Basics of Fluorescence Spectroscopy
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Lecture – 10

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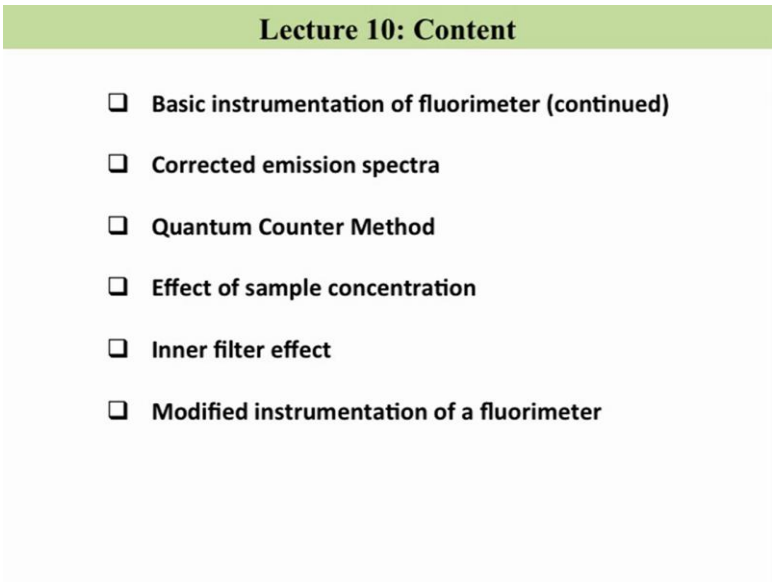

Welcome to the lecture number 10 of this course; basics of fluorescence spectroscopy. So, in the last lecture, we are discussing about the instrumentation of a fluorimeter, and I showed a very basic diagram of instrumentation of the fluorimeter. Let us see it once again.

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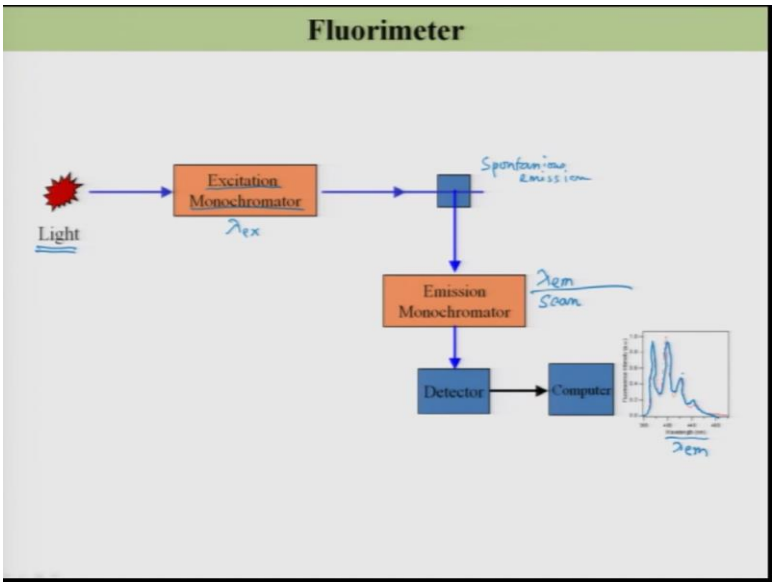

So, in this case, what I have? I have this light source that will be used for the excitation of your sample and then I have this excitation monochromator over here. And with this excitation monochromator, you can choose your lambda excitation value. Obviously, every molecule will not be going to absorbs as at the same excitation wavelength and that depends on the absorption spectra. So, in this case, this excitation monochromator will going to help you to choose the excited light wavelength you choose your a proper excitation light wavelength out of this spectra; output of this excitation lamp. So, this is directed towards the sample and I told you that this fluorescence is a spontaneous emission, here is spontaneous emission. And fluorescence is coming out and all the directions and we choose to collect this at a right angle compared to the excitation to avoid the scattering right, to avoid the scattering of this excitation light.

So, but this emission itself is a broad and for this molecules having different levels resolution levels and so on. So, to calculate, to measure the intensity of a particular a wavelength of emission, we need to again disperse the emission in a monochromator and detect the intensity of a corresponding and each wavelength individually by this detector. So, basically during the measurement, you keep this setting of this excitation monochromator fixed at your desired lambda excitation. However, you will going to scan this emission monochromator throughout the emission band, then you will get the intensity of emitted light at a every wavelength.

So, here is my lambda emission set by this emission monochromator and we need to scan it starting from a lower value to higher value or higher value to lower value at your wish. So, no problem, and then this detector a signal is collected in a computer. And what I have shown here is that wavelength like this is my lambda emission plotted against this fluorescence intensity right is plotted against this wavelength. And in this case, what you will see is a nice fluorescence band like this, this is for the (Refer Time: 03:19). So, you will get this emission spectrum. And then what I was discussing is that even you will record this emission spectra with this a nice fluorimeter. The emission spectra which we are going to record in this case are not correct emission spectra.

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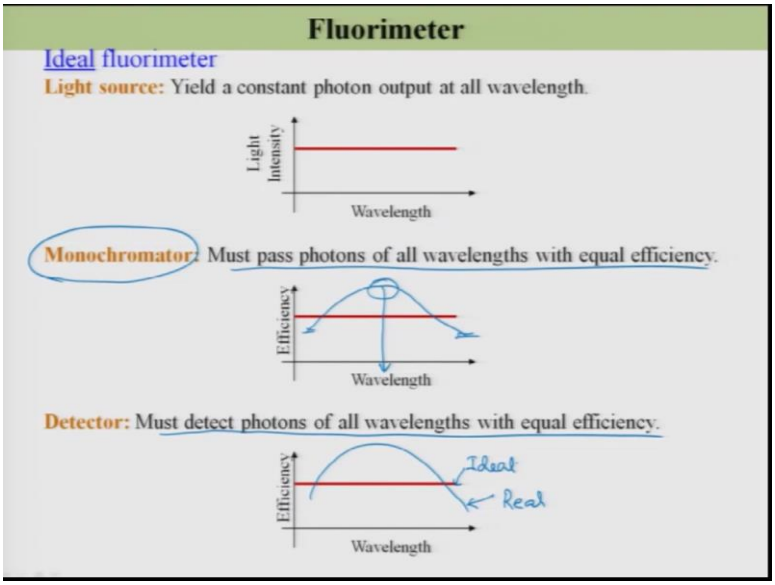

And the reason behind it is that the monochromator and the detector, they are not ideal. What I want to mean by ideal, ideal means for ideal monochromator right say ideal monochromator means it must pass photons of all wavelengths with equal efficiency. But in actual it is not; so ideally it should be a straight line, but in reality it is not like that it, it is something like this way. So, here the efficiency of this particular wavelength to pass through this monochromator is more compared to this wavelength and this wavelength. Similarly, for the detector ideal detector means mass detect photons of all wavelengths with equal efficiency. So, I must get a horizontal line like this way, but in reality this is not the case. So, the digital efficiency is also like a kind of this shape. So, this is the real and this red one is ideal.

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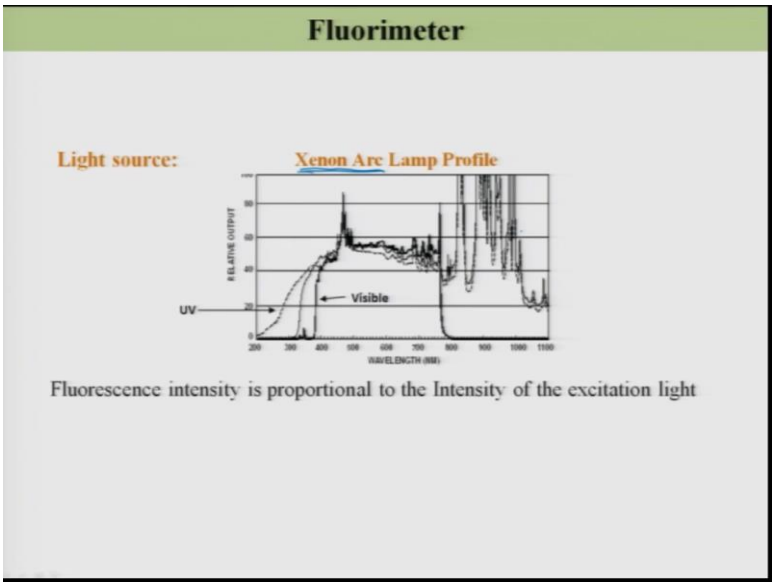

And what will be the problem in this. So, here just I want to show like also this light source is also a not a horizontal line like this. So, actually this is actual spectra of a xenon arc lamp, we had xenon arc lamp, the light source. You see this actually having lot of spikes and the shape is like a bell shaped with a lot of spikes over here.

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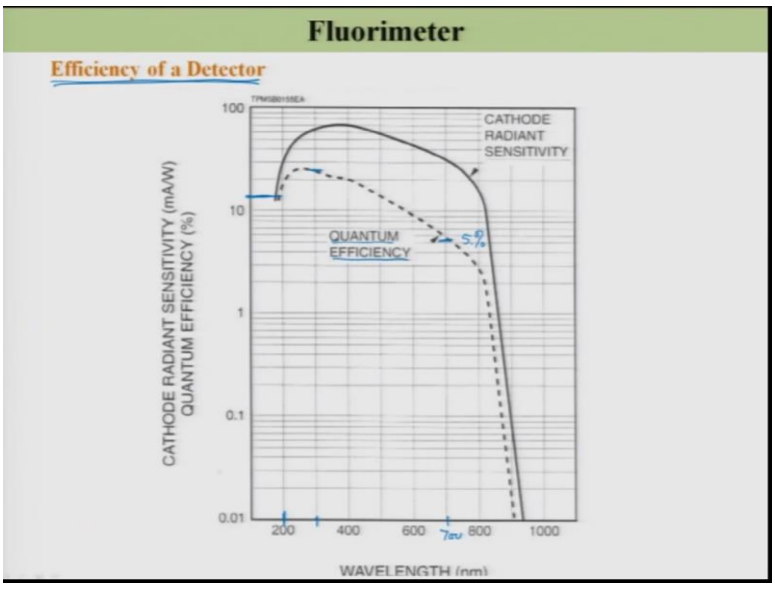

So, similarly here I showed the efficiency of a detector. See the efficiency of a detector this particular detector this is (Refer Time: 05:25) 9 to 8 forget about that a particular detector. So, here you see the efficiency is about 13, 14 percent at 200 nanometer, this is

the quantum efficiency. How efficiently it converts the photon to electrons that is it. However, the at 300 nanometer, you see the efficiency is about 30 percent; and you see at or say this 700 nanometer the efficiency is down to few percent 2, 3, 4, 5 is about 5 percent. So, efficiency is a function of the wavelength for this detector.

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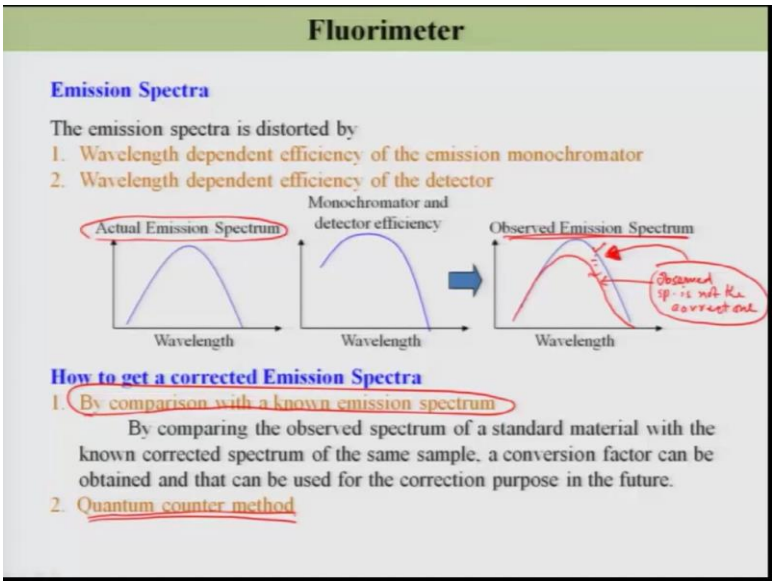

And for that what will happen if this is the actual emission spectra of your species right as I shown showed here this actual emission spectra let me change the color of this pink. So, this is the actual emission spectra of your species if is like this right, but together the monochromator and detector efficiency are not ideal, it is like this kind of shape right then this kind of shape will be multiplied with this and you will get your emission spectra accordingly. So, your observed emission spectra observed emission spectra will not be like this because of this factor. So, you are observed emission spectra will be something like this. It means that whatever you have observed is not correct. So, an observed spectrum is not correct one.

So, you need to convert these one to these one you need to correct this to this and that is known as corrected emission spectra after you will measure your emission spectra in your instrument, you have to do something at to produce the corrected emission spectra and that spectra is a real spectra of the molecule. So, and I briefly showed you that the one technique is by comparison with a known emission spectra of a molecule. If you know this is if it is already known to you, then you can just simply do this divided by

this, this divided by this. And then you will get this correction file whenever you will measure this emission spectra you just multiply that and you will get your corrected emission spectra.

There is another way to do this. Suppose, you are the person is doing the experiment for the first time. So, no corrected spectrum is known to you right then what will happen. So, you really have to do something to get the correct emission spectra is not it. So, this is another method which is called the quantum counter method. So, this quantum counter method are going to discuss after 5 minutes, because another important topic needs to be discussed before I will talked about this quantum counter method. What is that?

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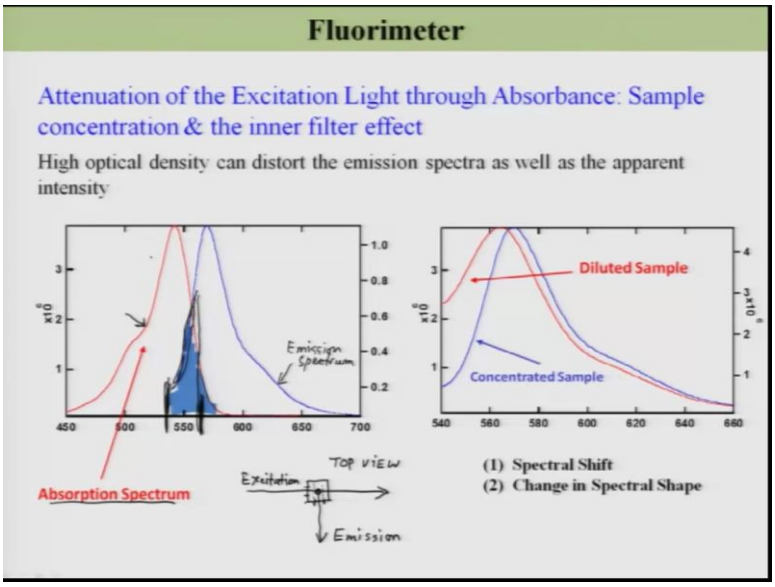

So, as you have seen that while measuring these emission spectra our geometry is a right angle just to I heard the scattering. So, here is my excitation light passing through my sample, this is my top view. The excitation light is passing through my sample and I am collecting emission from here this is my emission, and this is my excitation. So, in this case, I am going to collect this emission from the middle side. And also please note that we usually use a slit over here, and a slit over here to collect this emission, this is necessary for the dispersion in the more (Refer Time: 10:06).

So, using the slit, I am going to collect emission only from this region. Now, let us say this is the absorption spectra of the sample that red one, this is the absorption spectra of the sample that is that you put inside your sample chamber and this is the emission

spectra of the sample which is already known, this is my emission spectrum of the sample which is already known to us. Now, you see a considerable overlap in the absorption and emission of the sample. So, see here these overlap, there is a overlap over here. It means that if the concentration of the sample is high then the emitted light in this region means in this wavelength region can be reabsorbed by the other unexcited molecule present in the vicinity of the excited molecule, clear? So, if the concentration of your sample is high that means, absorbance is high then the emitted photon in this wavelength window could be reabsorbed by the molecules because it had large amount of molecules concentration is very high.

So, those molecules; those are not excited they are in the still in the ground state because only fraction of the molecule you can excite from ground state to the excited state. If you will increase the excitation light intensity then more molecule will going to excite, but not everything; not all. So, in this case, those whose are not excited there is a certain chance that those molecule will reabsorb it. So, when I will going to ensure that the emission intensity in this region, so emission intensity in this region whether this is a real one or it is absorbed somewhat absorbed by the unexcited molecule, I need to ensure that.

So, the best way to do that is you do that dilution experiment. Suppose you have taken the emission spectra with 10 micro molar, you take the emission spectra with 5 micro molar, 2 micro molar if they are same then obviously, there is not much re absorbance of the emitted photon. And you will be able to sure detail that the; my emission spectra in this region is correct in this region is correct no problem. Now, if it is not, then there will be a distortion in the emission intensity as I have shown over here.

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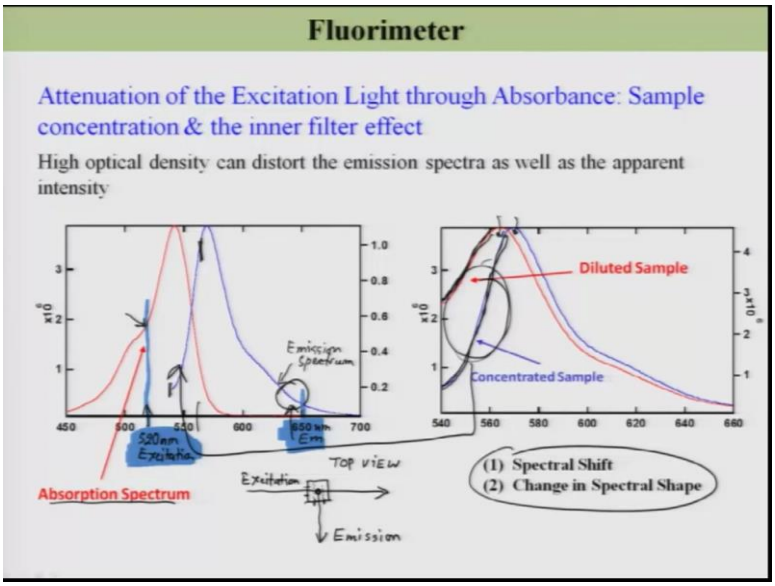

Suppose, this is the emission spectra you have recorded. So, you see the intensity around this region is low. When you have diluted the sample you see the emission intensity increase at this region the emission intense as this side the emission intensity increased when I normalize it both of them, there is a apparent shift in the emission maxima. So, as I said that one of the parameter of the fluorescence is emission maxima that will going also going to be distorted and that distortion is because of the re-absorption of the emitted photon by the sample and that is because the concentration of your sample is too high. So, you need to ensure that there is no re-absorbance and that can be easily done by the dilution experiment as I said.

So, you have to take a note on that. So, the problem will be spectral shift as I have written here, spectral shift change in the spectral shape. So, we need to be cautious while measuring these emission spectra. So, measurement of emission spectrum is not trivial is difficult. I can tell you is not like absorbance, absorbance is fine because it is just the ratio I 0 by I take a log plot it versus wavelength, but in this case it is not like that. So, you need to really take care of different aspect.

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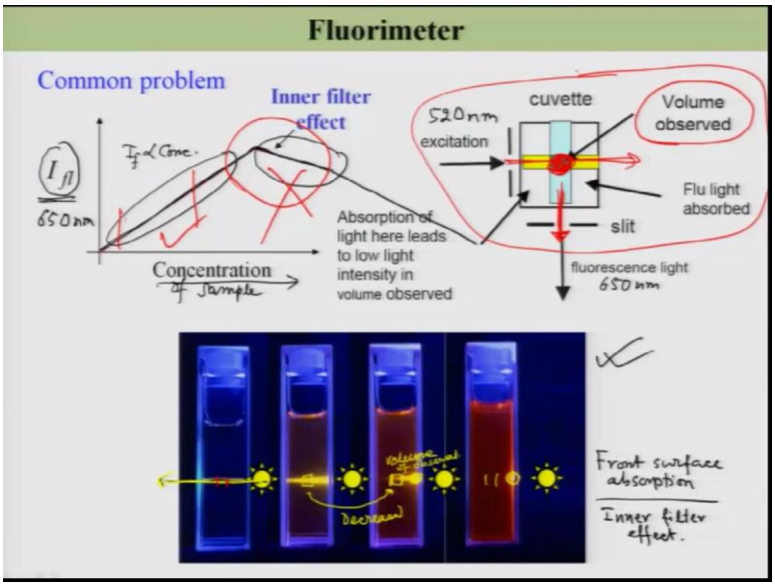

Nevertheless what will happen as I said earlier that when you will increase the intensity of excitation, your fluorescence intensity will increase when you will increase the concentration your fluorescence intensity will also increase I said that. But that is now if I plot the intensity of fluorescence versus concentration of sample concentration of sample I can also ended up with a situation that fluorescence intensity was increasing and then we started decreasing I know about this region I know this region because I know fluorescence intensity is proportional to the concentration.

But how I will going to explain this part. For example, these fluorescence intensity is not in the overlap region still what I said is that these fluorescence intensity may decrease in these region because there is a overlap over here, but what about this processing is there is no overlap there is no overlap with absorption spectra. So, re-absorption of this photon is not that way possible in this particular case. So, I am talking about this 650 nanometer still as in the next slide let us say it is the 650 nanometer, still you will going to see such kind of wavelength is it clear.

Obviously, you cannot ask any question to me now, but later during in that portal you can ask me, if there is any doubt. So, what in this particular experiment, in this particular experiment, I have set the excitation wavelength as let us say for example, 520 nanometer excitation and let us say 650 nanometer emissions. Please note it, I will highlight it. So, here I am exciting and here I am monitoring. And that what I am going

to do it over here 520 nanometer excitation and 650 nanometer emissions. Still what I will going to see is increase initially but then decrease and that can be explained with this photograph. When your solution is dilute, this excitation light will completely pass through. Let me change the color. This excitation light will completely pass through like this way. And as I said this is my geometry of my excitation. So, in this case what I do I excite like this and emission is collected from the mid part of this excitation. So, this is the volume of absorption, this is my volume of absorption for observed volume.

So, in this case, this observed volume is somewhere over here. And now if you increase the concentration of your sample, you see that the excitation light will find more molecule in this region. So, it will be excited and throughout the region, the excitation light intensity will not going to be equal same. So, if you increase the concentration more. So, these excitation light will be used to excite mostly the molecule over here. So, you see here the intensity of this region, your observed volume region, this region I am talking about volume of observation this intensity is less than this intensity. From here to here, increased because the number of molecule increase, but here from here to here, here to here decreased, because the excitation light intensity is not that strong that it overcome and it come till this position to excite the molecule because it is eaten up by the large quantity of the molecule present in the front surface of this sample cell.

If you increase more, then this excitation light even cannot penetrate it just stop over here. So, the there is no intensity. So, intensity will eventually drop to 0. In this case intensity will eventually drop to 0 like this. So, this is called the front surface absorption. And the re-absorption and along with this is known as inner filter effect that means, the fluorescence intensity is modulated because of the sample itself which is present inside the sample right sample itself, so inner filter effect. So, you have to be cautious while recording the emission spectrum while reporting the fluorescence intensity. So, you have to be in this region; if you go in this region then it is wrong, this region is correct.

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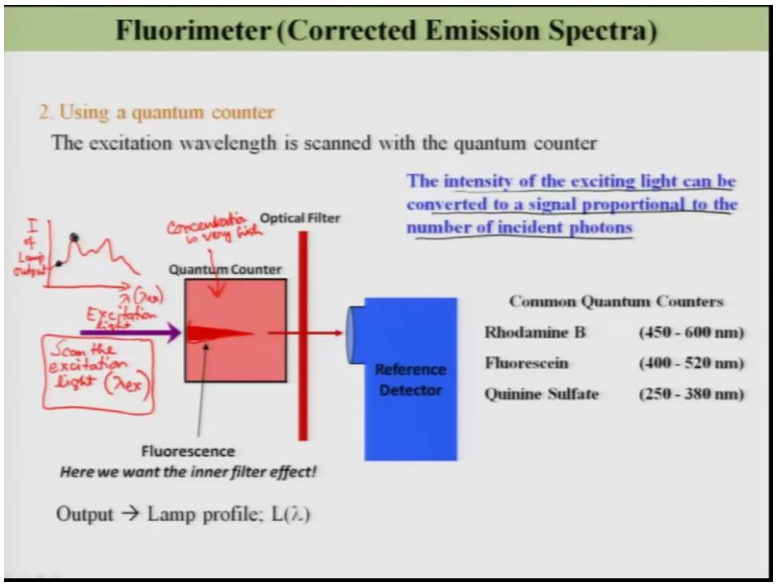

So, having this idea, let me show you that quantum counter right how to get the correct emission spectrum. In this case what will going to do, we will going to use a sample of my choice right and the concentration of the sample is very high, concentration of the sample is very high. So, in this case, the excitation light right this is my excitation light will be completely absorbed by the sample as shown over here completely absorbed by the sample. So, it will not going to pass through the sample. It will not going to pass through because whatever the molecule you have taken concentration is very high. And once it will be absorbed, you can get the emission either from the front surface of this. So, the total fluorescence intensity collected by so in this case the geometry is different if this geometry is not like that. So, please make sure in that quantum counter case the geometry is not like this, geometry is not like this, the geometry is different.

So, in this case, we will collect this emission right either in the front surface or some or from at a certain angle. So, the amount of here all the complete excitation light has been absorbed by the sample concentration is fixed because you have you know the concentration you have created a particular concentration of your sample over here. So, the fluorescence intensity coming out of this particular sample is proportional to the intensity of the excitation light.

Now, if you change the excitation light if you scan the excitation light wavelength. So, let us scan it scan the excitation light that means lambda excitation, I am going to scan it.

So, when I scan this excitation light what is the allowed region of the scanning, the allowed region of the scanning is same as the absorption spectra of the molecule that you have taken in this case, so that is my allowed region of the scanning. So, when you will scan, so depending on the spectra of the lamp output, the light intensity at different lambda excitation wavelength will vary right as simple as it is.

Let us draw the lamp spectra right for this allowed region of the lambda excitation. So, let me draw over here intensity of lamp output versus lambda. So, that lambda you will going to be used as lambda excitation. So, let us say that spectra at this region looks something like this. So, when you will use these lambda excitation, the total intensity observed by this quantum counter this system will be less compared to when you will use this much of intensity these wavelength. And as you change this wavelength, the total intensity coming out of this quantum counter will vary like that is not it. So, what I wanted to do I wanted to record the total fluorescence intensity in this detector, so and that is what I have written here the intensity of the exciting light can be converted to a signal proportional to the number of incident photon that means, a intensity of the incident light.

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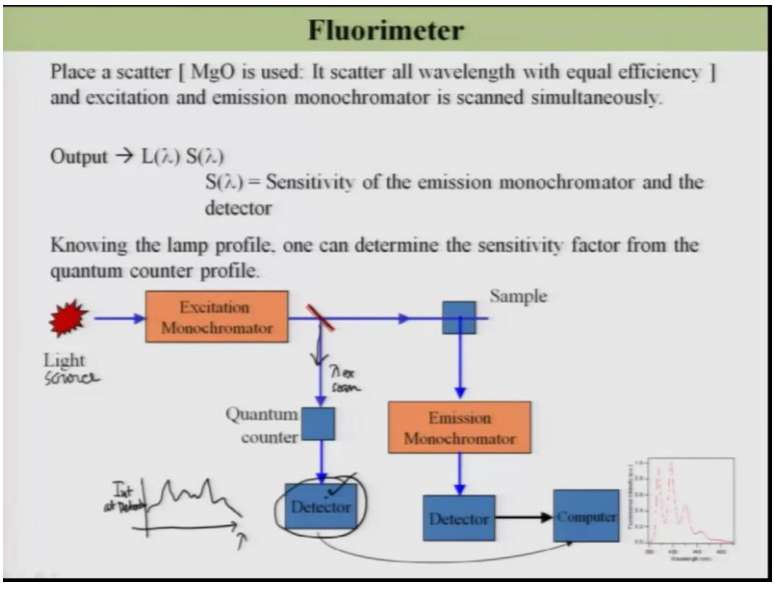

So, now if you scan, now in this case over here, now I have here you see here is my light source light source excitation monochromator and I have taken this light in this region. So, I have this quantum counter that is the choice of a molecule, where I want the inner

filter effect. So, no excitation light is passing through and I want to collect the complete emission there is some arrangement to do that I will not go into that detail. So, that the whole the complete emission is collected and that is detected by this detector by this detector.

So, in this case, the complete emission from this molecule will be detected. And as you change the excitation wavelength here lambda excitations scan. So, this then detector signal will also vary accordingly. So, if you plot the detector intensity at detector intensity at the detector then this detector intensity will also vary as per the lamp spectra in my lamp spectra what I showed like this way like this way. So, detector intensity will also vary like this way. So, ultimately what you will going to get is a similar profile like this here, not here this emission spectra and I said earlier that these detector the response of the detector is not same. So, I need to correct, do I need to correct here.

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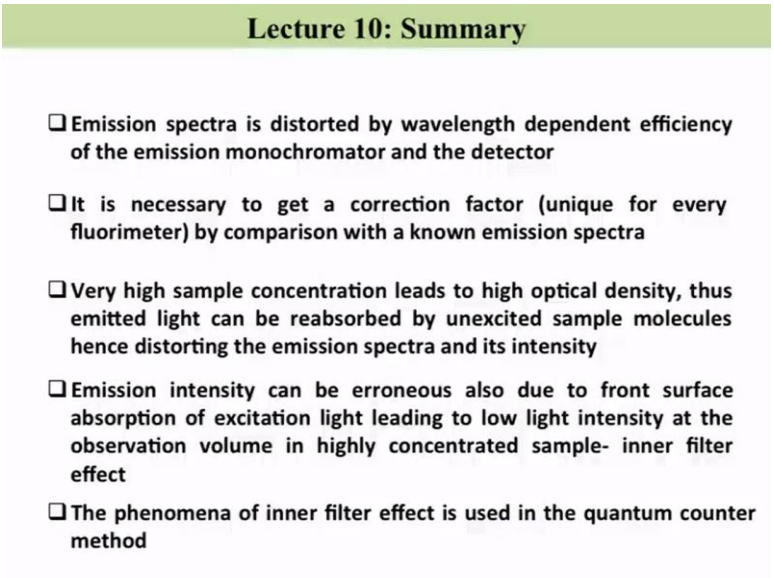

Do I need to correct the intensity for this particular detector or it is; that we will going to discuss in the next class.

Thank you very much.