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Lecture-6
Materials Characterization
Fundamentals of Optical 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 saw the live demonstration through videos. And I hope you have some basic idea about how this is being done in the light optical microscope setup. And today I am going to just start other variants of this optical microscope. So let us straight away begin this.

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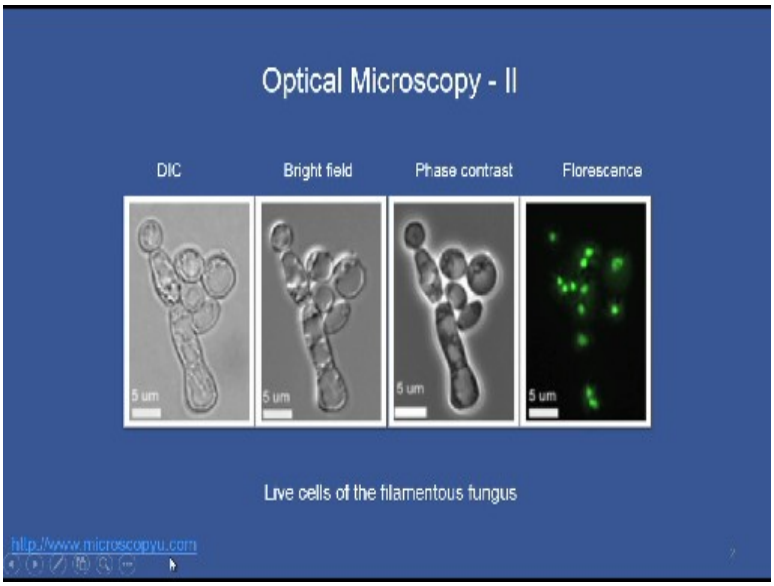

I would like to begin this with showing the slide. This is very interesting slide taken from microscopyu.com. The very purpose of showing the slide is look at this carefully this images are taken from a live cells of as a fungus. And you see that they are all at the same magnification, but you see the different type of contrast you obtain from a different variants of this optical microscope you see this is a bright field illumination which we have seen yesterday.

And the one which is marked as DIC is the differential interference contrast and what you have seen in the right side is the phase contrast; and finally is the florescence contrast. So it is very nice to see all the variants of the microscope is compared in one short that is why just I want to do and you notice.

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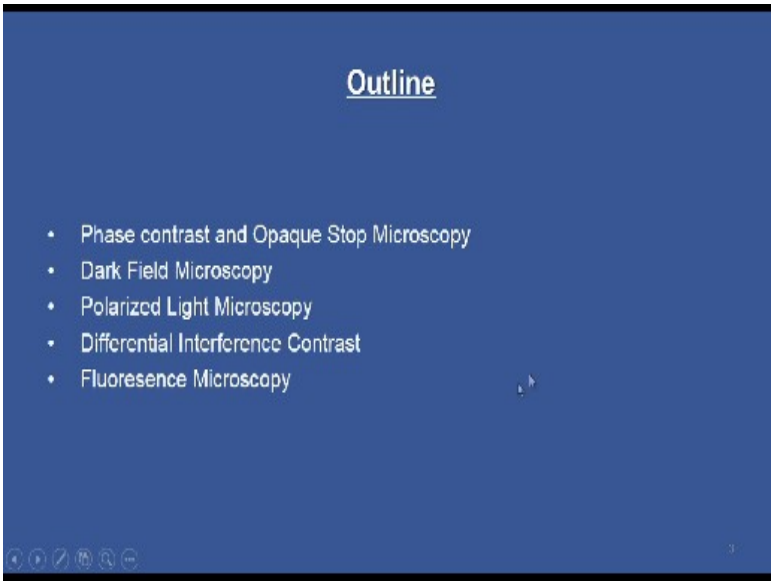

So now we will just move on to the lecture. So I will be just discussing the variants I will start with the phase contrast and the technique; opaque stop microscopy and then dark field microscopy. And we will go in this order discussion. And we will just show a similar live demonstrations like we have seen for bright field illumination and so forth all these variants. And before I just begin this phase contrast microscopy, we have very similar technique of phase contrast is opaque stop microscopy.

Opaque stop microscopy is, since we have just finished the bright field illumination; there is a slight modification from the bright field illumination 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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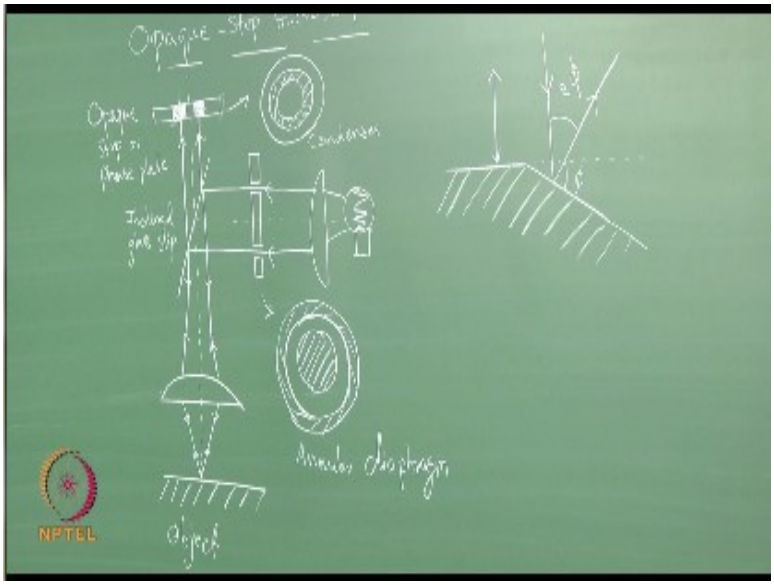

So let me draw the schematic of the opaque stop microscopy. So you have the light source here; and then you have the condenser; like we have drawn yesterday it is a glass slip which is inclined at 45°. And you have objective; and then you have the object; the light rays are first coming through this objective and then going back, like this. It is supposed to go to the eyepiece, like this. So this is inclined glass slip; and here we will introduce this an annulus which makes it different from the general bright field illumination.

So this will appear something like this, this is an annular diaphragm so there is a opening in angular opening and this is the disk and which is kept here. So only a cone light has been passed through 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 will appear something like this. Entirely opposite to this something like this so this will be opaque annual phase opaque part this is a disc. So this the diagram for this opaque stop microscope let me describe it once again; when the lines pass through the annual defame 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 enter the eyepiece set up it as being introduced by or it as being stopped by phase plate or opaque stop.

So the advantage when you have a oblique surface like this. Suppose this is the object surface which is having some unevenness and this is Ø this is an inclined phase at an angle Ø I am suppose the light falls through this it will be reflected this angle so what we are now trying to say here is a line 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 otherwise all the refracted or diffracted rays will escape this opaque stop.

And will give a illumination which is similar to dark field illumination ;but this is also something called as sensitive dark failure illumination, so this 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 as being discussed in this lesson. So now we will move no to the phase contrast microscope; very interesting technique and the you should ask why you need a phase contrast microscope.

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 the reflected beams is being manipulated in the phase contrast microscopic it is a special case of interference microscopy to find out the minute details which are not visible into the bright field illumination, so that is the very brief introduction to this phase contrast microscopy so let me repeat it a bright field illumination.

Some of the samples do not exhibit enough contrast. However the changes in the phase in the transmitted or reflected beams are manipulated by the phase contrast microscopic technique to in order to obtain the fine details.

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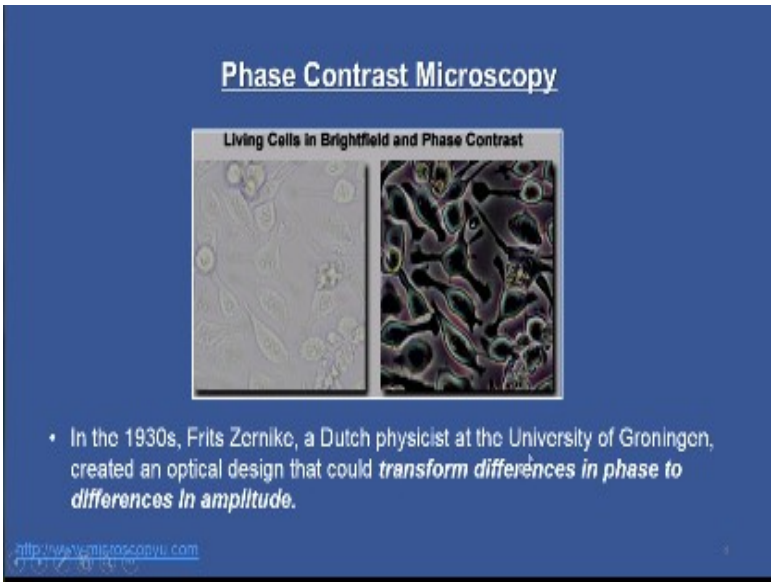

So now let us see what are the principles. Let me began with this slide this again very nice micrograph taken from this website; I just brought this just to give the idea of what kind of contrast enhancement on it is when you go to the phase contrast microscopy a brief introduction in 1930's Frits Zernike a Dutch physicist university of Groningen created an optical design that could transform difference in the phase to difference in amplitude. I just mention that there will be a small change in the phase of the object so the change in the phase are the difference in the phase is being transformed to difference of the amplitude in the image so difference in the phase in the object is manipulated to the difference in the amplitude in the image which will produce or which will enhance the contrast.

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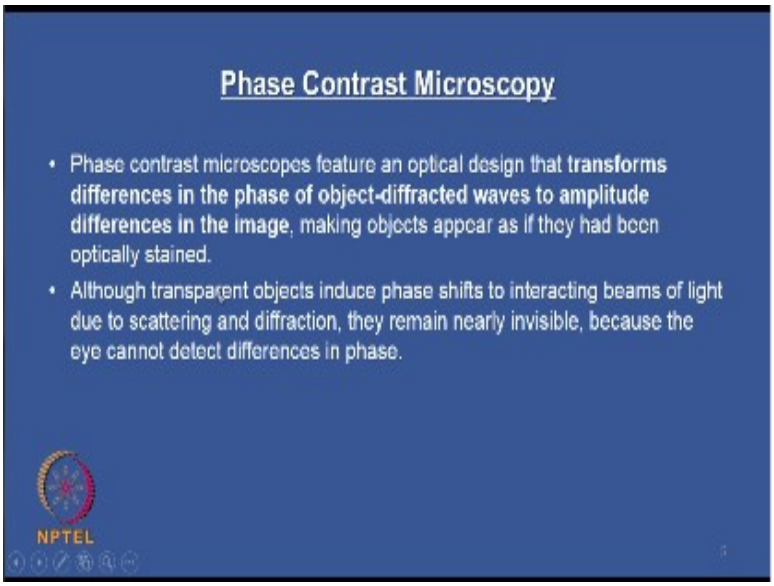

So we will now see that let us go through the introductory remarks: phase contrast microscope feature and optical design that transforms difference in the phase of the object diffracted waves to amplitude difference in the image. So it could be making an objects appear as if they are they had been optically stained; all though the transparence object reduced phase shapes to interacting beams of a light due to the scattering and diffraction they remain nearly invisible because the eye cannot the difference in phase.

So this is a unique feature of this that is why it is finding very extensive application that transparent objects. Which induce a phase shifts a very small shifts and we will see how it can be manipulated.

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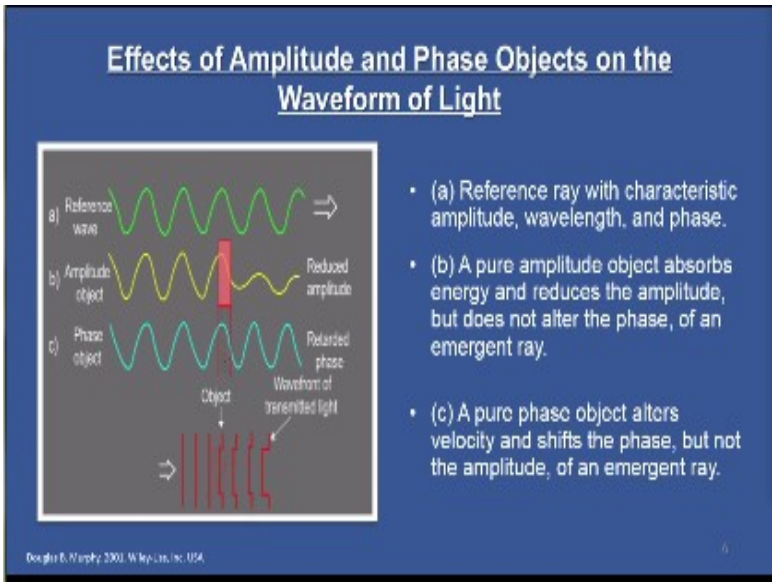

So let me first introduce the effect of amplitude and phase objects on the wave from a light, so in this lecture we will see something called amplitude object or phase object what do you mean by that? What is phase object what is amplitude object? So if you look at the slide the semantic nicely shows the difference between what is an amplitude object what is an phase object the semantic (a) shows a reference wave.

And semantic (b) shows an amplitude object this is an object and semantic (c) shows a phase object so what is the difference you see so you have the reference 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 except they are classified as amplitude objects.

And look at this (c) the wave interacts with the objects and comes out and 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 value? So any material, which change the

phase of the wave is called a phase object and then you see that the transmitted wave let as got a there is a change in the phase of this wave which is coming out of this phase object.

So that is what is written here the reference ray with the characteristic of amplitude wavelength and phase. A pure amplitude object absorbs energy and reduces 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 this very important in order to get the concept which we are going to see in the phase contrast microscopy. So let us make it clear about what is amplitude object and what is a phase object.

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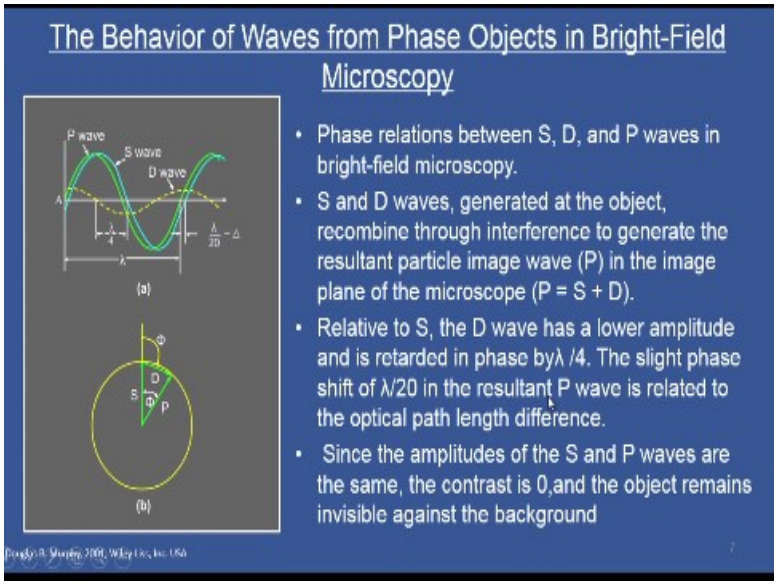

And what we are going to see in this slide is, the behavior of waves from phase objects in a bright field microscopy, so how the phase objects are going to be appearing in the bright field illumination , so you have to just have some background before you look into this schematic so let us assume that light wave is interacting with an object which goes undeviated 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 an D wave, so let this S and D wave interact or interfere in the image plane to form an resultant wave called P or a particle wave a P wave. So if you assume these things in mind then we can have a some kind of understanding about this schematic. So the resultant wave or particle wave is equal to P = S+B 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 λ /20 a retardation is only very small λ/20 and you have this diffracted wave which has been shifted significantly to the extent of λ/4.

So to understand this you look at this schematic carefully this distance is λ and then with you compare this λ distance with the other waves then you can see this the retardation you will be able to understand this retardation λ /20 or λ/4 and so on. So please remember we assume that these are the rays which are coming out of phase object when they are in the bright field illumination.

So let us see this phase relations between SD and P waves in the bright field microscopy. Please understand it is also important that we have seen some of the introductory concepts right in the coherent of illuminations, so you should have in this assumptions it is stated that your S and D and P will have a specific phase relations, that comes from the coherent of illuminations. So look at this schematic (b) the same thing is shown in the a polar coordinates.

Where you have this S and P in a vectorial form that the length of the vector represents the amplitudes of this waves and this angle ϕ represents the phase displacements and if it is the phase retardation it is clockwise direction and if it is phase advancement it is anti-clockwise direction; so that is how the same thing is what all we have seen in A is represent in a polar coordinate in B so now you look at the S and D wave generated at the object.

Recombine through the interference to generate the resultant particle image wave P if the image plane of the microscope that is P = S + D relative to S the D wave has the lower amplitude and it is retarded in phase by λ/4, the slide phase shift of λ/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 λ/20 and λ/4 and so on.

So now since the amplitudes of the S and P waves are same, the contrast is 0. So we are now please remember we are now talking about the phase objects which are how they look at in the or how they 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, so now we see how this phase contrast.

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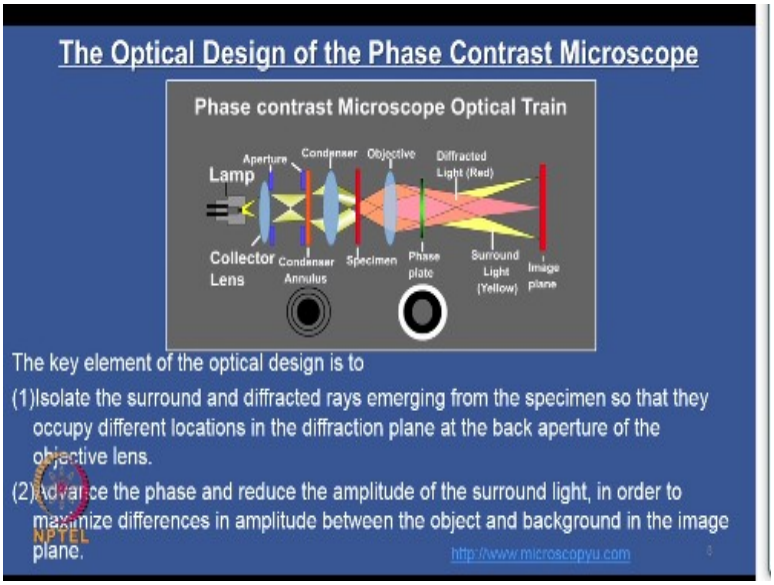

Optical design is kept In order to produce a contrast, look at this schematic you have the light source you have the aperture, lens and this is a condenser annulus like your opaque stop which I mention and then you have a condenser, specimen, objective and then a phase plate. The phase

plate is going to again alter the incoming or outgoing rays. We will see what the phase get does in much more detail.

And you have the rays which is coming out of this you have a surrounded light as well as the diffracted light and finally you see the image wave. The key element of the optical design is to isolate this surround and diffracted rays emerging from this specimen so that the occupied 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 you are the phase plate is doing?

Either it advances the phase or reduces the amplitude. See if you look at the earlier schematic to phase object was not producing contrast under bright field illumination because the amplitude of the S and P wave where almost similar. That is why the phase plate has to do a the job of either advance the phase sand 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 let us see how it is done.

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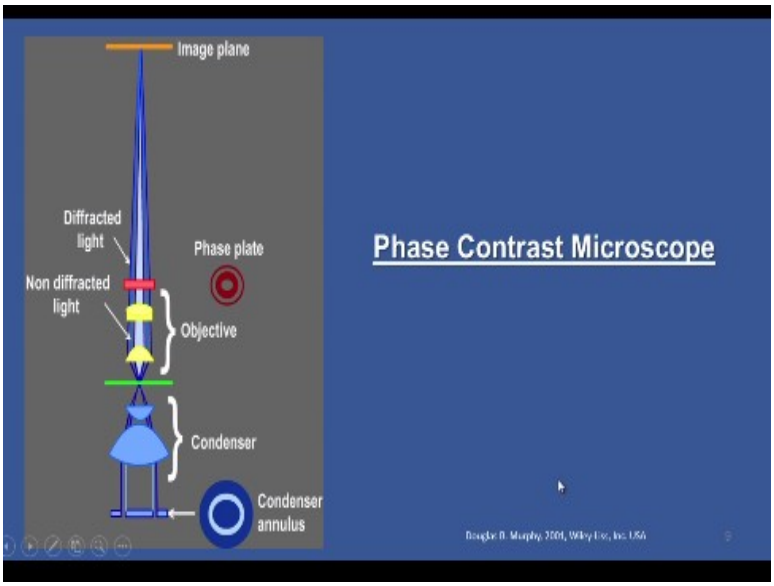

These are the similar design optical design which is shown in other reference so I will just brought it for a completion so you have the phase plate here again you have a condenser annulus here.

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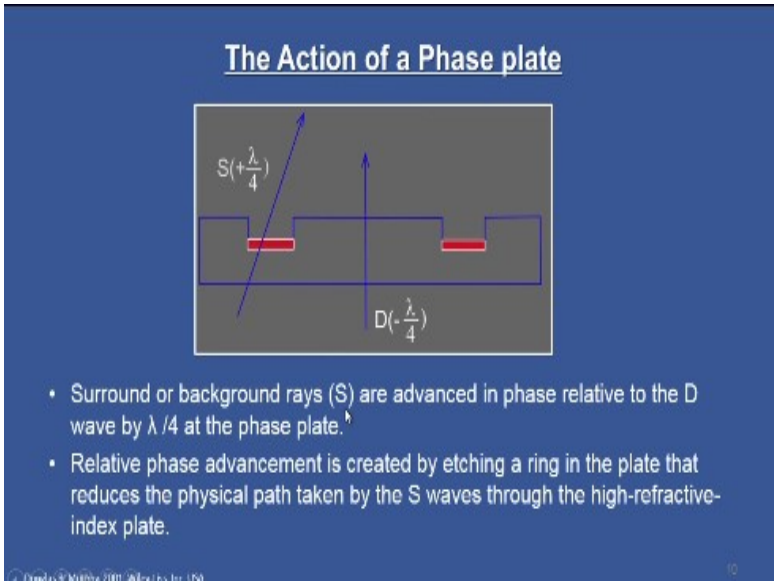

So let us look at the action of a phase plate so this is the phase plate a typical place plane to be have and you see that light rays are passing through this one is advanced this annulus ring this advancing the S ray by + λ/4 and the other region of the phase plate is retarding the wave by λ/4 so the surround and background or background rays S are advanced in phase related to that D wave by + λ/ 4 at the phase plate. The relative advancement is created by etching a ring in the plate that reduces the physical path taken by the S waves through the high refractive index plate.

So what is here is this is 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 reduced the physical path taken by the S waves through the high refractive index plate.

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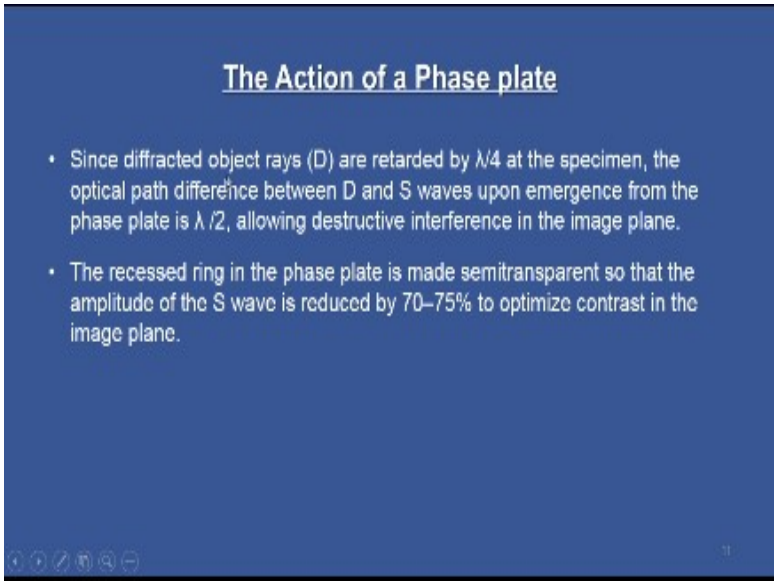

So the diffractive object rays D are retarded by λ/4 at the specimen the optical path difference between D and S waves upon emergence from the phase plate is λ/2 allowing destructive interference in the image plane. The recessed ring in the phase plate is made semi-transparent so that the amplitude of the S wave is reduced by 70-75% to optimize the contrast in the image plane.

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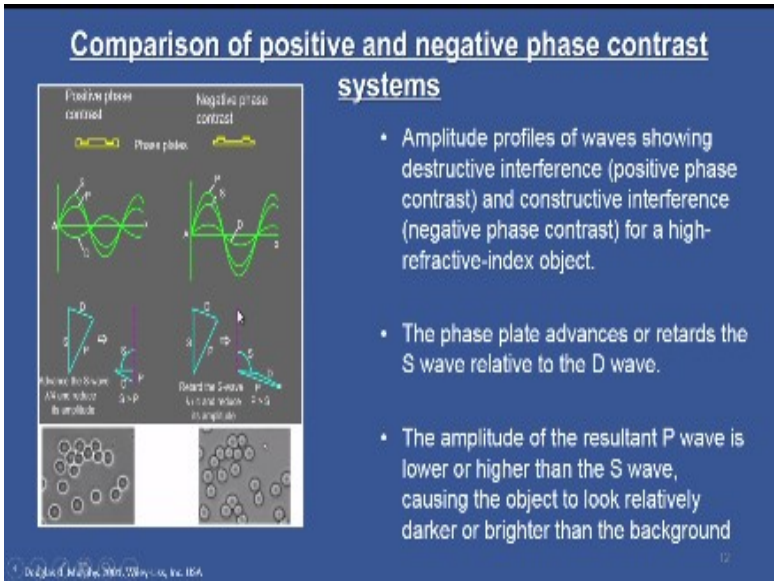

So now we will see the some of the other aspect of this phase plate, comparison of positive and negative phase contrast systems so you have this positive phase contrast plate and this is an negative phase contrast plate, so they do exactly the opposite actions in order to produce a different, different 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 annulus ring if you have an erched regions like this it is called which 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 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 exactly the polar coordinate diagram also shows that the angle is rotated counter clock wise because the S wave is advanced here it is retards it is a clockwise rotation. You can see that and you can see the figure at difference in the contrast enhancement of the objects because of this phase contrast effect.

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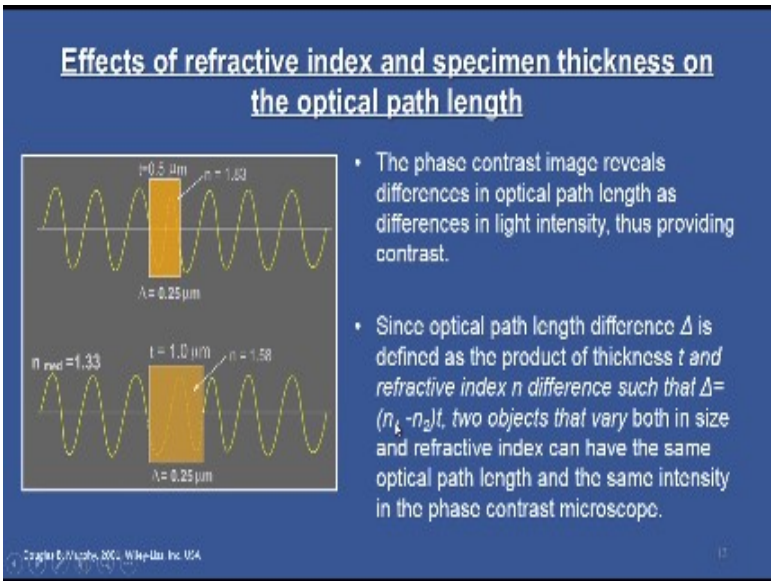

And let us now look at the effect of refractive index and the specimen thickness on the optical path length. We have already seen that what is optical path length and which just having a significant role in this phase contrast microscopy let us see what it is, the schematic shown here is an object with different thickness and different refractive index, okay so the phase contrast image reveals a difference in the optical path length as difference is the light intensity thus providing a contrast.

Since optical path length difference Δ is defined as the product of thickness t and the refractive index n difference such that Δ=(n1-n2)*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 Δ=(n1-n2) and t you can have two objects with different thickness and different refractive index to produce a similar intensity.

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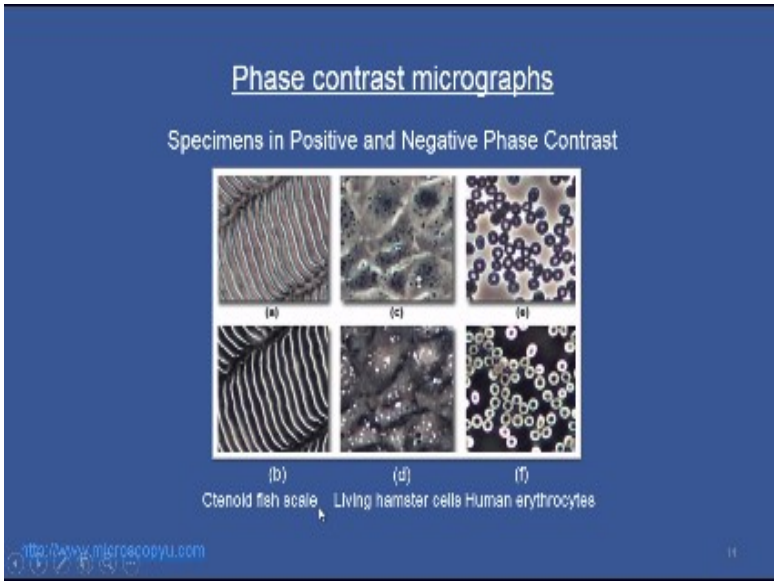

And this is some examples which I have taken from the website just to give you a flavor of what is this phase contrast effect the one is fish scale you can see the contrast difference a positive and negative phase contrast in the again a cell some of the cells and in a cells related to human eyes something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

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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 a powdered specimen and these are all glass microspheres so let us look at this glass microspheres in an optical or in a transmission optical electron microscope like I described in the last class, so the 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 a 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 an appropriate aperture right now we do not require a polarizer, so it is in a 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 this objects in a bright field mode then only we will see how the contrast is enhanced. So again we are setting up the another condenser apertures 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.

So you start looking at this details of this glass microspheres: and this is how it appears at a very low magnification and as we just increase the magnification. We will see slowly the minute details yeah right now the specimen is being focused to the best possible manner. Now we will look at some other regions; and this is about 200X trying to focus you see that all the microsphere start appearing slowly you have a complete distribution of the microspheres varying from different sizes.

We are trying to focus this down and then what now you are seeing is yeah it is a focus image and we are looking other details and what you see as a some color rings hallow is a diffraction hallow it is an 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 and top as well as bottom the case contrast aperture and filters and now you should appreciate this particular let me go back when you rotate this.

Condenser and 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 would we 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 the enhance in the contrast so we are trying to focus the image still. So what now you see is a the micro glass microspheres under the phase contrast mode and you also see this diffraction hallow which is an artifact it is not part of the material feature and what you will see is the.

enhanced contrast of the periphery. So you I hope you will appreciate this compared to the bright field illumination a significant improvement in the contrast because of the action of the phase plate. I will just go through this can through the sample and you can see that various features of

this glass microspheres. Now we will also see that some of the cross the cross section of these money plant stems 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 we just take the stem and then it is bring slice by the razor blade to 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 banyan tree banyan tree root and one to make as slices and we collected in the beaker and then you have to choose only very thin and transparent sample.

For the examination so the, the one which is floating on the water is suitable most suitable and good for microscopic analyses. So you take up one of this a slices and put it on a glass slide first you place some water bubble in order to hold that slice intact, and this is how it is done; and you see you can out two three places water like this then keep this one of the cross section taken from this the plant stem section and now you will see how it is appearing on the glass slide so this is how it is kept and couple of samples are specimens are there and you are row looking at the bright field illumination.

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

So quickly let us look at the other details at high banyan equation you can see that very nicely it is reveal and this is in another sample of the same stem in a so now we will look at the cross section of the money plant section in a bright field mode. It appears like this and then when you change to phase contrast mode to see that the kind of details you get this phenomenal you see this is now under phase contrast mode and which is 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 of the bright field illumination but they do appear very good under the phase contrast mode. So been that I will stop the lecture on the phase contrast microscopy and then I will continue this other variants of microscopic techniques in the next class thank you.

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