Optical Spectroscopy and Microscopy
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Lecture – 48
Fundamentals of Optical Measurements and Instrumentation

Hello and welcome to the lecture series on Optical spectroscopy and Microscopy. In the last lecture, we were talking about multi-photon excitation and how the multi-photon excitation can be used in the spatially resolved imaging, particularly to get 3-dimensional resolved structures of samples that you would like to investigate. In this perspective, we were looking at what happens when we shine light photons of lesser energy onto a system specifically around half the energy gap and the 1/3rd energy gap.

We saw that the molecule can observe two or more photons of lesser energy and that can still add and make up to the total energy gap and result in an excitation and we were looking at the schematics and then how do you represent that in a schematic and we conclude it by saying that such a excitation is very useful in multi-photon microscopy and microscopy in general, when the tissue or the sample that you are trying to look at has an UV absorption.

You do not want to shine the UV light and break the bones inside the sample and create damage or for that matter, if it is a scattering tissue, very much a scattering tissue and you want to reach deeper inside, then in both these cases going to this more of excitation is pretty useful when you are detecting fluorescence. So let us quickly take a look at the schematic.

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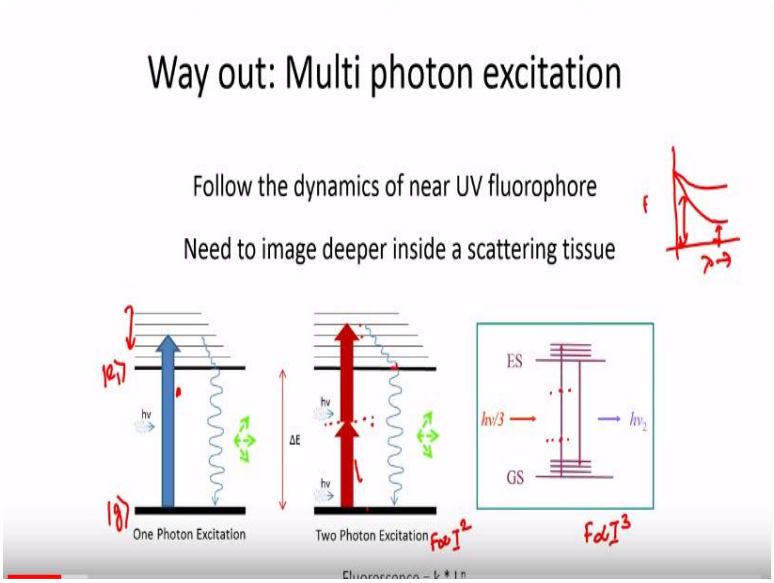

The conclusion was that in any of these, in this or this, any of this, the characteristic equation that describes fluorescence as a function of excitation intensity, would be F directly proportional to I to the power n, by the fluorescence K into i to the power n, so this is a very conclusive evidence to say that you are actually looking at photon process. You are actually looking at two photon excitation and fluorescence.

You are looking at the fluorescence and then measuring the amount of fluorescence as a function of incident intensity and when you plot the fluorescence of incident intensity more specifically, the log of the fluorescence where is the function of intensity, then what you will see is that you will have this straight line and the slope of that straight line would tell you the order of excitation.

This is very much doable job in our lab and the numbers you get very astoundingly tight, anywhere between 1.95 to 2.05, if you are using for two photon and similarly for three photon etc. Okay, if it is this consistent and we are talking about this as if it is a yet another process, then why do not we normally when we take the substance and put it in a spectrometer, why do not we see that often.

We do not talk about two photon excitation or excitation that is caused by photons that are half the energy or one third of the energy. Why do not we do that. The reason why we do not do that is because the probability of excitation right, you remember the proportionality constant here, fluorescence is directly proportional to this k times I to the power n. This proportionality constant for the sigma 2 and sigma 3 is progressively lower and just to give

you the feel, I am going to write down the order of the magnitude of this variant sigma, so far, sigma one is typically of the order of 10 to the power minus 18.

So, right now I am not stating the units, but you can actually you will see that is not the same. Simply because the k, or the sigma here, that is what the k is, basically f by i to the power n as the intensity keeps changing, the order of the intensity keeps changing, the unit does change, but there is a universal unit you should know that and it is called as GM, it is called Goeppert Mayers unit and it is given in name of the scientist who mathematically showed much before anybody could experimentally see it.

Mathematically predicted that such process will happen. She is a nuclear energy physicist. She predicted that such kind of processes should happen. The probability would not be 0, but it is small, but definitely greater than 0. So in that unit you can think of this as a having the magnitude of 10 to the power minus 18, while this is anywhere between 10 to the power minus 48 to 50, while this is the order of 10 to the power 92 to 95. So, this proportionality constant is indicative of the fact like how probable is such event.

You have to have a substantially higher amount of intensities to be able to see the effects of this 2-photon excitation. Well, if you need to have such a high intensity and the whole point of going to multi-photon excitation is to one of the point at least is to minimize the dimension, what is going on here. So to understand this, we need to pay attention to how this is generally done in a lab.

So, what we do is that we need very high intensities and there are ways to achieve this intensity. One, just like any other focal microscope, you are going to focus them, right. That is a spatial focus that we talk about. Other, also you can focus it in time. So, what do I mean by focusing time.

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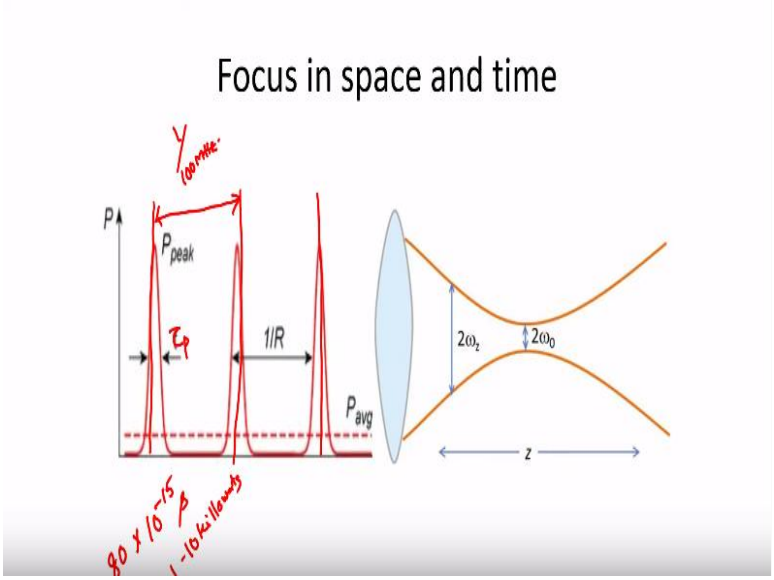

So, let us look at this graph here. So, what I am plotting here is the power or also you can think of that as the photon density, okay as a function of time. The dotted line here represents the average power, which is basically the laser that we use for causing this multi-photon excitation if it were to be continuously emitting the photons, right without any gap, that is where it would be. However, we are kind of magnified it to make this illustration legible, but if you were to draw really to the scale, they are actually simple line, just a small line with almost negligible thickness.

So in order to understand this, what I am going to do is, I am going to blow it up and then show it as a peak with we call that as tau p, that is pulse width and the gap between them is the time corresponding to that is 1/R. This is our repetition right. This is how you use an ultrafast laser in a microscope thing. The point of introducing the ultrafast lasers, there are multiple reasons and multiple ways that one can utilize them, multiple characteristic that are extremely useful, one of the dominant character being is to study something really occurring fast.

So, it allows you to study fast dynamic, ultrafast right because it is set it has a very short time profile, but then on the other hand, because of the shorter time profile, you also have this ability of focusing all of this light into a very short duration, extremely short duration we are talking about 10 to the power minus 15, and typically it is like 80 to 90 seconds reaching peak intensities of about a kilowatt or 1 to 10 kilowatts.

Now clearly, if I were to give 1 to 10 kilowatts of light to a sample, it will definitely burn off. It will fry, meaning the whole sample will go bad, however, the fact that it is a flash of light occurring for only a brief period, it is about 80 femtoseconds and for a large period of time, the lasers that we are talking about given by the cavity length and all that stuff, the repetition changes, but typical repetition rate that we are talking about is 100s of mHz.

So, given 1/100 mHz is the time period here, so the inter pulse interval is the order of few nanoseconds, as a result is a nanosecond gap and femtosecond pulse. So a large gap happens and as a result, the average power comes down quite drastically and thereby eliminating the damage to the sample and it is precisely if you want to fully utilize the multi-photon advantage, you need to be able to really focus the pulse or focus the light in time as well, apart from focusing it in space.

So we do that and send it through the lens. Now when you do this, deviating from the ray diagram and actually indicating the real profile of the beam here. When you do that, what happens is in the beam progressively becomes smaller and smaller reaches about 2 omega before it starts to diverge again. So, now these pulses that are in time gets focused also in space. Both of this increases the instantaneous intensity localized in space because the intensity is the number of photons per unit time, per unit area, so we have reduced both of them, we are talking here about the peak intensity, peak power.

So that peak intensity in this space is very high, being able to create or being able to initiate the excitations that we have discussed before which is the two photon and three photon processes. So, once again all that we are doing this is that we are giving that kind of density of pulses, that high number of pulses for a very brief period of time and nothing at all for a long period, much longer period so that the average intensity and then the like load or the hit load is much smaller on the sample.

Because of this what you have is a very localized why localized because you are also focusing in space, so if you focus in space then you can see that the intensity is really at the maxima at the peak time, right. Whenever the peak arrives here, the intensity is at the top of its value and then as we move away, even if it is a peak intensity that you are following, because of the spatially distribution you are going to loose that intensity so that this sets the

path or the feature by which the intensity changes across the propagation direction of the light as a result, the probability of excitation also changes.

The center being most probable and as we move away from the focus, you would see that the probability keeps falling down drastically. So what you can ask is that, given that my intensity is causing a probability that is one, then how does the rest of the light distribute. Now, it is actually very striking. Actually, look in this picture here, so what we have done in this lab is to actually take a curette fill it with fluorescence. Fluorescence is a substance that absorbs in the green region and emit in the red region.

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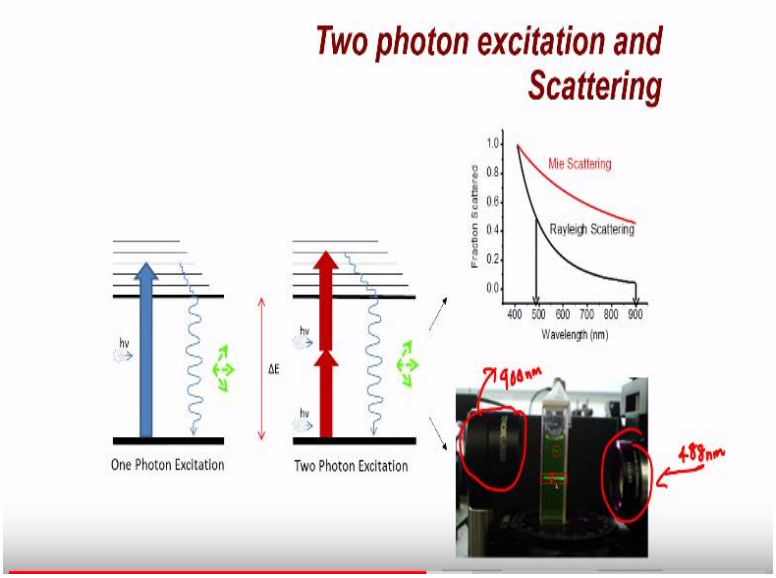

So, now what you see here is that we have seen this fluorescence before right, so we are focusing it down using two lenses, lens one here and then another lens, so inside this we are coming in with 488 nanometer laser, while here we are coming in with 900 nanometer laser, and what we are actually looking at is that when we focus, you will see this will be capable of eliciting one photon fluorescence and in fact you see that result, right.

It is all over the place and the entire path in which the light is traveling, while on the other hand when you are actually focusing light through this lens, which is focusing the infrared radiation, you see a very localized fluorescence excitation. This localization of fluorescence, allows you to do 3-dimensional resolve imaging without having to have a pinhole. If you have a fluorescence profile something like this where equal chance of getting exited all throughout, then you do need that pinhole discrimination to actually localize the photons that are originating from the focus. Otherwise, no other simple way to do that.

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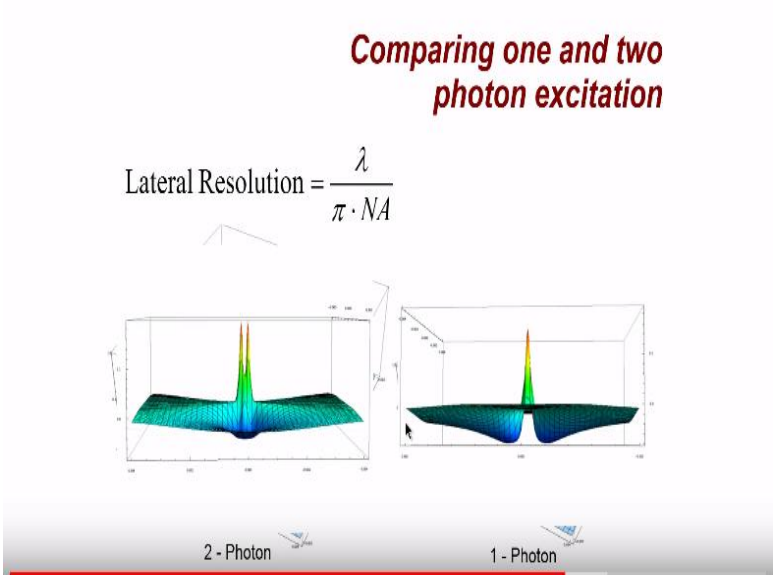

So, now if that is the case then why is this localization achieved, let us look a little bit more deeper. So now, we know that it is localized, now what are the properties of this multi-photon excitation that we need to consider before we restart using that for imaging or what for any other purpose. First thing is that, we know that the lateral resolution in a microscope is given by lambda by pi dot NA.

Now, if the lambda increases, the lateral resolution goes up, meaning, you will be able to only see larger particles and not smaller particles, which is what a good microscope would like to do. So, compared to one photon there, this is a common misconception that happens in the field where since you are using a larger wavelength, your lateral resolution would be compromised, will be twice as (()) (20:51).

However, the scenario is not that simple and trivial, so I am going to show you here the calculations that we have made, and dealt with the detail in that reference, but then I am going to bring out some of the salient features of this calculation. One, is that we ask the question at Z is equal to 0, let the probability of excitation be 1. Then, I would be able to plot out or write down an expression for the probability of excitation as a function of space.

So, when you do this, in this graph what we are actually seeing is distance as you move away from the focal spot, the focal spot is where my cursor is, where the central peak is 0, 0. So, as we move out what happens to the probability that the molecule gets excited by a 2 photon

means. So, if you compare that with one photon excitation, so clearly you see one photon is a little bit more sharper in terms of the width of the peak.

So, clearly this tells you that two photon process is the excitation profile is little broader, however, the interesting thing is if you actually look at the difference, that just quantify how much larger that the two photon excitation probability distribution compared to that of the one photon. So, you can think of this as a excitation probability distribution, so if you do that that is how it looks.

So, the peaks around the focus, right, means that is not surprising. We are having extra probability, so you are going to see more excitation around the focus in 2-photon. However, what is interesting is when you actually look at the 1-photon, so while the peaks around the focus is what we are doing here it is the lateral axis, you can think of this as the R axis. There are two axis, so I am going to talk about lateral and axial.

So, when I am talking about this, the first set of graphs, I was talking about you take a cross-section perpendicular to the direction of the propagation, this is how it looks like. Similarly, you should be able to take a cross-section allowing the Z axis and that is where we are really worried in terms of the localization and when you do that for 2-photon, that is how it looks. However, for one photon what you see is that may be this and this are more or less the same, however, what is interesting is that when you look at the excitation away from focus.

This increase in the excitation away from the focus even though the peak as defined by width as defined by reduction in the peak, or the Z at which the peak has come down to 1 by E is shorter. The actual excitation proceeds further thereby making this to have a larger volume compared to 2-photon.

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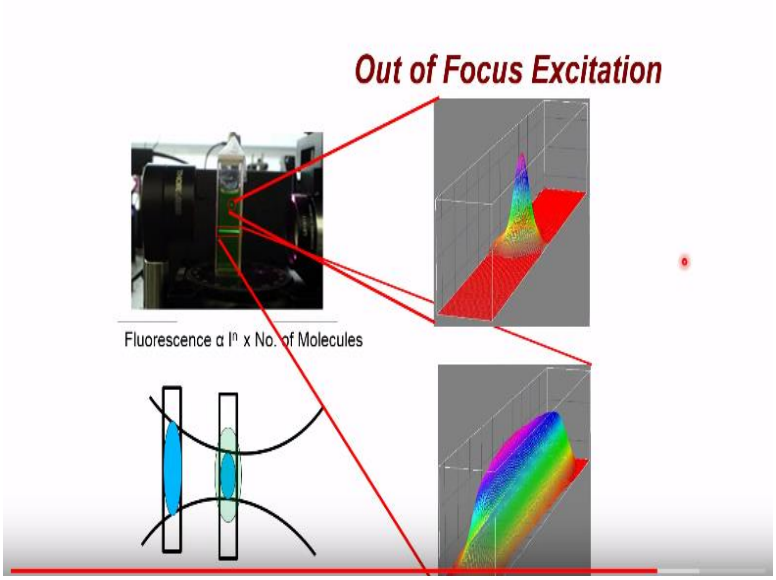

So the out of focus excitation are in some sense, it is the contrast that makes this really valuable and gives you the perception of localization of excitation and you can actually see that if I take this and then ask for a profile plot that is how it looks, which is very similar to initially what we have seen. It is a uniformly changing intensity, the density of the light, however, for the two photon it is very symmetric and it really did not matter.

I mean, you are actually looking at is really localized and it did not matter where whether you are before or after, but really localized in the Z plane, to tell you that it is the light, the excitation falls of almost to 0 than the focus even though cone here could be wider, but that is not what we have seen in theory. In theory, what you see is that both of them are very sharp, then why in this long line of continuous fluorescence, that is because it is not sufficient to take this alone.

There is also detection scheme, where we are actually taking this picture in an orthogonal direction to the excitation. The detector is setting at an orthogonal position, so you can actually spatially resolve in x, y this whole excitation spatial profile. There, let us look at what is happening. In an one photon excitation, so what you see is that as we focus the laser beam. So let us start with the n number of photons that are coming in as we focus the laser beam, the area of excitation comes down, so you gain in the intensity.

However, the number of molecules that are present inside the volume goes down, because concentration is kept constant all throughout, as a result, what has happened is that the total amount of fluorescence or the brightness that I see, remains the same whether I am looking at

this slice or the other slice. Because, this entire focal region, I mean the fluorescence looks very identical, in fact, that is what gives rise to this, not so localized, excitation beam present there.

On the other hand, because you are looking at the 2-photon, the loss in intensity as you go out because the area is broadened, goes a square as a result, nonlinearly falls. The probability nonlinearly falls and the number of molecules that are present also goes down, and as a result there is nothing to alter this, giving rise to a very localized excitation or increased contrast. So that is so vital in fact and what you can actually do is that.

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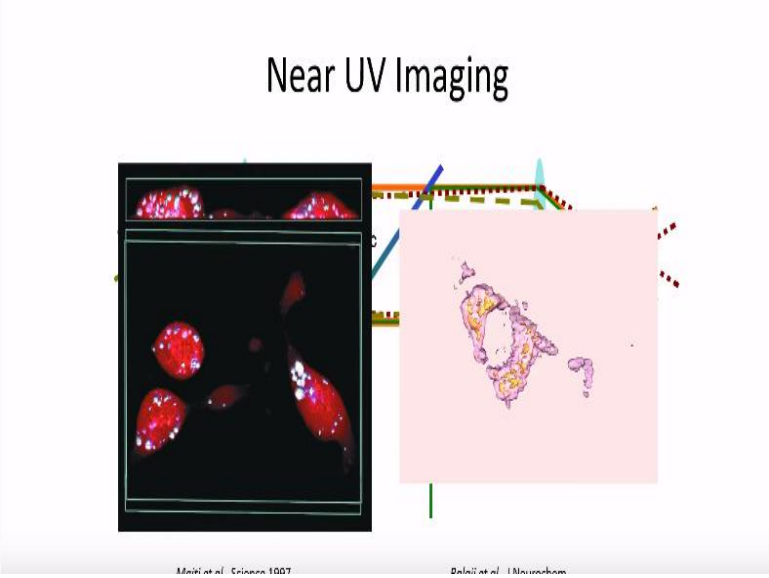

So now we have a very localized excitation and we do not need a pinhole anymore to actual detect the fluorescence all the time. All that I need to do is to actually collect all of this fluorescence, and I know that fluorescence originated only from the focus and like the one photon here. Since I know that the fluorescence originated only from the focus my localization accuracy is pretty good as a result I do not need the pinhole at all which means my spatial resolution comes not from the discrimination of a discriminatory detection but really localized excitation.

That really determines my localization or the resolution, in order to estimate that you have to actually take the I square and if you do that, what we see is that there is hardly any difference between the 2-photon or single photon fluorescence. People who are interested in knowing more, I would strongly recommend. Look at the reference that you are cited here. We have discussed it extensively there.

Now, the usefulness of this, it is not just saying that you can use it in a scattering sample or in a UV imaging people have studied it and these are serotonin, the molecule that I said they are absorbed in, so that what you see is a green is actually serotonin molecules present inside a vesicle in a mast cell and you can actually 3D resolve them and then the follow the dynamics and when you follow dynamics that is like an exocytosis in a live system that is a proof that you are actually killing the system itself and clearly you can very beautifully see this dynamics, and you can also in-live neuron.

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I mean not just a laboratory sample, but you are actually can do that in a live neuron and between the two and the three which is better, if you have to ask the question that again one can ask similar question about the resolution contrast, so forth and there is a objective way of determining this by actually measuring the contrast and what you see is that even though you do compromise a little bit on resolution, the gain that you have in contrast is so high that your three photon excitation is better if you want to actually want to study specific molecules particularly from native substances.

We can actually finish this lecture on the multi-photon microscopy right here, and then we will have the live lab session, where you will be able to show you how the lasers are and what is the light path and what happens when you are actually imaging using a multi-photon microscope in the lecture series covering the lab sessions. Thank you.