Fundamentals of optical and scanning electron microscopy

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Module – 01
Unit-3 Variants in the optical microscope and image formation
Lecture - 06
Opaque stop microscopy
Phase contrast microscopy

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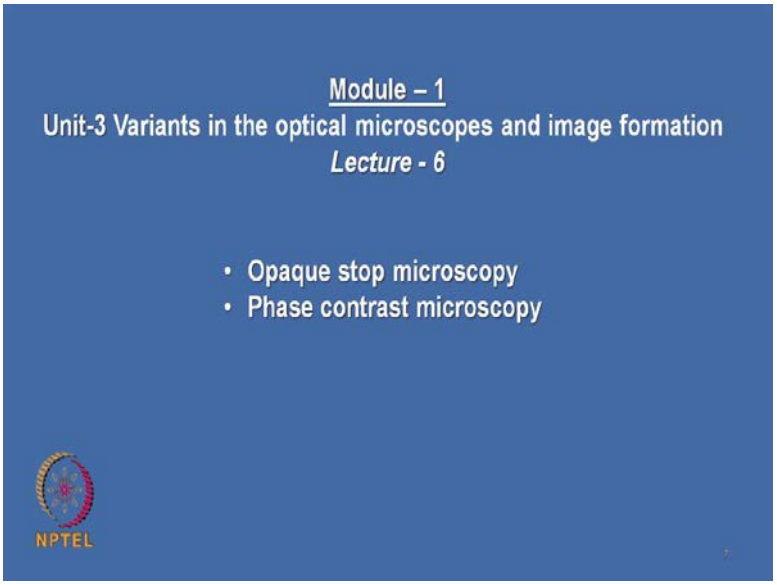

Hello, welcome back. In the last class, we just looked at the bright field illumination mode of optical microscope, and we looked at the theory involved as well as the some of the live demonstration through videos. And I hope you will have some basic idea about how this is being done in the light optical microscope setup. And today, I am going to just start the other variants of this optical microscope.

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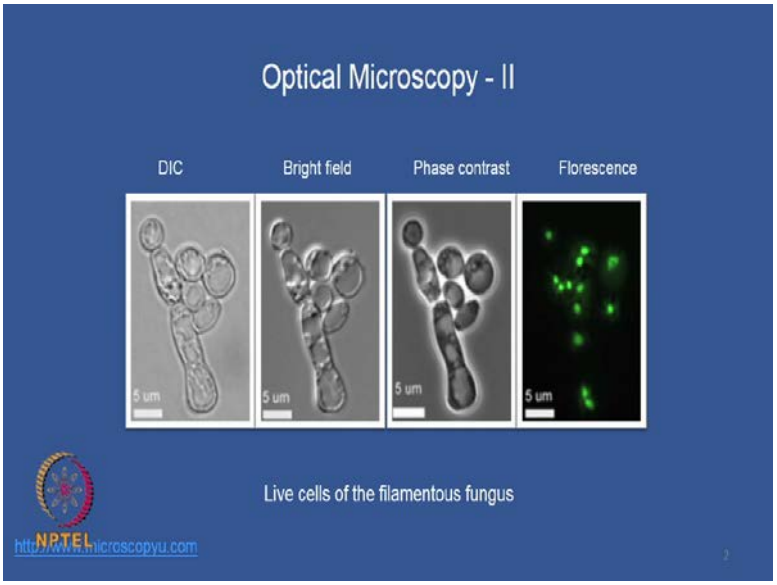

So, let us straight away begin this. I would like to begin this with showing the slide is very interesting slide taking from microscopy u dot com. The very purpose of showing the slide is if you look at this carefully, these images are taken from live cells of some fungus. And you see that they are all at the same magnification, but you see the different type of contrast you obtain from different variants of this optical microscope. You see this; this is a bright field illumination, which we have seen yesterday. And the one which is marked as DIC is differential interference contrast. And what you see in the right side is a phase contrast. And the finally, this is a florescence contrast.

So, it is very nice to see all the variants of the microscope is compared in a one shot that is why I just want to bring to your notice.

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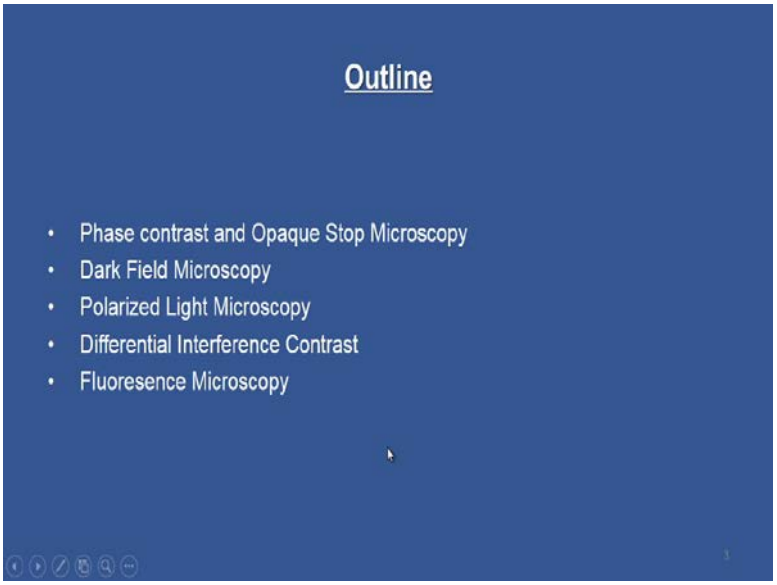

So, now we will just move onto our lecture. So, I will be just discussing the variants, I will start with the phase contrast and its similar technique opaque stop microscopy, and then dark field microscopy, I will go in this in this order, excuse me. And we will just show similar live demonstrations like we have seen for bright field illumination also for all this variants.

And before I just begin this phase contrast microscopy, a very similar technique of phase contrast is opaque stop microscopy. Opaque stop microscopy, since we have just finish the bright field illumination, it is the slight modification from the bright field illumination and just one additional aperture is introduced at the illumination source and then how it is useful that I will briefly discuss on the board, and then I will come back to this phase contrast microscopy.

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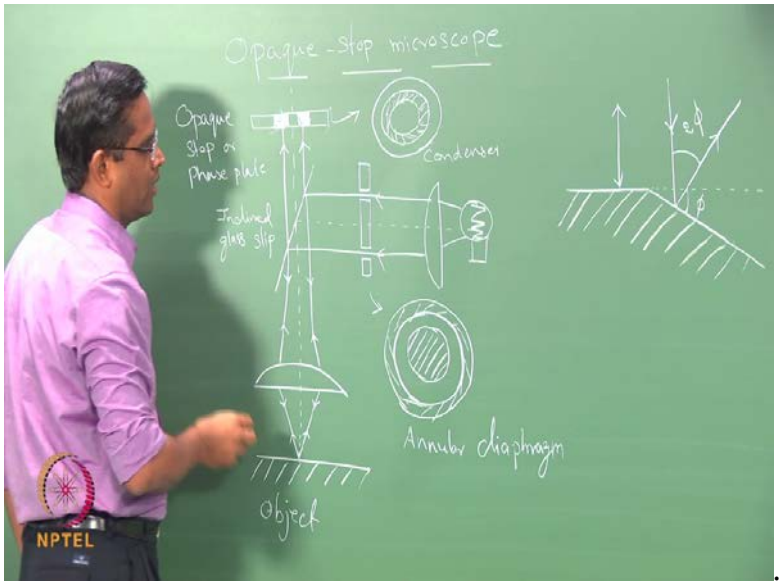

So, let me draw this schematic of the opaque stop microscopy. So, we will have the light source here and then you have the condenser. Like we have drawn yesterday, it is a glass slip, which is inclined at 45 degree then you have this is objective and then you have the object. So, light rays are first coming through this objective and then going back like this. Then it is suppose to go to the eyepiece like this. So, this is inclined glass slip.

And here we will introduce this is an annulus, which fix at different from the general bright field illumination. So, this will appears something like this. This is some an annular diaphragm willing this. So, there is now opening, an annular opening and this is a disk and which is kept here. So, only a cone of a length light is being passed though this microscope and then made reflected on the objective and then to the object. And then finally, when it collected this is something like this, this is opaque stop or a phase plate, so that it will appear something like this; entirely opposite to this, something like this. So, this will be opaque annular phase, opaque part and this is a disk.

So, this ray diagram for this opaque stop microscope let me describe it once again. When the light passes through the annular diaphragm, only the cone of light is being allowed to pass through the microscope, and then they are being reflected on the specimen and then it comes back; and before it enters the eyepiece setup, it has being introduced by or it has

been stopped by a phase plate or opaque stop. So, the advantage is when you have a oblique surface like this, suppose this is the object surface, which is having some unevenness, and this is phi, this is an inclined phases at an angle phi, and suppose if the light falls through this, it will be reflected this angle. So, what we what we are now trying to say here is a light which is reflected from the perpendicular surface will be stopped by this opaque stop or a phase contrast.

The light which is reflected from the inclined surface will escape this opaque stop or the otherwise the all the reflected or diffracted rays will escape this opaque stop and will give illumination, which is similar to a dark field illumination, but this, is also sometime called a sensitive dark field illumination. So, this is discussed along with this phase contrast, because phase contrast also uses a phase plate, but the concept is slightly different, since it is having a similar setup. So, it has being discussed in this lesson.

So, now we will move onto the phase contrast microscopy, very interesting technique. And then you should ask why you need a phase contrast microscopy. Some of the samples do not exhibit enough contrast in the bright field illumination, but somehow the phase changes that is introduced in the transmitted and their reflected beams is being manipulated in the phase contrast microscopy. It is a special case of interference microscopy to find out the minute details which are not visible under the bright field illumination, so that is the very brief introduction to this phase contrast microscopy. So, let me repeat in a bright field illuminations, some of the samples do not exhibit enough contrast. However, the changes in the phase in the transmitted or reflected beams are manipulated by this phase contrast microscopy technique in order to obtain the fine details.

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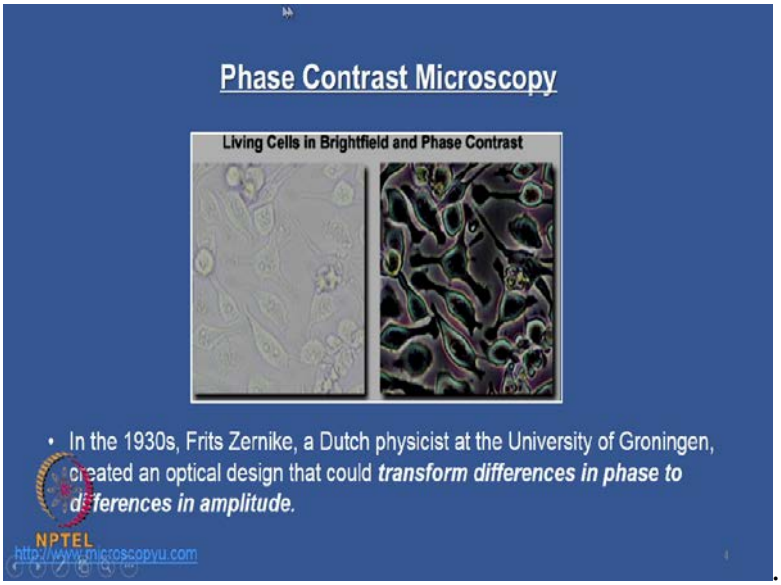

So, now let us see what are the principles. Let me begin with this slide. This is again a very nice micrograph taken from this website. I just brought this just you give you the idea of what kind of contrast enhancement one gets when you go to their phase contrast microscopy.

A brief introduction, in 1930s, Frits Zernike, a Dutch physicist at University of Groningen, created an optical design that could transform differences in the phase to differences in amplitude. I just mentioned that there will be a small change in the phase of the object. So, the change in the phase or that the differences in the phase is being transformed to differences in the amplitude in the image. So, differences in the phase, in the object are manipulated to the difference in the amplitude in the image which will produce or which will enhance the contrast.

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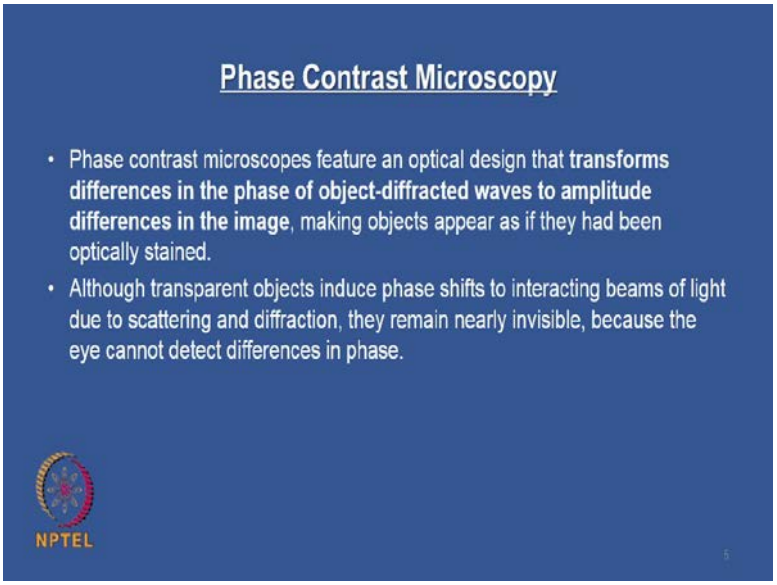

So, we will now see that. Let us go through the introductory remarks. Phase contrast microscopes feature an optical design that transforms differences in the phase of the object-diffracted waves to amplitude differences in the image, so it could be making an objects appear as if they had been optically stained.

Although the transparent objects induce phase shifts to interacting beams of a light due to the scattering and diffraction, they remain nearly invisible, because the eye cannot detect the difference in the phase. So, this is a unique feature of this that is why it is finding very extensive application in the transparent object which induce phase shifts, a very small phase shifts, and we will see how it can be manipulated.

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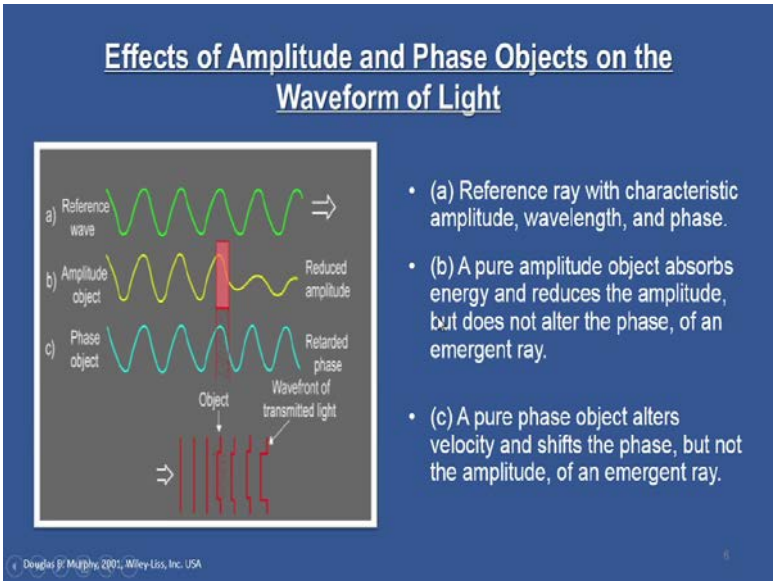

So, let me first introduce the effect of amplitude and phase objects on the waveform of light. So, in this lecture, we will see something called amplitude object or a phase object. What you mean by that, what is phase object, what is amplitude object. So, if you look at this slide, the schematic nicely shows the difference between what is an amplitude object, what is in phase object. The schematic a shows the references wave; and schematic b shows an amplitude object, this is some object; and schematic c shows a phase object. So, what is the difference you see? See, you have the references wave here, and amplitude object that means when the wave pass through this object your amplitude is getting influenced; in this case, it is reduced. So, this kind of behavior any material exhibit, they are classified as amplitude object.

And look at this c, the wave interacts with the objects and comes out, the amplitude does not change, but the phase is changed. What is the change; it is being retarded to some value. We will see what is the phase retardation and then what is it is value. So, any material which changed the phase of the wave is called a phase object and then you see that the transmitted wave light has got a edge, there is a change in the phase of this wave which is coming out of this phase object, so that is what is the written here the reference ray with the characteristic of amplitude, wavelength, and phase.

A pure amplitude object absorbs energy and reduces the amplitude, but does not alter the phase of an emergent ray that is b. A pure phase object alters the velocity and shifts the phase, but not the amplitude of an emergent ray. So, this understanding is very important in order to get the concept which we are going to see in the phase contrast microscopy.

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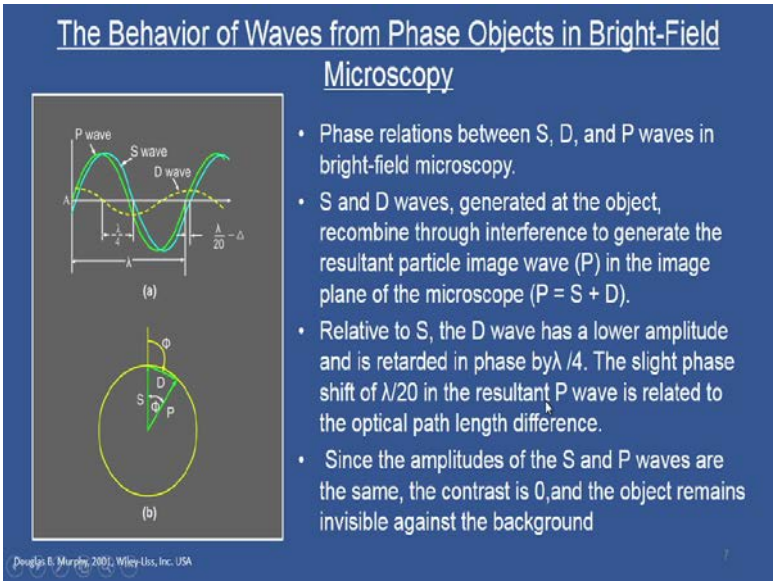

So, let us make it clear about what is amplitude object, and what is a phase object. And what we are going to see in this slide is the behavior of waves from phase objects in a bright field microscopy. So, how the phase objects is going to be appearing in a bright field illumination. So, you have to just have some background before you looking to this schematic. So, let us assume that a light wave is interacting with an object, which goes un deviated or surrounded wave called an S wave, but does not interact with the object that is S wave.

The next one is which is the wave which is getting transmitted or diffracted or refracted by the object that is let us assume that as a D wave. So, let this S and D wave interact or interfere in the image plane to form and a resultant wave called P or a particle wave - a P wave. So, if you assume these things in mind then we can have some kind of understanding about this schematic. So, the resultant wave or particle wave is equal to P is equal to S plus D.

So, with this background let us look at this schematic. You have the s wave and you have the P wave, these two waves have a similar amplitude, but shifted very small that is lambda by 20, the retardation is only very small lambda by 20. And you have this diffracted wave which has been shifted significantly to the extent of lambda by 4. So, to understand this, you look at this schematic carefully, see this distance is lambda, and then with you compare this lambda distance with the other waves. Then you can see this, the retardation, you will be able to understand this retardation lambda by 20 or lambda by 4 and so on. So, please remember we assume that these are the rays, which are coming out of a phase object when they are in the bright field illumination.

So, let us see this phase relations between S, D and P waves in the bright field microscopy. Please understand, it is also important that we have seen in some of the introductory concepts like you know coherence of illuminations. So, you should have in these assumptions, it is stated that your S, and D, and P will have a specific phase relations that comes from the coherence of illuminations. So, look at this schematic b, the same thing is shown in the polar coordinates where you have this S and P in a vectorial form. The length of the vector represents the amplitudes of these waves, and this angle phi represents the phase displacements. And if it is the phase retardation, it is clockwise direction; and if it is phase advancement, it is anticlockwise directions, so that is how the same thing is whatever we have seen is represent in a polar coordinating in b.

So, now you look at this. S and D waves generated at the object recombine through the interference to generate the resultant particle image wave P in the image plane of the microscope that is P equal to S plus D. Relative to S, the D wave has the lower amplitude and it is retarded in phase by lambda by 4. The slight phase shift of lambda by 20 in the resultant P wave is related to the optical path length difference. So, we have seen this optical path length in the introductory concepts. So, what we are now talking about is the optical path length difference is lambda by 20 and lambda by 4 and so on. So, now, since the amplitudes of the S and P waves are the same, the contrast is 0.

So, please remember we are now talking about the phase objects which are how they look at in the or how they will appear under the bright field illumination. Since the amplitude of the S and P waves are the same the contrast is 0 and the object remains

invisible against the background, so that is why a bright field illumination a phase object will not produce enough contrast. So, the very difference of going to the phase contrast microscopy is well understood by looking at this slide.

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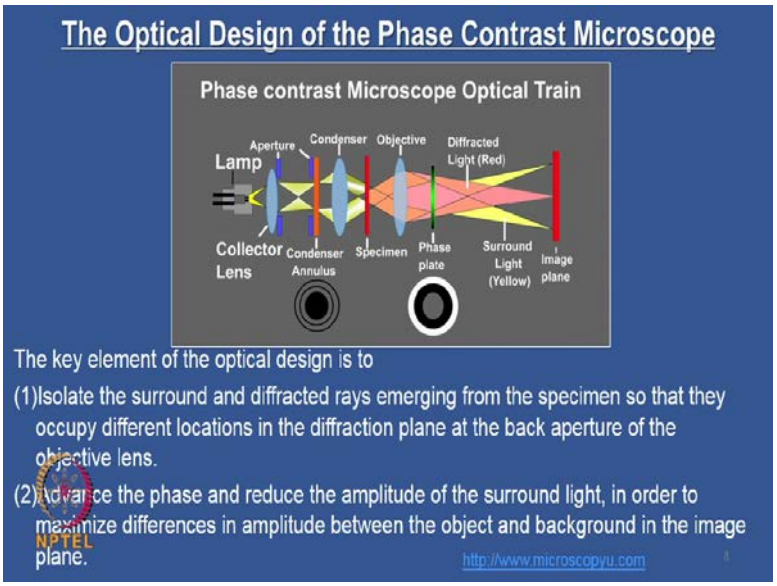

So, now will see how this phase contrast optical design is kept in order to produce a contrast. Look at this schematic; you have a light source, you have the aperture lens, and this is a condenser annulus, like your opaque stop which I mentioned. And then you have a condenser, specimen, objective, and then a phase plate. The phase plate is going to again alter the incoming or outgoing rays. We will see what the phase plate does in much more detail. And you have the rays which are coming out of this, you have a surrounded light as well as the diffracted light, and finally, you see the image plane.

The key element of the optical design is to isolate the surround and diffracted rays emerging from the specimen, so that they occupy different locations in the diffraction plane at the back aperture of the objective lens. Advance the phase and reduce the amplitude of the surrounding light, in order to maximize the difference in the amplitude between the object and the background in the image plane.

So, what is your the phase plate is doing either it advances the phase or reduce the amplitude. See if you look at the earliest schematic, the phase object was not producing contrast under bright field illumination, because the amplitude of at S and P wave were almost similar, that is why the phase plate has to do a job of either advance the phase and reduce the amplitude of the surrounding light, in order to maximize the difference in the amplitude between the object and the background in the image plane.

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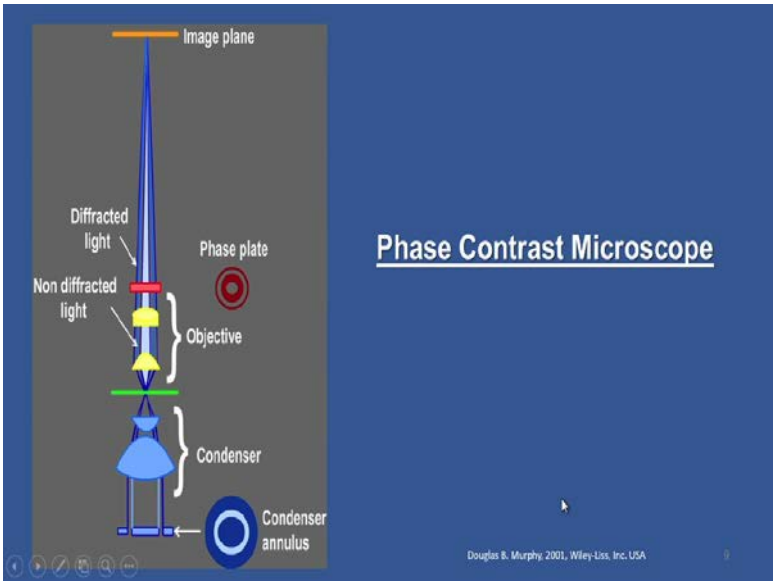

So, let us see how it is done. This is the similar design optical design, which is shown in another their reference. So, I just brought it for a completion. So, you have the phase plate here again you are a condenser annulus here.

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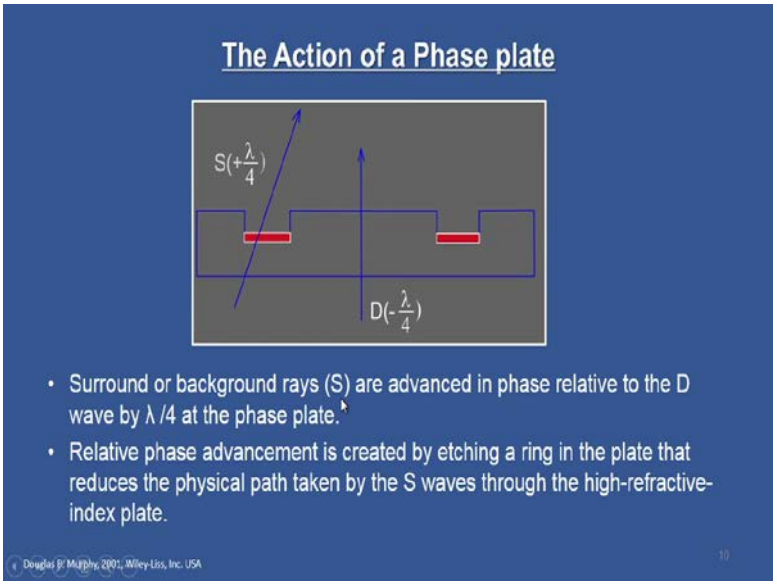

So, let us look at the action of a phase plate. So, this is a phase plate, a typical phase plate will have. And you see that light rays are passing through this; one is advanced, one this annulus ring is advancing the S ray by plus lambda by 4; and the other region of the phase plate is retarding the wave by lambda by 4.

So, the surround and or background rays (S) are advanced in phase relative to the D wave by lambda by 4 at the phase plate. The relative phase advancement is created by etching a ring in the plate that reduces the physical path taken by the S wave through the high refractive index plate. So, what is edging here? This is an annulus which is being created it is circular, it is a cross section that is why you are seeing in this view. So, what it does is, it reduces the physical path taken by the S waves through the high refractive index plate.

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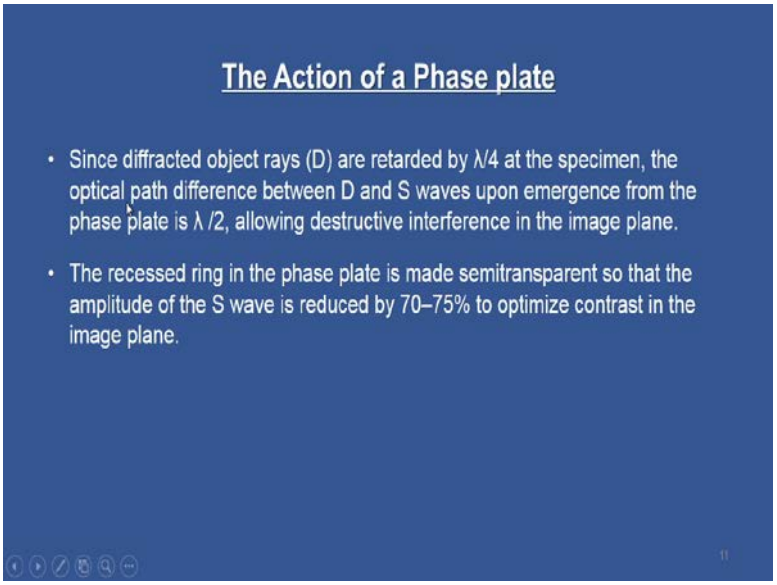

Since the diffracted object rays (D) are retarded by lambda by 4 at the specimen the optical path difference between D and S waves upon emergence from the phase plate is lambda by 2, allowing destructive interference in the image plane. The recessed ring in the phase plate is made semitransparent so that the amplitude of the S wave is reduced by 70 to 75 percent to optimize the contrast in the image plane.

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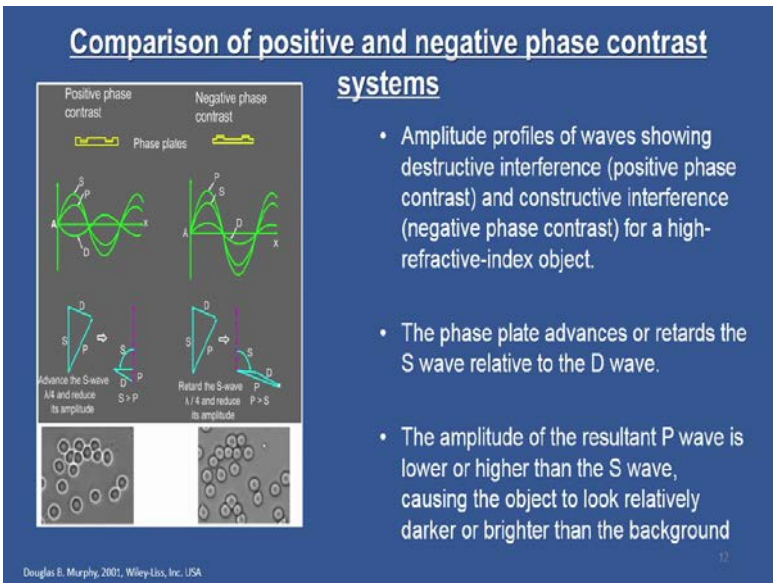

So, now we will see the some of the other aspect of this phase plate; comparison of positive and negative phase contrasts systems. So, you have this positive phase contrast plate and this is a negative phase contrast plate. So, they do exactly the opposite actions in order to produce a different, different contrast effects as you can see in the images.

Let us first go through the remarks. Amplitude profiles of waves showing a destructive interference that is a positive phase contrast and constructive interference is a negative phase contrast for a high refractive-index object. So, when you have in an analyze ring, if you have an etched regions like this, it is called, it will produce a negative phase contrast. When you have a projection like this in your phase plate, it will produce a constructive or positive sorry it will produce a positive phase contrast. The phase plate advances or retards the S wave relative to the D wave. The amplitude of the resultant P wave is lower or higher than the S wave, causing the object to look relatively darker or brighter than the background.

So, you can see this in this ray wave diagram, in the positive phase contrast, the S wave is being advanced; and in the negative phase contrast, the S wave is reduced that is exactly the polar coordinate diagram also shows that the angle is rotated counterclockwise because the S wave is advanced here, it is retards, it is a clockwise rotation you can see that. And you can see the clear difference in the contrast enhancement of the objects, because of this phase contrast effect.

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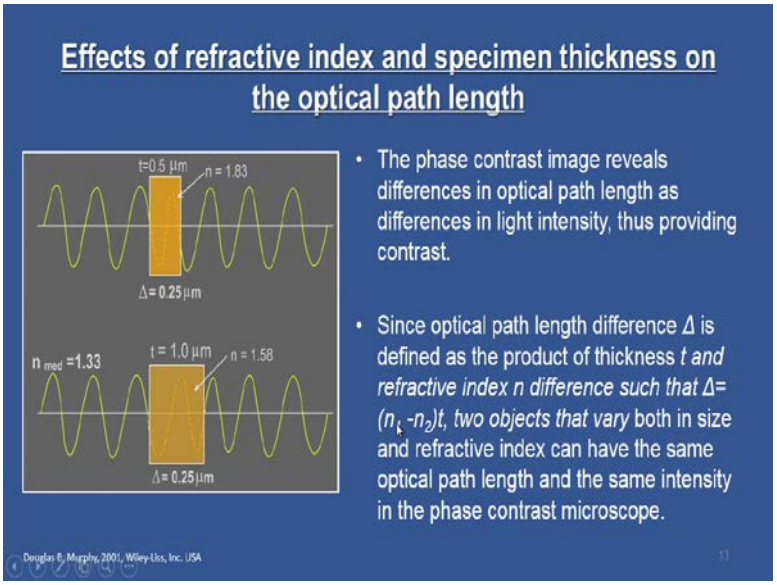

Let us now look at the effect of refractive index and the specimen thickness on the optical path length. We have already seen that what is optical path length, and which is having a significant role in this phase contrast microscopy. Let us see what it is.

The schematic shown here is an object with different thickness and different refractive index. So, the phase contrast image reveals differences in the optical path length as differences in the light intensity thus providing a contrast. Since optical path length difference delta is defined as the product of thickness t and the refractive index n difference such that delta equal to n 1 minus n 2 times t, two objects that vary both in size and refractive index can have the same optical path length and the same intensity in the phase contrast microscope. Since it has got this relation delta equal to n 1 minus n 2 and t, you can have two objects with different thickness and different refractive index to produces a similar intensity.

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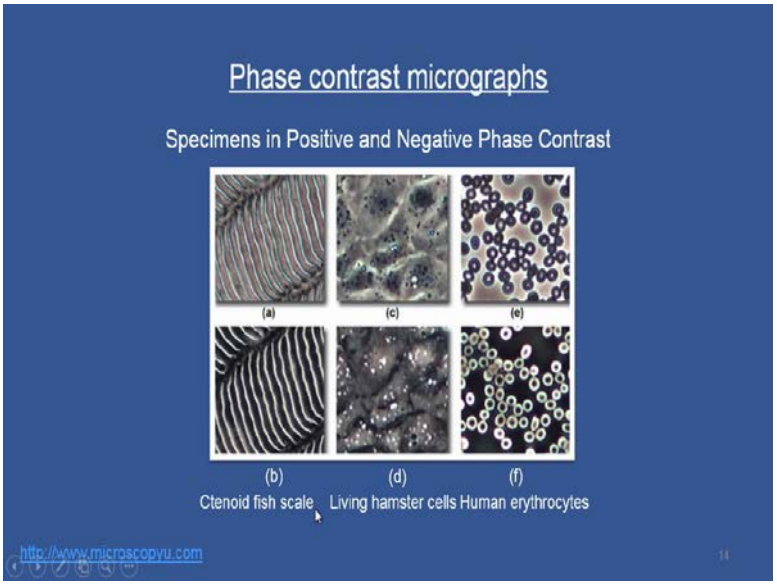

This is some examples, which I have a taken from the website just to give you a favor of what is this phase contrast effect. The one is a fish scale, you can see the contrast difference a positive and negative phase contrast in the; again the cells, some of the cells and then a cells related to human eyes something like that. So, the details are not important, but you look at I just want you to do appreciate the contrast difference.

So, now let me go to the microscope. And we will just look at some of the live example; how this is the phase contrast microscopy is performed in the real laboratory scale. So, what you are seeing is a powder specimen. And these are all glass micro spheres. So, let us look at this glass micro spheres in an optical or in a in a transmission optical electron microscope like I described in the last class. So, specimen is taken on the glass slide in a very small quantity, and then we will now load it on the microscope and then we will look at the details. So, now you can see that specimen is kept on the stage, and it is being brought to the optical axis. And now as I told in the last class, we have to choose the appropriate aperture, right now we do not require polarizer, so it is in the bright field mode. Now here we have to change, this apertures condenser apertures and filters to bright field mode.

And first we will look at these objects in a bright field mode then only we will see how the contrast is enhanced. So, again we are setting up the other condenser aperture which is below the objective to the bright field mode, and increase the intensity of light. And then we will look at the glass microspheres structure, how they appear in the monitor.

See you start looking at these the details of these glass microspheres and this is how it appears at very low magnification. And as we just increase the magnification, we will see slowly the minor details; right now the specimen is being focused to the best possible manner. Now, we will look at some other regions. And this is about 200 X trying to focus you see that all the microsphere start appearing slowly. We have a complete distribution of the microspheres varying from different sizes. We are trying to focus this now and then what now you are seeing is, it is a focused image, and we are looking at other details. And what you see as some color rings hallowed, it is diffraction hallow, it is artifact, this is not part of the material feature.

So, this is now we will change to phase contrast mode that means, you have to change the aperture both the top as well as bottom to the phase contrast aperture and filters. And now you should appreciate this particular, let me go back, when you rotate this condenser aperture and filters, and this particular aperture will have the phase plate, the phase plate will be inside. Similarly here also you will have the phase plate. What were we are have seen as a phase plate will be kept inside this along with this condenser apertures and filters.

So, now look at the images and also see enhance in the contrast. We are trying to focus the image still. So, what now you see is the glass microspheres under the phase contrast mode. And you also see this diffraction hallow which is an artifact which is not part of the material feature and what you see is the enhanced contrast of the periphery. So, I hope you will appreciate this compare to the bright field illumination a significant improvement in the contrast because of the action of the phase plate. I just go through this scan through the sample. And you can see that various features of this glass microspheres.

Now we will also see that some of the cross section of these money plant stem as well as the banyan tree root cross section under the transmission optical microscope to distinguish the contrast between bright field and phase contrast mode. It will be very interesting. So, you just take this stem and then and it is being sliced by the razor blade to a smallest possible thickness, and then you are suppose to put it in a floating water in the beaker. So, let me speed up this activity, this is done for the money plant stem now; earlier one was the banyan tree root. And once you make slices, and you get collected in the beaker, and then you have to choose only very thin and transparent sample for the examination. So, the one which is floating under water is suitable, most suitable and good for a microscopic analysis.

So, you take up one of these slices and put it on a glass slide. First, you place some water bubbles in order to hold that slice intact. And this is how it is done. And you can see you can put to the places water like this and then keep this one of the cross section taken from this the plant stem section. And now we will see how it is appearing on the glass slide. So, this how it is kept and couples of samples are, specimens are there and you are now looking at the bright field illumination. So, this is a bright field illumination.

Now we will change to phase contrast mode. And you see that a significant increase in the contrast. This is the banyan tree root cross section. You can see the details of the all the plant cells, the botanical information is a not known, but then our interest is to just look at the contrast enhancement, because of the difference in the optical density within this material, you are able to see this. This is water what we are seeing is white here is water, and this is your banyan tree root cross section.

So, quickly let us look at the other details at high magnification, you can see that very nicely it is revealed and this is another sample of the same stem. So, now, we will look at the cross section of the money plant section in a bright field mode. It appears like this. And in a change to phase contrast mode you will see that the kind of details you get is phenomenal. You see this is now and phase contrast mode and which is they the surrounding white region is water and you see the complete details of the cell much more clearly compare to the bright field illumination.

So, this is one of the now the good examples of specimens which do not exhibit good contrast into the bright field illumination, but they do appear very good under the phase contrast mode. So, with that, I will stop the lecture on the phase contrast microscopy, and then I will continue this other variants of microscopy techniques in the next class.

Thank you.