**Optical Spectroscopy and Microscopy**
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**Lecture 44**
**Fundamentals of Optical Measurements and Instrumentation**

Hello and welcome to this series of lectures on Optical Spectroscopy and Microscopy. We are looking into how the imaging equipment work and imaging microscope work and various aspects of that. In that light, the first thing that we are going to look at today is to understand a class of imaging systems. It is called the laser scanning systems or point scanning systems to be specific. So the way we will go about this is to kind of understand.

We have previously looked at a way of creating a localized excitation and detection right using confocal setup, using an aperture and a focus light. Be that or something else that we will be seeing later in terms of multiphoton excitation where we specifically create localized excitation. Either of this, that does not matter; howsoever it be you are able to localize your photons that you detect to a particular region okay, either through special filters the confocal apertures.

Using confocal apertures, you localize your photons originating from that point that is of interest to you in X, Y and Z in from the sample plane or by creating a localized excitation okay through multiphoton. Either way if you create, what you have done is that you have created an ability to look at a specific point in space and the photon that are emanating from that. However, for creating an image, as we will see in this set of lectures that you need to be able to do more than that right.

Clearly, you need to be able to create such representations as a function of space. I mean it is not just one point, but you need to be able to create a set of points, an array of points if you like and how are we achieving that and how do we go about devising an optical equipment that is capable of doing that. That is basically the essence of modern-day laser scanning microscope alright and when you are talking about point scan systems.

That is what we are going to see and clearly the approach that you are going to take us, we are going to define what an image means right in a very loose manner, but sufficient enough. It is a loose, but still sufficient enough to understand the instrumentation point of view, as less in terms of what limitations the instrument imposes on to that image itself okay. So let us go and then look at what the images and then say how we go about creating that.So if you are talking about an image.

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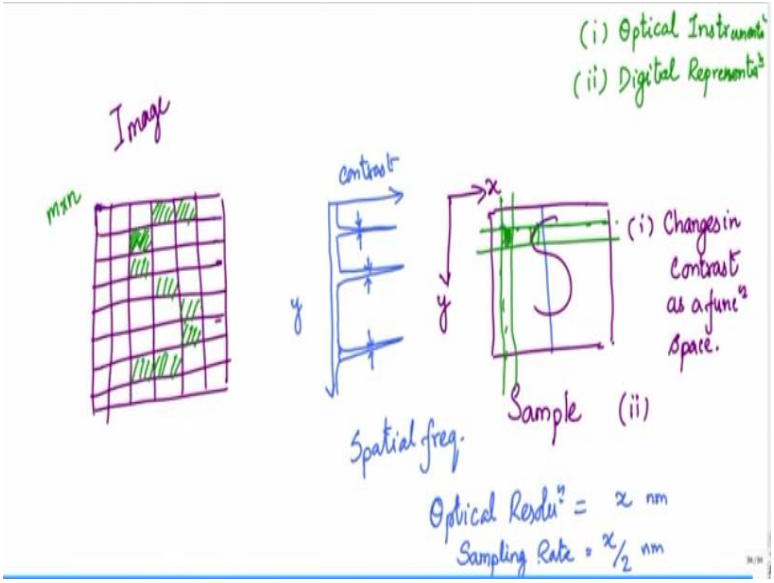


So then we can think of you having a sample alright and a sample could just be some structure in here structure like S, some arbitrary structure. So, now when you say that, so this is my sample alright. When I say that I am actually imaging, what I am actually dying to do is I am trying to create a representation, preferably a digital representation but image in general could be just a representation, could be any form of representation, but of the sample itself.

When I say that, sample has many features alright. The features starting from its curved bendy line and how do I actually, what do I mean by taking a representation of that right. So which feature am I actually looking at? So typically, when we are looking at imaging in the field of microscopy or in any other way like in a camera or wherever we are looking at, when we see an image what you are trying to do is that we are trying to see okay.

There is a change in contrast as a function of space okay. Contrast could be coming from optical detection of the light, is just it could be a simple transmission. That is what we do, when we are actually putting in a sample slide and under the microscope and then look at through transmission microscope right. That is optical detection which changes in the absorption, reflection or any of them that results in the throughput of the light right.

So the extent to which this transparency, I mean the sample is transparent could be changing as a function of space in X and Y. So if you capture that, then you call that as a transmission image, of course right. So there what you are the contrast is really the change in the absorptivity of the material or the sample as a function of space or it could be a fluorescence emission, often we see that right.

So we will be looking at fluorescence images in live systems or in soft condensed matter systems. So in a fluorescent sample what we are actually looking at is the changes in the intensity of the fluorescence that is happening across the space. Now this intensity per se could be changing because of so very many different reasons. One prominent thing could be the number of fluorophores in the space right or in the X-Y localization could be different.

As a result, the local intensity of the fluorescence could be changing or any factor that contributes to the density starting from the ability for the molecule tubs or if locally because of some perturbed environment are for different reasons, the fluorophore present, even though the floor for per se the number density could be exactly the same all throughout the space; however, the local environment in terms of there may be a quencher that is concentrated in space.

In which case, by imaging the fluorescence intensity, you in turn image the fluorescence quenchers concentration in space. You can do that or it could be just, that could be in the fluorescence quencher could be a very different stuff and one of them very prominently people use is through resonance energy transfer and things like that where there could be different reasons for taking away the fluorescence right. So you can also do such kind of imaging.

Or for that matter, you can generate a new kind of a signal okay, in response to localized excitation okay. So now then you can map that as a function of space, that signal. So depending on which of these you are following the contrast is going to change right. So you call a particular image based on the signal or the contrast mechanism that the physical phenomena that underlies the contrast variation across space.

So if the controls were not to be changing across space and there is no point imaging. You can just take one simple point measurement and they do not have to resolve in space right. The fact that you want to resolve in space means something is changing in space and whatever that is changing, we call it as a contrast and that contrast we are going to capture it. So now let us look at the sample here in the S. So what you are actually looking at is very plain and simple.

You can think of this as the light falling on this particular square and then the place where I have drawn the S is reflecting less compared to the other or you are actually looking at the monitor, then the place where you see it as a S is because the transmittance of that screen at that place is lesser. I mean amount of light that is coming out in the place where there is S is less compared to the other places, light, I am assuming it is being transmitted. So we need to capture this.

So that is one; that is changes in contrast as a function of space. Second is that how fast is it actually changing, like for example and I can actually take, I mean if I actually I can start zooming this and zooming particularly around this region, which is I keep going up, at some point you will start seeing that these are actually pixelated right. So I am going to go back and so you can actually go down to that individual pixels and then see that okay.

They are actually in isolated squares. They are coming and changing in isolated squares. So now when I say how fast, what I am asking is that if I were to take a profile right, in let us say. Let us call this as X axis and let us call this as Y axis and as I draw a line across my Y axis okay and then now along this line, I can actually ask how is my contrast changing. So clearly; if you look at it, so this is my Y right and then now in this plot what I am actually plotting is my contrast.

So clearly wherever it is where the yellow line is crossing the black, you are going to see that the contrast is high alright. I know that is going to happen at three different places, because it is being S okay. So now in some sense when I ask about the speed, what we are actually after is what is the sharpest peak that; we can actually measure right? That boils down to the resolution itself right; that the optical resolution.

I mean no matter however hard I try the extent to which I can focus my light right, that is fixed. That is given by our uncertainty principle given that I can only focus the light using a lens in one direction and then the largest angle that I can think of is about 90 degrees and that sets the limit on given a wavelength of light photon, then the localization accuracy of the position is limited for you right.

So localization, they will be spread over a certain space and we will not be able to tell any finer than that where the given photon is. On an average of course, you can say. You can actually now go ahead and since we know the functional form of this function, you can actually really, really, really nicely try to localize the peak or the most probable value of it and then thereby you can go below that spot size.

Of course, so that is how the super-resolution microscope enhance the resolution to make use of these ideas, so one way or the other, but then here what we are actually talking about is the optically limited microscopes. I mean when you are looking at the optically limited resolution and then that is going to determine how fine a change in contrast I will be able to detect and that you can think of as the frequency content of the signal.

This is just how fast the signal is changing or how fast the contrast is changing. So if you actually look at this, as we can actually take this and just to make ourselves, make it easier we can actually try to rotate it okay. Rotate the image, so let me see if I can actually rotate it. If I were to actually move it around and plot it, you can actually see that if your orientation right. So this for the editing, if we can actually show that it has been after it is rotated, that will be great at this point okay.

So now we can imagine that there is the rate, there is the sharpness with which these peaks are being portrayed reflects, how fast a change in contrast that you can actually see. Now this speed or this frequency at which the contrast changes, we measure in terms of something called as a spatial frequency and this is the spatial frequency at which the contrast is changing as a function of space.

This is the frequency at which the contrast is changing as a function of space, but then if you are going to take a representation of this, then how fast should my sampling be right. Now you can there is a pretty elaborate sampling theories that go around, but the essence of it is that you actually want to sample at following the Nyquist criterion or Nyquist sampling. So roughly speaking, you need to be at least twice or more, faster than the fastest frequency that you see.

So if your optical resolution is some, right optical resolution okay, now remember this is determined by uncertainty principle right. That is given by uncertainty or if you like the way formalism, then it is different by the diffraction limitedness of the limited focus spot size and that let us say is some X typically in nanometers, because this of the order of the wavelength and then what you want to do is that you are sampling right.

That you have to construct a grid here that is going to represent the sample itself and each square in this grid okay; now each square in this grid is going to have a value that is proportional to the contrast itself right. So if we want to do that, then the first question you want to ask is, how fine a grid should I be drawing? So that is related to the optical resolution that our microscope is capable of providing.

So where I said let us say if optical resolution is about x nanometer, then you need to be sampling at least all right. I mean you need to be sampling at a rate, your sampling rate needs to be greater than x by 2, in fact nanometers. Now this is very handy. Please note this and keep in mind. Because this is what is going to tell you or this is going to tell allow us to say that how fine a grid we should be constructing to represent the sample itself.

Anything more than that, you have a disadvantage of accumulating more noise; anything lesser than that you have the disadvantage of losing the information that possibly, which you could have collected all right. So now let us go into the representation itself, where I have been drawing the grids right. So now we talked about what size the grid should be right. Is this sampling rate essentially tells you okay?

So, now in real space, I am going to actually put in this grid here and once I put in my grid on and then this is my representation space right. So this is you can call this representation as image and in this image what I am actually going to do is that I am going to construct an imaginary grid on this real space right. So this is given by let us say an m cross in matrix or a grid. So now this m cross n grid, I am going to imagine is going to be on super laid, I mean overlaid on top of this sample.

So once you do this right, now we can think of, I am just going to draw one of this element. So now like that you have an imaginary grid that has been overlaid on top of the sample. Now at each of this sample, I am going to measure; I am going to integrate the entire contrast in the real space right. So in this place, there is really nothing there zero. So I am just not going to have anything. On the other hand, if you think of a place like this right, so there is some contrast.

So what is going to happen is, that you are going to actually fill that with some number proportional to the amount of contrast that we have. So if you keep doing that for this entire grid, then you can actually see that you might be able to generate some sort of a representation that is like that right. So clearly this is course. This is not super fine, because the way I have drawn it and that would mean that we have not sampled properly.

I mean sampled at the rate where you like to sample. I mean if your optical resolution is x, I said you should go x by 2, when we have not gone that high. Second is that there is no variation in the intensity along this curve. So I am actually having an uniform number, I mean if whatever the number that we get proportional to the contrast that remains exactly the same all over right. So but then the point is that you are being able to generate this matrix or to say a representation from the sample and then that you call it as an image.

So in principle, what you are actually trying to make is that I am going to take a snapshot of what is happening and that snapshot should be a representation, so that later for whatever the measurements that I am going to do, I do not have to actually do that on a real sample. Either because I mean it is happening at very fast rates, then you may not be able to make that measurement that fast.

But you can once you have the representation, you can actually extract this information from there or it is happening at a very, very microscopic level, it is impossible for us to actually be able to capture the entire thing in the whole space right. So if it is a too much of I mean very dense set of information coming distributed all over the space, then you may not be able to see it. Either way, you are actually getting an image and the idea of this imaging is to be able to generate a representation and that a representation acts like an image.

Now I told you that we are actually going to superimpose this grid and then actually go point by point and keep measuring the contrast okay. I have not told you how do we actually achieve this and what is the optics behind achieving such kind of a thing all right. So now that will be the focus, I mean that will be the next step and focus towards the constructing a microscope which can actually generate these representations.

So the goal number one is understanding the optical instrumentation. Goal number two is understanding how do we actually create that representation. So here I am actually going to talk about a digital representation. When I am actually talking about a digital representation, what goes inside a microscope and how do I visualize or how do go about thinking, I mean go about understanding an image and what is it means in terms of the numbers that they represent okay. So these two would be the focus for the next discussions.

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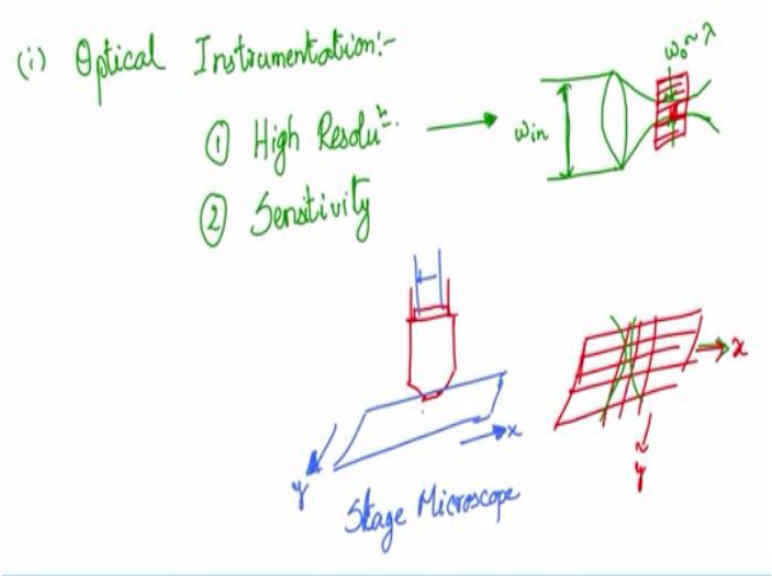


And first let us look at the optical instrumentation itself. So in order for us to understand the optical instrumentation, first we need to know what is the requirement that; we actually looked for? First thing is that we want to generate the highest resolution possible right. We want to extract the maximum amount of information that could be present in a sample, in a spatially resolved manner, so which would mean that we need to be able to have the requirement.

Number one would be high resolution okay and number two of course sensitivity. So now what we will do is that let us look at how we normally generate a high-resolution image. For this, we said if you want to get the smallest possible spot size or the focal spot. So that would mean we know from the lens equation. We want to go such that the width of the incoming beam right, we call that different ways, we can width of the incoming beam should be as large as possible.

Actually, you want to be matching the back aperture, the size of the lens itself, so that you end up creating a spot that is omega naught, which is of the order of lambda. The smallest possible omega naught that you can get. Now; that here is the problem. Now if you want to do this and then we are going to have a sample and the sample is going to be, let us say a plane or a cube and you want to create this, make this measurement as a function of space.

Then you need to be able to place this spot point by point. So one simple way you can think of is that, look you have you are a sample okay. So now and all that you are doing is that you are

actually focusing the beam at this point and then you can move your sample in x and y direction and then at each point you actually remember, how much was the contrast. You actually store how much was the contrast.

So now that is a very simple and primitive way of doing that and many times I mean in a modern day and some for some good reasons, we do such kind of imaging. These are called as stage scanning microscopes. Basically what you have is a lens, an objective lens, this is basically a combination lens and we have talked about this earlier. So a combination lens and that is being held onto a microscope and then the light, there is an incoming light.

The excitation light that comes and that is really wide, so that the objective lens can actually focus it to a tight spot and then below which you have your sample okay, sorry below which you have your sample and this is mounted on a microscope stage and this stage can be moved in x and y direction and as it is moving and making the measurement, I mean as it is moving and it is going to excite different places of the sample.

The light is going to represent excite the different places of the sample and then at each point, you actually measure the contrast and then store it and register it onto a matrix that is constructed m by n matrix that is constructed. So now this is good, but the problem with that is that you need to be able to move the stage and you need to have to move the stage and then the stage, mechanical stage is really hefty and heavy and they tend to be slow.

Unless your sample itself is huge and I mean nowadays, they are becoming really huge and there is no point actually to be able to use; to be able to move these stages when you are moving in a few 100ths of a micron size. If you are moving like few 10ths of a millimeter or centimeter, then yes, it can be helpful, fruitful to move the stage itself. So these are called the stage scanning microscopes and alternate to that is not to have to move the stage of the microscope the objective.

But the beam itself makes the beam actually scan all right. Now that has some issues, we will see it in the next class and particularly has to do with the fact that you need to be able to have a wide

beam that is coming into the lens and then still be able to move it in the x and y direction so that has its own technical difficulties and we are going to talk about what those technical difficulties are and how can we overcome that in a nice and elegant optical way and then we will look at a practical implementation of that into a microscope and that will be in the next class.