Basics of Fluorescence Spectroscopy
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Lecture – 37

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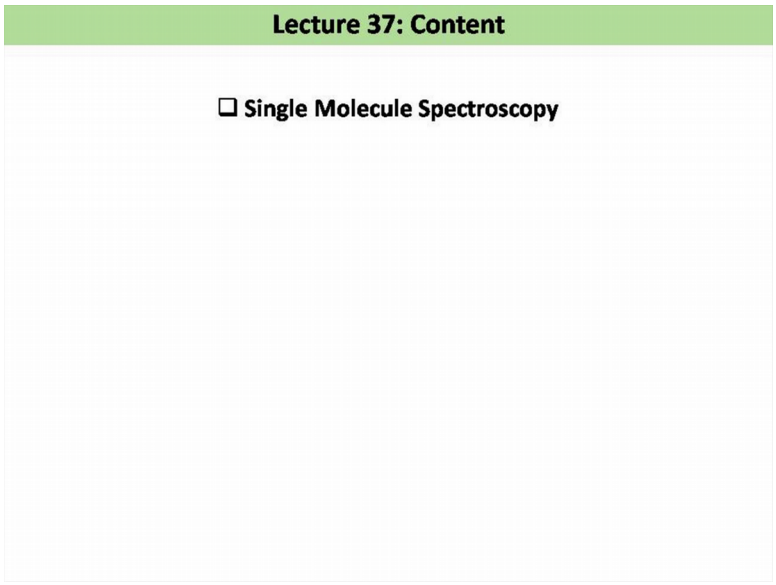

Welcome to lecture number 37. Today we will going to start one of our last discussion, right which is a single molecular fluorescence. Now first question which will come automatically that why we should bother about the single molecule, right? Till now what we are doing is the fluorescence response of a molecule when it is present in solution, but the concentration is in the order of typically is in the order of micro molar

So, we are going to have at least 6 into 10 to the power 17 molecules, right in a liter right? If it is a one micro molar so that means, we are getting a collective response from all that fluorophores present in the solution. Now in some case, right this kind of collective response of the molecule present in the solution, right? Which is known ensemble average fluorescence property of the molecule may be different from the single molecular response of this molecules present in the solution.

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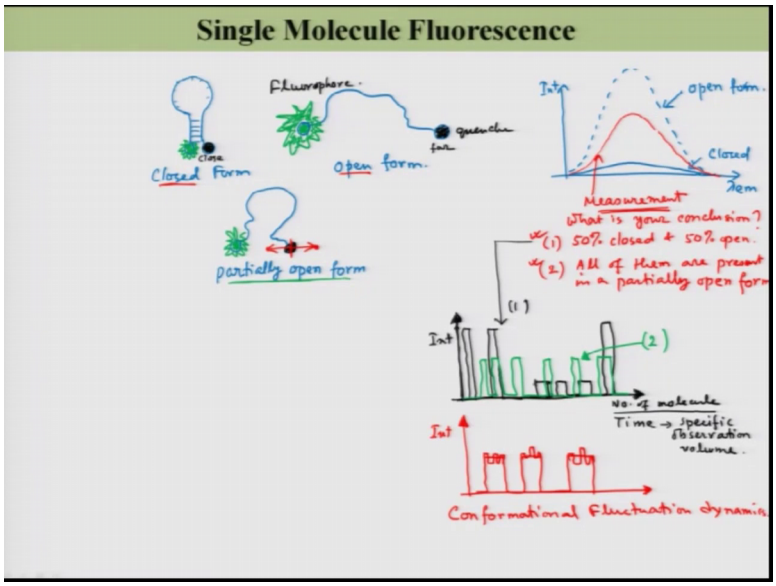

It will be clear let me take one example let us for example, you have such a molecule with you is made of some DNA base pair where these base pairs are pairing these are typically called the molecular beacon. So, here you have a fluorophore and here you have a quencher right. So, this one is your fluorophore, let us say it is showing you green color emission.

Now, once this quencher is present nearby then this quencher can quench the fluorescence of this, right? And the fluorescence intensity is very low. Let me consider that this is one of the structures of this molecular beacon present in the solution. The other structure of this molecular beacon which is present in the solution could be in the open form. So, this is my closed form and the other one is by open form. So, here is your fluorophore and here is your quencher.

Now, as the quencher molecule is far apart from this fluorophore; obviously, the fluorescence intensity in this case will be much more compare to the other case compare to the closed form right. So, this is your fluorophore and this is your quencher over here and this is your fluorophore and this I referred to as the open form.

Consider that I know the emission spectra of this closed form and this open form. Understandably the fluorescence intensity of this closed form will be much smaller and the fluorescence intensity of this open form is much stronger, right?

So, if I now draw the fluorescence intensity of this closed form and open form what you will going to see is, let us say this is the fluorescence spectrum of the closed form lambda emission verses intensity, this is the closed and let us say this is the fluorescence intensity of the open form.

Now, consider you have taken this molecule in a different solvent and what you are getting experimentally is something like this. Now my question is this is your measurement. So, my question is what is your conclusion? Number one, you can conclude it like this way that 50 percent of these particular molecule is present in the closed form and 50 percent of this molecule is present in the open form. And as a result 50 percent will show the low intensity, 50 percent will show the high intensity and all together it is showing the moderately intense fluorescence.

So, the option one is your 50 percent closed and 50 percent open, but there could be another situation because you are doing this experiment in a different solvent right. So, in that particular solvent it is also possible that all of them are having a particular form which is neither open form nor the close form it is intermediate. So, that the quenching is, right? The quenching of the fluorescence by this quencher is not that much permissible and you are getting a moderately intense fluorescence intensity.

For example what I want to say is that let us say this is the situation right, another type of form. So, I will name it as partial partially open form, is not fully open partially open form. So, in this case what you will going to get is moderate intense fluorescence, right? Because the quencher is not very far, not very close here is very close here is far, but here is not very far or very close.

So, probably these fluorescence intensity we will going to give you this moderately intense fluorescence intensity. So, the second option could be that all these molecules under investigation, right? You have certain concentration, but all of them are present in a partially open form right. So, all of them are present in a partially open form.

Now, if I ask you which one is correct of this one a one or 2? Which one is correct? If you are just simply doing the ensemble average fluorescence spectroscopy with this fluorescence spectroscopy it is impossible for you to tell which one is the correct explanation correct.

You may say that let me measure the anisotropy, even you measure the anisotropy as you know in this case as it is in the compact structure for the closed form then the steady state anisotropy will be smaller and in this open form the steady state anisotropy will be larger, but as mixer because in the anisotropy we were looking all the molecules together right. So, we cannot distinguish whether it is partially open or fully open is a mixer fully open and fully close or it is just a all of them are partially open. So, we cannot do.

The only solution is that we will going to we have to look these molecule individually. Suppose you are doing this experiment individually how I will come later, but consider that you have the technique So that you can measure the fluorescence intensity of such molecule one by one. Now you are measuring for the molecule number 1, right? Next you are measuring for molecule number 2 next you are measuring for molecule number 3 and so on and so on and so on.

In that case, what you will going to see? You will going to see 2 different depending on the 2 different case, right? If it is the case number one, right; then the fluorescence intensity versus the number of molecule. So, here is intensity here which molecule you are measuring; so number, number. So, you are measuring the molecule now number one molecule then second molecule then third molecule then forth molecule and fifth so on and so forth right.

So, number of molecule or I can change this number of molecule to something else, let us say you are measuring from this is your sample cell, right? And you are measuring from here and you let the molecule to come one by one here and let it go out of this. So, molecule is coming once it is entering in this region you can see fluorescence, because you are measuring from here and when the molecule will leave from here you will not be able to see the fluorescence.

So, say number one molecule is coming entering and it is going to give you the fluorescence and when it will leave you will not going to see any fluorescence. Then probably the second one will come it will enter here and then you will see the fluorescence and when it will leave from here then you will not going to see any fluorescence. So, this number of molecule I can for such kind of arrangement I can replace with the time right; so for the continuous monitoring process. So, here I can also refer this as a time for a specific observation volume.

In this case what we will going to see if it is the case one, right? You will see the fluorescence intensity probably well is 0 because none of the molecules were present inside this observation volume. Then fluorescence intensity will increase and then fluorescence intensity again decrease. Then again probably other molecular will enter fluorescence intensity increase. But when the third one will enter, right? When the third one will enter probably the fluorescence intensity is less, because it is now the close form which is entering. So, I should draw it properly.

So, the first one is entering fluorescence intensity is high then when it will leave fluorescence intensity will be less. When second one is entering let us say it is also the open form sorry, it is also the open form yes then fluorescence intensity is very high and when it will leave fluorescence intensity is less. When the third one will enter, right? Then fluorescence intensity is too low, because it is in the close form the forth one is like this, sixth fifth one is like, this sixth one probably like this and so on. So, you will get such kind of fluorescence intensity versus time plot for this observation volume. So, this is for the case one.

However in case 2 what you will going to see is, whenever the molecule will enter this observation volume the fluorescence intensity which you will get from this observation volume will be fixed, right? It will not going to defense molecule to molecule which molecule is entering because all of them are partially open, right? It is not like that some are fully open and some are fully closed. So, in this case what you will? It is not necessary that at the same time you will going to see the fluorescence burst, coming out of the observation volume it is not necessary right.

So, but whenever you will going to see it will be of moderate intensity like this way whenever it will come, clear? Like this. So, you see the basic difference. So, from your experiment if you look at that how this fluorescence fluctuation is happening you will be able to tell clearly that, what my proposition is number one is 50 percent close or 50 percent open is correct or whether my proposition that all of them are present in a partially open form is correct. So, for that what I need to see I need to see the fluorescence characteristic of a single molecule of this particular bio molecule right.

Moreover let us say I can also say that somehow I came to know that this is present in the partially open form, partially open form. Now I can think of that this is the structure

of the partially open form and these structures remain fixed, throughout the time. What I mean is that there is no fluctuation in the structure, but recent studies like this can be represented as a protein, right? Recent studies suggesting that the protein structure which we use to see in the text books, right? The PDB structure the crystallographic structure of the protein they are not, they are not rigid, right? In solution in solution they undergo a slow breathing type of motion, the time skill is very slow it is in the order of few micro second to tens or twenties or micro second right, but they are undergoing a slow breathing motion, right? And those breathing motions can be captured by using such kind of single molecular technique.

What I want to say is that the conformational fluctuation dynamics, right? May also change the fluorescence property of such kind of molecule, right? For example, if because of this conformational fluctuation these part this quencher will come little close or far from this original position. When this quencher will come at this position? Quenching will be more, when this one will go here then quenching will be less; that means, there will be some fluorescence intensity fluctuation, right? Within these molecule which is not much change in the structure.

Both are partially open form, but there is small change in the structure, right? Within this partially open form it is not that it is converting between the close form and open form not that big fluctuation, right? Small fluctuation, but that small fluctuation is good enough to change the fluorescence characteristics of this molecule right. So, such kind of slow conformational fluctuation dynamics can also be observed and in such case if you now do such kind of plot, what you will going to see? You will going to see such kind of diagram.

In all the cases it is similar intensity, but within these similar intensity, there is some sort of fluctuation there is some sort of fluctuation sometime it is high sometime it is low, right? Sometime like this. So, that can be used further to calculate the conformational fluctuation dynamics. So, just for your reference I will write here, this conformational fluctuation dynamics.

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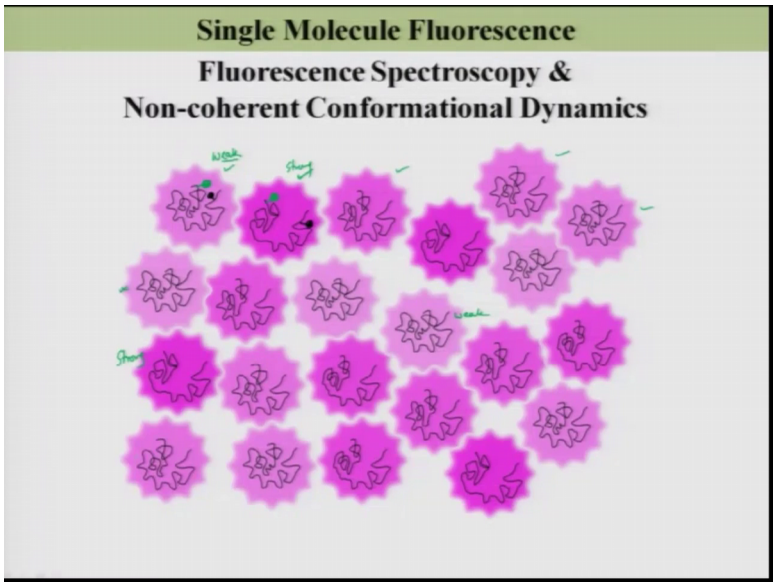

I have to make it more clear to you I have this PPT slide for you, here you see let us say this is my protein molecule, right? And these protein molecules the fluorophore is present in this location and the quencher is present at this location. So, when they are far apart I can see a strong fluorescence strong pink fluorescence, right? Just I have described like that and when they are closed by. So, in this case you see they are closed by that is what I have made this cartoon. So, when they are close by the fluorescence intensity is less.

So, in this case in the solution probably such molecule exist, such molecule also exist, such molecule also exist, such molecule exist and many, many such kind of such molecules will exist together, right? When one molecule is unfolding when one molecule is going from the close to open and many, many other form in between, right? Closed one and this is opened one, but many, many other form between that is my conformational fluctuation molecules are doing like this way right.

In case all the molecules are opening and closing opening and closing simultaneously in a coherent fashion; that means, one molecule when this molecule is doing like this molecule is also doing like this then when this molecule is like this, this molecule simultaneously like this right.

So, in that case in the coherent fashion if everything is going to do like this, then ensemble average picture is good enough to see the fluorescence fluctuation of this

molecule. Because all the molecules are changing it is conformation, right? Simultaneously right, in a coherent fashion right, but. So, then I will see the fluorescence intensity will increase then fluorescence intensity will decrease increase decrease and so on and so forth, but if it is not here in this particular scenario as I am showing here at a particular instance let us say this is a particular instance in time, these molecule you see look here, these molecule showing strong fluorescence this is weak fluorescence, this is strong, this is very strong, this is weak like here are different.

So, still as I can see by my eye in this particular PPT slide that this is strong. So, this must be open this is strong this must be open this is close. So, this is weak. So, this must be close and so on and so forth, but it is possible for me because I have only 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 220 only 22 such molecules are there.

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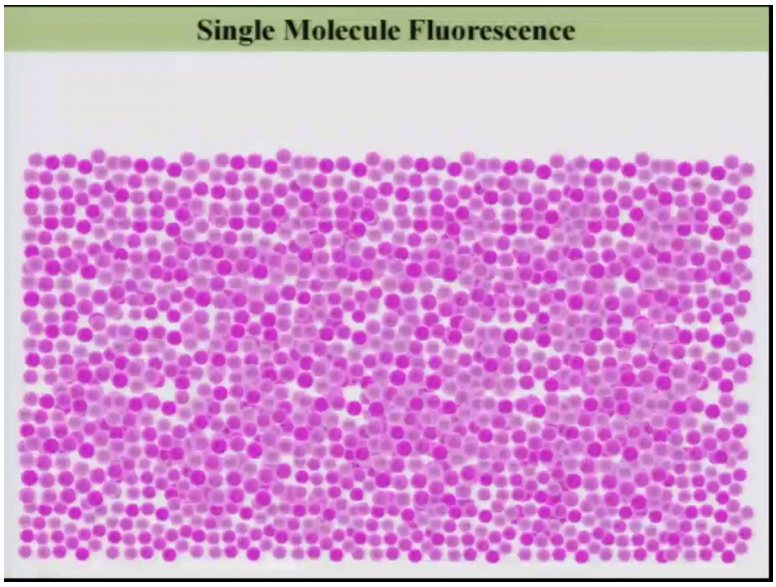

Consider you have Avogadro number let us say micro molar; so 10 to the power of 17 molecules per liter right. So, in such case let us see this animation. So, you have several, several such kind of molecules right. So, is a huge number 10 to the power 17 molecules are present in the solution.

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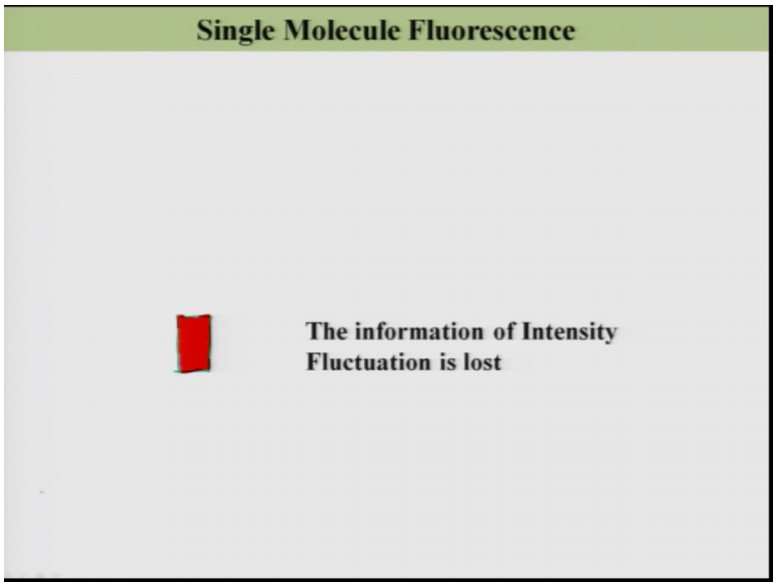

So, in this case what you will going to see from far apart? I cannot distinguish this different color of different bio molecules present in my sample; that means, the information of intensity fluctuation is now lost, right? I cannot see. So, for me that all these color are equal. From here to here it looks like a single color, right? I cannot distinguish this.

So, in this case what is the solution? My solution is that I cannot see these molecules in a ensemble average picture, right? Not together, if I am going to see all these bio molecules together then I will lose such kind of important information that is a fluctuation of the fluorescence intensity, right? With time what I have to do? I have to see such kind of bio molecule in the individually, individually I have to see this bio molecules. And that is my goal, how to achieve? That is what we will going to discuss here ok.

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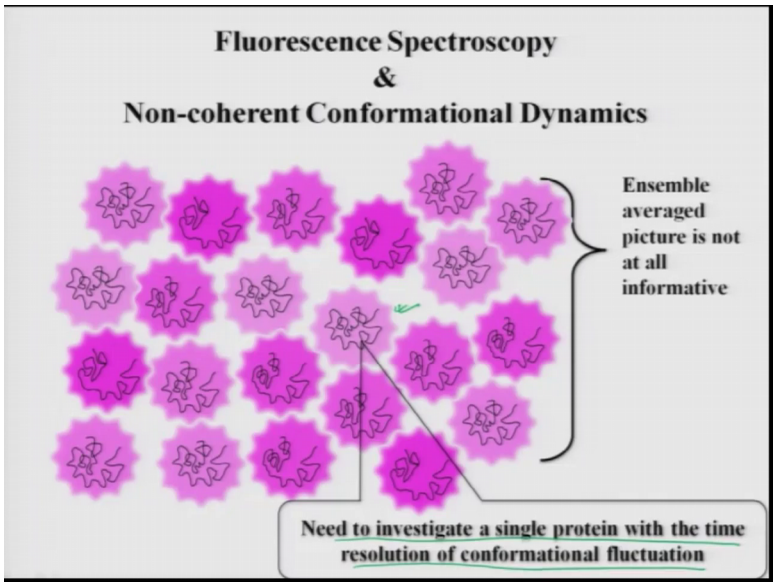

So, my aim is that see individual bio molecules, right? As I have shown here. So, we need to investigate a single protein in bio molecule with time resolution of the conformational fluctuation, right? If I am going to see only this one then I will be able to tell that with time how these bio molecules actually fluctuating if it is fluctuating; that means, it is fluorescence intensity must fluctuate, because I have a fluorophore over here and I have a quencher present over here. So, if it fluctuate it is process intensity must fluctuate.

So, if I just sitting over on these particular bio molecule and see how the fluorescence intensity is changing with time, then I should be able to comment on the conformational fluctuation dynamics of this bio molecule.

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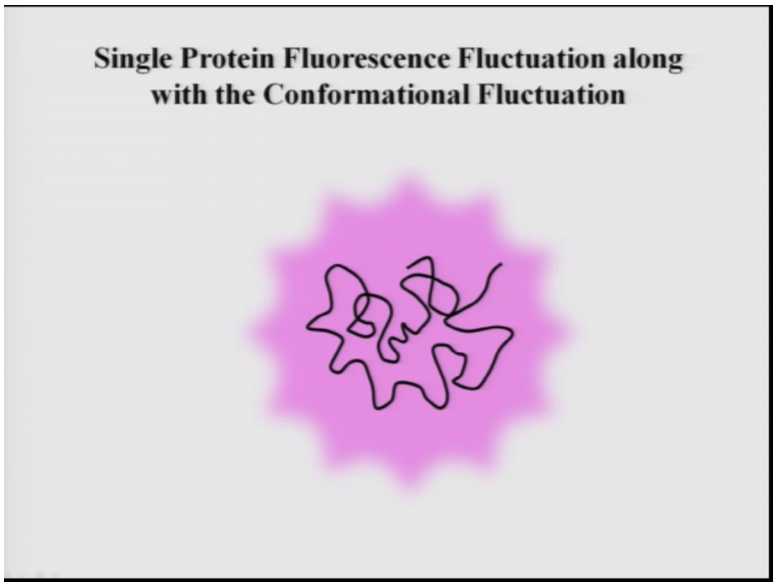

So, for to show this I have this animation with me, as you can see here that when this is in the close form fluorescence intensity is less when it is in the open form fluorescence intensity is more. You can see this as you are seeing only one molecule, as I showed earlier for several molecules such kind of fluctuation information is lost, because many, many molecules are present and because this is non-coherent in nature.

So, I showed you the importance of this fluorescence single molecule fluorescence. The first one is that you will be able to say that what kind of structure it is and the second one you can also able to determine that with time right, how the structure is changing.

Next I would like to show you another advantage of the single molecule spectroscopy by showing this type of reaction.

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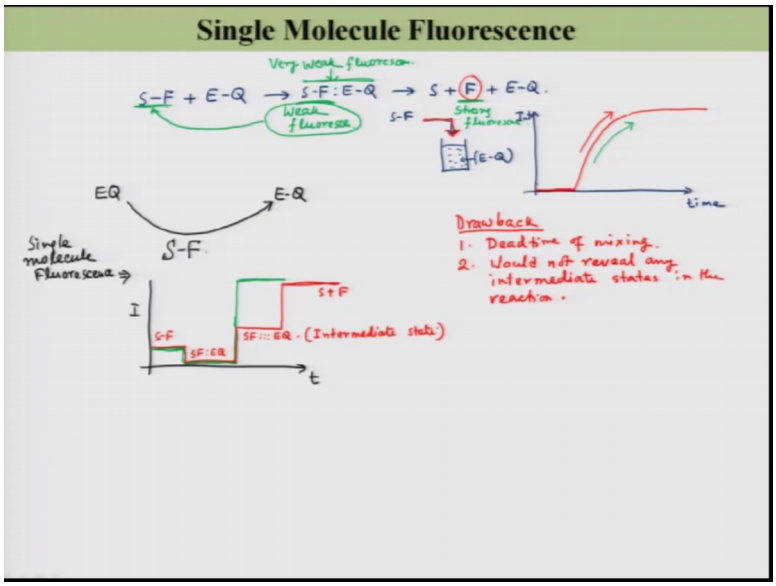

So, here let me have a reaction a substrate level with fluorophore. So, f is my fluorophore and it is getting bonded with this enzyme to form some enzyme substrate complex which is denoted like this E-Q and ultimately this is going to give you the product S F and this enzyme back right.

So, if you want to study the kinetics of this reaction, what you should do? You should, I am talking about our normal fluorometric assay of this enzymatic reaction. So, in that case what you should do? You should take your S F or S E-Q in the Q head, right? Let me take E-Q in the Q head and then if you add this S F initially, right? If you follow as a function of time the fluorescence intensity. Initially the fluorescence intensity must be 0, right? Let me change the color initially the fluorescence intensity must be 0 because E-Q, does not contain any fluorophore. So, fluorescence intensity should be 0 over here.

Once you add this S F in the mixer, right? Then the E-Q will react with S F to form this free fluorophore right. So, with time the fluorescence intensity of this will increase. And when the reaction will complete all the S F will be converted to S plus F and then fluorescence intensity will be maximum.

So, from here you will be able to say; what is the constant of this reaction. And you can now change the temperature; you can change the environment and see how the enzymatic reaction is actually depending on those cases.

But now you consider that when you are mixing S F with E-Q; obviously, you need some time, right? You need some time. So, whether you will be able to monitor this enzymatic reaction, right? That will be guided by the date time of mixing, right? This is the drawback of our, this setup right.

So, one of the drawback is dead time of mixing. And it will also not going to tell me right, whether there is a formation of any intermediate state for such kind of experiment would not reveal any intermediate states in the reaction right. So, what is the solution, right?

Solution is let us say if I have, solution is single molecule level spectroscopy single molecular fluorescence consider this situation that, you have S F sitting over here. And somehow you are just monitoring this S F right. So, E-Q will come and interact and will go right. So now if I plot, so this is my single molecule, right? You are monitoring single molecule fluorescence in this case. So, in this case if you just see it and look at these S F molecule as a function of time what you will going to see, right? S F this f is present, but it is attach to A S.

So, let me define this intensity as it is fluorescent, but weak fluorescence. And this F is strong fluorescence when it is free from S right. So, in this case what you will going to see is that there is some fluorescence intensity right. So, the fluorescence intensity is like this way let us say, but when this E-Q sorry, sorry, sorry, sorry, sorry, sorry. I should write this weak fluorescence not here, but here. But when it is this form; obviously, the Q is nearby so that means very weak fluorescence. So, this one is very weak fluorescence, this one

So, the fluorescence intensity of S F you are looking here, right? Is like this, but when this E-Q binds to S F the fluorescence intensity will decrease. So, you will going to see a decrease in the fluorescence intensity. And that fluorescence intensity in the lower low fluorescence intensity region will stay for some time. And then when the reaction will takes place when the reaction will finish, right? Then there will be a formation of S and F the free F. So, the free f fluorescence intensity will be there if you are still looking this F, right? If this f will go away then obviously will not be able to see, but consider that you are still looking this thing then the fluorescence intensity will increase like this, fluorescence intensity will increase right.

That means I have both my S F and E-Q in the solution, I am just only looking at the S F, all of them are there. So, if S F is alone, right? Then I see a moderately fluorescence intensity when a Q come and interacted with S F then fluorescence will decrease and that reaction will take it is own time and then when the reaction is over it is forming S plus F at the fluorescence intensity again will increase.

So, if I do this thing several, several times then I will get a clear idea about what is the rate of this enzymatic reaction. Here the plus point is that there is no dead time of mixing, right? Because all the substances are present in the solution beforehand only what you are doing you are monitoring the S F, right? E-Q is already present

So unlike this, if let us say the reaction will takes place in one second and your dead time you are mixing very slowly like it took 10 second then by the time you mix shake reaction is over, right? You would not be able to see such kind of rise. But in this case you will be able to see that such kind of reaction, because all the molecules are present reaction mixer.

Important information what you will going to see is that whether there is formation of any intermediate state. Let us say the fluorescence intensity of this reaction is not like what I have shown in this green, but it is like this, this one and then decreased and then is like this and then it increased and remain like these and then again increase and it become like this.

So, clearly I can tell that here is my S F here is my S plus F this for sure in between that S F must have interacted with E-Q that is why fluorescence intensity is less. And in this intermediate one the structure must be S F little bit of different type of interaction with E Q; that means, you will be able to see this intermediate state very easily if you monitor such kind of single molecular level fluorescence. Let me finish here and we will continue our discussion in the next lecture.

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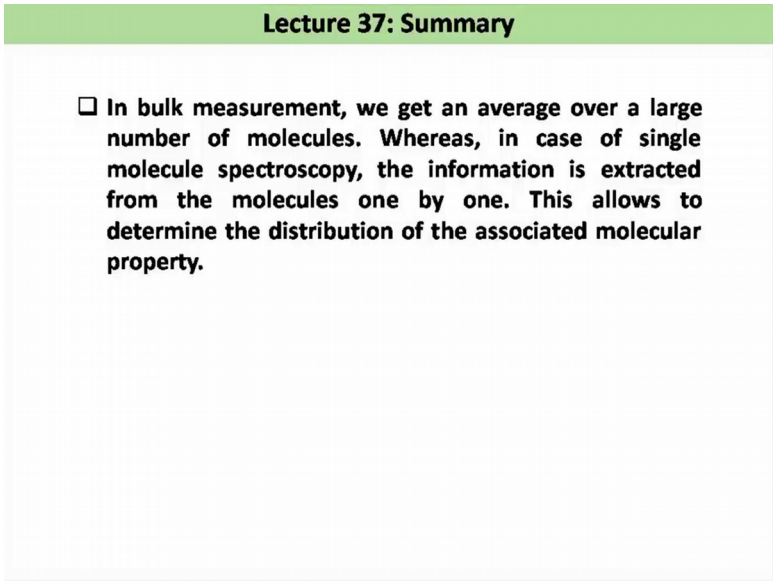

Thank you very much.