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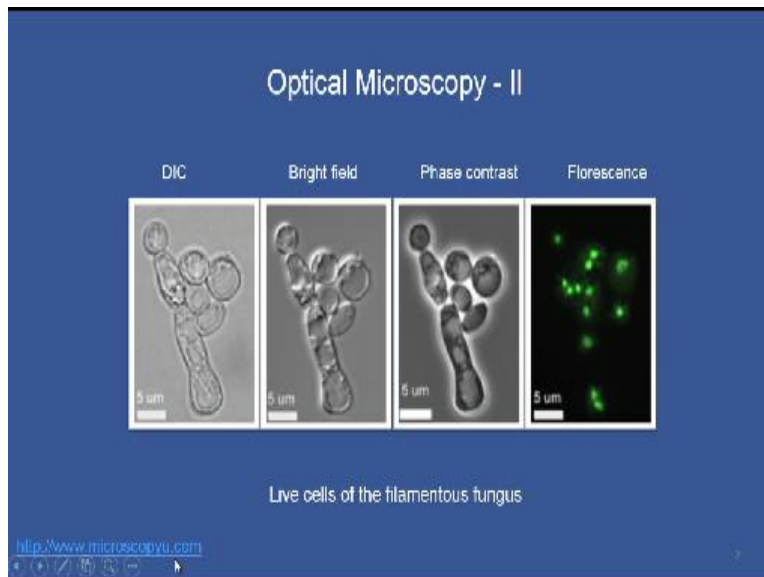
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**Lecture-6
Materials Characterization
Fundamentals of Optical microscopy**

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Hello welcome back in the last class we just looked at the right field illumination mode of optical microscope and we looked at the theory mold 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.

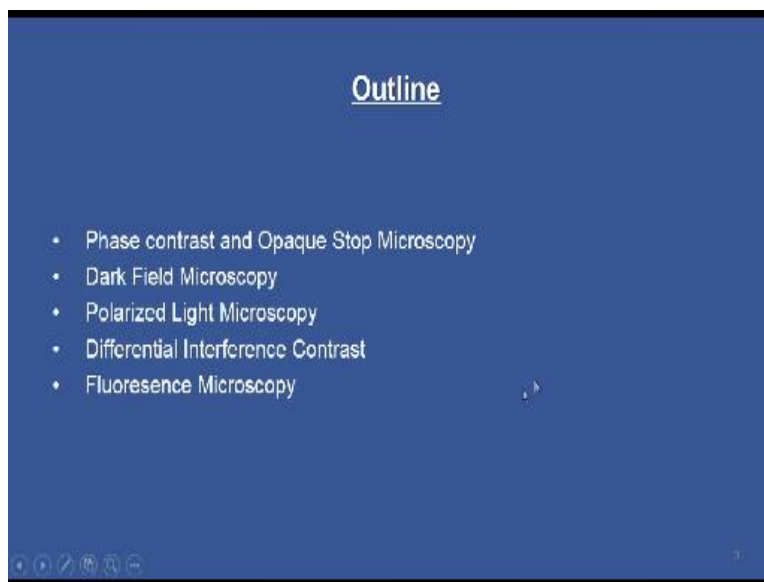
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I would like to begin this with showing the slide this is very interesting slide taken from microscopyu.com. The very purpose of showing the slide is look at this carefully this images are taken from a live cells of as a fungus. And you see that they are al 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 white field illumination which we have seen yesterday.

And the one which is more is the differential interference contrast and what you have seen in the right side is the phase contrast and finally 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.

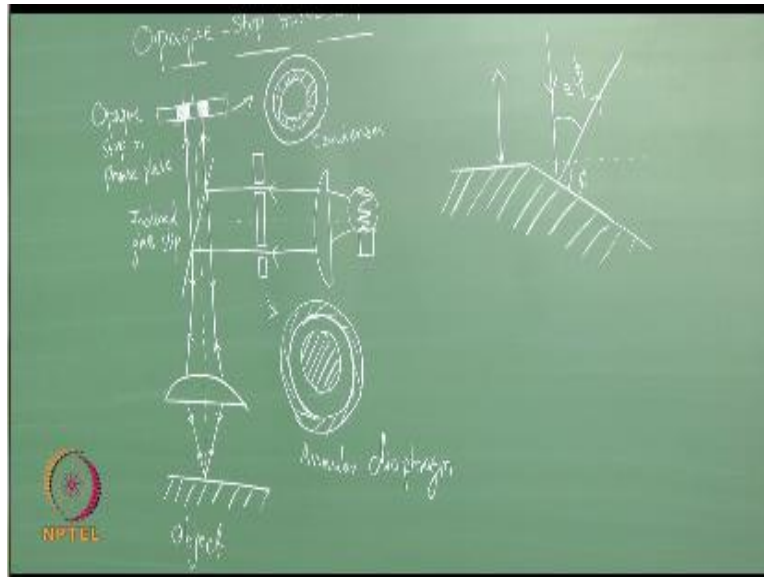
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So now we will just move on to the lecture. So I will be just discussing the variants I will start with the phase contrast and the technique opaque stop microscopy and then dark field microscopy and we will go in this order discussion. And we will just show a similar light 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 approach 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 line source here and then you have the condenser like we have drawn yesterday it is a glass 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. It is supposed to be like this, like this so this is inclined glass and here we will introduce this an analysis which picks at different from the general bright field illumination.

So this will get something like this, this is an angular 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 light, so that we look here something like this. Entirely opposite to this something like this so this will be opaque

annual phase opaque part this is at 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 are it as being stooped by FAS plate or opaque's stope.

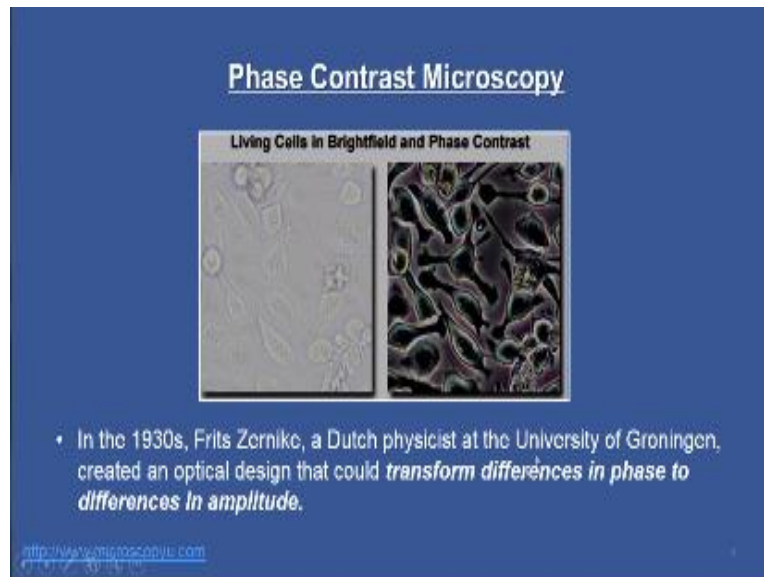
So the advantage when you have a oblique surface like this suppose this is the object surface which is having some unevenness and this is θ this is an entering phase at an angle θ I am suppose the line falls through this it will be reflected this angle so what we are now trying to say here is a line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

And will give a elimination which is similar to dark field elimination but this is also something called as sensitive dark failure elimination, so this discussed along with this phase contrast because phase contrast also uses a phase plate but the concept is lightly 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 except enough contrast in the bright field elimination but somehow the phase changes that is introduced in the transmitted and the reflected bearings is being manipulated in the phase contrast microscopic it is a special case interface microscopy to find out the minute details which are not visible into the bright field elimination, so that is the very brief introduction to this phase contrast microscopy so let me reaped it a bright field eliminations.

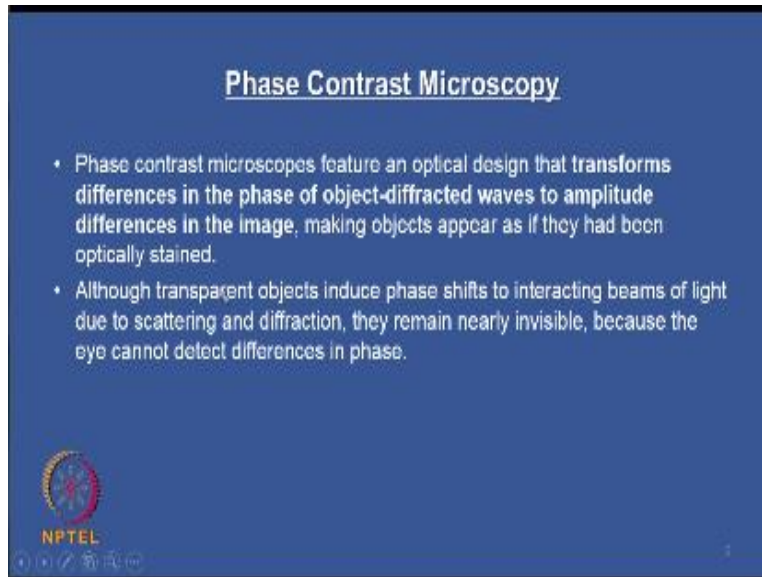
Some of the samples do not except enough contrast however the changes in the phase in the transmitted or reflected beams are manipulated by the phase contrast microscopic technique to in order to obtain the fine details.

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So now let us see what are the principles let me begin 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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Phase Contrast Microscopy

- Phase contrast microscopes feature an optical design that **transforms differences in the phase of object-diffracted waves to amplitude differences in the image**, making objects appear as if they had been optically stained.
- Although transparent objects induce phase shifts to interacting beams of light due to scattering and diffraction, they remain nearly invisible, because the eye cannot detect differences in phase.

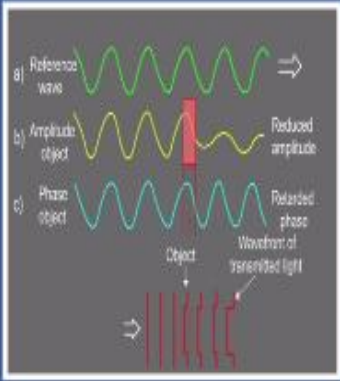

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So we will now see that let us go through the introductory remarks phase contrast microscope feature and optical design that transforms difference in the phase of the object diffracted waves to amplitude difference in the image so it could be making 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 of it which induce a phase shifts a very small shifts and we will see how it can be manipulated.

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Effects of Amplitude and Phase Objects on the Waveform of Light



- (a) Reference ray with characteristic amplitude, wavelength, and phase.
- (b) A pure amplitude object absorbs energy and reduces the amplitude, but does not alter the phase, of an emergent ray.
- (c) A pure phase object alters velocity and shifts the phase, but not the amplitude, of an emergent ray.

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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 pas 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 just not change but the phase is changed what are the change it is mean to tolerate to some value we will see what is the phase redefinition 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 led 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 wave length 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 microscopic. So let us make it clear about what is amplitude object and what is a phase object.

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The Behavior of Waves from Phase Objects in Bright-Field Microscopy

- Phase relations between S, D, and P waves in bright-field microscopy.
- S and D waves, generated at the object, recombine through interference to generate the resultant particle image wave (P) in the image plane of the microscope ($P = S + D$).
- Relative to S, the D wave has a lower amplitude and is retarded in phase by $\lambda/4$. The slight phase shift of $\lambda/20$ in the resultant P wave is related to the optical path length difference.
- Since the amplitudes of the S and P waves are the same, the contrast is 0, and the object remains invisible against the background.

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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 elimination, 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 unnecessary-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 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 $\lambda/20$ a retardation is only very small $\lambda/20$ and you have this diffracted wave which has been shifted significantly to the extent of $\lambda/4$.

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 $\lambda/20$ or $\lambda/4$ 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.

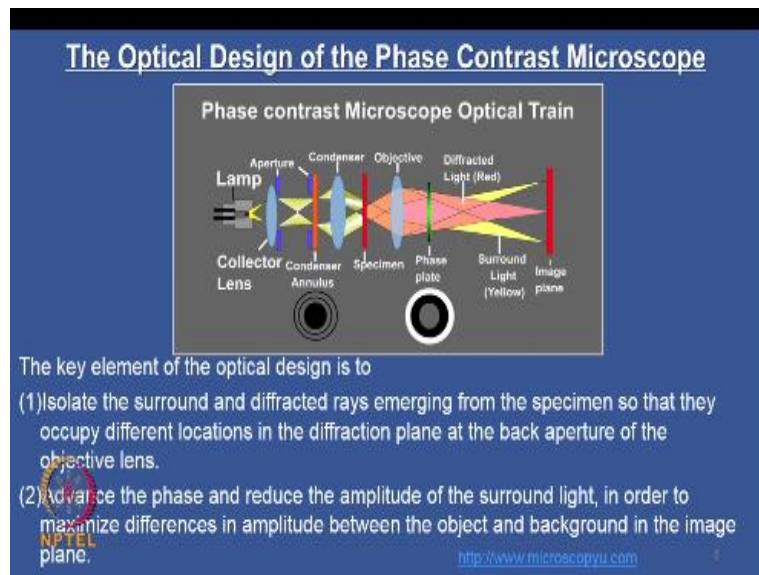
Where you have this S and P in a pictorial form that the length of the vector represents the amplitudes of this waves and this angle ϕ represents the phase displacements and if it is the phase retardation it is clockwise direction and if it is phase advancement it is anti-clockwise direction so that 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 $\lambda/4$, the slide phase shift of $\lambda/20$ 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 $\lambda/20$ and $\lambda/4$ and so on.

So now since the amplitudes of the S and P waves are seen the contrast is 0 so we are now please remember we are now talking about the these objects which are how they look at in the or how they appear under the bright filed 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 addressed.

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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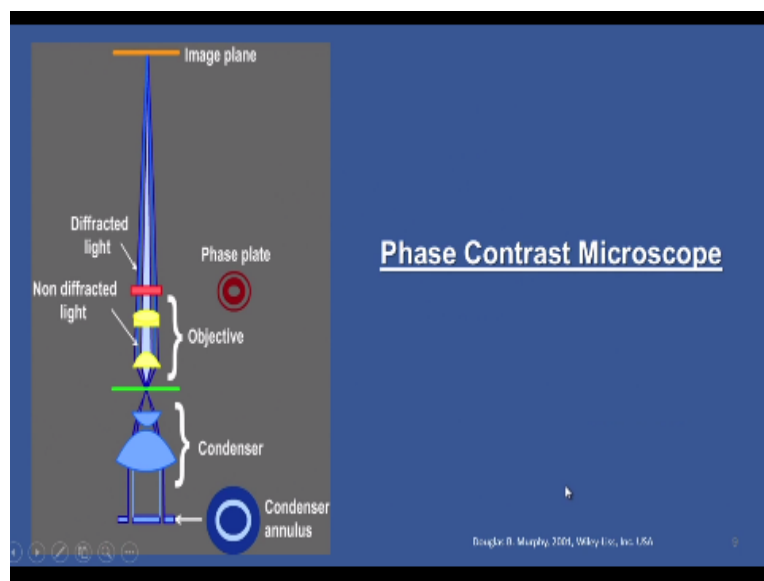
Optical design is kept In order to produce a contrast, look at this schematic you have the light source you have the aperture, lens and this is a condenser annulus like your optic stop which I mention and then you have a condenser, specimen, objective and then a phase plate the phase plate is going to 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 were almost similar that is why the phase plate has to do the 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. So let us see how it is done.

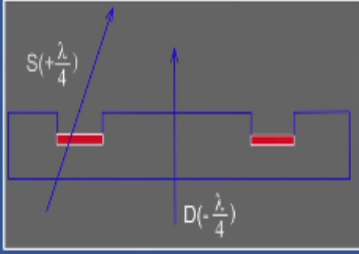
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These are the similar design optical design which is shown in other reference so I will just brought it for a completion so you have the phase plate here again you have a condition I know just here.

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The Action of a Phase plate

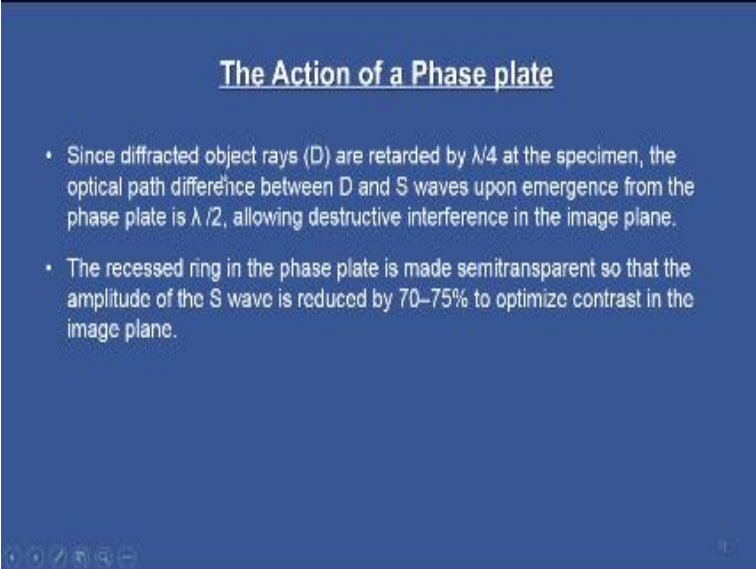


- Surround or background rays (S) are advanced in phase relative to the D wave by $\lambda/4$ at the phase plate.
- Relative phase advancement is created by etching a ring in the plate that reduces the physical path taken by the S waves through the high-refractive-index plate.

So 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 $+\lambda/4$ and the other region of the phase plate is retarding the wave by $\lambda/4$ so the surround and background or background rays S are advanced in phase related to that D wave by $+\lambda/4$ at the phase plate the relative advancement is created by 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 amyloses 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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The Action of a Phase plate

- Since diffracted object rays (D) are retarded by $\lambda/4$ at the specimen, the optical path difference between D and S waves upon emergence from the phase plate is $\lambda/2$, 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 70–75% to optimize contrast in the image plane.

So the diffractive object rays D are retarded by $\lambda/4$ at the specimen the optical path difference between D and S waves upon emergence from the phase plate is $\lambda/2$ 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 70-75% to optimize the contrast in the image plane.

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Comparison of positive and negative phase contrast systems

- Amplitude profiles of waves showing destructive interference (positive phase contrast) and constructive interference (negative phase contrast) for a high-refractive-index object.
- 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 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 edged 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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Effects of refractive index and specimen thickness on the optical path length

- The phase contrast image reveals differences in optical path length as differences in light intensity, thus providing contrast.
- Since optical path length difference Δ is defined as the product of thickness t and refractive index n difference such that $\Delta = (n_1 - n_2)t$, 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.

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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 $\Delta = (n_1 - n_2)t$, 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 $\Delta = (n_1 - n_2)t$ 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 coming and we are reflect other details and what you see as a some color rings hallow is a diffraction hallow it is a or the affect this is not part of the material future 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 and hands in the contrast so we are trying to focus the image still so what now you see is a the micro glass micro spheres under the phase contrast mode and you also see this diffraction hallow which is a more defect it is not part of the material feature and what you will see is the.

Their hands to contrast of the Perry, 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 micro spheres now we will also see that some of the cross the cross section of these manipulates stems as well as the Banyan tree root cross section under the transmission optical microscope 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 laser blade to smallest

possible thickness and then you are suppose to put it in a floating water in the figure so let me speed up this activity this is done for the manipulates stem now earlier one was banyan true 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 light first you place some water bubble in order to hold that slice in fact 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 light 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 manipulate section in a bright field mode we copy as 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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