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

Lecture - 40

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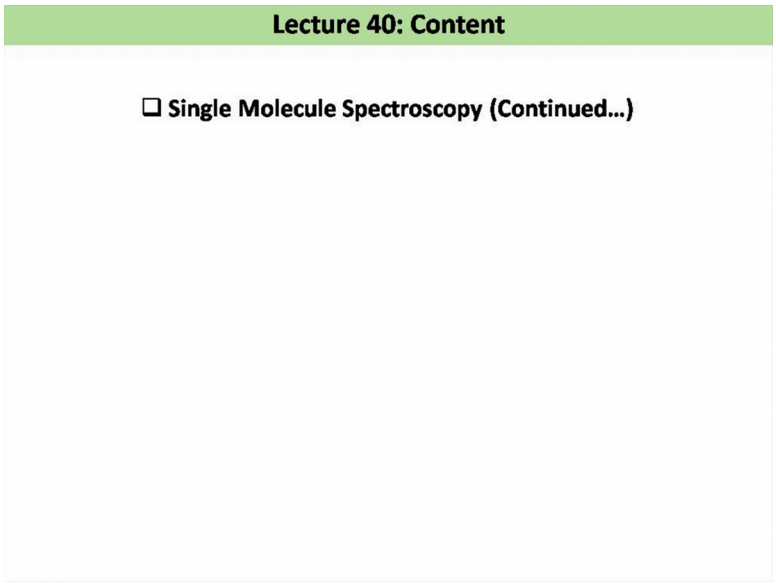

Welcome to lecture number 40. In the last lecture we have discussed about the total internal reflection geometry to achieve very tiny observation volume and we were also discussing about this confocal geometry to create the small observation volume.

In the confocal geometry I was showing you this diagram with this animation, that if I have this excitation light and this is my lens.

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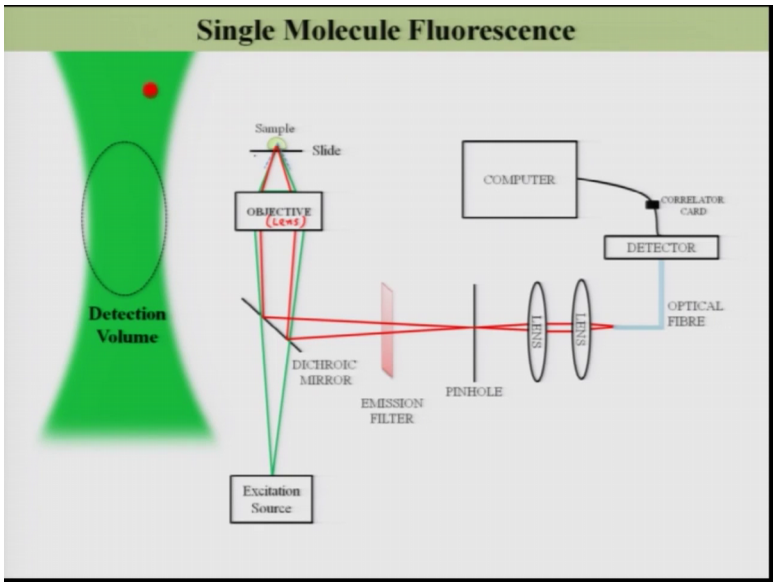

So, these molecule is actually defusing and when this molecule will enter this observation volume it will going to show the fluorescence right and when it will go out right then we will not going to see the florescence.

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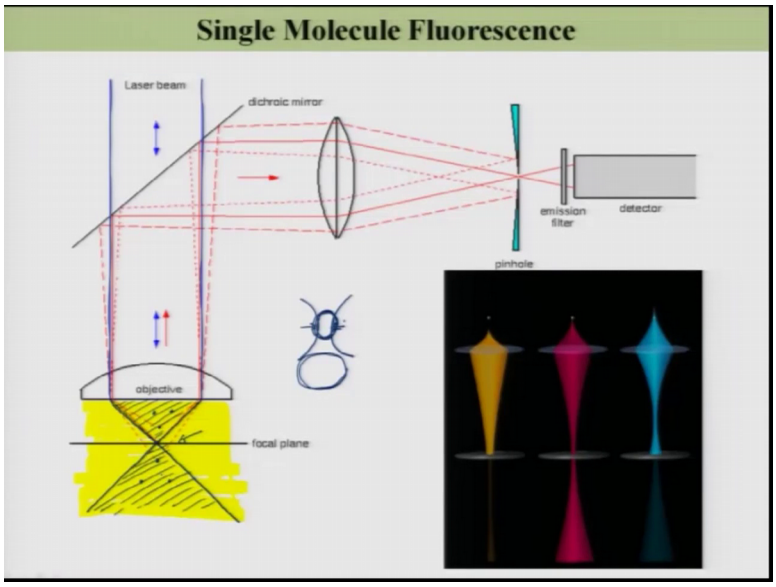

So, this is given here right, you see in this case this is my excitation laser beam right when it will reach this sample position, let me define this samples are present throughout Let us say this is your sample position starting from here everywhere is your sample and when. So, all are sample. So, if you just follow this excitation light, I will just take this

which colour is this blue one. So, I will just take the same colour and I will redraw this excitation light please follow. So, the excitation light is coming like this way, this way this way and it is exciting throughout this is the other side of this excitation light beam and then it will excite throughout; that means, whatever the molecules are present within these shaded region let me shade it nicely.

So, these shaded region will be excited whole shaded region will be excited, but; obviously, these volume is much more right it is not a like a femtoliter; 1 femtoliter. So, to get this one femtoliter I must restrict my observation to some part of that the tiniest part in the width of this excitation beam as I told you in the last class will be somewhere in the focal plane, that is the tiniest one right if you remember I showed you right. So, this is the spot size right and. So, the observation volume if I can restrict around this region then that will be the tiniest right. So, if you restrict your observation around this region, then the size the volume will be more than this that is for sure.

So, I need to do something over here so that right although the molecules which are present here or here or here or here everywhere the molecule will give fluorescence, but I will not going to detect the fluorescence from everywhere, I will going to detect the fluorescence only from a specific position right within this observation volume how to achieve that that is the question right.

It is simple that if you just put a pinhole over here right I just put a pinhole, now let me erase all those thing and you follow this line. As I told you that this blue one is your excitation safe divide excited; that means, the molecules which are present at these position that is also going to be excited the molecule which is present at these position that will also be excited, and the molecule which is present at these position that will also to be excited.

Now, you follow this line this line, the molecules which are present below the focal plane right after you putting the pinhole let us say first you follow the line, the molecules which are present at the focal plane. So, if you follow this line with a basic knowledge of your optics, you follow and you see this rayed actually passing through this pinhole and reaching the detector; that means, the fluorescence which is originating from this position this focal plane actually is reaching the detector, because this pinhole position is over here right.

Now, keeping this pinhole position same; however, do not move it now, keep the pinhole position same keeping this pinhole position same. Now if you follow the line the ray diagram of this light originating from this particular position you see the follow it here it will be something like this, and then it will go like this way this way and it is blocked. This part is blocked over this part is blocked over there right. So, it cannot reach the director.

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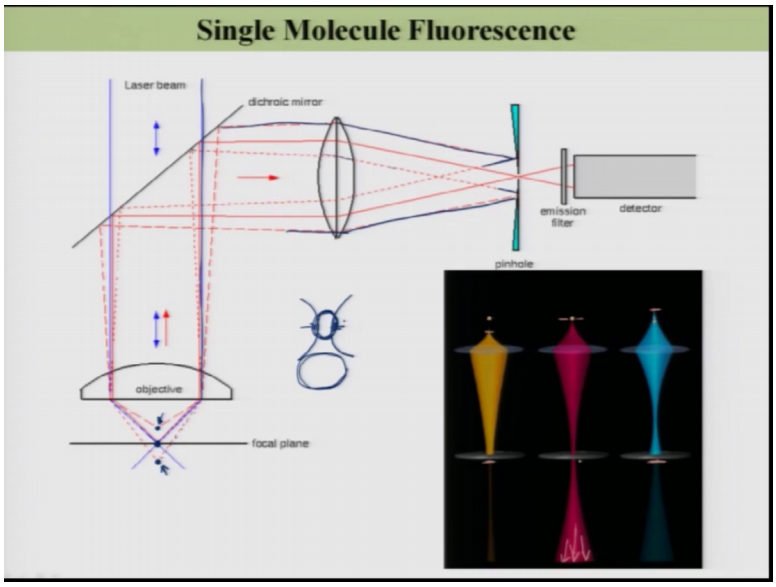

Now, if you consider this point right if the fluorescence originates from this particular point, you follow this line what you will going to see? It is now blocked at this position and this is now blocked at this position. So, the placing a pinhole at this position ensures me that the fluorescence which is originating very near to the focal plane only will be pass through this pinhole and that light only going to be detected by this detector. That same figure that same concept I have displayed over here in this case see here that this is your focal plane. So, when the signal is originating this is your focal plane right here this plane.

When the signal is originating from let us say here at this position it is not passing through this pinhole it is blocked. When the signal is originating from this position it is not passing through this pinhole it is blocked over here, is blocked over here, is blocked over here, but when the signal is originating from this focal plane this signal is passing through right see signal is passing through this pinhole right. And this is called this

confocal geometry. That means it is very simple we have objective, we have this lens, and exciting you can excite the sample throughout, but just by putting this pinhole you can actually restrict your observation volume.

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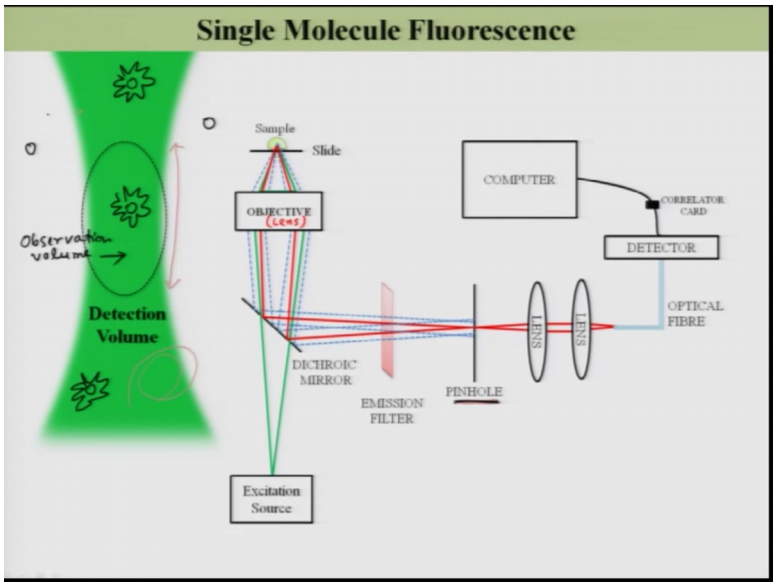

Now, I will go back to my this animation, now you see here, you once you excite your molecule we will start moving because the molecular motions is random right this is the Brownian motion. So, once it enter over here, then this signal is actually passing through this pinhole where with this pinhole and you will reaching the detector, but when the signal is like this when the molecule is present over here, then that signal is blocked when the molecule is present over here, then the signal is also blocked over here.

So, you will going to get the signal only when the molecule is present within these volume and the height of this will be guided by the width of this pinhole right. There is a direct relationship from this width of the pinhole and height and if you now consider that is a Gaussian shift of this laser beam then with using some mathematics you can actually calculate the volume of this observation right. So, it is called the detection volume or observation volume this region or observation volume. So, that is it.

If I can achieve this observation volume then I can see the single molecule. When the molecule will present inside this observation volume, then it is visible when the molecule is present outside. Outside if the molecule is present here it will be excited, it will going

to give you fluorescence, but it is not visible because of the because of this pinhole right once the molecule is here obviously, it will give the fluorescence then only it is visible.

If the molecule is present here right then it will not going to fluoresce, if the molecule is present here it will fluoresce, but it is not visible. If the molecule is present here will not going to fluoresce. So, I can restrict my observation and I can achieve this femtoliter type of observation volume.

Now, consider that you have some matrix let me draw this matrix over here, you have some matrix and you have all the molecules are present right throughout this matrix.

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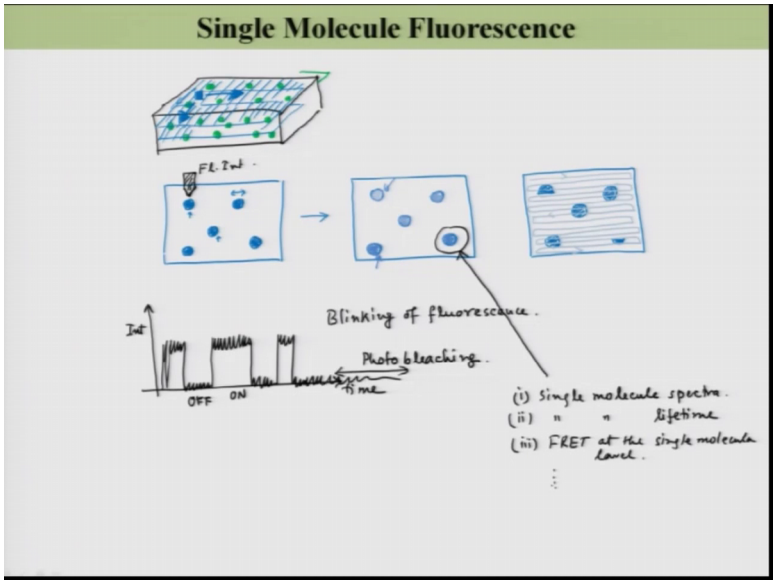

Let us say this is your matrix, let us say this is a polymer matrix and in this polymer matrix the molecules are present right and you have sufficiently dilute condition. So, that the this is between the two molecules are far apart; one molecule is here another molecule is here like this molecules are present, but as this is solid matrix this is in this plane right in this plane and if you just take a cross section probably you will see one molecule here, another molecule here, another molecule here, another molecule here, here, here, random, random distribution like this.

So, now if you want to image how the molecules are distributed in this matrix, what you will going to do? You will just put this slab polymer slab having all these molecules embedded inside in a confocal setup, right in the confocal set up you have this

observation volume, the observation volume is having certain width and certain height right the width is guided by this deflection limited focusing, which is the approximately half of this wavelength right. So, you will get a spot size like this way and then you will get a depth because of the presence of pinhole.

Let us say this is your resolution. So, if you scan what you will going to see? Either this layer of this molecule this is layer one right let us say and obviously, below that there will be another layer right below to this layer there will be another layer this is the layer by layer. So, depending on which one you are actually. So, this is the layer by layer thing. So, depending on the height of this observation volume to the sample you will be able to see one layer.

Now, if you look at one of this layer, what you will going to see? You will going to see these molecules because either because you will going to scan right this observation volume; the observation volume is here then you scan either in x y plane, you will scan in the x y direction. So, then we will going to image everything. So, after you scan whole thing at a particular plane out of this slab; obviously, you will going to see that one fluorophore is present over here, another is over here, another is present over here it is random I do not know I am just writing, I am just drawing it probably you will going to see such kind of image one is present over here.

Here please note that this spot size right is in the order of 200 300 micron; obviously, depends on the excitation wavelength, the light wavelength of the light you are using to get the spot size and that is called the average deflection limit; and in this case the spot size will be almost the half of this wavelength. If you are using 200, 400 nano meter light for this excitation, this spot size will be around 200 nano meter.

Obviously the molecules right they are not like 200 nano meter, they are about one yes 1 nano meter, but still you will get a bigger spot size because of this deflection limited focusing. But no problem if the intensity of this spot this spot is same as this spot is same as this spot is same as this spot; that means, I can conclude that each molecule sorry each spot you will have more a similar number of molecule and in this case the number is one right because you have taken a reasonably diluted condition. So, number will going to be equal to 1.

But if you look at this real image of this the first thing what you will going to see is this; this is these are the molecules right what you will going to see is that some of them are light, some of them are dark I mean it means that intensity is more and for some intensity is even more. So, there is some distribution in the intensity; does it mean that more than one molecule is present? Yes I have taken sufficiently dilute concentration, then it is unlikely that in one place two molecules will be there in the other place none of them is there right. For this even distribution of this molecule in this particular medium considering that there is no specific interaction and all the location in this matrix are equally probable for this type of molecule which is present in the system.

The reason behind such kind of difference in the fluorescence intensity from each of the spot maybe because of the different orientation of this molecule; as I said earlier during our discussion on fluorescence anisotropy that the direction of the polarization of this excitation light right will excite selectively or preferentially those molecules whose transition movements are parallel to the electric field of this excitation light right. So, it is possible that for this molecule the excitation is more favourable because its transition movement is oriented in parallely to the electric field of the excitation light. In this case it is not right it is possible. So, in this case the different molecules are not aligned in a similar fashion right.

Sometime if you take this kind of image, you would also see such kind of feature. Like this is your, this is not a full circle this is a half circle right. Let us say this is the full circle, this is again full circle, this is full and let us say this is half right you will also see such kind of image right. So, I am just considering intensity is that same just for the time being you will going to see. So, what is the reason for this? Because you are going to scan your laser right throughout this matrix here when you are scanning you are scanning like this let me change the colour quickly, you are scanning like this you are going from here to here you are coming like this and then like this and then like this and then like this right like this you are scanning and you are creating this image starting from here, here and then like this like this right.

It means that when you are seeing here this fluorescence is on, but when you are coming back it is off. That is the half circle is fluorescent not the full circle right; that means, if I sit on a particular fluorophore and keep on monitoring the fluorescence intensity, right it must show the on and off behaviour and exactly the same when you see it on a particular

fluorophore in this matrix right you can just focus on this one right you just focus on this one and monitor how the fluorescence intensity is changing with time right.

Then if you plot this fluorescence intensity with time what you will going to see is a kind of fluctuation. The fluorescence intensity is more and it is like this and then it become like this and stay time sometime from here, then again it will increase it will stay like this way and then again it will come back and stay like this and ultimately it will never come back it is 0 and this is called the blinking of fluorescence. This is basically because of this conversion of the molecule from the singlet state to the triplet state. When the molecule will go to the triplet state; obviously, it will take some time to come back from the singlet state and fluorescence is only from the singlet state that we already discussed right.

In the triplet state (Refer Time: 18:28) florescence is typically quenched by this different quencher present typically by the oxygen molecule dissolved in this your system. So, we will not going to see the (Refer Time: 18:29) fluorescence at the room temperature. So, once the molecule will go to the triplet state, it needs to come back and that takes time and that is called my off state.

So, here is my off state and here is my on state and so on. But if you keep on excite the molecule right many times then the molecule may destroy. And it is the very common feature and this part this part here the molecule will never come back to give you fluorescence. That means, the molecule is destroyed and this is called the photo bleaching.

So, these are the typical behaviour of this molecular fluorescence, but single molecule fluorescence. Now if you just sit over on a particular molecule right and you just keep on monitoring until and unless this molecule will go back; that means, photo bleached right then you can get several information about this molecule for example, you will going to see the single molecule spectra, you will be able to see single molecule lifetime, you can see the application of rate in the single molecular level and many many many other application is possible.

But I would like to tell you one important application which is not in the solid matrix, but in the solution state right and if this is my observation volume if this is my observation volume now you have a clear idea about this observation volume.

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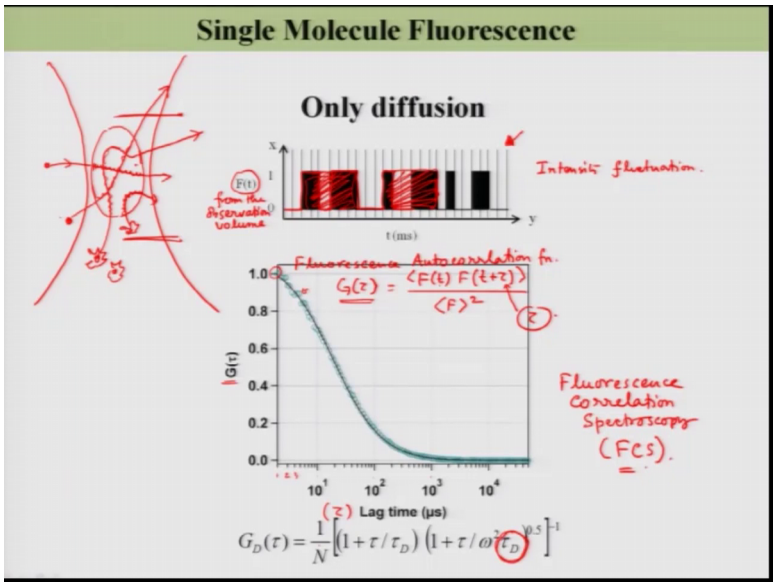

When the molecule will come within this observation volume is my molecular trajectory here the molecule is nonfluorescence the molecule fluorescence will be detected only when it is present within this observation volume, throughout this path right throughout this path the molecular fluorescence will be detected.

And the molecule right will take some time to cross this observation volume if the molecule is entering from this side and taking such kind of path then this fluorescence will be on for longer time than this. If the molecule is coming from this side and exists from this side, then the molecule then the fluorescence is on from for a shorter time right. So, here the fluorescence is on for a short time, on here the fluorescence is on for this time and in this case the fluorescence is on for a much longer time if the molecule will come like this way then the fluorescence is on for this time.

So, if you now monitor the fluorescence right as a function of time, then what you will going to see you will see the there is no fluorescence. This is the fluorescence intensity this F t is the fluorescence intensity from the observation volume right. So, in this case the fluorescence intensity is on for when it is present like this. So, fluorescence intensity is on and then fluorescence intensity off; that means, none of these molecules are present here.

If none of them is present although the molecule here is getting excited because your excitation light is like this right it is excited it is excited, but you will get the

fluorescence only when this is not excited right this is excited, but you will only get the fluorescence only when it is present within this observation volume, that height is determined by the width of the pinhole the size of the pinhole.

So, then again one the once the molecule will enter it will be like this, and then like this like this, like this and so on right now so there will be a fluorescence intensity fluctuation right I can simply write this as intensity fluctuation. Now you consider the diffusion of this molecule in and out of this observation volume is slow right then this self-similarity of this florescence fluctuation will be more because the molecule is present for the longer time within this observation volume the florescence is on and if it fluctuate rapidly, then the self-similarity of this fluorescence fluctuation will be less. So, that depends on how quickly the molecule is coming in and going in and coming out of this observation volume right.

So, if I consider a short time period for very short time period right; obviously, there will be lot of self similarity right. If I consider this, this fluctuation now and this fluctuation after a very small time; that means, the fluctuation should be very similar right then there is a lot of similarity, but if I now consider this fluctuation and same fluctuation after certain time after sometime, then the self similarity will be lost.

But if these fluctuation widths are more right if this is on for longer time let us say if it is on for this time this whole time region then it is off and then it is on for this whole time region. Then even for this case compared to the black one in this case after a certain time that self-similarity will remain very high right and that self-similarity is expressed by this fluorescence autocorrelation function and which is denoted as G tau and defined a, fluorescence intensity at time t plus tau average divided by average fluorescence intensity square right.

Now, if you change this tau value, you change this tau value right. So, the fluorescence intensity at time t and time t plus tau will be very very similar if the tau equal to 0; that means, it is the fluctuation will have very very similar fluctuation. Because it is same right F t and F t is same, but when I change this tau from 1 tau to 2 tau and sometime if I give1 microsecond, 2 microsecond, 10 microsecond then the self similarity will be lost right and you will get a lower value of G tau. So, G tau will start from a high value and G and it will ultimately will going to be0 when the value of tau is very very large and in

this case these G k of this G tau should be related to the how quickly the molecule is passing through this observation volume.

In other words it should be related with the how this what is the size of this fluorophore. If the size of the fluorophore is big then it will take some time to pass through this observation volume and that can be correlated easily. If the viscosity of the medium is high, then the molecule will pass through right this observation volume the longer time period and it will be it will be seen in such kind of G tau.

I will just show you this typical plot of this G tau as you can see here G tau is plotted against this; obviously, tau right this is tau is in the microsecond. So, here is your 1 microsecond, 2 microsecond, 3 like that 10 microsecond, 100 microsecond, 1000 microsecond and so on. So, you see this G tau value is very high is about one right is about 1 over here; that means, there is a lot of similarity between the F t and F t plus tau at 1 microsecond right, but as that tau value is changing its going to be 0, but there is no such similarity right at 10 to the power 3.

Now, if whatever I said I expressed this in mathematics right then it should be related with the diffusion time and if I show you that particular equation, now you see this G tau is related to this tau d that how fast this molecule is passing through this observation volume and this is guided by this particular equation right and where n is the number of molecules present in this observation volume. And you see that this experimental data those circles this experimental data are now fitted with this equation to get this value of tau d. Once you know the value of tau d from stokes Einstein relationship, you will be able to calculate; what is the volume of this molecule if you know the viscosity and temperature and vice versa.

So, this is a very important application in single molecule fluorescence, and this particular experiment is known fluorescence correlation spectroscopy. And is in short form it is known as FCS. So, we will finish here as our time is up.

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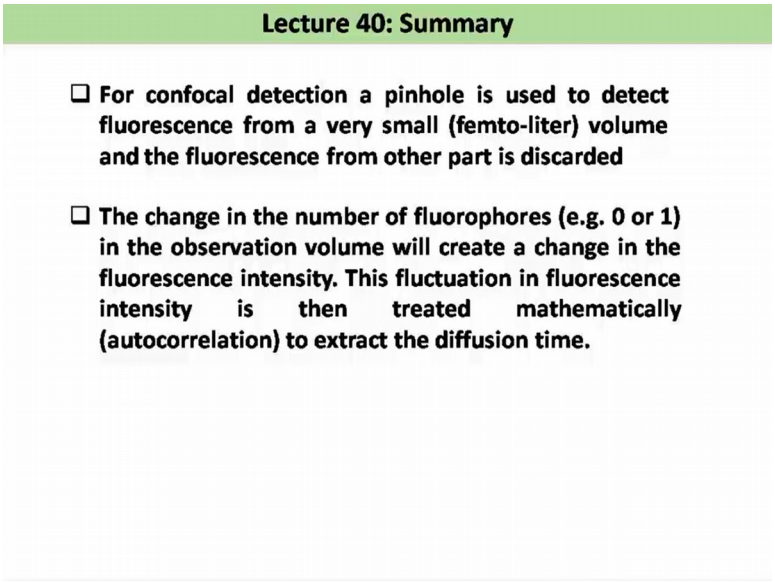

Thank you very much.