Basics of Fluorescence Spectroscopy
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Lecture – 30

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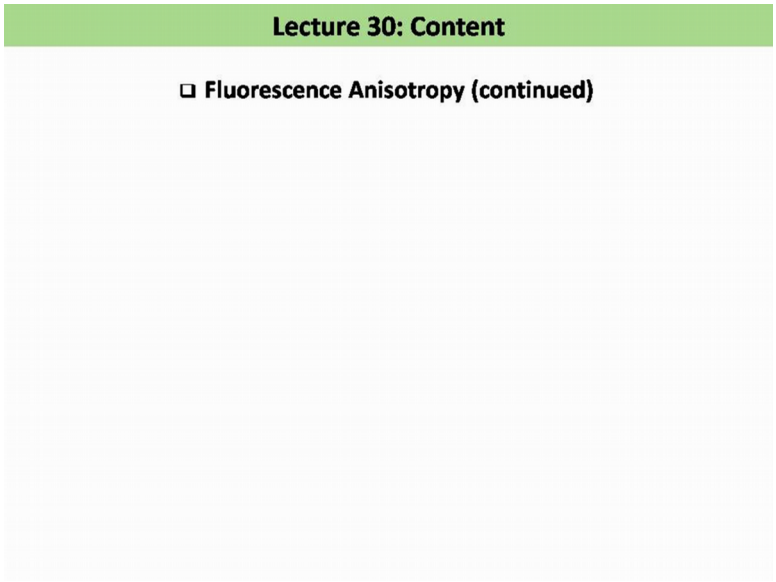

So, welcome back. Today we will going to start our lecture number 30. We are discussing fluorescence anisotropy and we are done till the Perrin equation. So, what is Perrin equation let me write it once again.

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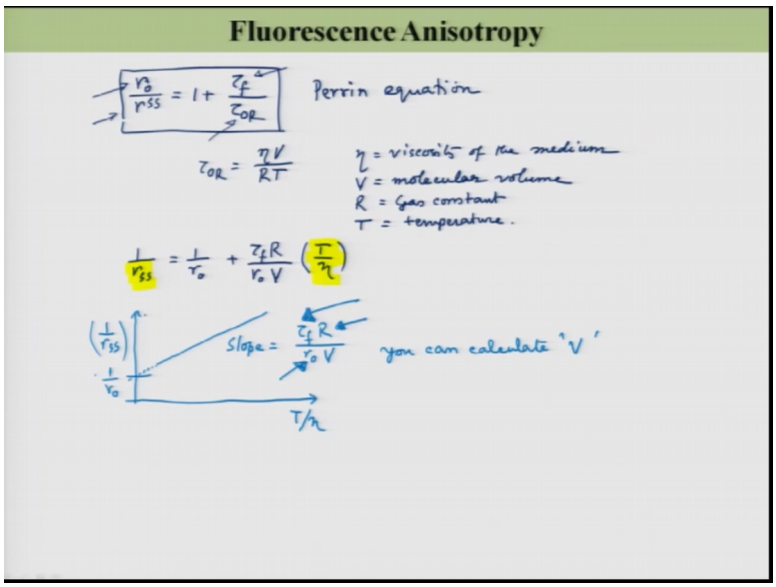

So, Perrin equation is written as r 0 by r ss equal to 1 plus tau f divided by tau or. So, where this r 0 value is initial anisotropy r ss my steady state anisotropy. And this tau f is my fluorescence lifetime, and tau over is orientational relaxation time.

So, from here what you can see is that, without if you have some idea about these value of r 0 right. And this r ss can be measured very easily from this fluoremeter, where you have this polariser and analyser right. Normal fluoremeter does not have this fluorophore polariser analyser, but if you can install a polariser analyser. So, that their excitation light is polarised light and the emission you are going to analyse which is the perpendicular direction and which is in the intensity and what is the intensity of the perpendicular direction and what is the intensity of the parallel direction.

Then you will be able to calculate this r steady state easily right. And if you have this knowledge of this fluorescence lifetime, then you will be able to calculate this tau over. Now if you recall that our stoke Einstein relationship, then this tau over can be written as tau over equal to eta v by R T right. This is my stoke Einstein relationship, where this eta is equal to the viscosity of the medium v is molecular volume, and R is my universal gas constant and T is my absolute temperature.

Now, if you plug in this expression for this tau or in the Perrin equation, what we will going to see is the following. 1 over r ss equal to 1 over r 0 plus tau f v by into r divided by r 0 into v into temperature by viscosity right. It means that now if I plot or this is this is the measurable quantity, this is your measurable quantity, these is the property at a particular temperature; obviously, you know the viscosity. So, then t by eta is known quantity and I can measure this r steady state for a different value of t by eta against the temperature; obviously, viscosity will change. So, t by eta will change and for different values of this t by eta I can measure this steady state anisotropy, and then I can plot it right.

So, let us plot that same. So, if I plot what you will going to see is 1 over r steady state versus t by eta. We will going to see a straight line and intercept will be equal to 1 over r 0. So, from here you will get some idea of this r 0 right. And then slope will be equal to tau f r by r 0 v. Now if tau f the fluorescence lifetime is known r; obviously, is a constant and r 0 if you have fair idea of this r 0 then you can calculate v that is the volume. And this is well used equation in application of the fluorescence anisotropy. So, from with

this you can actually determine the size of let us let us say you are working with a protein and this protein is getting denatured once the protein is getting denature the size will increase and if you have one fluorophore already attached to the protein then by measuring the fluorescence anisotropy; that means, the fluorescence this r ss value by measuring this r ss value what will be able to do you will be able to see that at this particular condition what is the volume of the protein or; that means, the size of the protein and so on right.

Now, one of the important thing here is that what will be the value of r 0 right. That is that is the biggest question right. So, that should be measured otherwise I cannot say anything about this. And we have seen that limiting value of r 0 you remember our first equation I have written r equal to i parallel minus i perpendicular divided by i parallel plus twice i perpendicular right. Now I am not talking about I am not including this g because considering the g equal to 1 g is that 1 then just a factor will be there right. So, considering the g value is equal to 1 and I am now doing all those thing.

Now, in case of r time dependence then this is nothing, but R T is equal to i parallel t minus i perpendicular t and so on. So; that means, at time t equal to t equal to 0 that is my initial anisotropy r 0 and that is whatever I have written here as r 0. So, in this case I have to determine what will be the value from that particular equation, when I said that this i parallel minus i perpendicular divided by i parallel plus twice i perpendicular; obviously, at time t equal to 0 there was no emission from this perpendicular component and there is only emission from this parallel component makes it r 0 equal to 1, but whether this is true and we are I also said that when this r value will change as time goes because the intensity of the perpendicular component will increase and parallel component will decrease because of the reorientations of the molecules or transition dipoles when at the excited state. So, as time goes there will be the intensity of the parallel and perpendicular components will be equal and in that case you will see that r will going to be 0, ultimately at a certain after a certain time.

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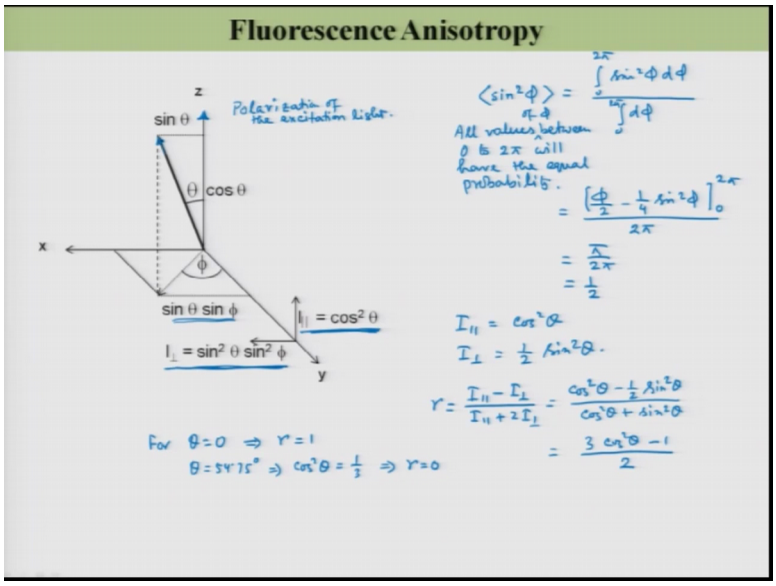

Now, let us see this things right in more detailed way. So, let us now consider that you have excited the molecules along z axis. So, z axis is your polarization for the excitation light. So, let me write here. So, polarization of the excitation light is along z, let us I consider it over here. So, if I consider that only those molecules that is I said the only the molecule whose transition moments are along the z axis they will be preferentially excited, that is what I said earlier again I am telling the same thing. So, those molecule will be excited and if the direction of the absorption transition moment and emission transition moments are same, like they are co linear then the emission will be also along z axis. That is my condition now I am imposing here the direction of the absorption transition moment and emission transition moment, they are co linear. Then the emission will be also along the z axis take a note on that.

Now, let me tell you that now another molecule has been rotated an angle theta right. And now I am looking at this molecule. So, now, this bold arrow is my molecule which has been rotated at an angle theta from the direction of the excitation, and now I am looking at this molecule. So, here you see the rotation of the molecule makes a component of this i parallel s cosine theta as you know and, but the molecule could can rotate like if this is my x z axis, the molecule can rotate from here to this side as well as from here to this side in all the cases the angle is theta this side angle is theta this side angle is theta. That means, I need to worry about all these different angles for a fixed value of theta; that means, if I assign this angle as pi then the value of pi should be 0 t 2

pi that is what I have written over here. So, now, here you see for this perpendicular component, this should be sin theta sin phi correct. So, the intensity those are the electric field. So, those are the intensity i parallel will be equal to cos square theta. And i perpendicular will be sin square theta sin square phi, but here also please note that we should take all values of sin square phi; that means, I should take the sin square phi average right. So, I should take the sin square phi average. So, let me find out that.

So, sin square phi average is equal to integration 0 to 2 pi sin square phi d phi divided by integration 0 to 2 pi d phi. So, and I already got this i parallel equal to like this i perpendicular is like this. So, if I can calculate this, if I can calculate this I can simply replace this sin square phi with this I can do as I said because that all values between all values of phi between 0 to 2 pi will have the equal probability. So, now, I can evaluate this integration 0 to 2 pi sin square phi d phi right that part.

So, if you integrate this then you will get phi by 2 minus 1 over 4 sin square phi limit I will put 0 to 2 pi, divided by this will going to this is simple this is just 2 pi. So, then this will going to be pi by 2 pi this is equal to half. So, as I got this sin square phi average is this half. So, I will simply write i parallel is equal to cosine square theta and i perpendicular equal to half sin square theta. You see that this is now half sin square theta.

now r is equal to i parallel minus i perpendicular divided i parallel plus twice i perpendicular, which will going to give me cos square theta minus half sin square theta divided by cosine square theta, plus 2 half and 2 this will cancels sin square theta. So, this is this is equal to 3. And then I will just simplify this, and what you will get is cosine square theta minus 1 divided by 2. Now as you can see here that for the value of theta if the theta equal to 0 right the theta value could be anything right. So, let us let me put for theta equal to 0; that means, it has not oriented because you have excited along the z axis and just immediately after excitation you are looking at the r value right. So, theta equal to 0. So, for theta equal to 0 this is nothing, but 3 minus 1 divided by 2, sorry this is equal to 1.

For theta equal to 0 the value of r equal to 1. So, here I go the initial anisotropy is one right and now as the molecule will rotate the theta will change theta will increase and it will increase and increase and ultimately at some value of theta then isotropy will be 0. So, when the cos square theta equal to 1 by 3 as you can see from this equation, that

when cos square let me write this thing this is important. So, when for theta equal to 0 r I got equal to 1 and when theta equal to 54.75 degree it means cos square theta equal to 1 third what you get you get r equal to 0 right; that means, the molecule is not necessarily has to orient itself 90 degree to make the anisotropy equal to 0. Only 54.7 degree orientations will make the anisotropy equal to 0.

Now, whatever I said here right is the ideal situation; that means, that your excitation light is polarised along z axis. Only the molecules which whose transition moment absorption transition moment is oriented along z axis only those will going to be excited like that. Now why and now this molecules will reorient them self and the theta value will change. I consider that the direction of the transition absorption transition and emission transitions are co linear. So, the emission will also come along that line right as I have excited and then I got this equation.

However it is not true that once you use a vertically polarised light or polarised light electric filed is oscillating along the z axis, that will only excite the molecules which are oriented along the z direction; obviously, there will be some component of even the electric field is like this and the molecules like this the component which is along this direction the electric the component of the electric field, which is the along this direction that will also be used to excite the molecule little bit. It is true that the those molecules which are oriented just perpendicular to the direction of the electric field of the excitation light those will not excited at all that is true, but there is a possibility that the molecules which are oriented at certain angle from the co linear geometry of compared to the excitation polarization, there will be some possibility of the excitation. And that is known as the excitation photo selection of the fluorophore, let me discuss these things over here.

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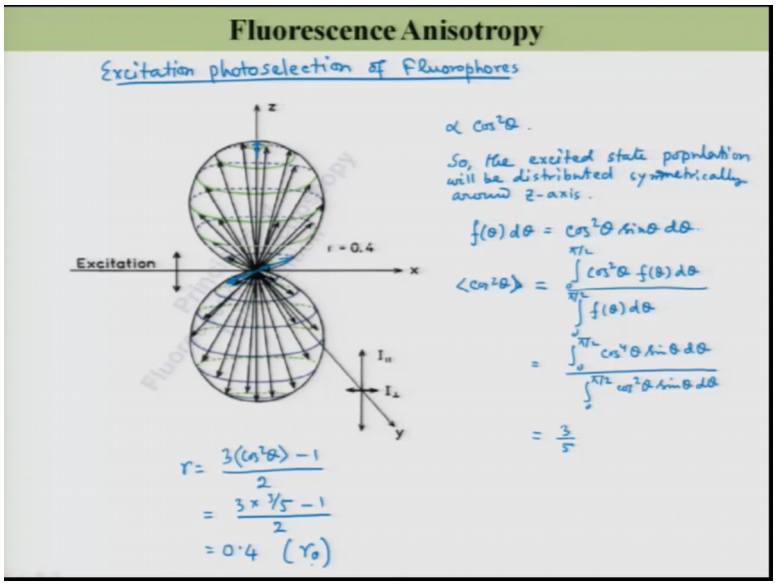

So, see this is the excitation photo selection of fluorophore. This is what I said. The molecules which are oriented like this way they will be excited that is sure, but the molecules which are oriented like this way will also some chance for the excitation right, that is what I said, and that that is proportional to the cos square theta, if the molecule is oriented at an angle theta from the polarisation of the excitation light right. So, that will be proportional to cosine square theta. So, let me write this over here. So, as you use let me write it like this way. So, the excited state population will be distributed symmetrically around z axis in this particular case.

So, that distribution right, I can write this as f theta d theta that is possible to show that is equal to cosine square theta sin theta d theta right. So, and this anisotropy is given by, I have already seen 3 cosine square theta minus 1 by 2 right. That is what I already got. So, in this a case what I will do let us continue, in this case that cosine square theta right not here in this case that cosine square theta in my earlier equation is that the value of theta is not a single value. Because now the theta value is different and that is my distribution over here what I said this is distributed. So, in a molecule, soan each molecules is make a different angle theta and for a different theta value all the 5 values are possible right.

So, this is a like a complicated situation and in this case I should not write the equation as cos square theta, but I should write the average cos square theta. So, the then I have to

take I have to take the average cos square theta right. So, for this average cos square theta what I will write, sorry average cos square theta equal to cos square theta f theta because this is my distribution f theta, d theta is my distribution and that integration from 0 to pi by 2, divided by 0 to pi by 2, f theta d theta. And if you evaluate this is integration 0 to pi by 2 cosine to the power 4 theta sin theta d theta divided by 0 to pi by 2 cos square theta sin theta d theta.

So, if you evaluate you will see that these value is 3 by 5 right. So, all this theta what is the distribution of theta is because of the excitation photo selection and this distribution is as this distribution is because of the excitation photo selection this is my initial value of the cos square theta average.

So, now if put this cos square theta average in my original equation, let us see that r equal to 3 cos square theta, but now this is average because I have so many things minus 1 divided by 2. So, then this is equal to for the initial value 3 into 5 minus 1 by 2 this is nothing, but 0.4. So, r 0 what I will get is a 0.4 and this is true for when the absorption and emission transition moments are co linear with each other.

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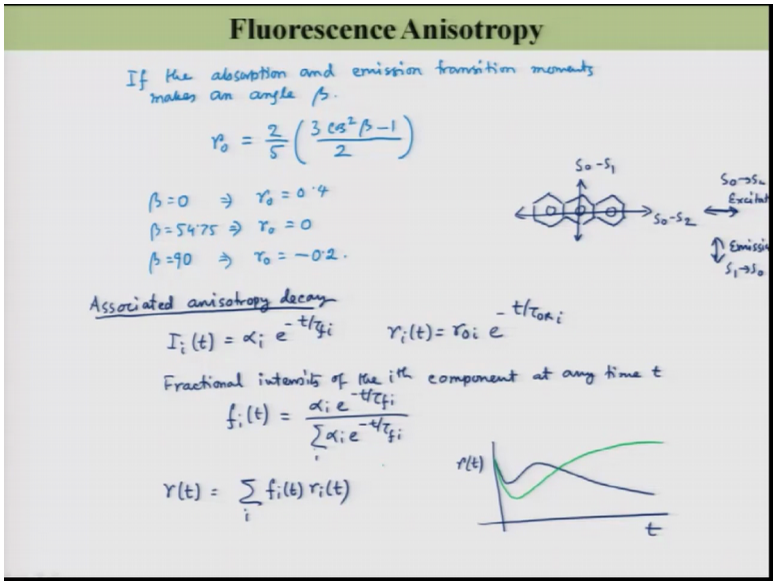

If they are not then this value will not like that. If not if the absorption and emission transition moments will make an angle beta in between them right.

Then this r 0 equation right now I am writing r 0 I am not writing r right now. So, r 0 will can be written as 2 by 5 to 3 cosine square beta divided by say minus 1 by 2 right. So, when this beta equal to 0; that means, they are co linear right immediately what you will going to see is that r 0 equal to 0.5, if beta equal to 54.75 then what will immediately get that r 0 equal to 0. If beta equal to 90 degree; that means, if this absorption and emissions are perpendicular to each other right, then for example, for example, let me give you example that will then it will be much clear to you.

Let us say I have this molecule our famous example many times I use this molecule enthroning right. And I told you this absorption for the s 0, s 1 is along this direction and s 0 s 2 is along this direction I consider that emission is also the same, but if you now excite from s 0 s 2 and, but you will get the emission from the s 0 s 1 from s 1 to s 0 because of the. So, you are exciting along this direction. So, here this direction is your excitation because you are exciting from s 0 to s 2, but emission is along this direction is different right. So, they are perpendicular to each other.

Now, if you excite emission is from s 1 to s 0, now if you excite from s 0 to s 1 and then s 1 to s 0, then they are co linear to each other right then they are collinear. So, in that case that is the value beta value is equal to 0, and in this case the beta value is equal to 90 degree. So, for beta value equal to 90 degree what you will get is this r 0 value equal to minus 0.2.

So, what you can see is that the value of r 0 or initial anisotropy or fundamental anisotropy right, it depends it depends on many factors right. Many factors means basically this is what is the angle between the absorption transition moment and the emission transition moment right. So, for a different molecules, this angle will be different. So, the value will be different. So, this has to be measured right and we can measure it by time resolve anisotropy decay, as I told you if you have a very high time resolution like a 10 to second time resolution using the fluorescence up conversion system then by putting this polariser in the 10 to second up conversion system either at the parallel or in the perpendicular position compared to the direction of the excitation or the polarisation of the excitation is lesser been. You will be able to find out the value of r at very early time with a very a high time resolution, and that will be going to be your r 0 value right.

So this all about this fluorescence anisotropy and as we have little more time then I think that we should continue our discussion on little more atmospheric of this fluorescence anisotropy which is the associated anisotropy decay, associated anisotropy decay. So, right now what I said is that the decay our anisotropy decay of a fluorophore is present in a particular media right, in a homogeneous media. Now I am thinking of that if I have 2 def 2 fluorophores right, but present together in a solution, but their environment are different their the environment of 2 different types of fluorophores are different, but they will going to give me give the contribution to the anisotropy decay then what will be the effect.

So, let us take this as that I have, i of the ith component is defined as alpha I. So, this my lifetime part right t by tau f i and my r t part r i t is equal to r 0 i into e to the power minus t by tau or i right. So, I can write the fractional intensity of the I; the component if it is only 2 then, first and second component at any time t. Just I write this as f i t equal to alpha I e to the power minus t by tau f i divided by sum over i alpha i e to the power minus t by tau f I; that means, the time dependent anisotropy that r t, r t should be given by sum over I because if it has more weight age the early time then the weight age of the r i t will be there, because it is now more weight age and in the later time.

So, I can simply write r t equal to sum over i f i t r i t. So, you see this is a complicated thing right and as expected the anisotropy will also be complicated, usually anisotropy looks like that is started from some value r 0 and exponentially decay, but in this case if you see this anisotropy decay, it will look something like this, but using such kind of formula you can analyse it without any problem. So, if I now plot R T as a function of t over here, then it could looks like this, like this. Or it could look like this. So, it does not look is anisotropy, but this is because of this 2 different fluorophore 2 or more different fluorophores present whose environments are quite different. One quick example is that the tryptophan right.

So, if you have tryptophan in a in a protein right when tryptophan is present in the protein the r t value right or this tau over value is very large because the protein is very large, but if it is now free right then the tau over value is really small and the lifetime is also quite different. So, for such kind of complicated cases what you will going to see such kind of associated anisotropy decay and you need to really careful you have to be really careful for the analysis of such kind of anisotropy decay. So, we will finish here.

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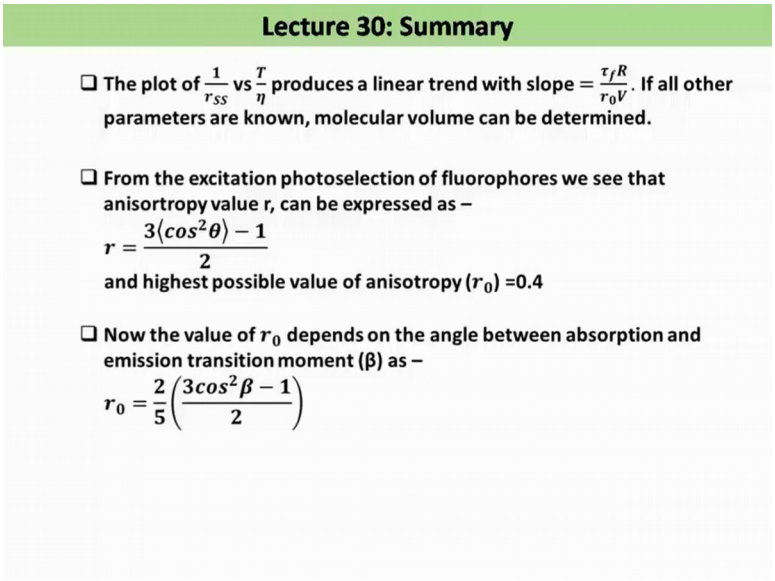

Thank you for your kind attention.