Basics of Fluorescence Spectroscopy
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Lecture – 39

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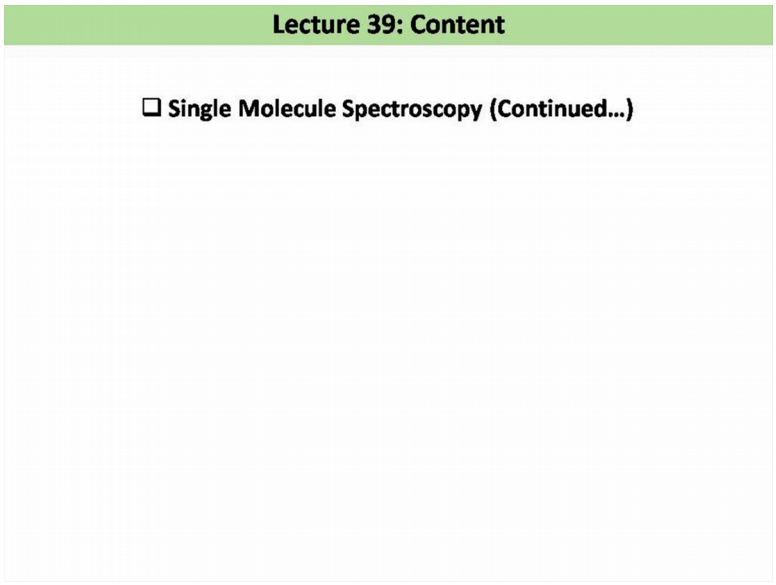

Welcome to lecture number 39. So, till the last lecture what we have done is the importance of the single molecule spectroscopy, right. And we have shown that what kind of extra information single molecule spectroscopy can give. And we also have seen that the small observation volume is essential to get the information of a single molecule. So now, question is that how to achieve such kind of small observation volume

As you remember I said that if you take 1 Rhodamine 6G molecule in 1 m l of ethylene glycol one of the solvent, right. Then the if the intensity of the fluorescence is 1 then the intensity of the Raman scattering by ethylene glycol molecules that 1 m l of ethylene glycol molecule would be 10 to the power 10. So obviously, you will not be able to see this one intensity of fluorescence in front of 10 to the power 10 intensity of the Raman scattering. But if you take 1 Rhodamine 6G molecule in 100 femtoliter of ethylene glycol then this intensity of the fluorescence and intensity of the Raman scattering will be very similar to each other.

And if you now take 1 Rhodamine 6G molecule in 1 femtoliter of the solvent here, it is here it is ethylene glycol, then in that case the fluorescence intensity will be 100 times more than the background Raman scattering and in and in this case you will be able to see the fluorescence from the Rhodamine 6G, because for this is single molecule, so the number of photons which are available from the single Rhodamine 6G molecule is very, very small. So, your background has to be small more. Importantly your solution, right has to be diluted enough so that only the small observation volume is not enough. Your solution has to be diluted enough so that this 1 femtoliter observation volume contains only one molecule of Rhodamine 6G.

If you put many, many of them if you increase the concentration then probably in that 1 femtoliter observation volume will contain more than 1 Rhodamine 6G molecule, then you will lose the single molecular level detection capability.

Now, the question is that how we will going to get this small observation volume which is 1 femtoliter right. So, if you considered a box like this way.

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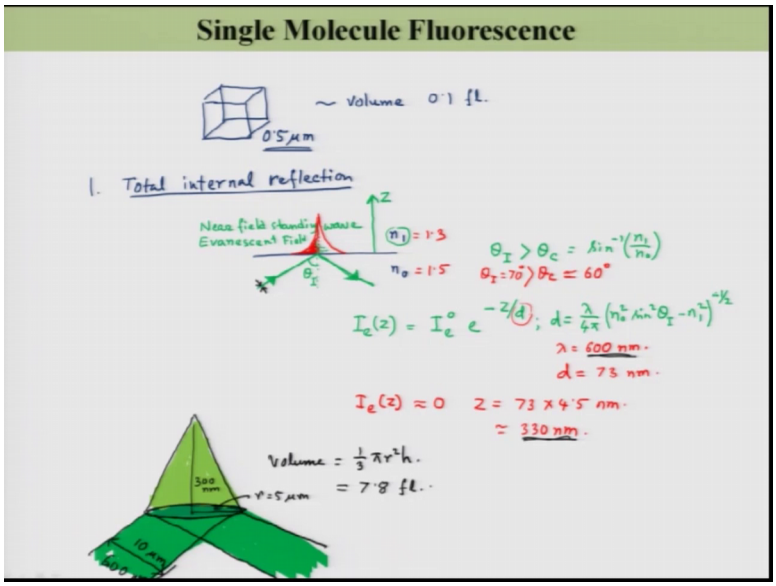

Let us say it is a cube, and if each side is about 0.5 micrometre. Then approximately the volume what you will going to see is about 0.1 femtoliter. So, you need similar type of dimension to be achieved. And so now, today we will going to discuss how to achieve such kind of small observation volume.

The first technique we shall discuss is based on total internal reflection method, total internal reflection method. And this we know that if I have 2 medium one with refractive index n 1 other with refractive index n 0. And then if I have incident light falling on this through medium with refractive medium n 0, towards the medium with refractive index n 1. And if this incident angle theta i is greater than the critical angle. So, if theta i is greater than theta c which is defined as sin inverse n 1 by n 0, then there will be total internal reflection right. So, the beam will be reflected in this way. So, this is my total internal reflection.

All the incident light will be reflected by the total internal reflection, if it satisfy the total internal reflection condition, but still what you will going to see is that one evanescent field around this region will be generated. Evanescent field around this region will be generated. So, this is nothing but near field standing wave, right. Just write here near field standing wave will be generated which is known as evanescent field, will be generated through the medium with refractive index n 1 right. So, you will going to get field out of this.

Now, the strength of this evanescent field, right actually decay you go away from this interface. Suppose this is your interface and this is your total internal reflection and this evanescent field will be generated here, but the strength of this field will exponentially decay as you move from the interface away of away from the interface right. So, this evanescent field strength right, decay exponentially. And that can be written like this way. So, e to the power minus z by d; obviously, it will start with some initial value of this field.

Let me write this is I e 0 and which is the electric field dependence as you move from away from the interface. So, here is my z, right. And in this case my variable is obviously this z. And d is given by, where d is given by lambda by 4 pi n 0 square sin square theta. So, this is my incident angle. So, theta i I should write minus n 1 square to the minus half right.

So, for example right, if let us say, let me consider this n 0 is equal to 1.5 some type of glass. Let us say this is 1.3 it is like water. So, I can readily calculate the theta c value from here. So, here theta c I can calculate, theta c will be around 60 degree right. So, if theta is greater than 60 degree right. So, let me consider that theta i is equal to 70 degree

I can choose that which is greater than theta c, then it will it will fulfil the total internal reflection condition.

In that case right, I can simply evaluate what is the value of d, right. Because I know in zeros I know theta i I know, I know n 1, but you see it also depends on the wavelength. So, I will fix the value of wavelength let us say for this case the value of the wavelength of the incident light is 600 nanometre. So, if you plug in all these different value, what you will going to see is the value of d. And if you calculate this you will get this value of d as 73 nanometre right.

So, you see this is d e 73 nanometre. And this d is over here, right. d is over here; that means, the intensity of the strength of this evanescent field will decrease exponentially, right. And it will be almost 0 at 4.5 times of this d value, right. This is the nature of this exponential function. So, what I want to say is that this evanescent field strength is high here, but these evanescent field will going to be 0 exponentially, see this is my exponential function, right. And do not forget that this is a symmetric, right. This is symmetric like this way right.

So, evanescent field strength is very high and then it is going to be 0, right, as the z will increase. So, I can simply tell that e I e z, right. Will be almost zero; that means, field strength is finish at a distance z equal to 73 into 4.5 nanometre which is roughly equal to 330 nanometre. So, I can simply right; that means, ultimately what you are going to see you are going to get a, I can describe this as roughly I can describe this as type of cone, right. Where this spot this is a spot this is a circle, right. This is the spot size of the incoming beam right.

Here that spot size whatever I have shown is little tiny, now I am expanding it. So now, the laser that light excitation light it is actually laser. So, this excitation light is coming like this way. This is important, let me show you. So, then here is your sorry, this is incident, right. And then, reflected, like this way reflected back under total internal reflection. So, what you are going to see is these is your spot size and because of this you are going to get this evanescent field. Evanescent field will decay exponentially, like this way this is your evanescent field; that means, above these interface thus place where right, this field exist this field does not exist below the interface. Above the interface the

field exist in this region. So, I have just assume that this feature right, this exponential decay will may will be like a cone.

Now, if the diameter of this excitation light is known, right. Then you know this diameter, right. And you know how to calculate the volume of this cone. So, volume is equal to 1 over 3 pi r square h. So, if I say the diameter of this incoming light right, is let us say real number. So, let us say it is 10 micrometre, right. Can be 10 micrometre I can create. So, it is a 10 micrometre and then this 10 micrometre light will come here. And it will undergo to total internal reflection like this, but this evanescent field will create.

So, then the radius it will be 5 micrometre and this height will be, I just have roughly calculated it around 300 nanometre. So, it will be around 300 nanometre, right. Or 0.3 micro mitre right. So, using this formula you will be, so here is my just situation, that 600 nanometre light, 600 nanometre light is making such kind of thing for the 600 nanometre I have calculated, what is the value of this height? Is above 300 nanometre.

And with that I will I want to calculate the volume. If you plug in all these values over here then what you will going to see that volume is 7.8 femtoliter. So, you see just by taking this total internal reflection condition you can achieve the observation volume of 7.8 femtoliter.

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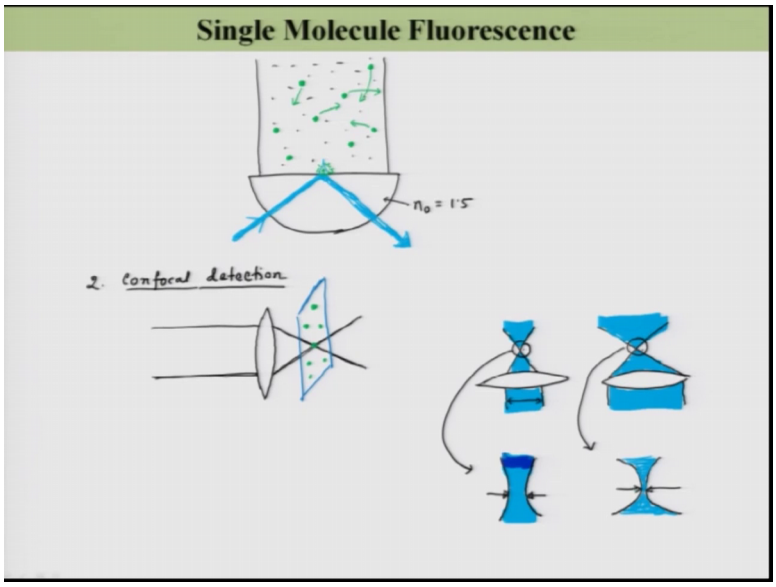

Now, let me show you that experimental setup in this case. Like, if you use a such kind of device, right. This is made of special type of glass, that is what I said this is the refractive index, and then your n 0 is equal to 1.5. And let us say you have set up your experiment by putting a sample cell over here, this is your sample cell. It is somehow attached, so if you have a sample, right, water solution of a sample. So, water solution is present throughout the sample, right. And you make a sufficiently dilute condition so that your fluorophores right, your fluorophores are present throughout, but in the sufficiently dilute diluted condition, right. These are your fluorophores.

Obviously this fluorophores will undergo Brownian motion which can from here to here it can go from here to there in motion, right all the random motion. But now if you use an excitation light, let us say this is your excitation light and you are creating your excitation light like this. So, the diameter of this incoming beam is not very high, because you want to achieve smaller volume if this diameter, if this diameter is high then the volume will be high right. So, you do not want that you want to achieve a smaller volume. So, you want a very tightly collimated beam and that will going to be focused reflected, that will be going to be reflected like this way.

So, if my drawing is it will be like this. So, here is your incoming here is your outgoing after total internal reflection, right. And what you have? You have created this evanescent field like this right. So, only when your fluorophores are present here then only you will going to see the fluorescence, right. Because this part of your sample this part of your sample I mean there is no one to excite. Here evanescent field having the similar wavelength of the excitation of this incoming light will be able to excite your fluorophore. Then you will get fluorescence only when your fluorophore will present inside this small observation volume, when the fluorophore is present here they are dark they are not fluorescing and when the fluorophore is present here, so you will get the fluorescence otherwise not. So, by this you can achieve this small observation volume.

The next one let to achieve this small observation volume is another method, the next method is called the co focal detection method, co focal detection method. In this case what we do is that we use a lens, right. Let me draw this lens over here, lens and this is your incoming excitation light. And you tightly focus this incoming excitation light to a very tiny spot, right. Best you can do is that diffraction limited focussing, the size of the spot is diffraction limited focusing and that you can do with a laser; so in all this

experiment. To achieve the small observation volume, right. The light source what is being used is a laser. Because for the conventional light source you cannot focus so tightly, right.

So, in this case you will get diffraction limited focussing. Now this is very much applicable if I, if I show you another version of this geometry it looks something like this. Suppose you have this lens over here. And your incoming beam diameter is this much. So, anyway this will going to focus right. So, if the colour of this laser is let us say blue. So, this part is blue and here is your blue, right. And here is your blue, right. That is the light.

Now if you zoom it out, zoom out this part, zoom out this part what you will going to really see is the following, right. And again this is your sorry, this is your that light; so in the focal plane, right. The diameter is this much, is your diameter. But if you do same thing, same lengths if you take and in this case let us say incoming beam of the same colour the diameter of the incoming is much more than this; obviously, it will again going to focus focal length is similar. So, it will focus in the similar position, right. Like this way. So, here the incoming beam is like this, this is your incoming beam and here is your beam and here is your beam, right.

Now if I zoom out this part what you will going to see is that it looks like this. So, here you see the spot size at the focal plane is much smaller than this, but it is diverging too much. So, here if I again colour it, so this is this is your excitation light, this is your excitation light. Now if you consider, this is your excitation light. Now if you consider that your molecules are present only at the focal plane. So, condition if I impose is the following that the molecules which are under investigation right, are present one a plane and that plane is this focal plane in this case.

So, in this case if you have molecule here, it is not visible if you have molecule here, it is not visible if you have molecule here it is not visible, but if you have molecule here it is visible, because it is focusing only in this focal plane. It is not visible, it is not visible, it is not visible. So, you can get a tiny spot, right. That time spot depends on many things basically the new medical aperture of this focussing lens and you will get a very, very tiny spot, but you cannot apply this thing to the solution, because in solution let me draw another setup for you for the solution case.

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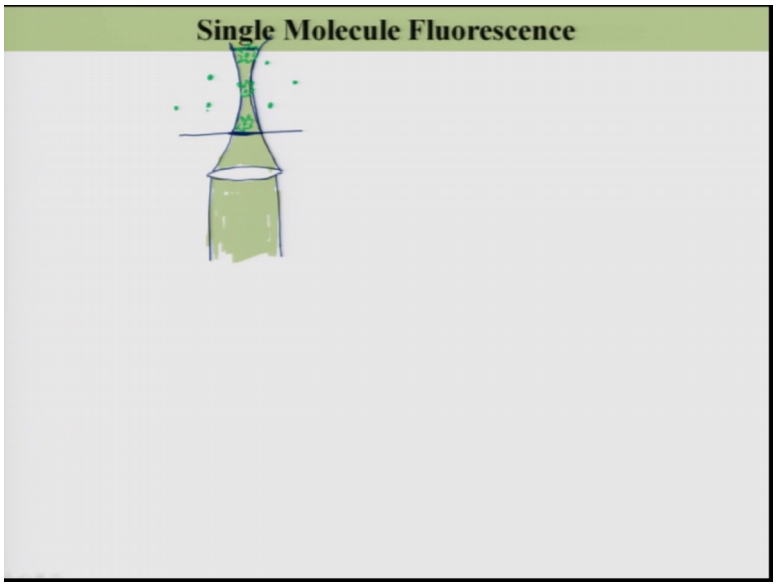

Let us say you have a sample stage like this and your that special lens which is called the objective lens is present over here, right. And you are having a (refer time: 24:00) incoming light over filled to the objective lens. And then this will going to focus, right. And this will going to focus it like this way, right. And your sample is present over here the whole position is your sample position, right. Your sample is present over all along over here, right. On top of this sample stage and this excitation light let us says like this, this is your excitation light, this is your excitation light. So, it will going to focus like this way, is not it? It will going to focus like this way.

Now, you see all over this region, all over this region starting from here to, here to, here to here all over this region the molecules are being excited if the fluorophore is present. If the fluorophore is present here, if fluorophore is present here, present here they are not exited, but if the fluorophore is present here, it is excited it is going to give fluorescence. It will going to give fluorescence, this will going to give fluorescence. If the fluorophore is present here this will going to give sorry, this will not going to give fluorescence. If it is here it will not, it will not right.

So, in this case you will going to see the fluorescence from the entire region. And obviously, then that means your observation volume is really large. If you now decrease the concentration of the molecule in the sample, but your observation volume is more

right; that means, your background Raman scattering will be much more. So, your aim your aim is not fulfilled that aim is to create a small observation volume.

When it was a just a thin layer of sample which is present in only 2 dimension then; obviously, there is no problem, I can achieve the small observation volume because nothing was on the top nothing was below that plane right. But in this case many, many molecules are there specially solvent molecules. So, the observation volume is important in this case. That can be achieved by this co focal method, what is this co focal method? For this I have one animation.

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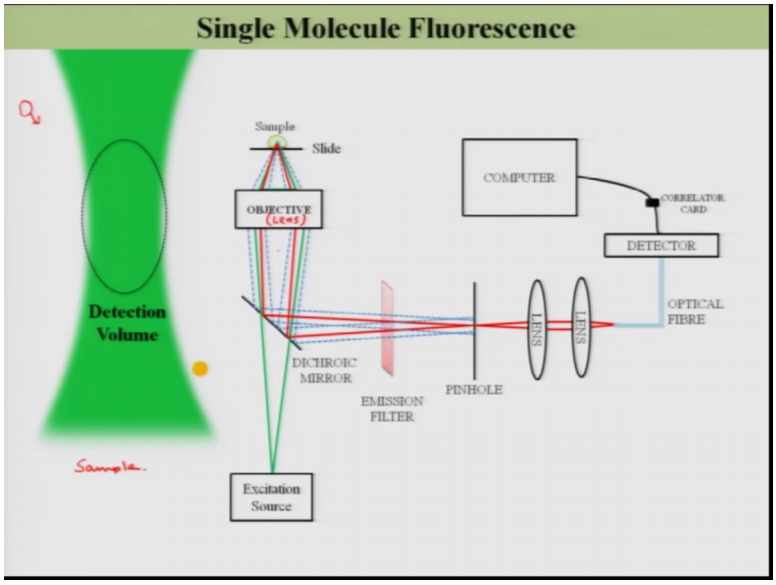

So, here you see this particular molecule is shown over here which is coloured as yellow, right. These molecules will start defusing right. So, you look at here once you excite with your excitation source as I said that most of the case this is the laser. So, this is focused with this special type of planes I said this is my lens special type of lens called objective lens and it will be focussed on the sample.

So, in the sample position this is my sample. In the sample position the excitation light will look something like this. Now once these molecule will come in this green region, once the molecule will come inside, here is also sample, this is sample because sample is large right. But this excitation light is only present, right in a smaller region within this sample.

So, in this case when this molecule will come and enter you are getting your florescence. And you see the fluorescence can come and when the molecule will go out of this excitation region then there is no fluorescence. But when the molecule comes in over here again the molecule show the fluorescence again molecule come in it is showing the fluorescence and so on and so forth.

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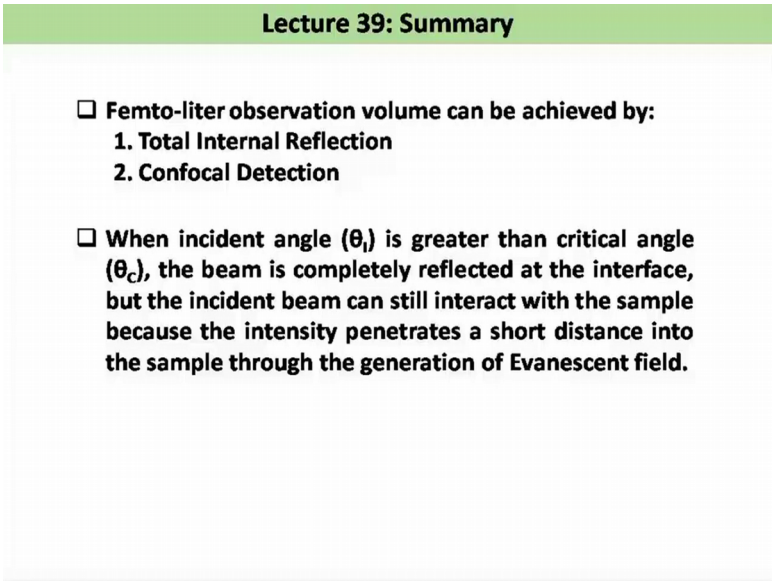

As time is up let me finish over here. And we will continue our discussion on the next lecture.

Thank you very much.