Optical Spectroscopy and Microscopy
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Lecture – 42
Fundamentals of Optical Measurements and Instrumentation

Hello and welcome to the lecture series on optical spectroscopy and microscopy, in this section of the course we are actually covering devices, we were talking about telescopes and how to implement that telescope using a tool and system and a mirrors in a laboratory setting we saw that how you can actually move the mirrors and the goggles of the alignment etc. Today, in this lecture what we will see is that what goes on in setting up a confocal detection, alright.

So, first I will explain what is the confocal detection followed by the setup itself, optical setup itself in terms of paper and pen in terms of the theoretical understanding of how the confocal detection works using Ray diagrams and then we will see this in a laboratory setup, very similar to how we had seen the telescope and then we talked about the telescope and then went and see the laboratory setup, okay.

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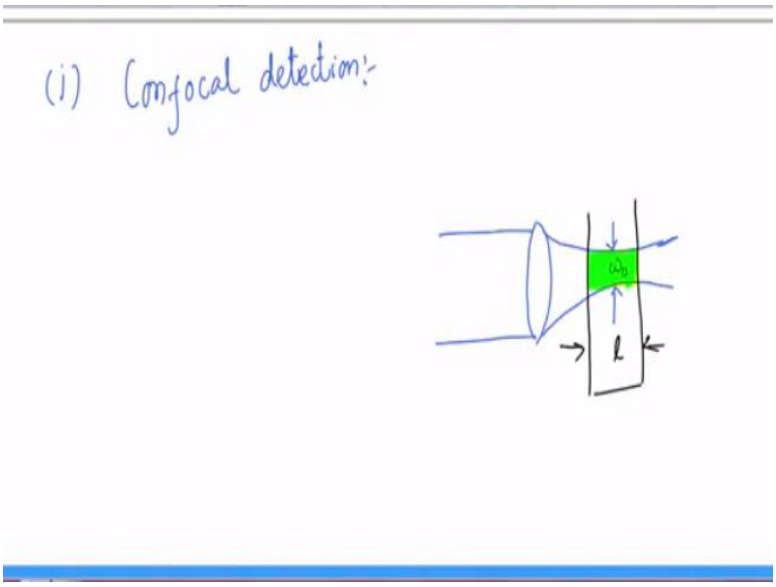

So, the confocal detection we can think of as a deduction that sorry, a confocal detection per se we can think of as a localized detection; what is happening okay, confocal detection we know that when we take a beam of light and then focus it using a lens, we can actually localize the beam of light okay, incident beam of light to a small volume in space right, we

know that if you send the beam through the lens, if it is a Gaussian; if it had to be a Gaussian beam, then we know that the at the focal point the spot that we follow will be omega 0.

And this omega 0 is more or less a constant over a region and we call over a range of Z values and we call that as the Rayleigh range, so now the confocal detection is our ability to detect the light or the light photon that are originating from this volume okay, predominantly originating from this volume, often when you are actually trying to detect in a spatially resolved manner, you need to be able to localize the photons that are originating.

The fluorescence that are originating in all from all over the space to a particular volume and in this case, we want to maximize that to that of the excitation volume, so that the deduction that we are actually looking at the fluorescence coming from a localized space; localized subspace of the sample which is fluorescing okay, so imagine you have taken an laser beam in this case, blue laser light.

And then putting it through a lens and then let us say we are having a cuvette and in that cuvette we have fluorescein alright, so you will have yellow, green fluorescence that will be coming out and so that is a green fluorescence that comes out from here, from this volume and we want to restrict our I mean okay, the way I have drawn it, it is actually the cuvette is; so I am going to redraw the focus spot.

Basically, what you want to do is; you want to collect the fluorescence only from this region and not anywhere else, so the way I have drawn it clearly what you can see is that the excitation itself is offering a very high selection which basically, restriction of the so, if the cuvette is of the length l and you are really range itself is of the length of the cuvette, then there is not much of a problem you can actually go ahead and collect the entire fluorescence and it will correspond to a fluorescence originating from that localized volume.

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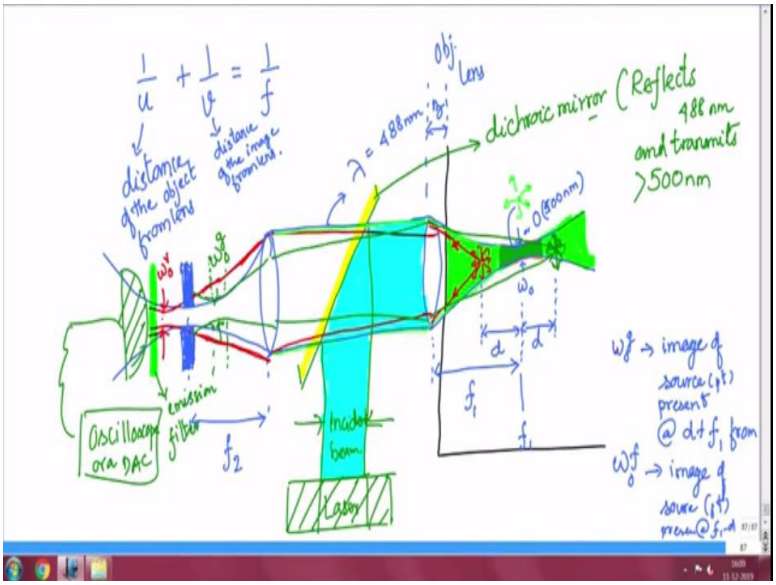

However, what happens is that we want to probe volumes that are of that is typically, diffraction limited, typically of a femto liter volume in which case typically, the geometry or the setup that you will see would be more or less it is a pretty much the same except now, the sizes; are the size of the beam and the focus would be very different right, let us say we are coming in with blue light, okay and blue laser light.

And the idea here is that when we are going to focus; our focus is pretty tight meaning, almost close to the diffraction limit and we know the diffraction limit is of the order of the wavelength of the light itself and the blue light that we are talking here is say okay, so that this lambda is from let us say argon ion laser, then it is 488 nanometers okay. So, now if you focus it using a lens down to a spot, now that spot is going to be very minuscule, okay.

So, even this is pretty large but the spot that we are going to look at will be after order of okay, because you remember lambda by; find the lambda by NA it is of the order of that, so we would be of the order of the lambda we are looking at, NA is typically close to that of the refractive index of a medium that is used as an immersion medium for this lens, we will talk about this lens sense etc., in a minute.

But the order of this would be in microns or 100's of this would be of about let us say 500 nanometers okay, given this what happens is that your same cuvette, if I were to draw would be spawning an area which is much, much larger right, this is about half a micron and we are talking about a cuvette that will be typical that will be huge in the same scale if I have to draw, I would not even be able to represent in this scale.

So, in such a case you can think; you can see that the whole of this medium right, the whole of this path of the light you can think of being under the excitation light, so we can think of the fluorescence, okay being originating from all over this place because the medium itself is present all over the place, though with what it looks like as of a higher intensity near the focus, which would mean that if I were to collect the fluorescence from this such a sample or such a set up, then the fluorescent photon in principle could have originated from any place not necessarily only from the omega.

I mean the region surrounding the focal right, the region surrounding the focus let us represent that by this yellow highlighter on top of this so, oh sorry okay, so let us say we would be; we would like to restrict our detection to only to this Rayleigh range okay, so now how will we achieve that. The set up that allows us to do that is called as a confocal setup now, what the claim is that if you actually can let us say put in a lens, another lens okay, such that the light that is emanating from here.

So, I think about the fluorophore that is present at this point okay, other point here right, that this fluorophore is emitting light in all 4 pi direction right because it is the probability of emission is isotropic right, so we know that the fluorescence the probability of emission is isotropic and then the fluorescence is depends on a few things which is the amount of substance that is present, the ability of the molecule to observe the light.

And the ability of the molecule to emit the absorbed light, right and in nowhere we see that an isotropic in with respect to the direction of the detection as a result, it is you have an equal probability of detecting the photon in entire 4 pi directions or solid angle, what happens is that a fraction of that corresponding to this the solid angle subtended by this lens at that focus, that fraction to that of the 4 pi comes in and gets collected.

So, it travels back, okay and that gets collected by this lens, since it is coming from the focus okay, now that light gets collimated back. Now, how do I; what tells me whether it will be collimated or not, let us go back to our simple lens equation, so the lens equation tells you that 1 divided by u, the distance of the plus 1 divided by v equals 1 divided by f, where u is the distance of the object from the lens.

And this v being the distance of the image from the lens, while f is the focal length itself, so clearly when you when somebody originates, when the object is present at the focus your image will be formed at infinity, so this is the or in other words distance of the image from lens. So, if u happens to be at; u or v either of them happens to be at f, the other one will be at infinity.

Or in other words, if you have an object present at infinity, then the rays emanate; light rays emanating from them will at the lens will look as if they are parallel and when they pass through the lens will focus at a point f and similarly, if you have an fluorophore located at the focus emitting the light, then that light when it passes through the I mean, passes back through the same lens, will come out parallel.

And since we have this lens here, another lens here will get focused back, now depending on the okay, so now this distance is f2 and this is f1, so now at this place if you have to actually put it in aperture alright, whose diameter equals to that of the diameter of the beam, then what happens is that only the light that is originating from the focus would be able to make it through while all other light say, for example I mean to represent in red colour.

The light that are originating from a point source that is present before the focus okay, so the light is getting emitted by this point source again in all four 4 direction. Now, what is happening is this light source, the red light is originating from a point before the focus, as a result if you look at this equation you will see that the light that is coming out from this lens would be diverging.

Or in other words would be if able to form the image on the; because on the side where the object itself is; the fluorescent object itself is; what it means is that the light will be diverging and if since it is a diverging light and that is coming and hitting this lens alright, unlike the parallel beam of light since it is a diverging light that is actually coming and then hitting this lens.

The focus of such a divergent beam would be further down here alright, so in order to make a; so what it means is that it is going to form the focus somewhat like that there by I mean, where it is trying to find from the focus here, so let us see, so if you carefully notice what

happens is that the aperture here right, so let us fill it out, the aperture here would actually block this beam of light.

As a result, you will see that there is a selective loss to the light that is I mean, the light ray that is traveling that is; that corresponds to the light source that is originating from before the focus and similarly, if you were to think of a light ray that is actually originating after the focus okay, so a dark green now, what you will see is that okay, so again you can think of photons originating with the same 4pi direction.

And then unlike the divergent rays that are emanating from this light source in this green, what you will see is that the light rays actually originating from this place would rather travel such that your; it is coming away from the focus, so I am going to draw it somewhat like this, so they will start converging right because they will form a focus somewhere between infinity and the lens on this side of the lens.

So, they would be converging in fact and then when they reach this lens, this lens makes them converge even faster, so let us say they get focused around this place, so in again what you will see is that the focus of the green spot, so let us call that as omega 0 g and let us call this as omega 0 r, okay. So, the omega 0 g is the focal or the image of the focus point or image of the point source that is present at distance D from the focus.

So, while this the omega 0 r is actually I will call that as image of light source or a point okay, in this case point present at d + f1 from the lens, this you can think of away from the focus and the omega 0 f is the image of the source of the point source okay, present at f1 – d, okay, so either of these images if you see gets blocked by the light source, the only I mean the way we have put the light; the aperture is such that only the light that is emerging from the focus actually makes it through without any loss.

While all other points suffer a loss, it is not that they do not reach but then what depending on how far they are from the focal point you will have an increased or they will suffer a loss in their intensity of the photons that are getting collected from, there by what it all means is that you have introduced a selective deduction, your deduction now is very selective. Now, if you want to actually focus your image or if you want to actually collect the light from a different point, you have one of the 2 options.

You can either move this lens back and forth with respect to the sample okay, what you are doing then is you are actually moving the distance, the relative distance of this lens we will call this as an objective lens because it is close to the object or the sample here and the relative, the movement of the relative distance between these 2 are change in the relative distance between these 2 changes the focal spot or the light ray, the point source that will be forming an image at the aperture, okay.

So, you could think of moving this lens distance d away from the sample in which case what you will see is that the image of the red point right, red point in our image, red fluorescent source would be formed at the aperture and the blue; the omega 0, the original omega 0 that we have drawn, the light source emitting I mean, emerging I mean, emanating from there, they would be getting blocked by this aperture.

Similarly, you can also move ahead assuming that you can actually; assuming there are no steric hindrance and you move forward by a distance d, then the green light source indicated by the green arrows; dark green arrows, the light rays from them would make it through the aperture while the red and then the blue points would get blocked.

So, this way of detecting right, this way of imparting a selectivity in deduction such that we can actually look at specifically at one of the I mean, a sub volume okay, in here what we are trying to correlate as a focal plane to the focal plane, so these points of the object that are present at the in the object plane and that of the aperture plane, they are called the confocal points.

Basically, they are the use and (()) (29:16), okay so they are the confocal points as far as you keep your aperture such that it is at the confocal point of the excitation volume which you want to keep do, then what you are doing is you are maximally collecting the light maximally and selectively collecting the light from the light sources that are present at the focal volume or that within the Rayleigh range of the sample.

While by moving this lens, you can actually decide where exactly in the sample this focal volume can be localized. Now, let us think a little bit more and then say what happens, if I start moving the aperture itself, instead of moving the lens if I were to move the aperture,

what happens? In principle, you would if you had to have light coming from all over the like a luminescence not like a fluorescence but in luminescence present.

Then they are of completely equivalent motion either you move this lens or this aperture, the first lens and objective lens or your aperture they would or the relative distance between the aperture and this lens you would probably get an established and equivalence, however what you see is in fluorescence when you are actually moving the aperture let us say instead of being present here, if you had to place the aperture such that it corresponds to omega 0g, okay.

Then it is true that you are actually looking at the light emanating from a point d, ahead of the focal plane, so you would say that okay I am selectively looking at that point, however the point to be remembered are the one, the main difference here is that if you ask the excitation probability of a fluorophore present there, that is lesser than that of the fluorophore that is present at this place.

This is because the excitation light itself is focused by this lens l1 and then since it is away from the focus, the probability; the intensity is less because the numbers of photons are the same and then you are localizing that number of photons to a larger area compared to that of the focal plane. As a result, the probability of excitation is lesser for this dark green, so you would see lesser amount of light.

Apart from this or of this, I am not also talking about one other aspect, which is the size of the spot itself okay, we will talk about this later in the laboratory we are actually we will be able to see this Airy disks and the shape of the; these images, there we would talk about it or and then we will come back once we are talking about confocal microscope not just the detection, confocal microscopy we would pay a little bit more attention on the size of this.

And how do we estimate that and all that stuff but right now, assuming that being not a factor then even without that you will see there is a difference in the amount of light that we will collect for the same fluorophore and of the same amount of light that is incident through the sample. Now, all along I have not mentioned few of the important components when it comes to practically setting up this confocal detection.

One of them being our ability to send in this blue light itself, the 488 nanometer light itself right, so where is the laser originating from; clearly, the laser cannot be present here right, in this end of it because you have to focus it and then of course that is not I mean, that is not making it through one code but that is not very fruitful but instead what happens is that the laser is indeed located at a direction orthogonal to that of this path.

And then the beam that is coming from this laser, okay that gets deflected okay, so this is a big you can think of this is a laser head, okay and that is where incident beam okay, since we said it is 488 nanometer the incident beam, it is a wide beam, so you could make it wider by using a telescope and in fact, we will have to typically it is that is how it is done, so the beam that is emanating from here gets reflected by a special kind of a mirror we have seen this.

We call it as a dichroic mirror and the property of this dichroic is such that it is going to reflect the blue in this case; in our case it is the excitation light while the fluorescence that is being collected by the lens goes through alright, so that it gets transmitted okay, this is a special that kind of a mirror is a special kind of a mirror, we call it as a dichroic mirror. So, let us okay, so now dichroic mirror.

Now, this dichroic reflects in the geometry that I have drawn, we have chosen the dichroic such that it reflects 488 nanometer and transmits everything above 500 nanometer which means the fluorescence which is red shifted will go through while the excitation beam will get reflected. Now, we will see in detail how exactly to choose this dichroic and what does it mean to say that it is reflected I mean, is it 0 or 1.

Is it going to reflect on not reflect, what you realize is that it is not quite 0 or 1 but there is a quantitative way of actually determining this, hold on for; let us hold on those details for a little bit but the point here is dominantly it is going to reflect the beam; excitation beam and dominantly it is going to transmit all the fluorescence, okay and when it transmits even after it is transmitting selectively more of the fluorescence, you will have some amount of reminiscent scattered excitation light.

So, typically what you would do is that before putting in a detector alright or for our confocal detection alright, so the detector you would like to place it somewhere here okay such that the entire light is falling on this detector okay and it is connected to either an oscilloscope or

digital data acquisition card okay. So, now and then to a monitor and so forth, the idea here is actually you can measure the amount of light that is falling on this detector.

Now, before it falls on this detector you also will have an emission filter alright, so this actually filters out any residual light that may be present okay, any residual excitation light that may be present following the scattering can will be removed by this emission filter typically, these are banned force filters, we will again talk about how do we choose this emission filters and dichroic (()) (39:26) in a minute.

But the idea here is that we could actually use this kind of a setup to specifically localize your photons to and detect I mean, 0 in on photons that are originating from a specific location in the sample, so that is about the confocal detection process itself, what we will see in the next class is that how do we actually go about selecting this dichroic filters and the emission filters and what process I mean, how do we actually choose them practically in the lab.

So, this will be useful even when we are actually going to do build up a setup and then say and think about which are all the components are need to be put in okay, we will see that in the next class.