Optical Spectroscopy and Microscopy
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Lecture – 47
Fundamentals of Optical Measurements and Instrumentation

Hello and welcome to the lecture series an optical spectroscopy and microscopy, in this part of the course we were actually looking at the various different equipment's that are coming from the basic principles that we have learned and that can be built using this light source, routing mechanisms and then the detector, the schematic and one among that is the microscope itself.

These are; I mean we were talking about the laser scanning microscope particularly, we were talking about the confocal principle and then how using a confocal principle, we can 3 dimensionally resolve, we can image sample in a 3 dimensionally resolved manner. The latest in this kind of technology is the advent of multi photon excitation, on use of this multi-photon excitation in imaging called multi-photon microscopy.

And so to understand the principle and the advantages of why one would like to go for the multi photon microscope and then when would you use a multi photon microscope, let us look back at our confocal basics itself and revisit, understand a little bit about how we generate the image and stuff in there, so that we can better understand what we cannot do there and how multi-photon excitation allows you to overcome those limitations alright.

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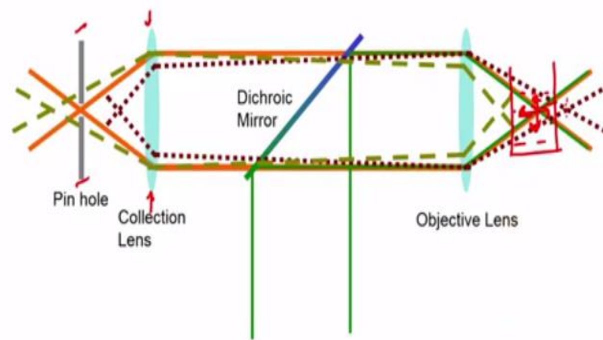

So, let us go into the lecture, so as we know that confocal principle can be summarized very schematic I mean, in schematically using these minimal elements right, you have a collection lens I mean and an objective lens, they from the lens pair l1, l2 so that we can define confocal points, right and then you have excitation beam of light typically, that is a laser in here represented by the green line through the ray diagram.

And we are focusing this excitation beam; focusing this excitation beam using an objective lens on to a sample alright, now the sample you can actually think of being placed somewhere here, okay and you have a solution that is present here. So, now what we are going to do; as we focus the light alright, when we focus, as we focus the light into the sample, the sample is going to; I mean the sample is going to get excited.

And then emit the fluorescence from when it comes back to the ground state, this fluorescence is collected by the objective lens and then we have a dichroic mirror as you know already as we have seen before has the character of reflecting the green wavelength and passing the red wavelength right, it is a when you can have it other way too but in this example, the way we have drawn it, we would like it to reflect the lower; we would like to reflect the lower wavelength, the visible light.

And then let the higher wavelength pass through red shifted fluorescence pass through, so when it does what you have is a parallel beam of light, it is parallel because you are collecting the light from the focus, as we had taken a point in the sample and at this point we

are talking about fluorescence originating from here, okay. So, it is this arrow that we are trying to represent by fluorescence.

And the fluorescence from this point is being collected back by the objective lens collimated meaning, the rays are parallel and then the collection lens focuses it down to a one spot. The reason why we are able to segregate a point from; a point that is located at the focus versus a point that is located before and after is very simple, as you imagine a point that is present outside okay, in this I mean, the way we have drawn it, it is outside the sample.

Imagine that if the sample were to extend here, I am just; we just kind of exaggerating, then if you do the ray diagram, so since it is going to come outside of the focus, it is away from the focus it is not between the focus in the lens but it is away from the focus, then the light that is coming out from the back; from the back aperture of the objective lens is going to converge okay.

The ray is unlike the rays before, the red solid lines that we used to represent the rays originating from the focus, instead of that now these dotted lines representing the fluorescence originating from a point that is away from the focus okay, so that is going to converge. Now, this would be made to converge, since it is already converging and now you are having another lens, a collection lens that is converging it even further.

Now, when it converges even further what is going to happen is that the focal spot will be before the focal spot defined by the orange lines right because it is already converging, so you are going to make it even more, it is going to convert it faster. In the same way, we can also look at ask the question about what happens to a light, so the fluorescence light that is emitted by a source that is between the focal plane and the objective lens, okay.

Now, if you do that then what you are actually going to see is that the since it is originating before the objective lens, what you are see I mean, it is originating from a point between the focal length and the objective lens, what you will see that it is going to diverge and as it diverges the and it passes through the dichroic making sure we have a quite enough dichroic, then it passes through the dichroic and then on wide enough lens.

And this lens is going to can make the fluorescence converge at a point away from the point defined by the orange lines; orange solid lines, so clearly you can see you have mapped these 3 points in the image plane that is defined by the collection lens. So, now if I want to actually look at one of the planes I mean, typically we want to look at the focal planes as we have seen explained before because the contrast is better there.

Because it forms the smallest size that you can actually see and so on and so forth, so given this to maximize the contrast and to detect the fluorescence originating from the focus, then the job is very simple all we need to do is to place an aperture right that is this aperture; this aperture right after the; right at the place where there is the convergence of the orange solid lines which is the focal point for the; it is a focal point at the image plane.

So that, that light goes; the light originating from the focus goes pass the pinhole and hindered while that of the originating from; while those light that are originating from the fluorophores present away; away here refers to a distance measured from the objective lens, so away from the focus is going to expand and only a small fraction goes through while that is closer to the objective lens, the light originating from the points that are closer to the objective lens are going to not converge fast enough to make it through the pinhole, okay.

Either way you have this discrimination coming in because of the placement of the pinhole and what you are actually doing is you are actively rejecting out the fluorescence originating from out of focus planes right, that are not present at the focal planes. So, now this is critical to recognize that the resolution basically comes from the; I mean, the detection discrimination, you are actually detecting the emission with discriminatory pinhole present there.

So that you reject out the other light now, this is wonderful because it allows you to localize your photons; the fluorescence photons either to the I mean, to the focal plane and then also restrict in x and y axis too, in the lateral axis because through the focus, so now that allows you to construct this 3D resolved images however, the problem is I mean, it is pretty simple actually, we will come back to that.

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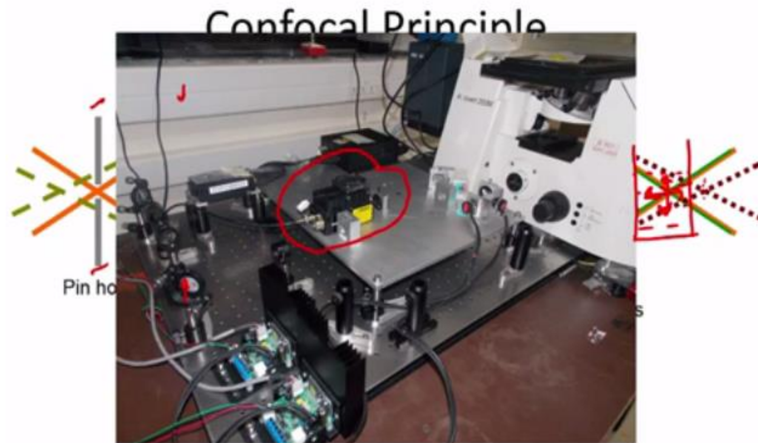

Axial resolution through emission discrimination

It is pretty simple in that you can actually do this kind of a construction in the lab, very easy, very simple construction and that is how that is a very preliminary I mean, very least number of optical component containing system and you can probably get it up and running in about a half a day already, if you have things all in place attach it to microscope just that the microscope body that you see here is just to place your sample and stuff like that.

Other than that you really do not, I mean and hold the objective lens, other than that there is no real purpose there because the imaging is all done at by using the photomultiplier tube that is present in this picture about here and then connecting I mean, correlating the photon; the amount of fluorescence that you measure in the photomultiplier tube to that of the position of the focal, focus spot on the sample, okay.

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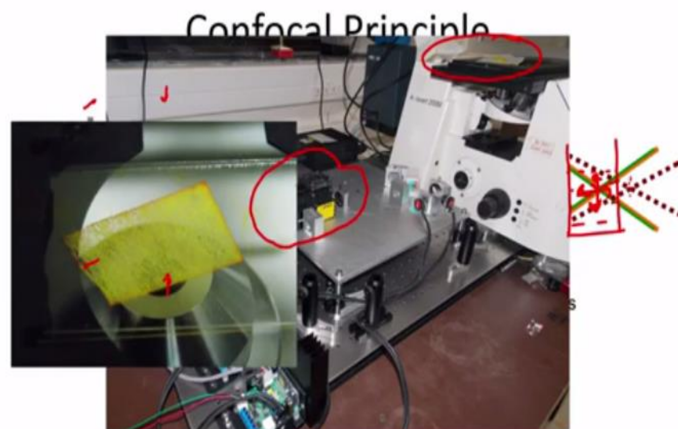

Axial resolution through emission discrimination

So, it is pretty simple in fact, as I said you can easily do that and this is just to illustrate the point that if you close in and zoom in onto the; this region, you will see a small tissue paper that has been kept and that is what, that is where your sample would go right, that is a fluorescent dipped tissue and you will see it is pretty easy to put together I mean, if you know what you are doing it is a really half a day job to actually put together. So, it is that easy, very, very useful robust however, it has its own limitations; number 1 like if you want to since the actual resolution is through the emission discrimination.

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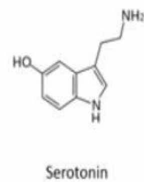
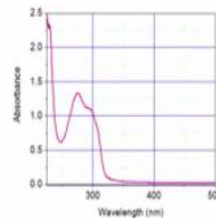
The Limits

a) Imaging near UV fluorophores:





Follow the dynamics of these molecules

If you actually would like to; I am sorry, if you would like to image substances that are scattering by nature in nature, you would be facing a lot of trouble because your emitted photons do not necessarily now follow a defined path as a result, if you like to I mean, they do follow a defined path that not the defined path set by the geometric optics that we have, made use of to construct this optical device.

But they can actually, typically they diverge more than what you would expect as a result, your ability to collect the fluorescence from the focal point goes down drastically, the further you go further is your detector away from your collection I mean, the further is your collection optics from your sample, the more you lose the light and in fact it becomes prohibitively impossible to detect anything beyond about a 40 micron thickness in a live tissue.

Because that is; that at that level the amount of scattering is way too much that the light, hardly any light reaches for you to actually discriminate and say that okay, I have I can

actually look at the photons originating from the focus. So, then what do we do; we need to find some other alternatives, so that is one. Second problem is that many times in live systems what we see is that the fluorophores that we want to probe observe light in the ultraviolet region.

So, you cannot imagine an ultraviolet laser being used to focus the light into a living tissue right, your ultraviolet light into living tissue can got, can cause a huge amount of damage, breaking the bonds and particularly if you think of that light passing through all through the sample right, it is a; remember you do not necessarily have much of a say in where the I mean, where the light can excite during this typical confocal where it is you are using a light to match the energy gap.

So, if you match the energy gap all throughout its path; all throughout its path until the focus and beyond the light is going to excite though with the differential amount at the focus will be more probability of excitation while outside it is less and so forth as a result, you might see some kind of spatial localization however, excitation is all throughout, we will see that in a little bit more detail in few slide from down.

But you end up causing this ultraviolet excitation all throughout that is very bad that is not good for the tissue, so if you want to image near UV fluorophore okay, not even just UV but near UV because we can, we know that the emission is typically red shifted, so even if your emitted light is in the visible near UV, then you have to use the excitation that is close to UV.

So, it is not possible and many neurotransmitter molecules and particularly here I am giving an example of a serotonin which even see can be nicely imaged using the multi-photon microscope that we will be describing in a minute and you can see that the excitation is in the UV range because 400 nanometre is and above you are calling it as a visible and it is very much inside the UV range.

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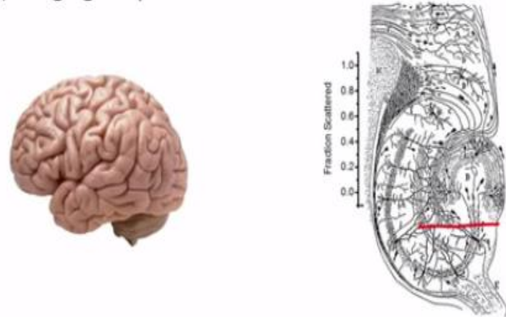

So, then if you and then as I telling you that if you want to image inside the brain as many of us would like to do in neuroscience to ask questions regarding what happens to a molecule or what happens in the brain in response to an activity that you are (()) (18:23) and so on and so forth because of the scattering nature of the brain, you will not be able to use a regular one photon scheme here and you will be limit, I mean you can use it but it will be very limited in the resolution.

So, then in these cases and this is just showing you as a function of wavelength of course, if you somehow, some function of wavelength if you look at the fraction scattered, whichever the scattering model that you like and you take it, what you will see is that at as we go higher in wavelength your, the scattering coefficient, I mean the amount of scattering that you see is reduced quite drastically, it is not linearly reduced.

So, your ability to reach deeper tissues, deeper regions are, is high and it can be quite beneficial if somehow we could excite the molecules using the longer wavelength regions, okay. So, now how do we go about doing this?

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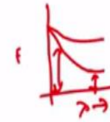
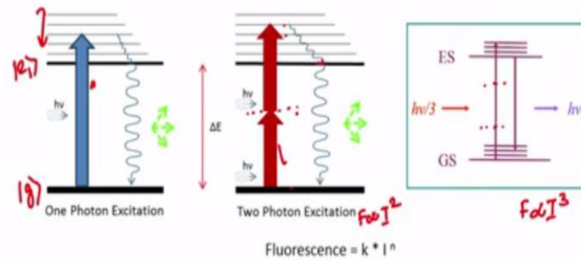
Way out: Multi photon excitation

Follow the dynamics of near UV fluorophore

Need to image deeper inside a scattering tissue





Fluorescence = k * I ⁿ

In order to do this, first is the feasibility of; of course feasibility of a excitation itself, right so, one of the ways that it can be done is through what is called some multi-photon excitation wherein what you are; so, what you are going to do is the following; now you can see here set of images right, so where we are try to represent the electronic energies in this black lines, the thicker black line represents the ground state.

And this is a first excited state, so if you like it in our notation this could be g and this could be e1, so now these are all our vibrational states; vibronic states, vibrational and rotational states, okay. Now, when you shine a light the molecule goes to the absorb satellite and goes to the excited state and we know this is our simplified version of the Jablonski diagram, wherein you see this relaxation and then it comes back emitting longer wavelength and the lower energy fluorescence photo.

Now this is not the only way to cross this excitation, you can also do this which is you come in with lesser than I mean, you come in with roughly half the energy of the delta e right, so your individual photons now here have only half the energy okay, now if you remember our very initial, the beginning lectures where we were estimating the probability of interaction or excitation, you will see that you would remember that we have taken the first term in the; I mean, the second term, first order correction term to the EK; the coefficient EK in deriving the Fermi golden rule.

You can actually go ahead and do this for the next order, when we do that we will be able to see that there will be a nonzero probability I mean, very low the extremely low probability

but a nonzero probability yet of these photons, the photons of lesser energy roughly half the energy of the band gap getting absorbed interacts with the molecule and they get absorbed but for the molecule to actually do anything meaningful, it needs to observe 2 of that to actually reach to the excited state.

So, that is what we represent by 2 of these arrows, so 2; it is a 2 consecutive absorption; sequential absorption right, one followed by the other, so one way not the, not necessarily the way to look at it, one way is to actually think of that the first photon takes the molecule from the ground state to some kind of a virtual state that is halfway between the final and the ground state and then since it is a virtual state, the lifetime is pretty, pretty small.

Within that if the next excitation, next interaction were to happen then you are going to; see this 2 photons are being absorbed by this molecule resulting in a 2 photon absorption taking the molecule from the ground state to the excited state. Remember, when we actually looked at the matrix element and we were actually giving the symmetry arguments to determine whether a given excitation is possible at all or interaction is possible at all based on the symmetry of the initial and the final states.

In that spirit, if you actually look at it we already know that the molecule can get, can absorb 1 photon and then go to the excited state, which means that sets the limits on the kind of parity that these 2 can; these 2 states can have, we have already seen that. Now, here what we are looking at is that there is modification to that I mean, modification meaning that the excited state cannot change its symmetry right, the excited state has the I mean symmetry just that has like this.

And the ground state has to have symmetry exactly like that, which means we know that the ground and the excited state need to be of opposite parity, cannot be of same, in which case we would expect in going by this law. hat logic we would expect ground state and virtual states should be of opposite parity and the virtual state and the excited state should be of opposite parity which will end up making the excited state and the ground state be of same parity.

So, what is going on here; what it turns out is that in the molecules where there is the centre of symmetry, right and you will always see that the exact stage; the exact state where the

molecule reaches okay, is slightly different from the state where the molecule will reach by 1 photon excitation. Again, we might probably see this little bit more detail in later it just to, so that you understand the fact that I was constantly saying that it is close to but not exactly half the energy right, it is close to half.

The reason is because of this, one of the reason is because of this and once it reaches the excited state, it does not really matter because either it reaches here or here is going to relax by send to the excited state; relax down to the excited state, come down to the excited state by radiating this energy to the solvents and from once it reaches here, then the molecule does not have a history of whether it went; it came that to point through 1 photon or through 2 photon, which means the fluorescence that you see here will not have the emitted photon.

The photon that you get here would not; the photon by itself would not have any history about whether it has originated because of 2 photon excitation because of 1 photon excitation in general. Similarly, you can also think of exciting a molecule in through 3 photon process, wherein you are going to talk in terms of 2 kinds of virtual states. Now, the advantage of this is first immediately you know that they are of lower energy, which means of higher wavelengths, so the scattering already; the scattering problem is already taken care for you alright.

So, I mean if you remember the graph of the fraction scattered for both for Rayleigh as well as for me, of any day as we increased the lambda, the fraction that is scattered is lower for higher lambda, then further lower, so scattering is lower. Second; now think of this problem of UV right, so what you have done is that normally with a 1 photon, you need a UV radiation, UV of a higher energy radiation.

But then now what you have done is that because of this multi photonics excitation you are going with lesser energy considerably, lesser like if for 3 photon, it is actually 3 times lesser I mean, roughly it is 1/3rd of that energy which clearly puts it into the visible radiation, light radiation, visible light range as a result, you can actually shine the visible light and cause take the molecule from the ground state to an excited state that normally would require a UV, okay.

Now, nevertheless it can come back to the ground state by emitting UV that is a I mean, come close to the violet region but nevertheless you definitely can excite the molecule by shining visible light. This is very, very critical please keep that in mind, one of the characteristic of this is that if you had to measure the fluorescence alright, the total amount of light produced by a given population of molecules as a function of the varying light intensity.

Then it has the characteristic relationship which is fluorescence is equal to some constant proportionality constant times I to the power n, alright where n is the order of excitation for 2 photon f would be directly proportional to rf is equal to k to the power I square and for 3 photon, f is equal to k to the power I cube. Now, this is one of the advantages, this is the wavelength advantage that we talked about.

In the next lecture we will actually look into this a little bit more and then see that apart from the wavelength advantage, there is a localization advantage, how it comes about and what does it mean for our microscopic or imaging method and how one go about thinking of which method of absorption or excitation to adopt, we will see all of this in the following lecture and then we will also get to see this in action, the setup at least how what it means in a lab setting in a lab course alright, thank you and see you in the next lecture.