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Lecture – 8
Materials Characterization
Fundamentals of Optical microscopy

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Hello everyone welcome back to this course of material characterization. In the last class we have just looked at the optical system of polarized light microscopy. And then we have identified and appreciated the merits of polarized light and its superiority over the right-field elimination. In today's class we will look at the another variant of the optical microscope called differential interference contrast microscopy. Let us see what is this microscopy.

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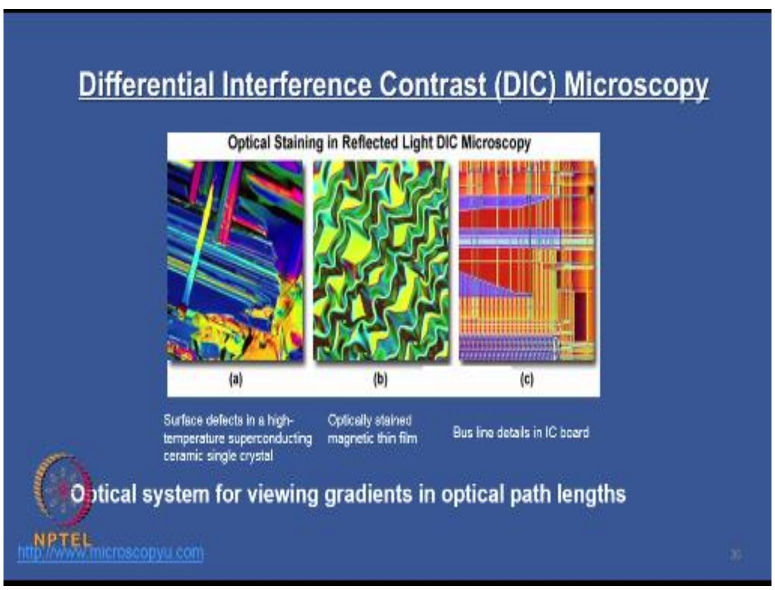

Look at this slide as usual I have picked up some of the classical images from this website microscope you not come you see that the first image shows that surface defects in a high temperature superconducting ceramic single crystal you can see that the kind of clarity and the contrast you get in the ceramic surface in through DIC. And the image B is of magnetic thin films and you may see is a bus line details in an icy board.

So these are some of the best micrographs one can achieve through this DIC. We will see that what is the principle of this technique and I have written here it is an optical system for viewing gradients in the optical path lengths. You see in one of the previous class we just saw a phase contrast microscopy which is an optical system that converts the optical path difference in a specimen to the contrast in the object of the image.

Here DIC is an optical system that is used to view the gradient in the optical path length or optical path difference. The image is produced by these systems are very distinct and relief like and shadow cast appearance a property of the image which makes it to appear like a three-dimensional and real. So that is why it is having some superior quality over the rest of the variants of the optical microscopes. So now let us get into the details of this DIC microscopic system.

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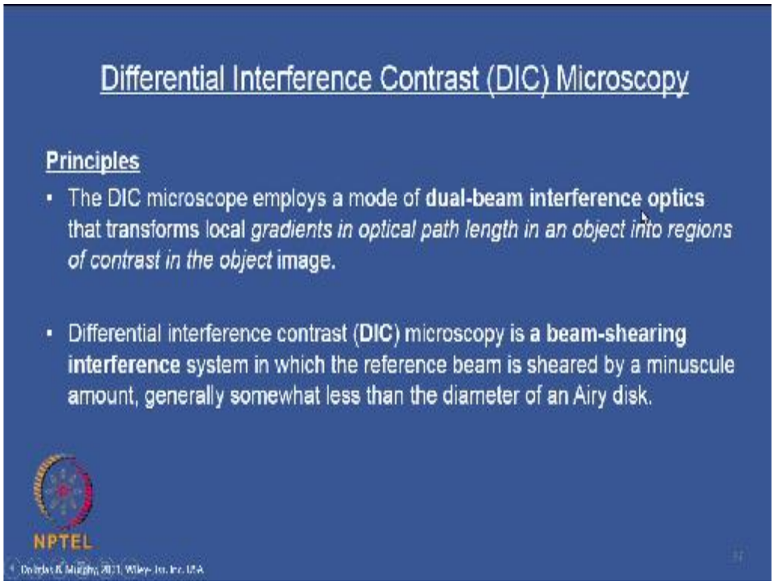

The DIC microscope employs a mode of dual-beam interference optics that transforms local gradients in the optical path length into an object into regions of contrast in the object image. Differential interference contrast microscopy is a beam shearing interference system in which the reference beam is sheared by a miniscule amount, generally somewhat less than the diameter of an array disk.

So please remember in contrast to the phase contrast microscopy this is an optical system viewing the gradients in the optical path length in the object into regions of contrast in the object image, that is it is converting the gradients in the optical path length into a contrast of the image. We will see how it is being carried out.

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Let us look at the other remarks on the principles of this technique the technique produces a monochromatic shadow cast image that effectively displays the gradient of optical paths for both high and low spatial frequencies present in the specimen. Those regions of specimen where the optical path differences increase along a refractive sorry, along a reference direction appear brighter or darker while regions where the path differences decrease appear in reverse contrast. As the gradient of the optical path difference grows steeper image contrast is dramatically increased.

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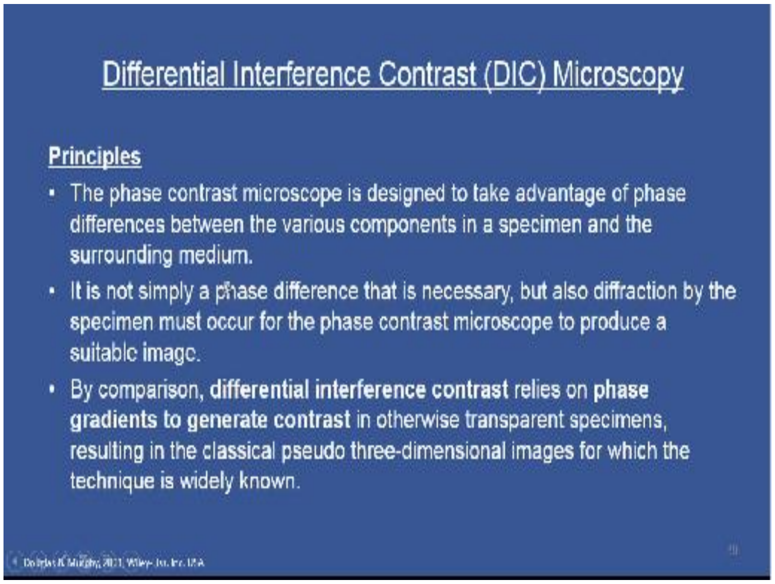

The phase contrast microscope is designed to take advantage of a phase differences between the various components in the specimen and the surrounding medium. It is not simply a phase difference that is necessary but also diffraction by the specimen must occur for the phase contrast microscope to produce a suitable image. By comparison, differential interference contrast relies on phase gradients to generate contrast in otherwise transparent specimens resulting in the classical pseudo three-dimensional images for which the technique is widely known.

But it is not that it is only popular for a transparent specimen it has been widely used for the effect specimens also. We will see the practical examples I will take you to the lab and then as usual I show the demonstration on the opaque examples how you can appreciate how this DIC is improving the contrast.

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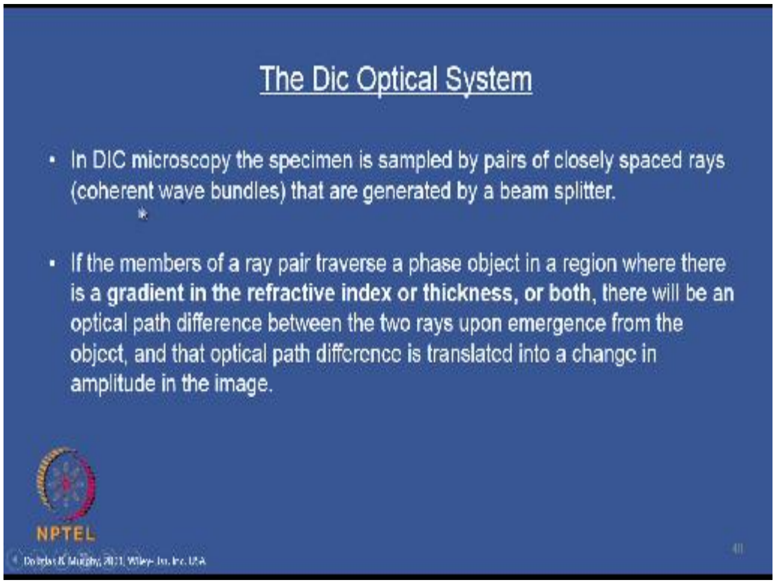

So let us look at the optical system in DIC microscopy the specimen is sampled by pairs of closely spaced rays that is coherent wave bundles that are generated by a beam splitter. If the members of the of a ray pair traverse a phase object in a region where there is a gradient in the refractive index or thickness or both there will be an optical path difference between the two rays upon emergence from the object, and that optical path difference is translated into a change in amplitude in the image.

So this is very important you have to look at this two lines again and again it is worth doing that. The image contrast basically produced from the gradient of the refractive index or thickness are both. So we will not be able to distinguish from where the contrast is coming from whether to thickness or the optical path difference. We will see why we are saying so in the coming slides.

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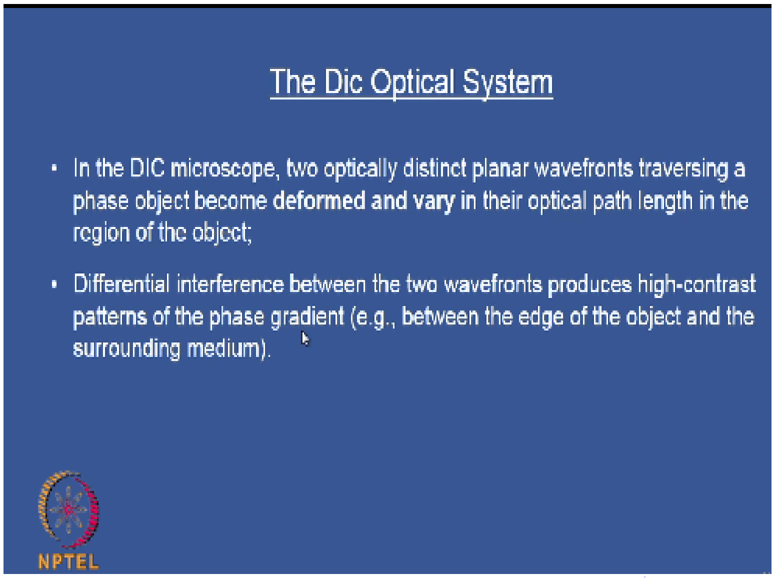

In DIC microscope, two optically distinct planar wavefronts traversing a phase object become deformed and vary in their optical path length in the region of the object. Differential interference between the two wavefronts produces high contrast patterns of the phase gradient example between the edge of the object and the surrounding medium. So you see we have also seen that a beam splitter in the polarize light microscope similar splitter is being used here also. In fact the polarizer set up is also a integral part of this DIC system we will see how it is.

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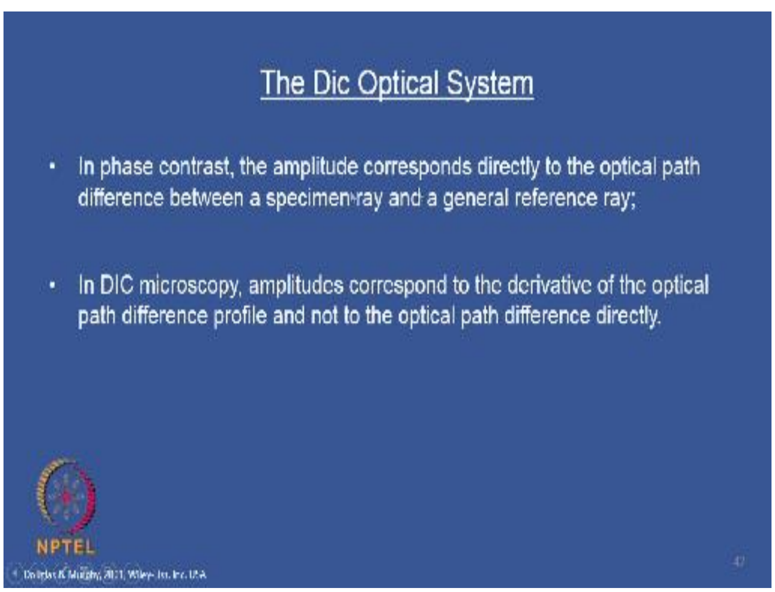

In phase contrast the amplitude corresponds directly to the optical path difference between a specimen ray and a general reference ray. In DIC microscopy amplitudes correspond to the derivative of the optical path difference profile and not to the optical path difference directly that is when the name differential interference because it is the derivative of the optical path difference profile. This is what is shown in the schematic.

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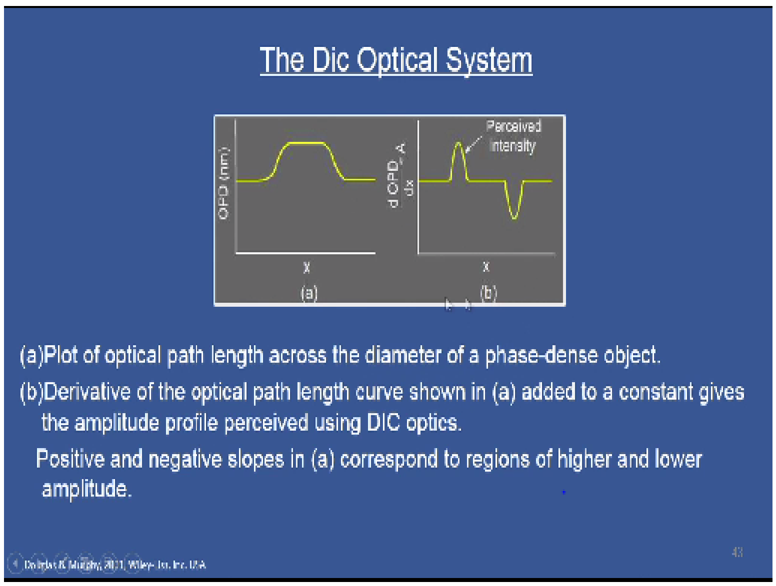

Please pay attention to this schematic this is optical path difference in nanometers this is a distance and you see that the profile like this that is a plot of optical path length across the diameter of a phase dense object. Why the curve appears like this, because any light travels through a phase object it will deflect or it will do something to do that light wave that is what we have seen in the previous lectures that is some phase shift will happen.

So look at the schematic B the derivative of optical path length curve shown in A added to the constant gives the amplitude profile perceived using DIC optics. So you can see that it is a derivative differential of optical path difference over a distance of small distance DX vs the distance this is you have a perceived positive intensity and this is a perceived negative intensity.

So you see that positive negative slopes in A corresponds to region of higher and lower amplitude. So this is the key schematic which explains the name of differential interference contrast.

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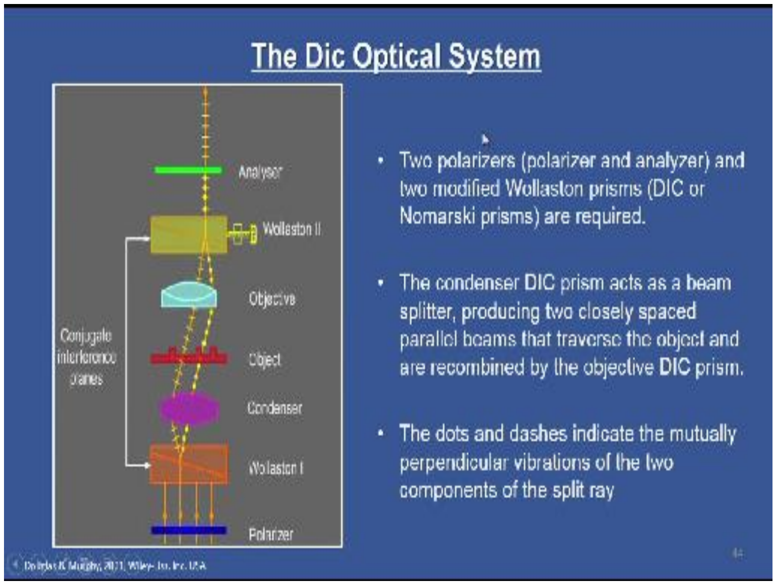

We will now look at the DIC optical system it is similar to what we are already seen then what is new here we will see. Two polarizers that is polarizer and analyzer which we are familiar with already and two modified Wollaston prisms that is DIC are Nomarski prisms are required you have a new prism here called modified Wollaston prism or DIC or Nomarski some, you have the condenser DIC prism act as a beam splitter producing two closely spaced parallel beams that traverse the object and are recombined by the objective DIC prism.

So you have condenser DIC prism that is shown here and you have objective DIC prism here this is what we have seen. As usual the dots and the dash indicates the mutually perpendicular vibrations of the two components of the split ray, these two rays are nothing but what we have seen in a polarize light it is an ordinary ray and extraordinary ray which will have this electric vector by vibrating in a mutually perpendicular direction to each other that is the same thing here you do not have to really get confused.

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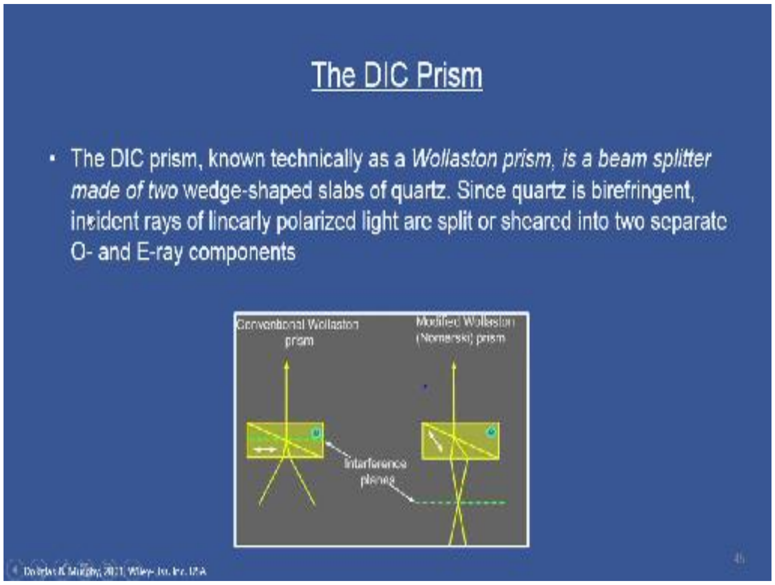

Now let us see what is this DIC prism does the DIC prism known technically as a Wollaston prism is a beam splitter made of two wedge-shaped slabs of quartz. Since course is a by refrigerant incident rays of linearly polarized light are split are sheared into two separate ordinary and extraordinary ray components.

So this is a schematic which clearly shows what is the conventional Wollaston prism you see that the two wedge-shaped slaps of quartz crystal you can see the vibration direction is marked here this is in this direction another is a perpendicular direction marked which is going through the the computer screen okay, that is.

And what you see here is the center is interference plane and what is modified Wollaston prism are Nomarski prism you have the two bed shaped slabs are there, but it is the orientation is little oblique and what is the consequence of that, because the orientation is oblique your interference plane is shifted out of this prism. So that is what it is, so your Nomarski prism is nothing but modified Wollaston prism.

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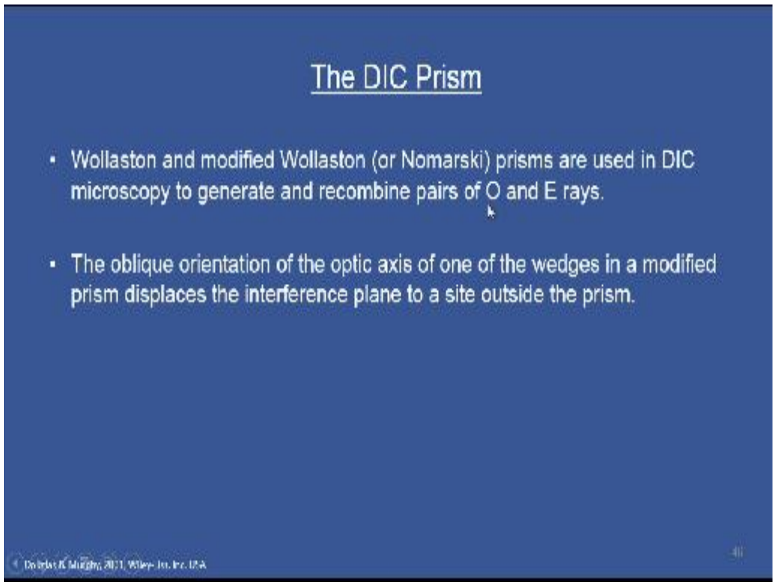

The Wollaston and modified Wollaston are Nomarski prisms are used in DIC microscopy to generate and recombine pairs of ordinary and extraordinary days the oblique orientation of the optic axis of one of the wedges in the modified prism displaces the interference plane to a site outside the prism.

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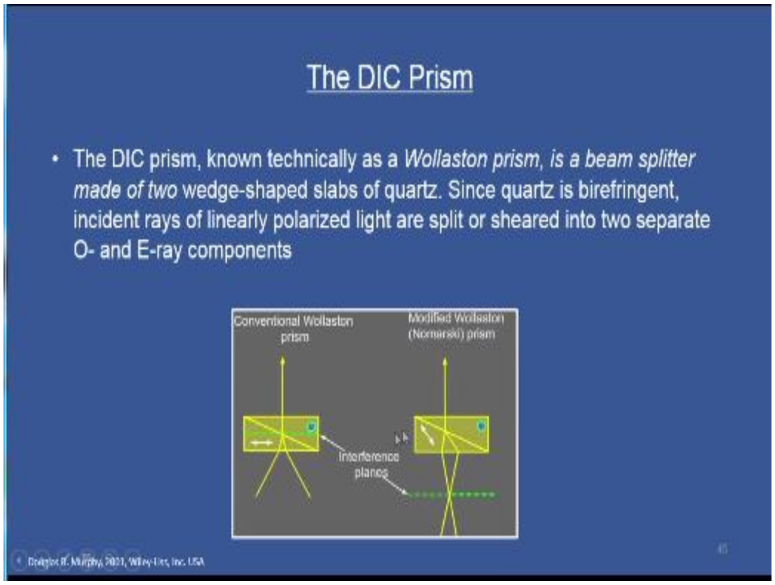

So this is what I have shown here.

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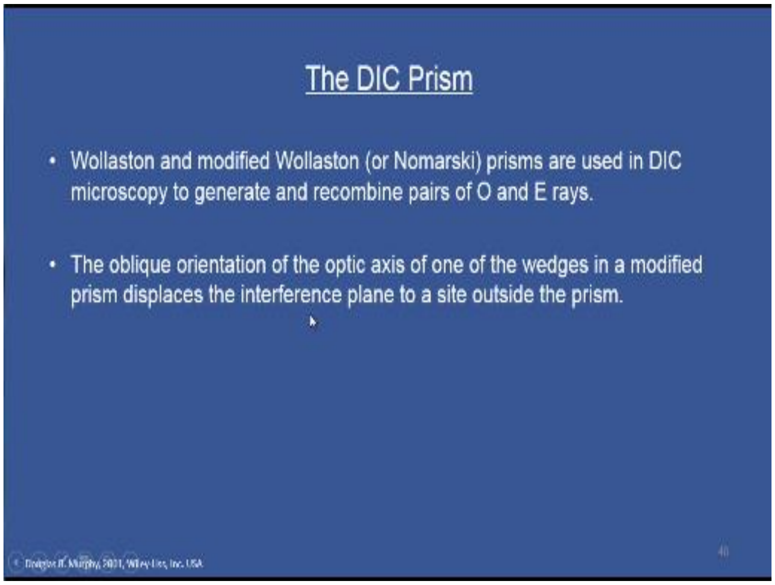

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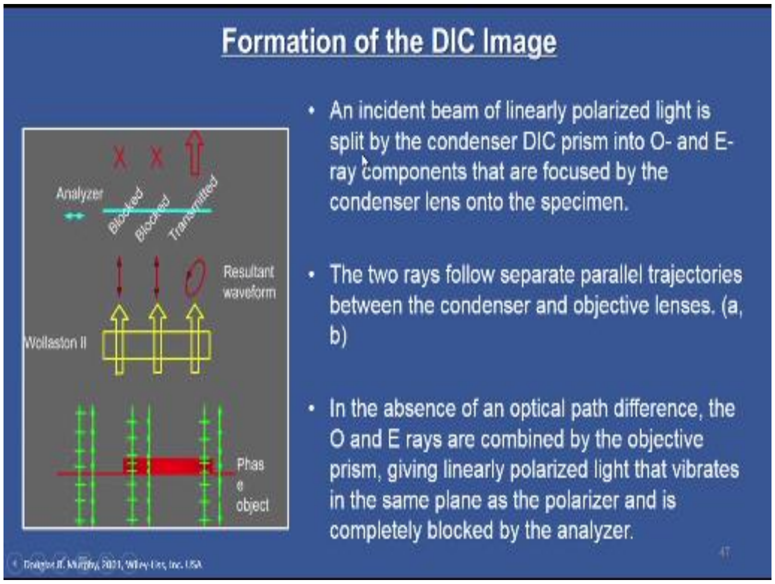

So now let us look at how the image is formed in the DIC microscope an incident beam of linearly polarized light is split by a condenser DIC prism into ordinary and external extra ordinary ray components that are focused by the condenser lens into the onto the specimen the to raise follow a separate parallel projector is like this between the condenser and objective lens a and B these two schematic in the absence of an optical path difference the ordinary and extraordinary race are combined by the object prism giving linearly polarized light that vibrates in the same plane as the polarizer and it is completely blocked by the analyzer.

So look at this schematic this is these are the two rays which is coming from the beam splitter or Nomarski prism sorry it is up from the beam splitter then you have this object phase object and let us assume that there is no optical path difference in the felt by these two rays then the ray goes like this and they are blocked by the analyzer because the polarized light that vibrates in the same plane as the polarizer and it is completely blocked that is the reason whereas if there is a optical path difference in the object and which affects this and then these two rays will interfere and then emerge out as a elliptically polarized light in three dimension.

And which will be partially transmitted through this analyzer as a resultant wave form and that is how it will form an image we will see the actual example in the optical microscopy lab.

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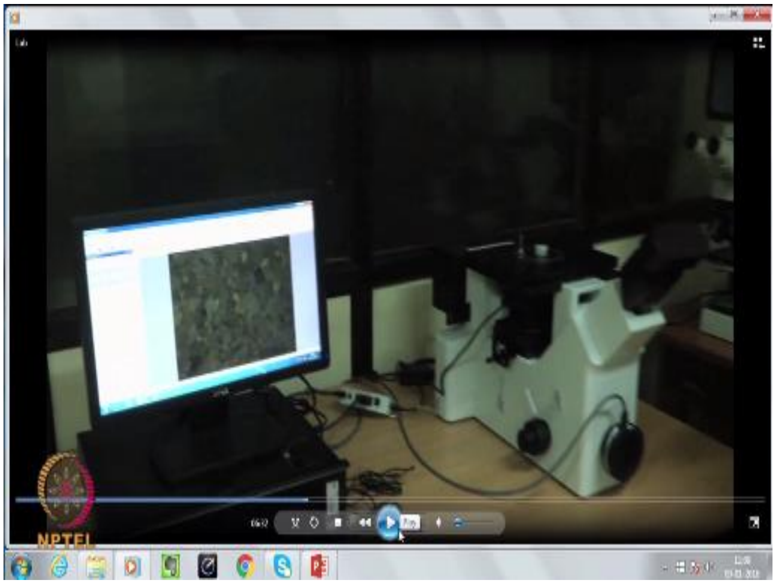

I will now go to the we will continue from this video optical microscopy now we will demonstrate the DIC what is the object which you are now seeing is the DIC setup you can see that you have this DIC prism or Nomarski prism or modified Wollaston prism which can be inserted into this optical axis and then you can see that there is an adjustable screw now we will see that what kind of contrast you get out of this microscopic.

So you see that this is a similar specimen that is at a titanium alloy what you are now seeing is like you know differential interference contrast you can see that all your boundaries and other regions appear like white and then it gives a shadow you are getting kind of a 3D projection or a surface leaf by this set up and we can also see that the twinning in the grains which will be much more clear when we go to higher magnification, and let us slowly increase the magnification.

Yeah now it is getting focused so you can appreciate that the kind of contrast you get at little higher magnification you see that all this twins and then defects appear as if they are the three-dimensional images you can see that a dark and a bright line which makes all this constituents microstructure constant as if they are at 3D we will see how this contrast is produced and we also appreciate the moment you change the analysis orientation the color of the micros micrograph changes that means it will block a particular wavelength and then you will start seeing the particular color.

So you can keep on changing the orientation of the under laser depending upon the orientation and it will block a particular wavelength of the light and then you see a multiple colored image that is also where an advantage of this DIC system now I will go to the blackboard and then draw some schematic.

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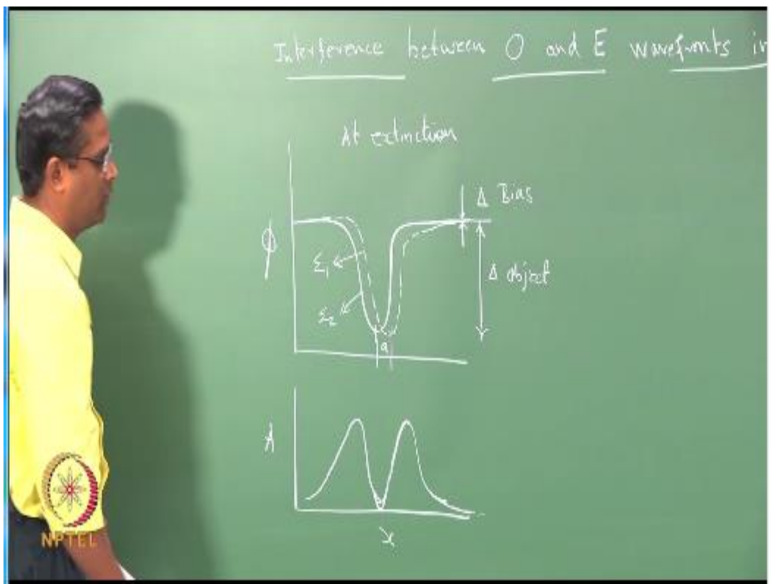

Of ordinary and extraordinary wave fronts in the image plane we are now going to talk about the interaction of this ordinary and extraordinary wave which is coming out of this beam which is coming out of the beam splitter and then through specimen this is the distance between these two let us call this Σ_1 and this is Σ_2 nothing but ordinary and extraordinary ray and Δ bias and this is Δ object.

This situation is at extinction that means your polarizer and analyzer they are at the mutually perpendicular direction and we have also seen in the polarized light microscope as well as DIC microscope if you turn the analyzer to the particular orientation you can see only a dark background and there will not be any image contrast that condition is extinction.

So this is corresponding to this is a phase of this to raise and the Δ for this that is a phase shift for this or retardation I would say is 0 here and you can see that corresponding this is phase versus distance this is an corresponding amplitude versus distance you see that so you see that the since these two rays are not retarded enough that is Δ is almost 0 so you see that there is no I amplitude contrast here this is almost equal to 0. So it should come like this corresponds to 0 the amplitude is 0 so there is no contrast.

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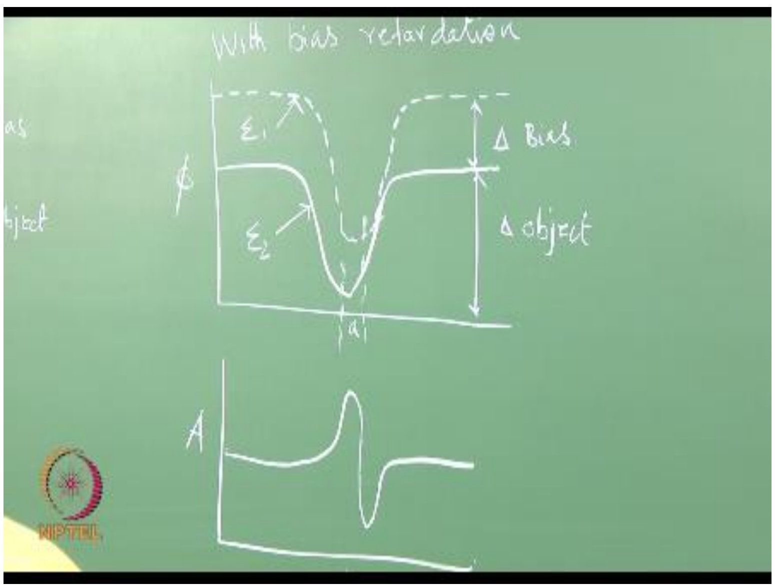

So with a bias biased retardation what happens this is Δ for object and then look at the corresponding amplitude versus distance the region correspond to these two you see that so with the biased retardation between to raise that is ordinary rare and extraordinary ray you see that Δ is increased that is adoration is the measure of retardation Δ.

So you see the corresponding amplitude which is increased to the peak and then decreased so that means what you will see the objects under the microscope at one side it will appear brighter the other side it will appear darker so the brighter side belong to this peak the darker side belong to this position that is rough okay, so this bright and dark contrast of the constituents and the micros micrograph will give you a three-dimensional appeal and with the grey background so that is how you appreciate the image contrast indeed DIC microscopy.

So now we will look at or we will move on to the last variants of the optical microscopy called fluorescence microscopy.

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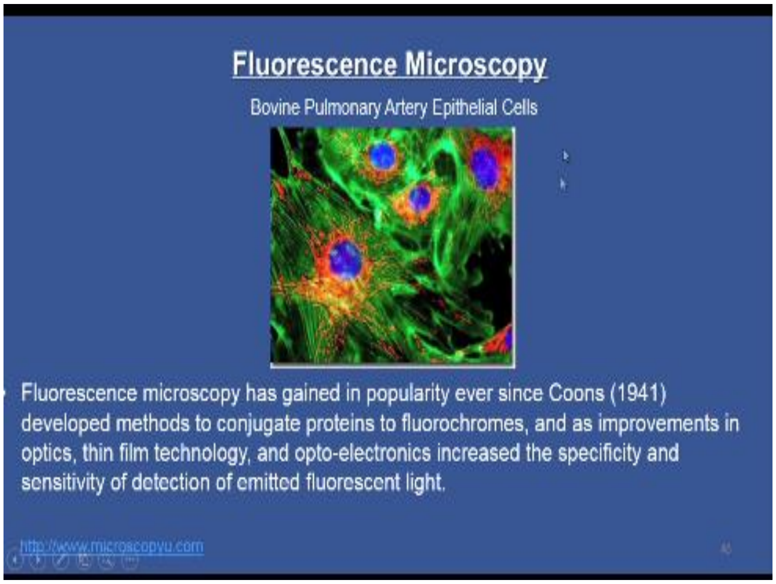

Look at this image which again taken from this website, the fluorescence microscopy has gained in popularity ever since cones 1941 developed methods to conjugate proteins to fluorochromes and as improvements in the optics thin film technology and optoelectronics increase the specificity and sensitivity of detection of emitted fluorescent light, we will see very briefly what is the principles of this microscopic technique.

Mostly it is being used in as mentioned it is thin film technology biological sample and transparent samples and so on we will now go through the principles and applications very briefly.

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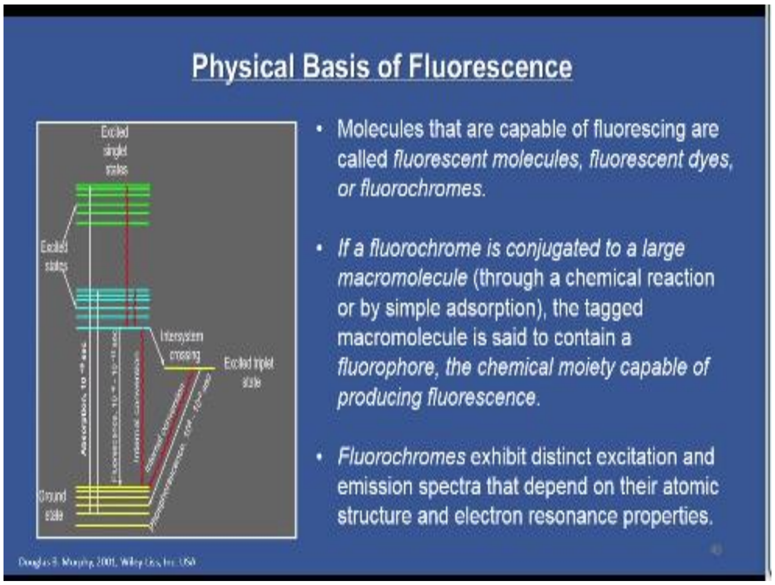

So let us review the physical basis of fluorescence I hope you have some idea about fluorescence or might have heard in your earlier classes, this particular schematic is known as Jablonski diagram, so if the fluorescent molecule absorbs the photons and then and these are all the energy states and we will first see the remarks and then we come back to this individual transformation, molecules that are capable of fluorescing are called fluorescent molecules.

Fluorescent dyes or fluorochromes, if a fluorochrome is conjugated to a large macromolecule through a chemical reaction or by symbol absorption the tagged macro molecule is said to contain a fluorophore the chemical quality capable of producing fluorescence, fluorochromes exhibit distinct excitation and emission spectra that depend on their atomic structure and electron resonance properties.

So if you look at the schematic you see this these are all the energy states once the photons are absorbed the electrons move to an excited states and you have some characteristic excited single states and excited triplet state and so on and you can see that a lot of phenomenon absorption and fluorescence internal conversion and then sorry yeah internal intersystem crossing and phosphorescence and so on. So what is that the fluorescence microscopy to do with this.

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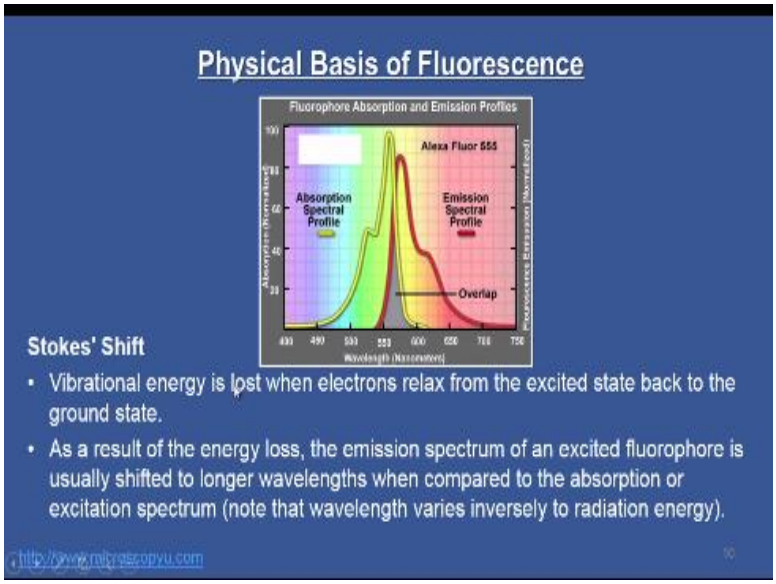

We will see that so there is something called a Stoke shift that means once the absorption of the photon takes place, the vibration energy is lost when the electrons relax from the excited state back to the ground state, as a result of energy loss the emission spectrum of an excited fluorophore is usually shifted to a longer wavelength when compared to the absorption or excitation spectrum.

Note that wave length varies inversely to the radiation energy, so you see that the schematic spectrum absorption spectral profile you see this yellow line and this is an emission spectra low profile or some kind of fluorophore you see that the emission spectrum as got higher wavelengths as compared to the absorption spectrum, so the primary idea is to separate these two and then see what kind of information one can get from this radiation at fluorescent radiation.

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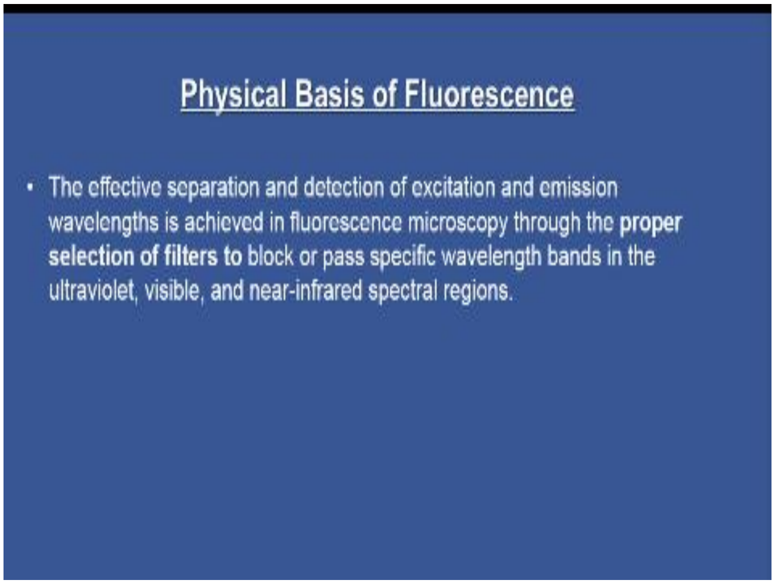

So the effective separation and detection of excitation and emission wavelength is achieved in fluorescence microscopy through the proper selection of filters to block or pause specific wavelength bands in the ultraviolet visible and near-infrared spectral regions, so you should recall that in an introduction class we have also seen some of these filters and that their characteristics. In this particular technique these filters are going to be used we will see how.

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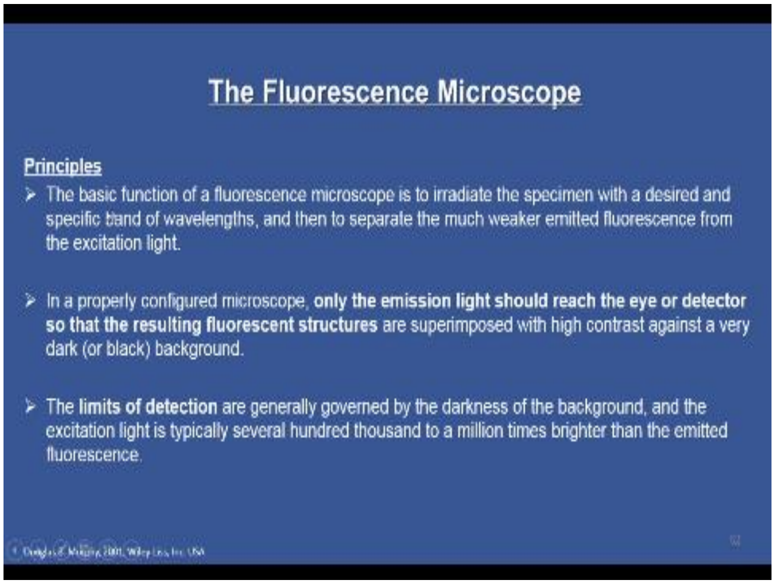

The basic function of a fluorescence microscope is to radiate the specimen with the with the desired and specific band of wavelengths and then to separate the much weaker emitted fluorescence from the excited light, in a properly configured microscope only the emission light should reach the eye or detector so that the resulting fluorescent structures are superimposed with high contrast against a very dark or a black background.

The limits of detection are generally governed by the darkness of the background and the excitation light is typically several hundred thousand to a million times brighter than the emitted fluorescence.

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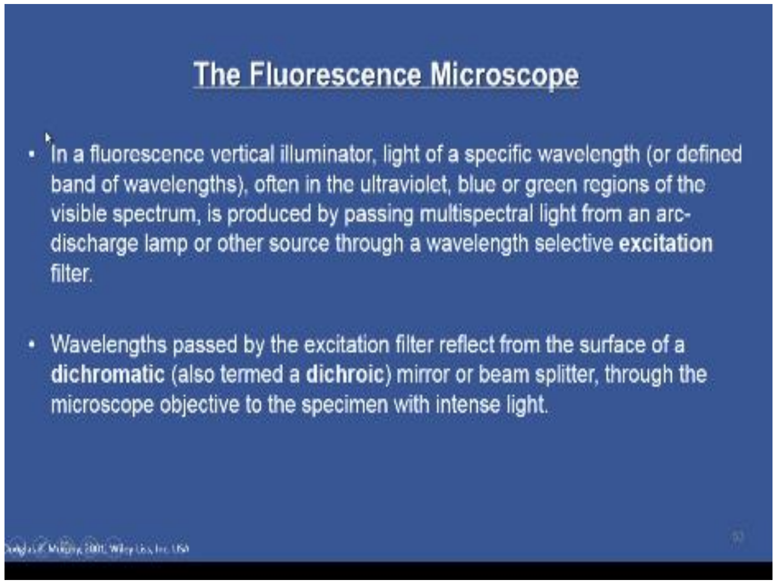

In a fluorescence vertical illuminator light of a specific wavelength or a defined band of wavelengths often in the ultraviolet blue or green regions of the visible spectrum is produced by passing multispectral light from an or discharge lamp or other source through a wavelength selective excitation filter, wavelengths passed by the excitation filter reflect from the surface of the dichromatic also termed as dichroic mirror or beam splitter through the microscope objective to the specimen with the intense light.

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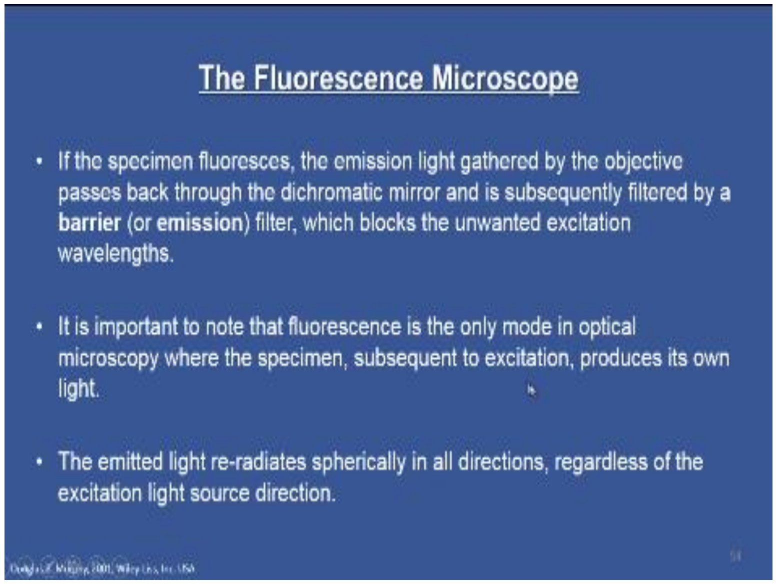

If the specimen fluoresces the emission light gathered by the objective passes back through the dichromatic mirror and it is subsequently filtered by a barrier or omission filter which blocks the unwanted excited wavelengths or excitation wavelengths, it is important to note that fluorescence is the only mode in optical microscopy where the specimen subsequent to excitation produces its own light the emitted light re- radiates spherically in all directions regardless of the excitation light source direction.

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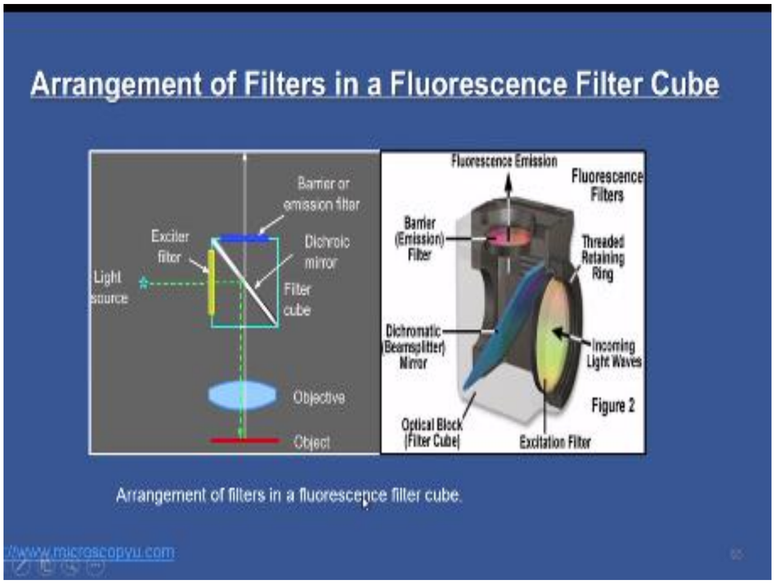

And this is the schematic which shows the arrangement of filters in the fluorescence filter cube you can see that this is a light source which goes through an excited filter and then you have the dichroic mirror and then you have emission filter and then a filter cube this is a very simple setup and then we see how this works.

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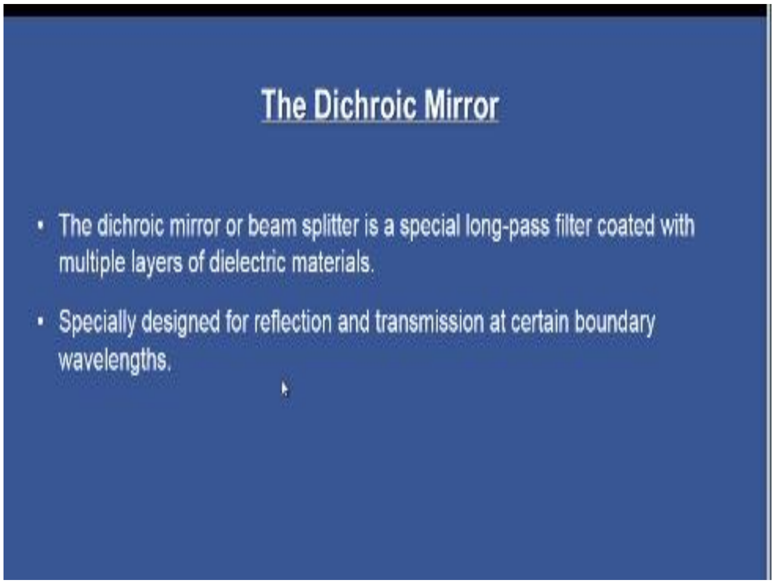

If you look at the function of dichroic mirror the dichroic mirror or a beam splitter is a special a long pass filter coated with multiple layers of dielectric materials especially designed for reflection and transmission at certain boundary wavelengths, so it is a kind of a long pass filter.

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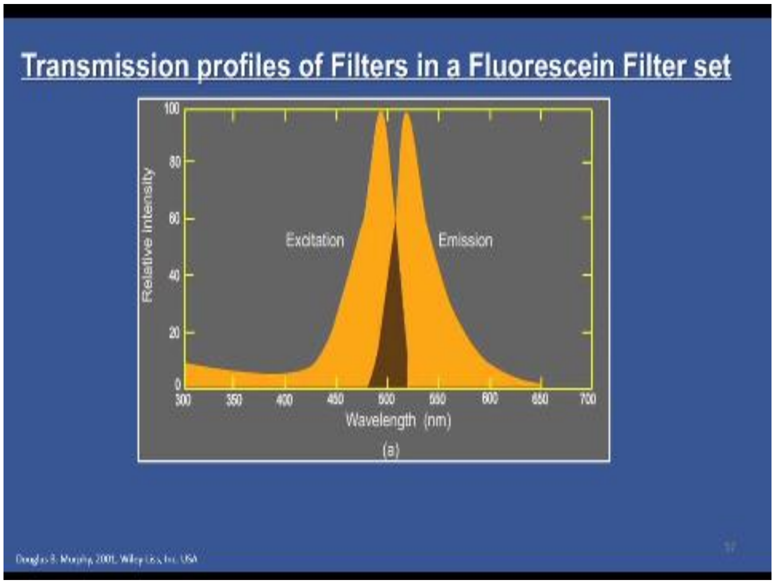

And let us look at what kind of action it does and this is the a transmission profiles of filters in a fluoresce in filter set so this is schematic spectrum you see that you have an excitation spectrum and an emission spectrum and in a fluorescent microscopy as we have seen we have to separate these two as much as possible and we will now see a three a different kind of high performance filter how they perform the filtering.

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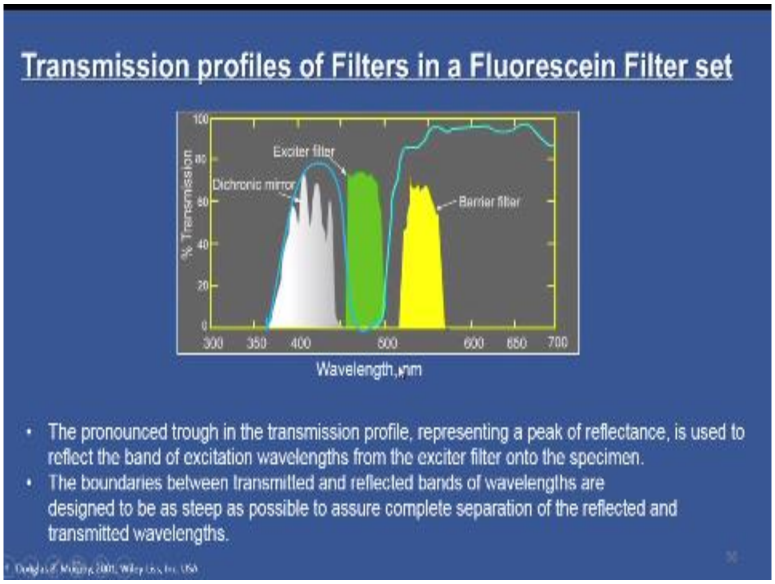

Look at this schematic plot where percentage transmission versus wavelength is plotted for the three high-performance filter, so one is barrier filter and whereas excited filter and what you are seeing here with this blue line which also includes this white region is a kind of die chromic or di- chronic mirror which has very sharp filtering capability we will see that the remarks first and then waited back to the spectrum again.

The pronounced stuff in the transmission profile representing a peak of reflectance is used to reflect the band of excitation wavelength from the exciter filter onto the specimen so this is very sharp you have the you see that one hundred percent 0% transmission means one hundred percent reflection of exciter filter which will be falling onto the specimen, the boundaries between transmitted and reflected bands of wavelengths are designed to be as steep as possible to assure complete separation of the reflected and transmitted wave length.

You can see that you have a complete Steep wavelength I mean a steep a transmission difference here goes almost ninety percent which separates the excitation radiation versus the transmitted radiation so which is very high performance filter this is how it acts it transmits or it reflects one hundred percent the excitation spectrum and then it that means it blocks here transmission is zero but it almost transmits ninety-five percent of the transmitted wave length in the in our case it is a fluorescent radiation.

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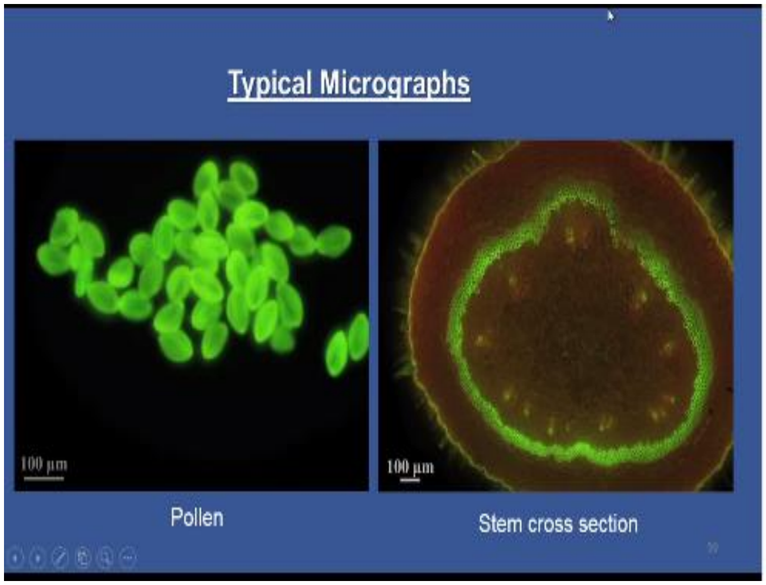

And these are the typical micrograph of obtained through fluorescent microscopy what I have shown in the earlier classes in using a transmission optical microscope these images were taken and this is an image of a pollen people do would have biological people would have done some experiment with this pollen is a kind of a powder which comes from trees, grass all the most of the plants and this is the one of the banyan tree root cross-section which we have seen in the phase contrast microscope as well as bright field illumination.

It is a similar root which appears under the fluorescence microscopy like this, so I think with this we will stop the discussion on all the optical variants we will now move on to a next topic that is how to prepare the samples for optical microscopy, so in next class I will take a different steps one has to go through to obtain a good specimen in order to get a very good micrograph and also we will see in a laboratory demonstration how each of the sample preparation steps are being followed to prepare a opaque specimen thank you.

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