Analytical Technologies in Biotechnology
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Module - 1
Microscopy
Lecture - 3
Dark-field and Phase Contrast Microscopy

In previous two lectures, we have discussed about the various concepts in the basic microscopy. These concepts will be encountered in the coming lectures, those were if you can recall they were like absorption, refraction, diffraction, magnification, contrast, resolving power, and so on. We discussed about geometric optics and physical optics and how the formation of image takes place was explained also, we have discussed about the compound microscope, various components of compound microscope like if you could recall, and there is a source of elimination. The condenser lens which condenses the light, the objective lens through which intermediate image formation takes place, and finally ocular or eye peas which forms the final image which will, which will be finally captured by the eye.

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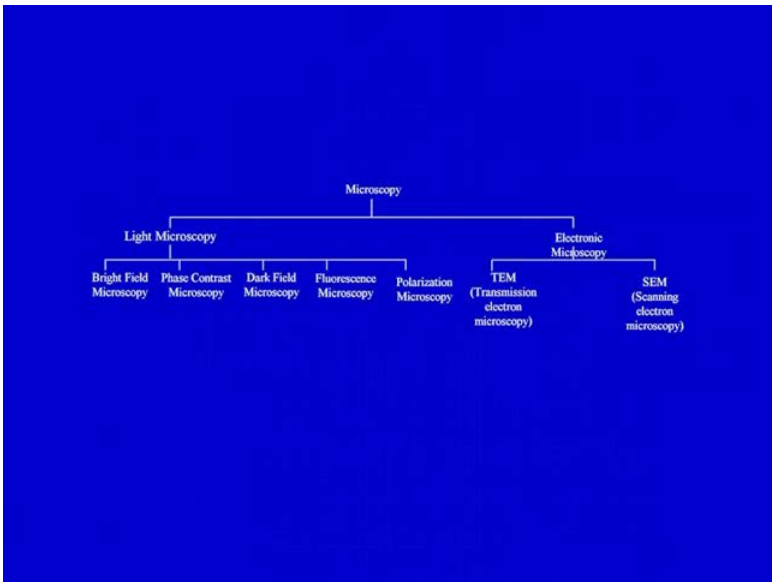

Now, both in here in subsequent lectures, we are going to discuss about specific microscopy techniques in both, light microscopy and electron microscopy. Now, light microscopy and electron microscopy, if they are compared, the basic concept remains the

same, but there are certainly specific differences between the two techniques. Light microscopy; for example, light source is simple halogen lamp or simple white light is utilised, in contrast electron in microscopy, you have deem of electrons which is generated from electron gun as we will discuss it later.

Then lenses, the light microscopy, the lenses are glass lenses or optical lenses as we call them where as in electron microscope. You have the electromagnetic lenses which are all we will be utilising, all like condenser objective, but they are electromagnetic lenses like microscopy. We do not have an enclosed system, you do not need vacuum, but in electron microscopy you need vacuum.

So, it is like enclosed chamber which is evacuated, so there are whole lot of differences between light microscopy and electron microscopy in terms of resolution. Also, you get much higher resolution many fold higher resolution in electron microscopy because the wavelength of electrons can be modulated and it is much lower than the light microscopy. In light microscopy, at best you can go is slightly less than the 0.2 micro metre which will reveal only details, but on electron microscopy you can go to much higher details. Another important part or compare, if you compare light microscopy electron microscope is the specimen preparation, electron microscopy requires extensive specimen preparation where you cannot use live cells and they have to be dehydrated.

So, then you have to add the mass to the specimen, so those electrons can be diffracted, whereas in light microscopy both stained and live cells could be utilised. For example, certain techniques uses steam cells, where certain other techniques contrast can be created by changing or creating certain optical phenomena where you can visualise the object as we will discuss later on.

Now, in subsequent lectures, we are going to discuss specific techniques and let us introduce you to these techniques here first in light microscopy. We are going to discuss bright field microscopy, dark field microscopy, phase contrast microscopy, polarisation microscopy, fluorescence microscopy and an electron microscope. We are going to discuss the transmission electron microscopy and scanning electron microscopy.

Now, bright Field microscopy as it says that the background is bright and damage which is formed is relatively darker than the background. Now, if you could recall when the light from condenser passes to specimen, there are two kinds of lights which will come

out of specimen into the objective, one is diffracted or altered light, another will be unaltered or direct light or undeviated light.

Now, a non altered or direct light forms the background, which is bright and the image formation is relatively dark. Now, here many biological specimens are transparent, so staining is a must in many cases, but only problem is that staining you cannot use live cells because staining kills the cells and also a lot of processing is required. Now, other techniques which we are going to discuss in the in the lectures will not be requiring the staining of the specimen rather there be contrast will be created with other methods which we are going to discuss.

Dark field microscopy is first such technique whereas it says there the background be dark and bright image formed against a dark background will discuss in detail how this works out how the image formation takes place in dark field microscopy. The next one will be phase contrast microscopy which is a very valuable technique for cell and molecular biology people. Now, here a simple optical trick is utilised to create contrast, I think all of you know that when the rays of light passes through a specimen. They encounter different components of the specimen and depending on the optical density of the components, the light will be diffracted accordingly and it would be retarded because of that phenomenon.

Now, this creates or this leads to the phase differences, that is the waves are now in comparison to the original wave, there is Phase changes have been taking place, now this face change cannot be seen by our eyes because these are not intensity differences. So, in this a simple optical trick leads to the changes in face to the intensity differences that is faced changes translated into intensity differences and we will discuss in detail how it is done. In brief, like the scientist or physicist who did that he used one direct beam, that is reference beam and the diffracted beam which would later combined to form click contrast. We will see how that is done in detail next technique which also does not require a staining is the polarisation microscopy.

Now, polarisation microscopy is a very useful technique for certain samples which cannot be stained even they sometimes cannot be visualised in Phase contrast microscopy because as the face increments are not large enough to create intensity differences. Now, these are polarisation microscopy utilises accessories like polariser

and analyser. Here, you require a sample which has a particular structural arrangement like either the individual particles are arranged in a parallel array or informal stacked disks.

Now, this leads to a particular phenomenon called form birefringence by these arranged particles, where a polarised light will be allowed to pass only if it is parallel to their individual arrangement. So, this particular technique is very useful for certain applications and utilised for those samples which cannot be visualised by staining or by other techniques like Phase contrast microscopy.

Next one which we are going to discuss is fluorescence microscopy, fluorescence microscopy utilises the phenomenon of fluorescence, where a fluorescent substance absorbs the smaller wavelength particular wavelengths. Then this emits the longer wavelengths which could be, which are invisible region. This phenomenon could be utilised for lot of different applications like locating a particular substance like protein or D N A or other macromolecules or other components, various components at different times. So, this is very useful technique, now fluorescence microscopy requires the use of certain fluorescence substance, because not many biological substances are fluorescent in it.

So, you have to have external force to be utilised in this other extension of fluorescence microscopy is co focal microscopy where you can use thick specimen or tissues. Remember, all these techniques right from light microscopy and electron microscopy requires thin slices of specimen which are in micrometres and nanometres in electron microscopy and because otherwise absorption of light or other problems will occur in image formation. So, in co focal microscopy, you can use sticky specimen and it utilises fluorescence optics.

Now, here you can focus at different times or you can restore the whole specimen in depth, or in sites you can take various pictures which are umpteen numbers of images, which can be later combined to give three dimensional look or three dimensional picture of the whole specimen. So, that is a very good technique for visualising three dimensional structures also in phase contrast microscopy will be extension of a phase contrast which we are going to discuss is differential interference contrast microscopy. You have worked or three dimensional quality images are obtained because of certain

interference techniques being involved in here where you use a lot of prisons and other accessories to make that happen. So, these were the techniques which will be utilised in electron in the light microscopy.

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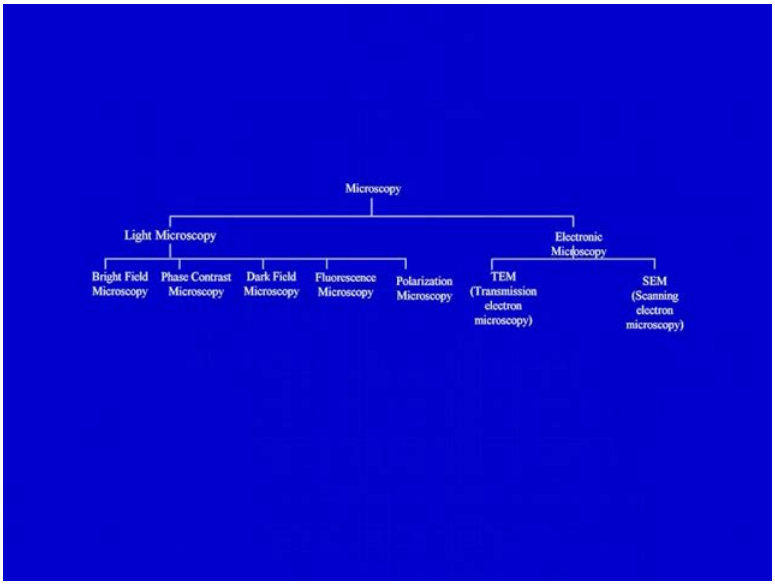

Now, the two techniques which we are going to discuss an electron microscopy are T E M and S E M that is transmission electron microscopy and scanning electron microscopy. Now, as it says transmission electron microscopy, the image will be formed by transmitted electrons through specimen and the image is formed due to the transmitted electrons from the specimen hitting the fluorescent screen. Here, a very thin slice like I said nanometre has to be there and there should be a diffractive material in the specimen in forms or form for heavy atoms which are added during the staining and sample preparation there is extensive sample preparation for electron microscopy.

In scanning electron microscopy, rather looking for the details internal details of the specimen. Here, surface details are visualised or recorded, so here rather than a transmitted electron here backscattered electrons which may be elastic or non elastic scattering those electrons be collected for image formation and again for retaining the surface.

There are special specimen preparation techniques, so this was a little introduction to the different techniques of microscopy enlightened electron both which we are going to discuss subsequently. So, let us start with some of the light microscopy techniques will

start with the bright field microscopy. Now, this is one of the simplest optical microscopy illumination techniques and mostly used in compound microscopes like I said earlier. The term bright field is derived from the fact that specimen is relatively dark contrasted by bright surrounding because of the dark because of the direct light which forms a bright background a sample is eliminated from below.

Those are transmitted light and observe from above like I have shown you in the compound microscope that will be the particular arrangement or in the microscope. Now, contrast is created by the absorbance of some of the transmitted light in dense area of sample. Now, like I said as few biological absorb light, a staining is required to enhance the contrast. For example, they might be certain coloured substances or which might absorb light, but otherwise the value it is very hard to visualise or make visible the details of the specimen and bright field microscopy and for staining. There are a lot of different materials dyes which are there to create contrast and will be discussing this in the end of the Light microscopy section in the specimen preparation.

Now, bright field microscopy may use either critical or Kohler illumination and it consists of a light source which is a simple halogen lamp condenser objective and ocular lens or it could be camera to view the samples. So, this is we are not going to much detail because it is a simple illumination technique and it is a very direct technique which is a widely used and observing the stained specimens. Now, let us move onto the next technique which where staining of the sample is not required and rather what you do is, there are certain optical tricks you applies to create contrast. Now, in dark field microscopy the bright image of object is produced against a dark background.

Many objects, now having refractive indices very, very close to their surroundings are very difficult to image in bright field microscopy. To view these things there are certain tech optical techniques have been developed, dark field microscopy is very useful for counting small particles and observing living unstained preparations. How does image formation takes place in dark field microscopy, so before we explain it on the figure let us understand little bit here.

Synthetical elimination, if you could recall like forms are very solid cone at object plane, that is light coming from condenser forms a solid cone at the object plane as we have discussed earlier. Now, supposing if there is no object in the object plane, so an absence

of an object causes field to be brightly illuminated uniformly. Now, here what is done is there is an accessory which is an opaque disk with transparent annulus is inserted just below the objective or in a condenser which causes the cone of light to become hollow. So, what you do is annular like disk or annual aperture opaque disk containing annular aperture inserted below the condenser lens and so what will happen a hollow cone of light will be illuminating the object plane.

Now, if there is no object then it will emerge as another hollow cone through objective. Now, annulus is made sufficiently that is a aperture where apertures are present in the open disk. It is sufficiently large that the hollow cone of light which is surrounding the object will not enter the objectives. So, objective is made smaller, so as to understand this no direct light is entering the objective. Now, what will happen if there is an object placed in the object plane, if there is object which is placed in or specimen placed in the object plane then certain diffraction is bound to happen and some of the diffractive race of certain orders will enter the objective?

So, what will happen to a bright image because these are light which is entering the objective and will be formed against a dark background because direct light does not enters there will be a dark background. But, bright light and certain orders of diffraction enter the objective and they will form a bright image against a dark background. So, the outcome is that you get exceptional contrast, but very limited details here and mainly many details are lost because you have avoided zero order light which is direct light. Now, depending on light intensity and decreed to which this internal reflections have been eliminated the quality of the picture will be or image will better.

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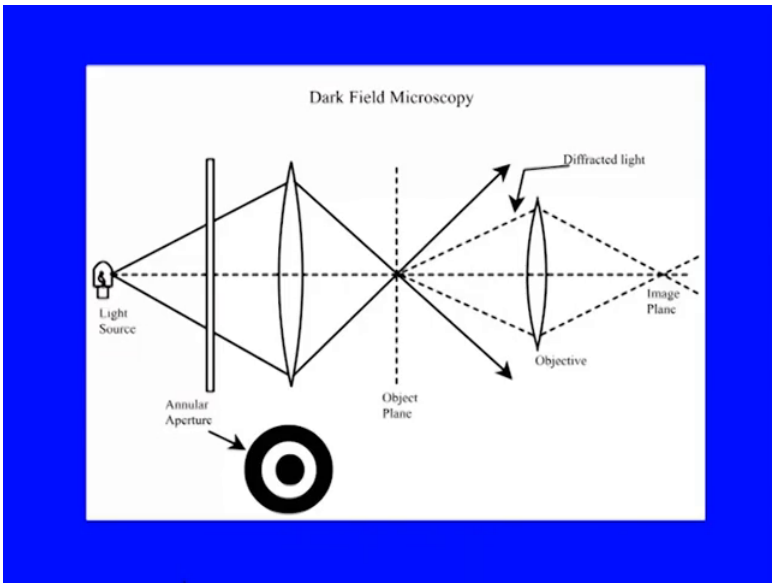

Now, in dark field microscopy if you see the optics of this, this figure shows you the optics of dark field microscopy you can see here the light from the light source is allowed to pass to the condenser, but you have inserted a annular aperture here. Now, these here are not shown here in this, but if you see here there is a whole or there is an area, this is like aperture is there in this particular disk. Now, the light passes only from this area and not from the other part which is opaque here. So, what you get you get, hollow cone where this is part is not eliminated here and you get a hollow cone of light illuminating the object plane.

Now, this hollow cone of light in absence of an object will directly go and will not enter objective because you can see the size of the objective numerical aperture of the objective is smaller. Now, if object is present there be a diffracted light certain diffracted light is just one light train we have made, but there is certain diffracted light which will enter and the objective and will form an image in the image play now this will be the bright image against a dark background, so you will get a very good contrast here. Now, this particular technique is used, this is ideal for objects which are minute living and they have very close refractive index with the surrounding. For example, living aquatic organisms die atoms small insect's bones fibres bacteria and cells in animal tissue culture can be used here.

No biological specimens could also be utilised here which include chemical crystals, or thin sections of polymers ceramics having a small are enclosed or other are different specimens could be utilised. So, dark field microscopy is quite good for particular applications, but not good to get too much of details here.

For example, it will be a very good technique to count the number of particles, like say number of viruses or other particles, but if you want details then you have to use other techniques. So, this was about dark field microscopy, now let us move onto the next one, that is phase contrast microscopy. Now, phase contrast microscopy is one of the most valuable techniques in the field of cell and molecular biology and here the contrast the way contrast has been created is a is very fascinating to observe. Now, this particular technique was first described in nineteen thirty four by the Dutchman Fitzgerald and for which she was awarded Nobel Prize for physics in 53.

Now, this is a contrast enhancing optical technique for transparent and unstained specimen like living cells sub cellular organelles microorganisms etcetera. Remember, one thing has to be taken into account that thick specimens are not suitable for this technique. Another thing is the samples or specimen must be non-absorbing, because that will create intensity differences and will lead to a poor image. Now, in Phase Contrast microscopy, minute differences in phases will understand how that minute difference in phases is created which might result due to very small differences in refractive indices of the components of a specimen through which light passes.

These are translated into intensity differences remember the phase differences cannot be observed by our eyes, but intensity differences can be observed by our eyes. So, this is very important, now here Fitzgerald used a reference beam to create this whole thing, let us see how this is done. For example, consider a transparent object in a transparent medium and it is being illuminated by parallel light falling perpendicular on that disk. Now, what will happen if the refractive indices of the two are different with the light tends to differ and phase due to retardation of light.

Now, disk remains still invisible due to the inability of eyes or the photographic film to detect different phases. So, what we are seeing here that though something is happening, but you are not able to see this, now in presence of an object diffraction through the object will also contribute towards image formation, but again how do we visualise this.

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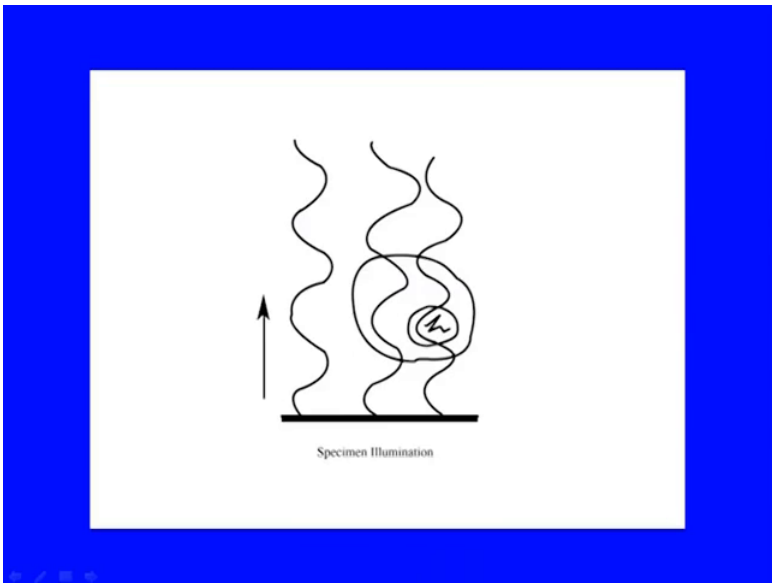

If you see this picture this is here to show you that how the retardation of wave will occur if you see here this is a direct wave, which is going on when it passes through the specimen here then the different components, like this is maybe liquid component here. This component contains certain more material or has higher optical density you can see differential retardation of the wave here like here it is more retarded. Now, here again another thing is that when it passes it will be diffracted in different directions and the diffraction will also depend on how they are placed out dense an optical object is. So, these things will lead to the face differences.

So, when this Phase difference, what we have to do is, it has to be translated into intensity differences, how it is done. Let us get in here, so what is done is you place an annular aperture like dark field microscopy what we have done we are putting our annular aperture below the condenser lens. Likewise, here also will be placing our annular aperture or facing in focal plane below the condenser and which will result in a hollow cone, we cannot do a innocence call it a hollow cone. But to understand that does not mean that hollow cone is completely a hollow cone is illuminating the specimen, but to understand this we say it is a hollow cone of light illuminating the specimen.

Now, when you this light passes through condenser annulus and it will pass through the objective. It will make the image of the condenser annulus at that plate of particular place of faceplate is introduced that is after the objective where the conduct condenser

annulus will form its image of faceplate is introduced. So, these are conjugate arrangement that is it has to be completely coinciding the faceplate has two exactly coincide with the image of the condenser annulus because then only this optical phenomena could be obscure.

Now, let us see what happens then once you have done that then all light rays or light wave speakership passing through condenser annulus will be either advanced. I think I will just show you that on my screen that what is face ring, what faceplate is before we go any further, so please pay attention to your screen, so like I was saying they will the face ring and there is a phase plate.

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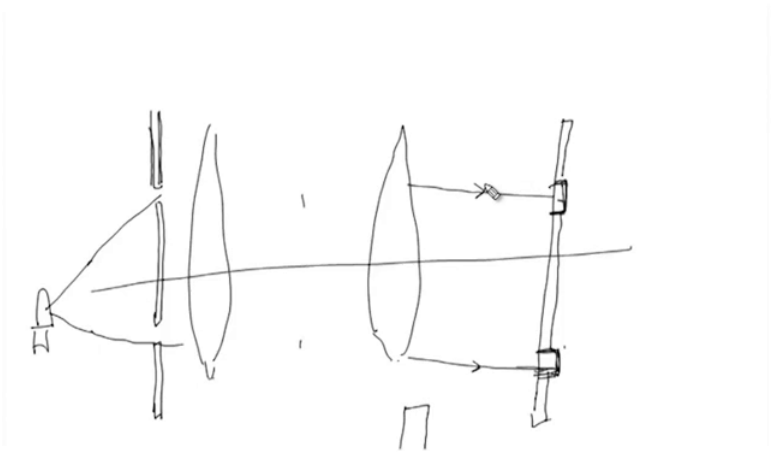

Now, face ring or annular aperture is something which is like this that you have created two apertures in here through which the light will pass which will be parallel light or just we are showing it here through the light source. Now, this could be we can say that there is annular aperture that is there is a place aperture through which the light is passing.

Now, if you if we have to say face ring has to be formed after the objective lens the face ring will look something like this and it will completely coincide with this that is these two apertures. Now, what is has been done in this face ring that you have there are two kinds of face rings could be one is at exactly where apertures there you have deposited an extra layer another at two places, so what will happen when extra layer is there? The light rays which comes from the objective like they will be condenser first here then they

will be if I make it like this be object plane. Then they will be objective lens and through objective lens, these light rays will pass through these two places.

So, what will happen is when the wave is going through this place here then this direct light, there will be two lights, one is diffracted light another is undiffracted light. Now, this diffracted light which is an undeviated light will pass through this faceplate, if it is layered faceplate then what will happen it will be wave will be retarded by one quarter wavelengths. So, layering is done in such a way that it will be retarded by one quarter wavelengths.

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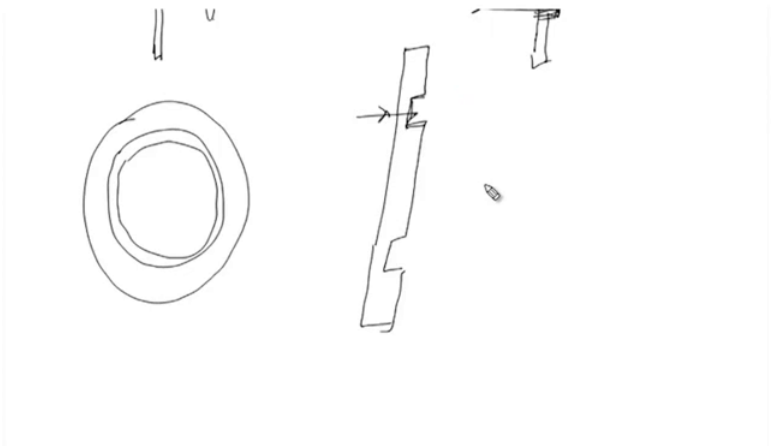

There could be another arrangement which could be rather than rather than a layering, it could be grooved, there could be a groove could be stilled here. Now, this groove is still here then what happens that this groove will lead to the advancement of because you have less material in here, so the light ray passing through this will be advanced by one quarter wavelengths.

So, it is done in such a way that either in layered form it is retarded one quarter wavelength or it is advanced one quarter wavelength, so this is just to make understand that how face ring or annular aperture and the face plate is playing a role in here. Now, let us move on again to our lights here.

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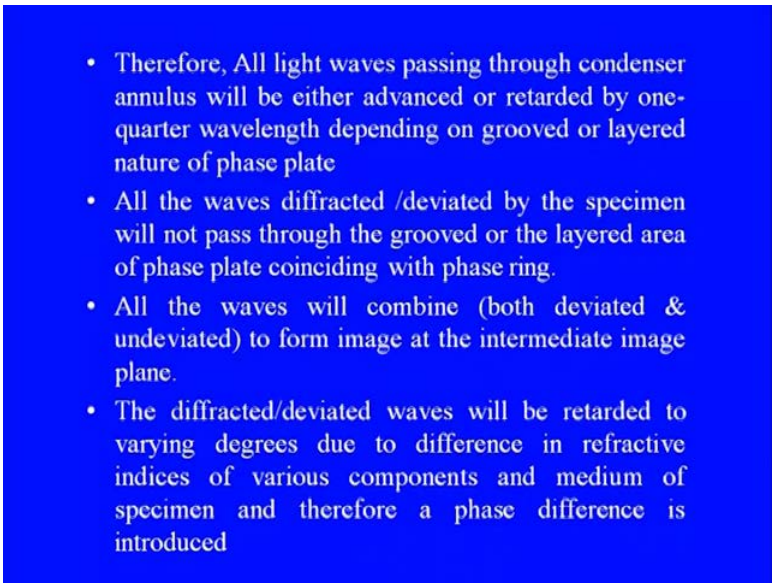

Now, here is what we have tried to understand in this that all light waves passing through condenser annulus will be either advanced retarded by one quarter wavelength depending on your passing through groove or layered nature of faceplate. Now, all wave diffracted or deviated because when you have a specimen then diffraction is autoworker. All wave diffracted deviated by the specimen will not pass through the grooved or the layered area, we will see in the figure later on.

Now, here diffracted rays will be retarded to a different extent because your specimen will contain various kinds of things. For example, if say you are observing a single cell or a thin layer of cells, a cell will contain cytosol also, it will contain nuclei also it will contain Golgi. It will contain mitochondria are it will contain fat droplets also it will contain ribosome is also all of these will retard because of differences in refractive indices will retard it differentially, so you will get the rays or wave frames with different faces here.

So, what happens that all wave which are diffracted which are retarded to a different extents will pass through the other part of the faceplate and also mainly from other parts of faceplate rather than grooved or layered area, but they will. It does not mean, it will pass through grooved or layered area, but direct rays will pass from grooved or layered area and they will be retarded or advanced. Now, all the waves will combine both deviated and undeviated to form image at the intermediate image plane.

Now, diffracted and deviated wave will be retarded to varying degree a like I said due to differences and this will create a particularly create different intensity, differences will see how that is done, when the phase difference of diffracted wave due to specimen. Now, here you have to understand that as we have seen the direct light passes through grooved or layered, there is one quarter wavelength retardation or a advancement as per the whether it is grooved or if it is layered there will be retardation if is grooved.

It will be advancement likewise when the wave pass through the specimen and they are diffracted. Then there also the retardation takes place and which is assumed to be approximately one quarter wavelength like I said, different refractive indices will a retarded differentially, but one quarter wavelength could be understood as a standard thing approximately. Now, when this is combined with the phase difference created by faceplate the two things will happen, one the object will appear bright against a relatively dark background, so what does that means that due to constructive interference.

So, what does that mean is when undeviated light is retarded, if you remember if it is a layered faceplate already the undeviated light or direct light is retarded one quarter wavelength. Now, many superimpose this with the like one quarter wavelength retarded, now diffracted wave than both gives a constructed reference. Therefore, due to constructive interference, you will get a wave which will be more brighter relatively to the background. Other thing can happen that is second option could be that object would be dark white dark against the bright background because undeviated light has been advanced by the faceplate that is grooved face plate. That is called positive phase contrast, earlier one was called negative phase contrast where it was layered.

So, when undeviated light is advanced by one quarter wavelength and deviated light is retarded by one quarter wavelength, when they combine in the image plane they are out of phase by one and half wavelength that is the condition for destructive interference. So, you will get a dark image against a relatively bright background, so you will get a very good contrast. Let us understand these combination of waves like I was talking about and in this figure you be able to understand this.

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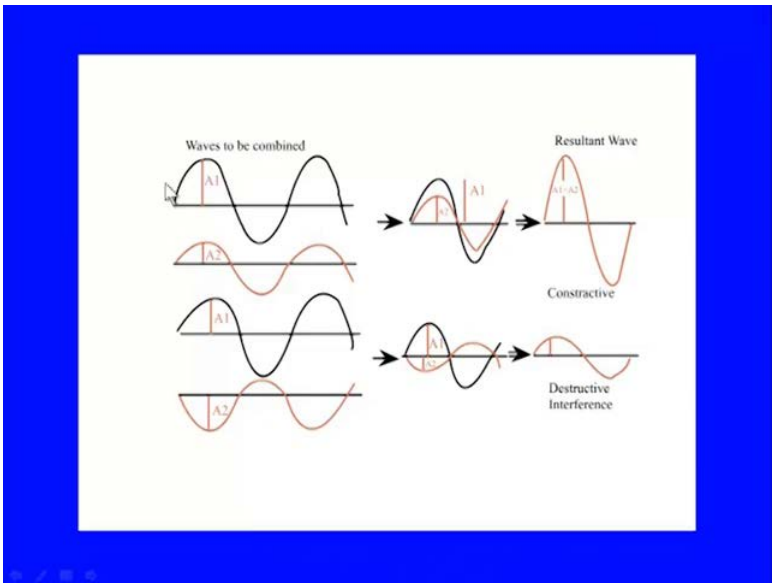

If you see here, if you say this is a direct wave which is being going and which is retarded one quarter wavelength and this is the another wave which is also retarded one quarter wavelength because of specimen. Now, when these two waves combine then the amplitude is bound to be increased and so the brightness the resultant wave will have higher amplitude and therefore, higher intensity. It should be a constructive interference which will show up as the as the bright image against a dark background relatively dark background.

We cannot say it is a grey background, we cannot say it is a very dark in contrast when there is an advancement in the light ray or the wave due to the groove nature of the faceplate. Then this another deviated light which is already one quarter wavelength deviate retarded which is approximately value than both are out of phase by 180 degree. Finally, what you get resultant wave is of lesser amplitude and so lesser intensity and so you get relatively dark images against a bright background, so these are two phenomenons where you can either use a negative phase contrast or positive phase contrast.

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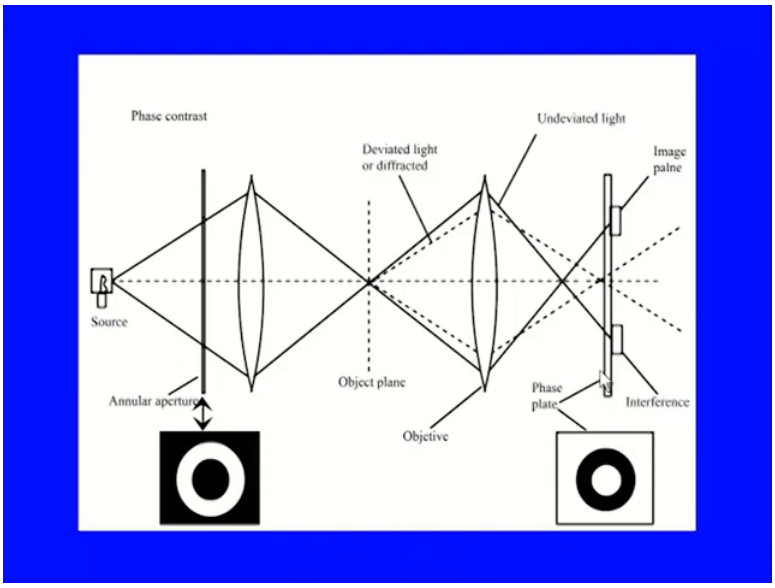

Now, to understand this now optics of the phase contrast microscopy like I have already discussed, but we can understand by this figure. You can see here there is a annular aperture like it was in the dark field microscopy and this is like area where through the light will pass, it passes through condenser lens and illuminates the object plane by supposedly hollow cone of light.

Now, deviated light will pass through other parts of the faceplate this is faceplate and the undeviated light passes through the phase where layered or grooved part of the faceplate. Now, certain times when intensity is has to be balanced then objective could be covered with certain a like alumina films or other things which does not change the face to decrease the intensity of the undeviated light. Now, these ones will combine in the image plane and they will produce the images which are either dark meat against bright background or bright image against dark background as per the phase contrast microscopy technique you have used.

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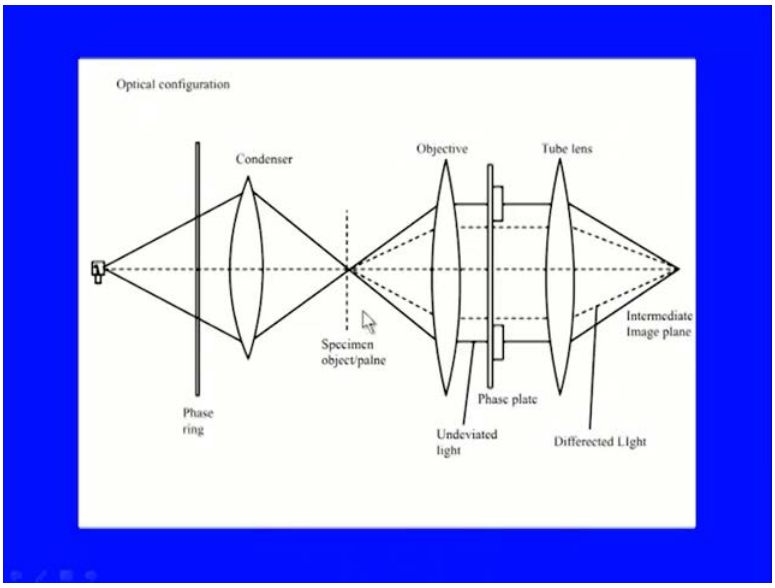

So, now if you see here an optical configuration this is like last time what I have seen this simple optical configuration of phase contrast, but if you are utilising infinity corrected optics than just to show how in the infinity space these accessories optical accessories are added. If you can see here a faceplate is being added between the objective and tube lengths and so parallel rays, which are like focused at Infinity passes through objective and then they are finally, they are focused by tube lens. So, it is the same optics as I have shown you earlier.

If we go back and understand this whole thing that simple optical trick has led to the creation of contrast through, which you can see the different specimen different components of the specimen very clearly like I said, the all different components will be bright or dark relatively. And to a different extent because of different retardation of the waves and as the combine and image plane they form of very highly contrasted image. But there are certain disadvantages also of phase contrast microscopy which needs to be looked into a major disadvantage of phase contrast microscopy is the unavoidable bright hallows which are surrounding the specimen and they are mainly caused due to many factors one is the diffraction by faceplate.

Now, this particular problem what happens is if you see our if you will observe the different images from phase contrast microscopy. It is a very valuable very good technique here cytosol of cytoplasmic contrast with nuclei, nuclei will contrast with other

sub cellular organelles present in here and there very nice pictures are opted. But if you see the membranes than the surrounding of the specimen will certainly see bright hallows, and sometimes it is hard to distinguish a certain parts of the components of in that particular area and due to bright hallow diffraction hallows.

Now, problem of hallows have been solved to a great is extent or completely in another interference microscopy technique, it is like phase contrast microscopy technique which is called differential interference contrast microscopy. This uses a complex lens system with prisms and other things to generate diffract, where diffracted in and undiffracted lights are combined later on and we are going to discuss in upcoming lectures this particular technique.

So, apart from bright hallows which are formed is a very good technique obscure details, now here not only that there are a lot of different applications of this technique like for example, visualisation of sub cellular organelles or tissues or microorganisms. The details of this could be obscured by phase contrast microscopy and you can use a single cells or thin layer of cells to observe that without staining. Remember staining is not allowed in this as staining will create intensity differences, so you require a non-absorbing specimen.

In this case dynamic mobility of a mitochondria and mitotic chromosomes etcetera can be followed very nicely like you can pictured that all can video record the whole phenomenon as it is going on in real time. This has been used for diagnosis of tumour cells; it has been used for many other applications in the field of virology bacteriology haematology and other areas of biological sciences. So, phase contrast microscopy is one of the most important techniques in microscopic to some of the lecture this particular lecture what we have discussed today is three techniques one is bright field microscopy, dark field microscopy and phase contrast microscopy.

Now, bright field microscopy there is a bright background and a dark images formed relatively dark images formed staining is required for transparent objects or specimen and most biological specimens are transparent in dark field microscopy in contrast to bright field here. The background is dark and your images bright, but only thing is because of avoiding of or because of not inclusion non-inclusion of direct light zero order light lot of details are lost. So, it is a very good technique you get very good

contrast, but has limited applications like for example, you can use it for counting in other things. So, dark field microscopy has a particular use, but it cannot give you a lot of details.

Now, third technique which we discussed today's phase contrast microscopy phase contrast microscopy has a really revolutionised cell biology observation of cells in cell biology. This has led to observation of many phenomenon's which could be dynamic motion of the movement or the mitochondria, or a mitotic chromosomes are different phenomenon's which are going during this particular process of diagnosis of lot of different kinds of cells has been done and lot of other applications had been given here.

The contrast is develop by a phase ring annular aperture and a faceplate, which conjugate to the face ring and faceplate of when deviated undeviated light passes through the faceplate. It could be it is advanced retarded and that leads to the constructive or destructive interference with the diffracted light in the image plane and leads to the image formation of very high a good contrast is obtained. You are able to see our images in vivo an in vivo condition without any problem only background is bright hallows, non absorbing specimens has to be used and you cannot use thick specimens. So, will end this lecture today here and will continue our discussion in the next lecture.

Thank you.