fBiophotonics
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Module 06: Bioimaging: Principles and Applications
Lecture 27: Microscopy Techniques

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Welcome back. So, let us continue with different microscopy techniques, again this is the general information given. So, in the last class we saw that using a compound microscope you can simply magnify the image of a particular specific object. Simple microscope is just a lens, compound microscope uses two times, two different lenses, one is an objective, one is an eyepiece and both of them magnifies and the result that you see, the resultant image is a magnified image of the first image that got magnified from the object, so it is twice magnified or more than that.

So, now that you have the image you now need to further polish it, polish in the sense that you want to make it better, you want to give finishing touches, you want to see how you can better or improve the resolution that we discussed in last class, how you can make it with a better contrast, better brightness. Now, all of these things depend to a significant term, significantly on the type of sample that you are looking at.

Obviously, if you have a metal sample, you are looking at a piece of gold jewelry under a microscope it will scatter or it will reflect light differently than if you are looking into say tissue paper, an oil cloth or a paint or some kind of a biological organism, what kind of biological organism, where it has taken from, it if is a tissue, if it is just a cellular structure, if it is an organ, if it is some kind of exoskeleton say for example, you are looking at skin, you are seeking looking at nails, I know skins are not exoskeleton, nails are, so nails, hair.

So, all of these things will have firstly an uneven surface, so when you are trying to focus different depth will give you different amount of focus and hence, there will be different kinds of images or aberration or problems, then their illumination or the light that falls onto them they will scatter it differently. So, depending on your sample we have tried to resolve or modify our microscopy techniques, prominent one which I personally used is the dark field microscopy.

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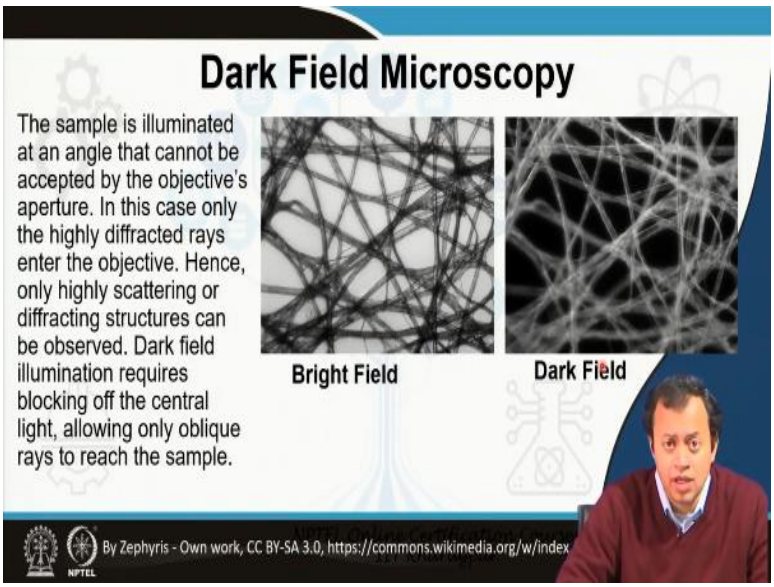

Dark field microscopy, I have personally used to look at metallic nanostructure, so I make very, very small structures made up of gold, atomic scale structure or slightly bigger than that and try to shine light through them to see their scattering. Now, it may so happen that you have different types of metallic structures which shine quite a lot of light

and all of their scattered light which scatters quite a lot of light not shine, when you shine light the scatter.

They are scattering all of them jumble up and you get a direct spot, you get a jumbled combined average spot where you are unable to distinguish between one point from another, they are not all one together, they are different and your resolution is also good but too much of light coming from all of different areas have overwhelmed the detector, have overwhelmed the eyepiece and you are unable to see or you are unable to distinguish.

You are unable to distinguish between one part or another because all of them is like seeing ten different light bulbs, all simultaneously and looking into them, ten different light bulbs all connected together, all adjacent to one another and all of them are illuminated and it may feel like it is just one big light bulb.

So, we therefore go for dark field microscopy, what dark field microscopy does, it uses some kind of a barrier, it is some kind of a barrier and it closes the most central point. So, if you have the lens like that you put a barrier here, so that only the light coming from the highly diffracted light rays enter the objective.

So, dark field illumination requires blocking of the central light, so you have a lens, the light is coming from here, you have blocked it, just block a little bit portion, so only light that can come at an oblique incidence, that can come at oblique incidence can actually go into the objective, allowing only oblique rays to reach the sample.

Example, I will give you example. So, this is the bright field image of a tissue paper, so these are the strands, these are the straws that make up the entire tissue paper matrix and this is the bright field where all of them are illuminated together. And this is the dark field image, we have taken the same sample, same tissue paper and this is a bright field image where all of the light, all area is illuminated and here only the light that is scattered from this strand certain oblique incidents are coming.

So, thereby you see these areas where there are not strands they are not reflecting because these parts are block, light coming from this diffract and form an oblique angle to reach

the detector. So, if you have highly scattering material, these are not but still they can be utilized, highly scattering material you use dark field microscopy, you use dark field microscopy and it sometimes help. Obviously, it has its own disadvantages, when you are blocking some amount of light, when you are blocking some amount of light obviously, the area is becoming darker.

So, it is like putting a curtain in front of your window, you use curtain in front of your window, why, to block some of the light but then if it becomes too dark you have to switch on external light to see. So, it is something of a similar principle, if it is too much sun outside you might put some amount of blocking window, curtain to block the light outside but it has its own significant disadvantage, you might have to switch on external light, increase the light power and that can harm your sample.

But if you are like me who are looking into very, very scattering objects like metallic structures, metallic nano structures, dark field is important. So, again as I said it depends on the type of sample that you are going to look at. What do you think we use when we are looking into biological samples? If I want to see certain cells shall I go for bright field imaging, where all of the cells are illuminated or shall I go for a dark field imaging?

I am looking at a cell, say blood cell, I have taken a drop of blood put it under a petri dish and I want to see, I want to just see the drop of blood, see if I can resolve the red blood cells, white blood cells etcetera from my microscope, what kind of illumination do, would you like to put a block, would you like to put a curtain, would you like to block the central light. Remember, blood has hemoglobin, hemoglobin has iron do you think iron scatter light? Think about it.

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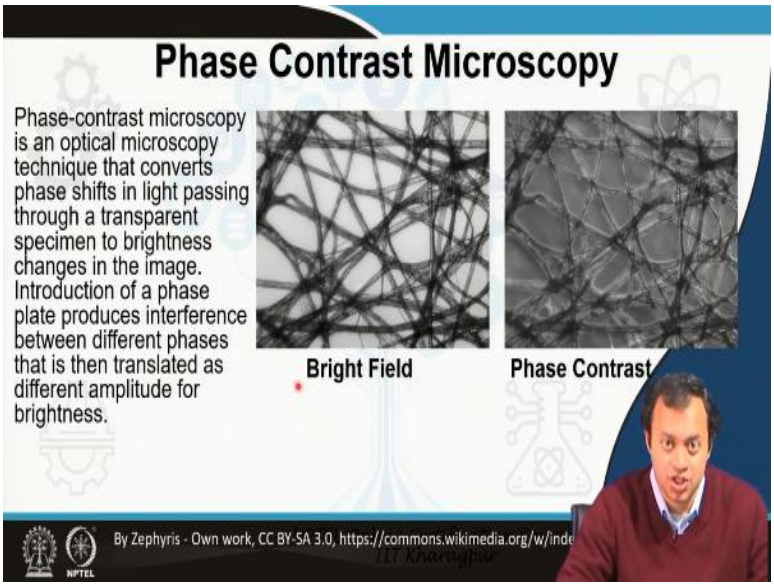

Similarly, you have phase contrast microscopy, where there is a phase shift between light coming from different areas, so there the light which is hitting this area has a different phase as compared to the light heating as this area. So, difference in the phases they pass through phase plate, we put some kind of a phase plate that differentiates more between the two different lights of two different phases coming to it.

We introduce a phase plate, previously we used a curtain here we are introducing a phase plate that produces interference between different phases, so the light coming from this has a particular phase, you know what phases I have discussed it in first class. And the light coming from this has a different phase and they interfere and the difference between the two phases are translated in different amplitude of brightness, thereby you see some of the finer structures.

So, these are the two most common dark field microscopy and phase contrast microscopy, this is again the same thing, same tissue paper which is seen under a bright field as well as phase contrast microscopy, it depends on you what exactly you are trying to look at. And what kind of sample you have, what exactly you are trying to find and thereby you go for all these different modes of microscopy.

And, yes, I have used Wikimedia as images because you do know that we cannot simply copy paste images from scientific journals and books because of the copyright violation, we have to have produce images which are free and if you think that several of my images are from Wikipedia or Wikimedia commons.

Well, the reason being they are free, I ask you to look into other different beautiful images of bright field phase contrast, dark field, just to a Google search its only that I cannot put it in my lecture and disseminate because that will be gross violation of copyright, you can see them but you cannot distribute them or you cannot utilize them for your own profit.

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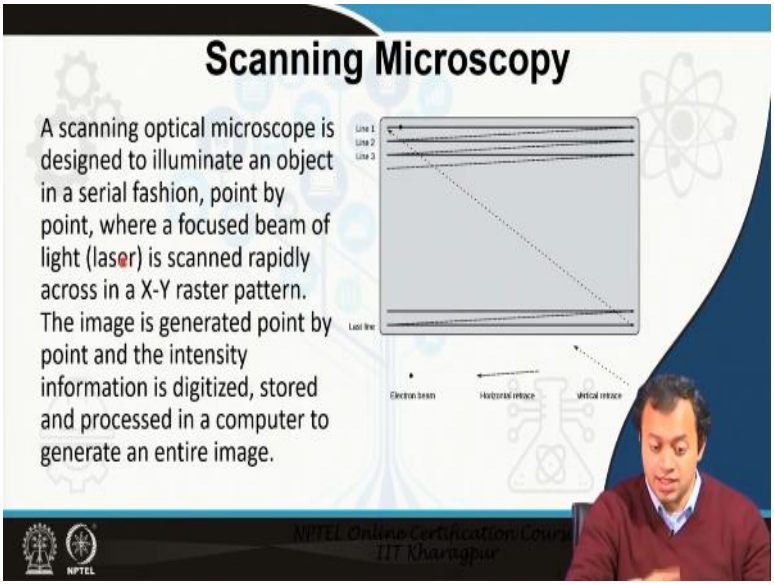

So, now comes the most important part, scanning microscopy. So, pay attention this is going to get interesting, so instead of you have a sample and you are sending a light to illuminate the entire sample, what we can do, we can scan the sample with a point light source, why, because your sample, especially in a biological matter if you are looking at a tissue or an organ or cell it is not going to be flat, it is not going to be smooth.

It will have depths and curves and there will be different type of scattering at different area, when you shine a complete light you get an average information out of it both in

transmission as well as reflection. So, what we can do we can scan it, in an optical microscope usually a laser light is used as a point.

So, if this is your entire sample, if this is your entire sample you illuminate specific areas of the sample in an x, y direction using a point source of light. So, this is your point source of light instead of illuminating the entire area at the same time, you are going to send a light here instance one, instance two, instance three, instance four, in x and then again come back to y and then again come back to.

So, this is y, this is x direction and you will move your laser scanner, you will move your laser pointer very rapidly in this particular direction and then you take an image from each single point, so you can think of it as pixilated, you can think of it at each single pixel, you can get the information digitized, pixilation store and process in a computer to get the whole entire information, this is scanning microscopy.

It is mostly used in scanning electron microscopy ACM all of you must have heard of scanning electron microscopes but we used it in optical microscope as well, in fact, I cannot prove it but people say that it started scanning optical microscope first then scanning electron microscope which at this present moment I cannot prove, but that is usually what my intuition says but I might be wrong.

So, scanning microscope instead of illuminating the entire surface with a light bulb and taking an average information of the reflected light or the transmitted light that has gone through, is illuminating every single point, the point what is the total point depends on your spot size, depending on how big your laser point is.

So, say you have an area like this 1 centimeter by 1 centimeter, you have a laser whose spot size, the size of the laser beam that is heating, say 1 micron, so you are 1 micron, 1 micron, 1 micron, 1 micron you will go in x direction, then go y you have a scan like this and take an information, take an image from just this part, this part, this part, this part digitized all these information, corroborate, analyze, compare, coagulate and process it and produce the whole information and produce the whole information, this is scanning microscopy.

You must have seen scanning printers, these old printers where there is a paper you must have seen in India especially, rail tickets are printed, if you have ever the pleasure of going and standing in a line and buying a train ticket, a train journey ticket, remember the printers where your train tickets are printed or at least it used to get printed few years ago, you put a paper, well the ticket paper and the scanner goes like this dropping information, dropping ink, so it is something like that but much faster using a laser thing.

So, that is scanning and we basically ok use raster scan then vector scan, raster scan is like this vector scan can be anyway. This actually used electrons, this is the image for electron beam but here we are using light or laser per se, this scanning microscopy is very much related to confocal microscopy.

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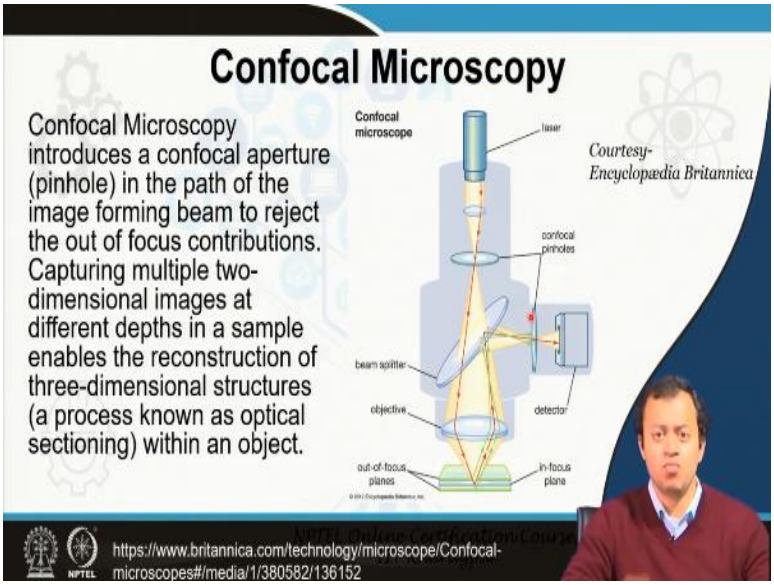

You must have seen confocal several times. Confocal microscopy introduces a pinhole, an aperture in the path of the image to reject out of focus contribution. Now, here also pay attention. So, confocal microscope, this is your microscope, this is your detector that utilizes a pin hole that utilizes a confocal aperture, that utilizes another curtain which has small hole in between, that hole only allows the specific light which is focused.

So, when you are sending light, laser light even to out of focus and in focus planes, different areas of the plane. One part of the plane will be in focus, one part of the plane

will be out of focus because this is not smooth, these are a tissue will have multiple depths, different types of heights.

So, the light that is reflected or the light that is scattered couple of them will be focused, here this particular red ray is focused, the light that is coming from out of focus planes are non focused, which when is put into a confocal pinhole and this is acting as a filter, this is acting as a filter which has a very, very small point very, very small hole, pin hole and it allows only the focused light that has come, so that your resolution become better.

So, confocal microscope is a spatial filtering microscopy, spatial as in space based, as in length based. So, it uses a pinhole to allow only the focused light, the light that is coming out after scattering or out after getting reflected from the sample, especially in a non-smooth different height sample, few of the light waves are in focus, few of the lights are out of focus, it only takes the focused light.

Now, combine confocal microscopy with scanning microscopy. Scanning confocal microscopy or laser scanning confocal microscopy, can you combine these two and think what happens. You have a sample, you have a sample like this, very large area. You are focusing a pin point light source, 1 micrometer, 2 micrometer, 20 micrometer usually spot size at 50 micrometer, 50 micrometer spot size the beam size is hitting, this area 50-micron meter area or somehow that area is illuminated.

In that area not, all portion is focusing all the light that is coming out, not all portion is properly, completely, uniformly focused, some of them will be defocused, some of them will be in focus, it is going into a pinhole, the pinhole is rejecting the defocused lights and only taking the focused light out into the detector and this thing is repeated all over the sample.

This thing is repeated all over the sample and you are using computer analysis, using image processing all of that, you are reconstructing the entire image of the entire sample from all these specific points. Meaning, confocal microscopy, laser scanning confocal microscopy that utilizes fluorescence at a bit, I will talk to fluorescence later, but laser

scanning confocal microscopy ensures, ensures that the different areas, different points of the samples are illuminated separately, not one at a time.

And the confocal microscopy ensures that only the focused light, only the focused light is coming to the detector, thereby the defocus lights are rejected, thereby you are increasing the contrast, you are increasing the resolution, you are reducing, you are increasing basically the signal to noise ratio.

Now, there is no requirement that confocal microscopy has to be always associated with scanning microscopy or vice versa, scanning microscopy can be without having a pin hole in front of the detector, but when you combine these two techniques, when you combine these two techniques confocal microscopy and scanning microscopy using a laser, laser scanning confocal microscopy you get beautiful images with high resolution, better contrast, better brightness, higher signal to noise ratio.

At the end of the day what we need is a signal to noise ratio, the light that is focused is signal, the light that is coming which is defocused is noise. So, when you have reduced the noise, when you have reduced the defocused signal you have automatically increase the signal to noise ratio. So, that is the overall point, so thereby you increase the resolution and you try to look into more and more different materials, different samples.

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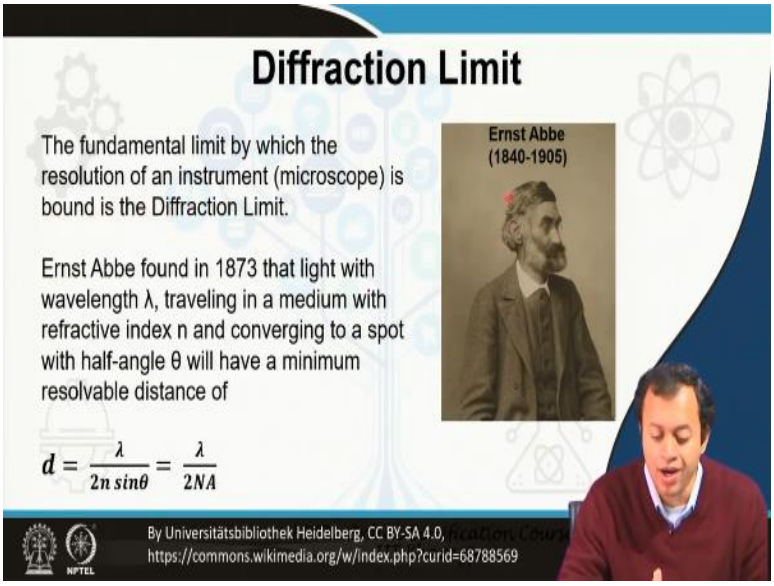

However, there is a small problem, there is a small problem and that is the diffraction limit. There is a fundamental limit under which the microscope, the resolution of a microscope is based on, meaning, I am not talking about aberration or error in making lenses or the problem in the lens or the manufacturing defect or anything. There is a fundamental limit which limits or which bounds the resolution of an instrument.

This gentleman Ernst Abbe in 1873 working at I believe university of Vienna figured out that the minimum distance, remember resolution, the minimum distance a microscope can actually resolved is given by this particular formula lambda by 2n sineθ, n is the refractive index usually 1 air, sineθ the same cone half angle basically 2 by NA.

The maximum value of sineθ can be 1, so and n is also 1, so lambda by 2 NA is approximately equal to lambda by 2, meaning what is the significance of that? If you are trying to utilize say you are trying to, you have a laser which has a wavelength of 500 nanometer, so 500 nanometers visible say it comes around green light.

So, or say you have a light of 600 nanometer, 600, 700 nanometers, say red light, red light 700 nanometer, 650, 700 nanometer is the wavelength of light, is the laser light since this is red, I am using 700, 650, 700 nanometer red light is the light of your laser, is the wavelength of your laser by which you are illuminating your sample.

The minimum distance you can resolved is lambda by 2, 700 by 2 that is 350 nanometer, meaning if there are two objects that are less than 350 nanometer apart from one another, your instrument, your microscope no matter how beautifully it is made, no matter if the lens is perfect, no matter if the lens is converging every single beam of ray falling into it irrespective of phase, irrespective of amplitude, irrespective of energy, irrespective of strength, no matter what it will still not be able to resolve it.

It will still consider any two points less than 350 nanometer that is lambda by 2, that is 700 by 2 as one single object. That is the fundamental limit, that is the fundamental limit called diffraction limit or Abby limit, given by d lambda by 2 NA.

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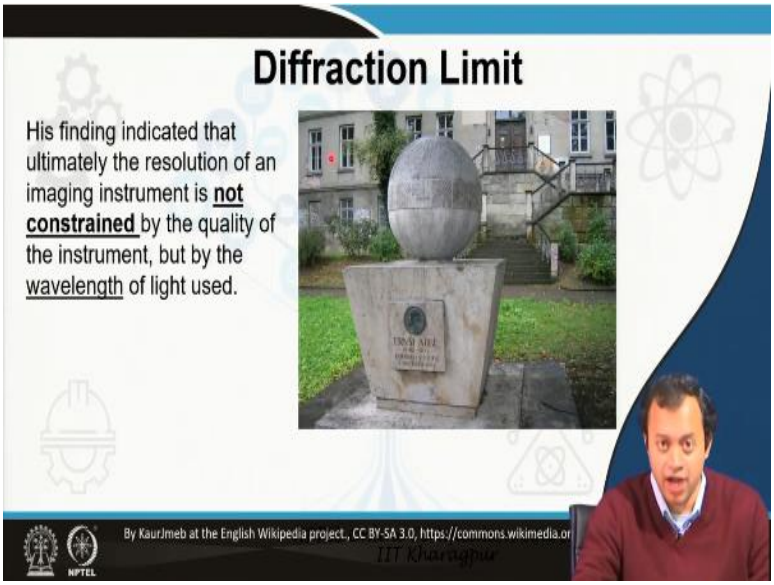

So, it is depending not on the imaging instrument but on the wavelength of light used, remember this there is a fundamental physical limit, just like you have fundamental physical limit, you cannot ah go faster than the speed of light, several different types of limits are there. Similarly, you cannot, basically light will diffract, meaning if the wavelength of light is very, very large compared to the slit, compared to the object, compared to the difference, compared to the pinhole light will not be able to resolve, light will consider it as a single object.

If there are two objects whose size, whose differences, whose distance between them are half or less than half, then the entire wavelength of light, then the entire wavelength of light, light will diffract, light will bend around the corner, diffraction is a property of light and light will not be able to distinguish these two as separate things.

So, we have a problem, so Ernst Abbe was so famous that, you perhaps have heard of the companies Zeiss or Zees they make microscope. So, Ernst Abbe was one of the I think co-founder or a partner of the Zeiss and this is the university of Vienna, this is the memorial plaque where this formula d is equal to lambda by 2 n sineθ, this is a very, very important formula for all microscopies.

Do not worry I am not going to discuss the life and times of Ernst Abbe, I have been criticized that in laser class I discussed quite a lot about the lives of scientists and that wasted several people's time, is just that I think laser is such an important discovery in human life, in human civilization that we should all know how we came to that its equivalent to discovering of wheel or agriculture but many people criticize it

So, I am not going to discuss about Ernst Abbe live, it is just that you become that popular and you become that famous that they made a plaque out of in your memorial and they put the formula that you have discovered in your name d is equal to lambda by 2 n sin theta. Most significant, perhaps the most significant formula in the life of microscopes.

So, basically it tells you that no matter how good your instrument is there is a fundamental limit that it can only image up till this distance, up till this resolution and no more, it can only image this much minimum resolution and no more. Photonics technologies breaks this limit, in my opinion the most important, significant achievement of photonics based optical imaging techniques is that we can bypass diffraction limit and can image one single molecule using light beams which are not twice but 10 times or even if I be little bit boastful 100 times larger than its size.

Photonics technologies are important because we break the diffraction limit, we bypass the diffraction limit, I have done it myself, it is not that I am somewhat special, any photonics engineer knows how to do it, any photonics person have used it but that is the point where photonics comes in microscopy.

Remember, I told you from the very beginning photonics is not incorporating microscopy, microscopy is a Bonafede field, a very strong field and not just optical microscopy, ion beam, electron beam microscopy they are going on for a very long time and it is a very, very strong and important field.

Photonics is just a part of it it taking just the optical microscopy part of it and then improving it by breaking the diffraction limit, by breaking or bypassing the diffraction limit where we are able to analyze, where we are able to image materials, points, dots,

spots who are separated at a distance much, much less than the overall wavelength of light. That is what photonics can do, I will tell you how in the next class with examples.

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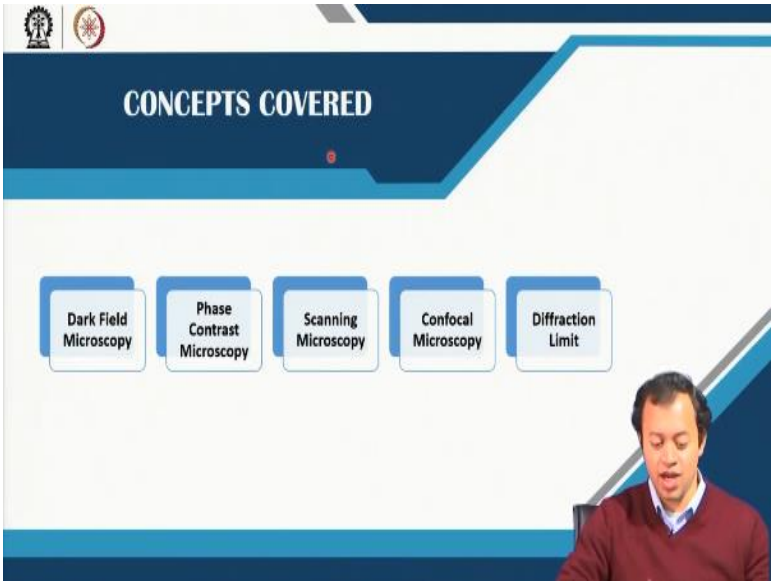

And these are the topics that I discussed.

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What I want to show you is, read this particular reference number 2, Beyond the diffraction limit in Nature Photonics, I have given you the doi, this is a very nice article

that got published in nature photonics, 2009 article Beyond the diffraction limit. Just try to see what are the different ways, I will be telling you no doubt about it, that is these two classes are merely the trailer, I will be discussing in detail in the next three classes what we can do and what are the future of imaging, bioimaging according to biophotonics.

But it will be better if you just go through these particular references, beyond the diffraction limit, published in the very prestigious journal Nature Photonics. So, with this I will conclude today's class and I will see you in next class. Thank you, thank you very much.