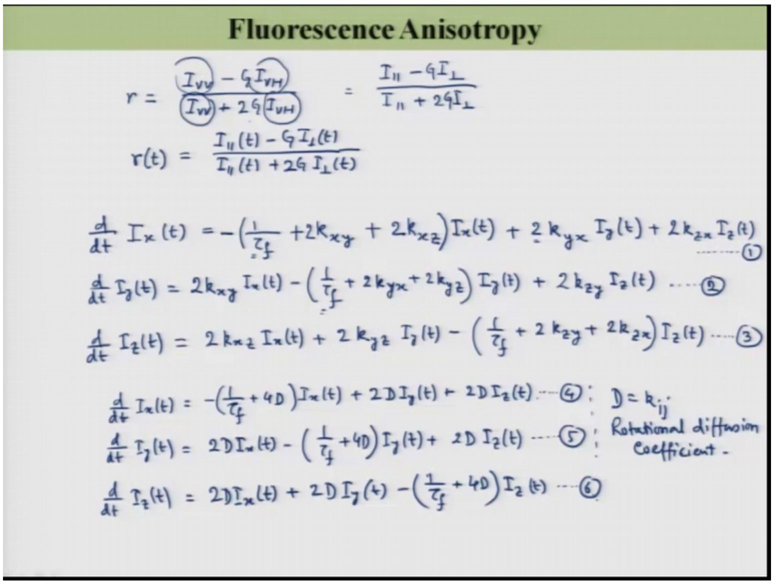
$$I_{HV} = I_\perp S_V$$

$$\frac{I_{HV}}{I_{HH}} = \frac{S_V}{S_H} \cdot \frac{I_\perp}{I_\perp} = \frac{S_V}{S_H} = \textcircled{G}$$

$$r = \frac{I_{VV} - G I_{VH}}{I_{VV} + 2G I_{VH}}.$$

So, basically what will going to measure, you will going to measure the intensity in the detector right. So, and these intensity in the detector should be multiplied that v H part I v H part should be multiplied with these factor G factor which is nothing, but the sensitivity of the detector for the differently polarised light.

(Refer Slide Time: 00:52)



Fluorescence Anisotropy

$$r = \frac{I_{VV} - G I_{VH}}{I_{VV} + 2G I_{VH}} = \frac{I_{\parallel} - G I_{\perp}}{I_{\parallel} + 2G I_{\perp}}$$

$$r(t) = \frac{I_{\parallel}(t) - G I_{\perp}(t)}{I_{\parallel}(t) + 2G I_{\perp}(t)}$$

$$\frac{d}{dt} I_x(t) = -\left(\frac{1}{\tau_f} + 2k_{xy} + 2k_{xz}\right) I_x(t) + 2k_{yx} I_y(t) + 2k_{zx} I_z(t) \cdots\cdots \textcircled{1}$$

$$\frac{d}{dt} I_y(t) = 2k_{xy} I_x(t) - \left(\frac{1}{\tau_f} + 2k_{yx} + 2k_{yz}\right) I_y(t) + 2k_{zy} I_z(t) \cdots \textcircled{2}$$

$$\frac{d}{dt} I_z(t) = 2k_{xz} I_x(t) + 2k_{yz} I_y(t) - \left(\frac{1}{\tau_f} + 2k_{zy} + 2k_{zx}\right) I_z(t) \cdots \textcircled{3}$$

$$\frac{d}{dt} I_x(t) = -\left(\frac{1}{\tau_f} + 4D\right) I_x(t) + 2D I_y(t) + 2D I_z(t) \cdots \textcircled{4}$$

$$\frac{d}{dt} I_y(t) = 2D I_x(t) - \left(\frac{1}{\tau_f} + 4D\right) I_y(t) + 2D I_z(t) \cdots \textcircled{5}$$

$$\frac{d}{dt} I_z(t) = 2D I_x(t) + 2D I_y(t) - \left(\frac{1}{\tau_f} + 4D\right) I_z(t) \cdots \textcircled{6}$$

$D = k_{ij}$ Rotational diffusion coefficient.

So, I can write here. So, r should be equal to I v v minus G I v H divided by I v v plus 2 G I v H. So, these are the quantity which is measurable in the detector right and these G value these G value will find out from the horizontally polarised excitation light source.

Now, if I write I can also write this as equal to I parallel minus I perpendicular because this is that original value I parallel plus 2 I perpendicular because this is the original value this is before reaching the monochromator, after reaching the monochromator it become v H and v v right. So, this become v H and this become v v and there is all the problem right the sensitivity problems. So, I put this G, but generally we do not care so, much and we always write G over here 2 G I perpendicular we generally do not care. So, it looks like that this is actually the measured intensity at the detector, but actually the measured intensity at the detector is this one I v v I H v I H v I H h like that, but we generally do not care. So, in most of the book what will see it is written that I parallel minus G I perpendicular divided by I parallel plus 2 G I perpendicular.

Assume that this is the measured intensity in the detector; this is not the intensity just after the emission after the samples are not that it is the measured intensity, but I can also tell if I said clearly then it is to write these things, if I do not say clearly then it is not to write that ok. So, let us me write this one now my question is that what is the time dependency of this, I have started with time dependency I said that I will excite this sample with delta pulse excitation all the fluorophores whose transition moment is parallel to the electric field of the excitation light source that will be preferentially excited to the excited state, and the excited state will be polarised emission will emit the polarised emission acts as time goes, the molecule will reorient them self right. Originally it was excited like this way now as time goes molecule will reorient like this like this way or that way or this way whatever the ways right.

The molecule will reorient themself and if that reorientation time is less than the lifetime then after the molecular will reorient you will get the emission from those molecules. So, then emission will be unpolarised emission right. So, I will go from the situation excited state is anisotropic to a excited state is isotropic oriental orientation ally isotropic. So, if now if I want to measure the time dependency right and then I change it to the c w mode now want to again come back to time dependency right. So, if I want to again come back to the time dependency this equation r equal to I parallel minus G I perpendicular that equation I can simply write as in function of time. So, here r will be the function of time that will be I parallel will be a function of time minus G is not a function of time; G is fixed for a particular instrument because it is the sensitivity of the monochromator and the detector towards the horizontally and vertically polarised sample the difference the ratio of the sensitivity.

So, G I perpendicular this is a function of t, divided by I parallel which is a function of t plus 2 G I perpendicular which is a function of t. Now if I now calculate this r value at time 0 let us say just after excitation, the r value should be more as time goes this r value will keep on decrease and decrease and decrease and at longer time very long time right as I said that probably all the fluorophore will reorient themself and it will randomised right. So, then polarise, then the fluorescence will be unpolarised completely and you will get the r equal to 0. So, looks like that if I now plot the r t versus t it should start from a high value right and from looking at this equation it looks that the value should be equal to 1 and then it will go to 0 right when I parallel equal to I perpendicular then the value will go to 0 right.

Let us now see this exact form of this I parallel t, I perpendicular t and let us now try to find out what will be the functional form of our t, for that let me imagine that I have a number of molecules in my solution and for a arbitrary polarization of excitation that arbitrary polarization of excitation could be along x axis could be along y axis could be along z axis. So, I will not specify right now I will specify it later, but for any arbitrary polarization of excitation I can write, the intensity of each direction how the intensity will vary with time. The variation of intensity with time for every direction I have the 3 directions here right either x or y or z. So, let me write these things over here.

For this I x which is the function of time the d d t of that how this is changing with time right. If now consider the molecules which is in the excited state is like let us say this is my x axis. So, the molecule is like this way. So, simply the molecule will come back from the excited state to the ground state. So, the intensity along these axis will decrease. So, if I have d d t of the along x right I should have a rate constant of deactivation, rate constant of deactivation from the excited state to the ground state and that should be the negative multiplied by the concentration this fluorescence intensity along the excitation direction.

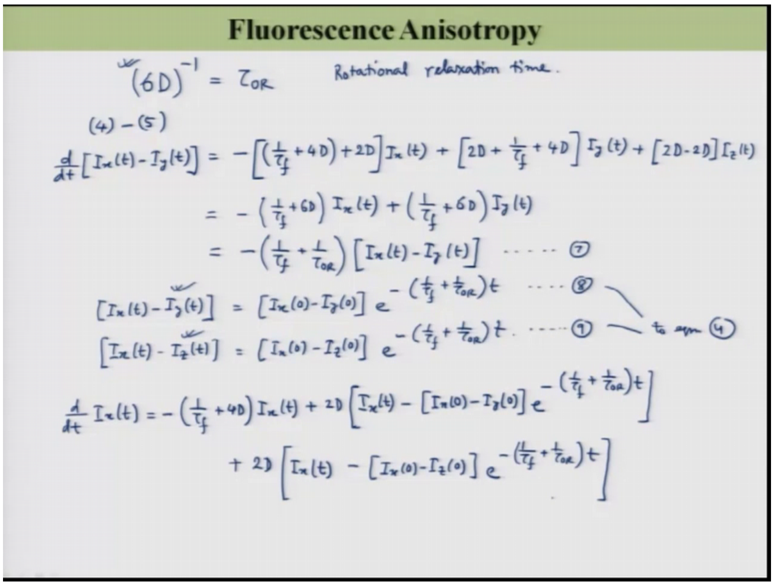
So, I have this what is that rate constant is k r plus k n r right k r plus k n r is 1 over tau. So, I can have this 1 over tau f minus 1 over tau f and what other things are there see here. So, I have excited over here and it can come back from the excited state to the ground state. So, fluorescence intensity will decrease that is the that 1 over tau f is accounted for that it can move like this way then fluorescence intensity of these axis will increase, but these axis will decrease that x axis will decrease. So, move movement of the molecule from x to y or movement or rotation of the molecule from x to z both will decrease the fluorescence intensity along x axis.

So, let me write that as rate constant of motion of the molecule from x to y. So, this is again minus and again this is decrease rate constant of rotation of the molecule from x direction to z direction right. Now again I will tell you that the molecule can move rotate from x to y x y like this way or that way. So, both clock wise and anti clock wise. So, in both case I have this 2 and I will take simply common take common this minus sign. So, it will be like this to I x t. So, this is my deactivation, but I have said that I have excited with arbitrary polarization of excitation. So, these molecules are excited, these are excited these are excited. So, those when these molecules are excited they will all they can also come back to this x axis y to x then it will contribute to the fluorescence intensity along x axis. So, I also should write this as a plus term over here. So, then I will write here plus 2 k this is rate constant of reorientation from y to x right. So, k y 2 x and this 2 term I have written again over here because of this clock wise and anti clock wise I y t plus from the z to x right. So, 2 k z x, I z t right I can write this.

Similarly, I can write d d t of I y t equal to rest follows simply as I said for this d d t of I x t. So, the rest of the things is very similar I am just writing it over here; k x y I x t minus 1 over tau f here see here these tau f and these tau f they has to be same because they are same molecule right plus 2 k y x this is from y 2 x, plus 2 k y z this is from y to z I y t plus 2 k z 2 y I, I z t and I can write this as the third one k of I z, t s 2 k x z I x t plus 2 k y z, I y t minus 1 over tau f plus 2 k from z to I this 1 right plus 2 k z x, I z t right I got this one 2 3 equations right the equation number one this time I am putting this equation number because later I need this number for to refer otherwise it will be difficult to follow 1 2 3.

Now, as I said rotation like this way or rotation this way or rotation this way or rotation that way they should be same because this is just the solvent and this molecules either rotating in this way or that way. So, these k x y should be equal to k x G should be equal to k y G should be equal to k a x and like that all value should be same right. So, let us replace these all these values with a one constant and that constant lets named it as d right. So, I need to rewrite all this equation which is difficult, but let me write it. So, d d t I x t equal to 1 over tau f plus 4 D I x t plus 2 D I y t plus 2 D I z t right. So, here I said that here D equal to all those different k I s. So, which is rotational diffusion coefficient right. Let me put as equation number 4 and d d t of I y t is equal to 2 D I x t minus 1 over tau f plus 4 D I y t plus 2 D I z t lets say this is equation number 5 and d d t of z t 2 D I x t 2 D I y t minus 1 over tau f plus 4 D into I z let us say this is equation number 6.

(Refer Slide Time: 15:05)



Fluorescence Anisotropy

$$\overset{\checkmark}{(6D)^{-1}} = \tau_{OR}$$ Rotational relaxation time.

(4) − (5)

$$\frac{d}{dt}\left[I_x(t) - I_y(t)\right] = -\left[\left(\frac{1}{\tau_f} + 4D\right) + 2D\right] I_x(t) + \left[2D + \frac{1}{\tau_f} + 4D\right] I_y(t) + \left[2D - 2D\right] I_z(t)$$

$$= -\left(\frac{1}{\tau_f} + 6D\right) I_x(t) + \left(\frac{1}{\tau_f} + 6D\right) I_y(t)$$

$$= -\left(\frac{1}{\tau_f} + \frac{1}{\tau_{OR}}\right)\left[I_x(t) - I_y(t)\right] \quad \cdots\cdots ⑦$$

$$\left[I_x(t) - \overset{\checkmark}{I_y(t)}\right] = \left[I_x(0) - I_y(0)\right] e^{-\left(\frac{1}{\tau_f} + \frac{1}{\tau_{OR}}\right)t} \quad \cdots\cdots ⑧$$

$$\left[I_x(t) - \overset{\checkmark}{I_z(t)}\right] = \left[I_x(0) - I_z(0)\right] e^{-\left(\frac{1}{\tau_f} + \frac{1}{\tau_{OR}}\right)t} \quad \cdots\cdots ⑨$$

to eqn ④

$$\frac{d}{dt} I_x(t) = -\left(\frac{1}{\tau_f} + 4D\right) I_x(t) + 2D\left[I_x(t) - \left[I_x(0) - I_y(0)\right] e^{-\left(\frac{1}{\tau_f} + \frac{1}{\tau_{OR}}\right)t}\right]$$

$$+ 2D\left[I_x(t) - \left[I_x(0) - I_z(0)\right] e^{-\left(\frac{1}{\tau_f} + \frac{1}{\tau_{OR}}\right)t}\right]$$

Now these D right this is rotational diffusion of coefficient right if you multiply it by 6 right and you take inverse of that that is known as tau or I am not telling from where it is coming, but you take it granted right. So, you d you multiply it by 6 take this inverse this tau or. So, this tau or is called the rotational relaxation time. So, the other equation this D I can replace with this later on right that I will do later on. So, now, what I will do you see here this 4 minus 5 I will do this 4 minus 5, let me do this 4 minus 5.

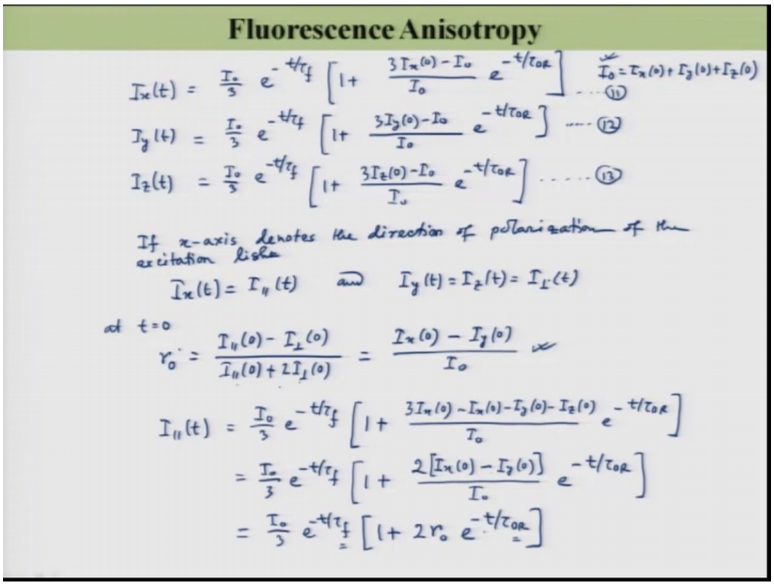
So, equation 4 minus equation 5 if I do so, linear operator d d t I x t, I y t is equal to minus what you will get 1 over tau f plus 4 D right here you get 1 over tau over 4 D from this x and from here you get 2 D right then I will have minus 2 D whole thing here I x t and for the I y I will also get this term, but this will be 2 D plus 1 over tau f plus 4 D right I y t because here you see here minus I am taking this minus sign and I will have this z term also. So, plus, but they will cancel you see this 2 D minus 2 D right I z. So, if I do it this will be equal to minus 1 over tau f plus 6 D. So, there is some mistake yeah minus 1 over tau f minus 4 D minus 2 D. So, then this should be plus. So, then 6 D to I x t plus 1 over tau f plus 6 D I y t and here this is nothing right. So, then this will be equal to minus 1 over tau f plus 1 over tau or see here this relation these I took outside. So, then this is equal to I x t minus I y t ok.

Now, what I will do I will do the 4 minus. So, I have done the 4 minus 5, now I will do I can do that x and z. So, then it is equal to 4 minus 6 I can do right 4 minus 6 I can do, but before doing that let us integrate this. So, if I integrate the result will be I x t minus I y t equal to I x 0 minus. So, these are this is the my initial intensity into e to the power minus 1 over tau f plus 1 over tau or 2 D. So, for this 4 and 5 I got these type of equation from 4 and 6 I will get this type of equation after doing all these steps I z t this will be equal to I x 0 minus I z 0, e to the power minus 1 over tau f plus 1 over tau over into t. So, from here I got what is this I y t see here. So, this one I know from here this one I know, now if you put the value of I y t and I z t here and here you will get everything in terms of I x, I x here I x here these 2 will be replaced I x I x. So, now, let us put that.

The value of I y and I z to that equation. So, what you will get d t let me name it as 7 this one is 8, this one is 9 and now putting the value of this 2 to equation 4 what will have is d d t of I x t equal to minus 1 over tau f plus this, this I am writing as 4 D because I did not have this 6 D term which I can replace till now, plus 2 D I x t minus I x 0 minus I y 0 to e to the power minus 1 over tau f plus 1 over tau or right t will be here and whole thing will this square bracket right. So, this is for this y term and I should also write the z term. So, here my 2 D I x t minus I x 0 minus I z 0, e to the power minus 1 over tau f plus 1 over tau over t square bracket close. So, I got this.

Now, you can solve it. So, you see this is big equation. So, I do not want to solve it here. So, I will just write this answer. So, if you solve it.

(Refer Slide Time: 22:58)



Fluorescence Anisotropy

$$I_x(t) = \frac{I_0}{3} e^{-t/\tau_f}\left[1 + \frac{3I_x(0) - I_0}{I_0} e^{-t/\tau_{OR}}\right] \quad \cdots ⑪ \qquad I_0 = I_x(0) + I_y(0) + I_z(0)$$

$$I_y(t) = \frac{I_0}{3} e^{-t/\tau_f}\left[1 + \frac{3I_y(0) - I_0}{I_0} e^{-t/\tau_{OR}}\right] \quad \cdots ⑫$$

$$I_z(t) = \frac{I_0}{3} e^{-t/\tau_f}\left[1 + \frac{3I_z(0) - I_0}{I_0} e^{-t/\tau_{OR}}\right] \quad \cdots ⑬$$

If x-axis denotes the direction of polarization of the excitation light

$$I_x(t) = I_\parallel(t) \quad \text{and} \quad I_y(t) = I_z(t) = I_\perp(t)$$

at $t=0$

$$r_0 = \frac{I_\parallel(0) - I_\perp(0)}{I_\parallel(0) + 2I_\perp(0)} = \frac{I_x(0) - I_y(0)}{I_0}$$

$$I_\parallel(t) = \frac{I_0}{3} e^{-t/\tau_f}\left[1 + \frac{3I_x(0) - I_x(0) - I_y(0) - I_z(0)}{I_0} e^{-t/\tau_{OR}}\right]$$

$$= \frac{I_0}{3} e^{-t/\tau_f}\left[1 + \frac{2[I_x(0) - I_y(0)]}{I_0} e^{-t/\tau_{OR}}\right]$$

$$= \frac{I_0}{3} e^{-t/\tau_f}\left[1 + 2r_0 e^{-t/\tau_{OR}}\right]$$

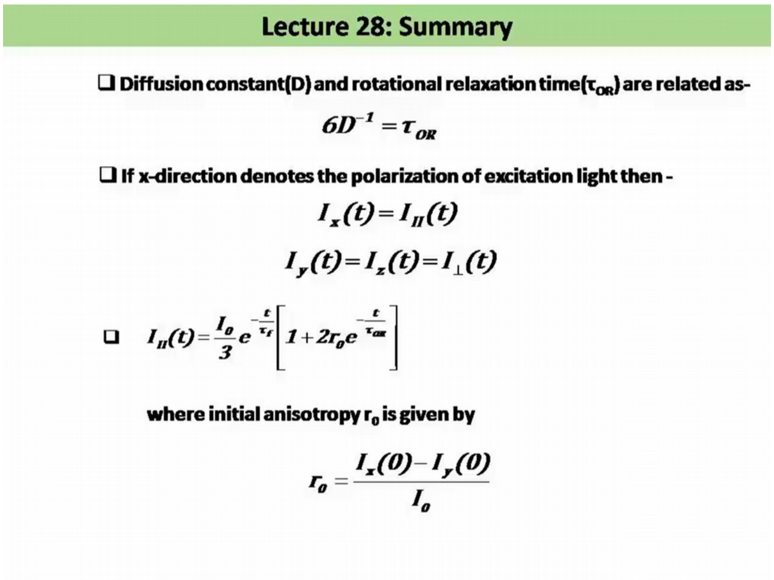
So, what you will get is I x t equal to I 0 by 3 e to the power minus t by tau f into 1plus 3 I x 0 minus I 0 divided by I 0 e to the power minus t by tau or simply we will get this where this I 0 equal to I should write that right I x 0 plus I y 0 plus I z 0. So, similarly you can write for I y t and obviously, you can write for I z t. So, I y t it will looks like I 0 y similar equation e to the power minus t by tau f, 14 plus 3 I y 0 minus I 0 divided by I 0 e to the power minus t by tau or here you will get let me name it as I have this is 8 9 this is 10 then this is 11, this is 12, this is I 0 by 3, tau f t by tau f 1 plus 3 I z 0 minus I 0 by I 0 e to the power minus t by tau or this is my 13 right.

Now, I will put this condition, now if my x axis is the polarization excitation right and the excitation light is polarised along x axis then I can simply write I x e then these are the emission I am telling if the polarization of the excitation light is along x axis, then I x will be I parallel I y will be I perpendicular and I z will be I perpendicular that is my condition. So, under that condition, I am writing if x axis denotes the direction of polarization of the excitation light and then I can write I x t is equal to I parallel t, and I y t should be equal to I z t is equal to I perpendicular t. So, for this at t equal to 0, I can simply write this r 0 right I parallel 0 minus I perpendicular 0 by I parallel 0 minus twice I perpendicular 0. So, here if I assume it like a x. So, I can write I x 0, this perpendicular either y or z let me write I y 0 divided by this is I x 0 and this is I y and I z sorry sorry this is plus. So, I x plus I y by plus I z right, this is nothing, but this I 0. So, this is equal to I 0 right.

Now if I want to calculate this I parallel t then this is nothing, but I x t right. So, I will simply write this equation I x t. So, this will be equal to I 0 by 3, e to the power minus t by tau f into one plus 3 x 0 minus I 0 right. So, now, I will write here 3 I x 0 I 0 I will replace with I x 0 plus I y 0 plus I z 0. So, minus I x 0 minus I y 0 minus I z 0 divided by I have I 0 let it let me write this as I 0 itself e to the power minus t by tau or. So, this is equal to I 0 by 3, e to the power minus t by tau f. So, here you see it will cancel. So, I 0 one I 0 and this I 0 will cancel, 2 I 0 looks like. So, here it is 1 plus 2 I x 0 minus I y 0 and I z 0 that they are same right. So, I will write here I y 0 to common divided by I 0 e to the power minus t by tau or here you see I x 0 minus I y 0 by I 0 this is nothing, but r 0.

So, here I 0 by 3 e to the power minus t by tau f 1 plus 2 r 0 e to the power minus t by tau or. So, I parallel is telling that these intensity at the parallel to the excitation intensity of the fluorescence which is parallel to the excitation polarization will decay like this way I have 2 component 2 term here one is tau f another is tau or tau f is fluorescence lifetime and then tau or is the reorientational time right or orientational relaxation time right. So, it is like that, similarly next day I will calculate this I perpendicular t and then I will see when I will plug in this I parallel and I perpendicular to my original equation of anisotropy how the r t will looks like.

(Refer Slide Time: 30:04)



Lecture 28: Summary

- ☐ Diffusion constant(D) and rotational relaxation time(τ_{OR}) are related as-

$$6D^{-1} = \tau_{OR}$$

- ☐ If x-direction denotes the polarization of excitation light then -

$$I_x(t) = I_{II}(t)$$

$$I_y(t) = I_z(t) = I_{\perp}(t)$$

- ☐ $I_{II}(t) = \frac{I_0}{3} e^{-\frac{t}{\tau_f}} \left[1 + 2r_0 e^{-\frac{t}{\tau_{OR}}} \right]$

where initial anisotropy r_0 is given by

$$r_0 = \frac{I_x(0) - I_y(0)}{I_0}$$

Thank you very much.