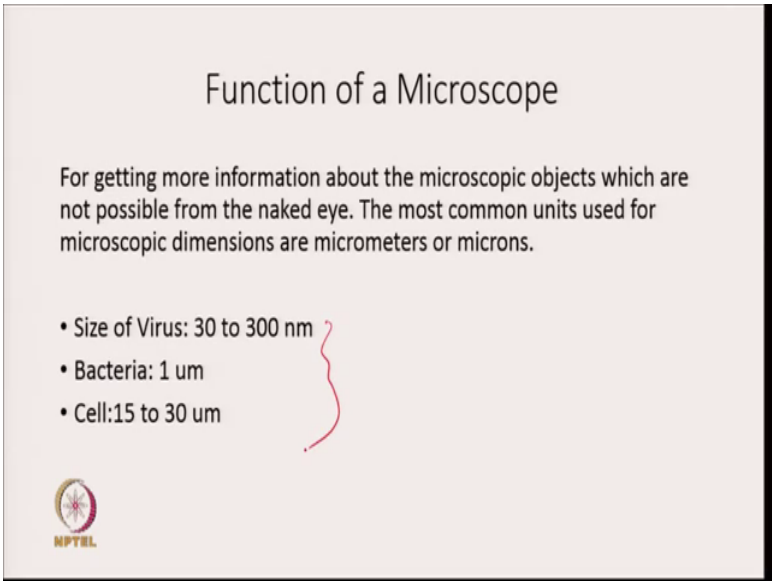
Spectroscopic Techniques for Pharmaceutical & Biopharmaceutical Industries
Professor Shashank Deep
Department of Chemistry
Indian Institute of Technology Delhi
Lecture 27
Microscopy

Hello students, welcome to the lecture 27 of this course. In the last lecture I have discussed about fluorescence spectroscopy. We discussed both steady state and lifetime measurement. In this lecture, I will discuss about principle of microscopy and then I will discuss about the fluorescence microscopy.

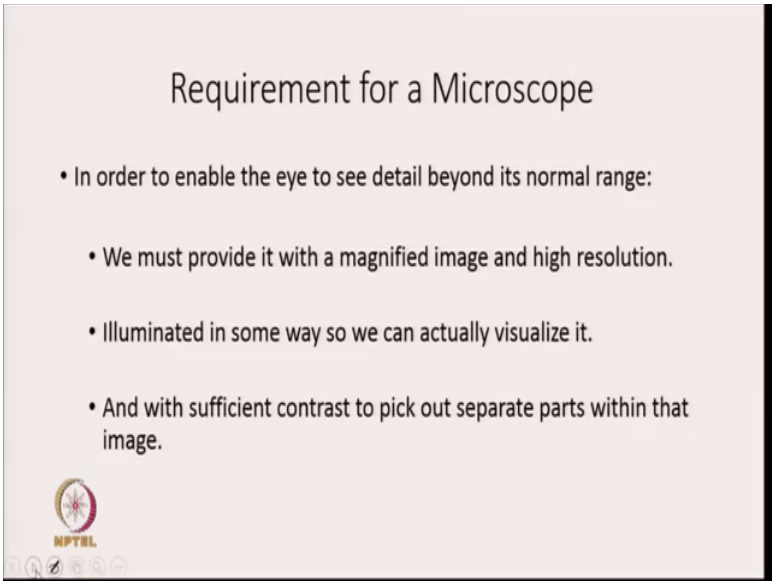
(Refer Slide Time: 0:50)



So, first thing to know is, what is the function of microscope, and almost all of you must be knowing, what is the function of microscope? We get more information about microscopic objects, which are not possible from the naked eye. So, anything, any small object, any microscopic object, which we cannot see through naked eye, we can get information about those objects from a microscope.

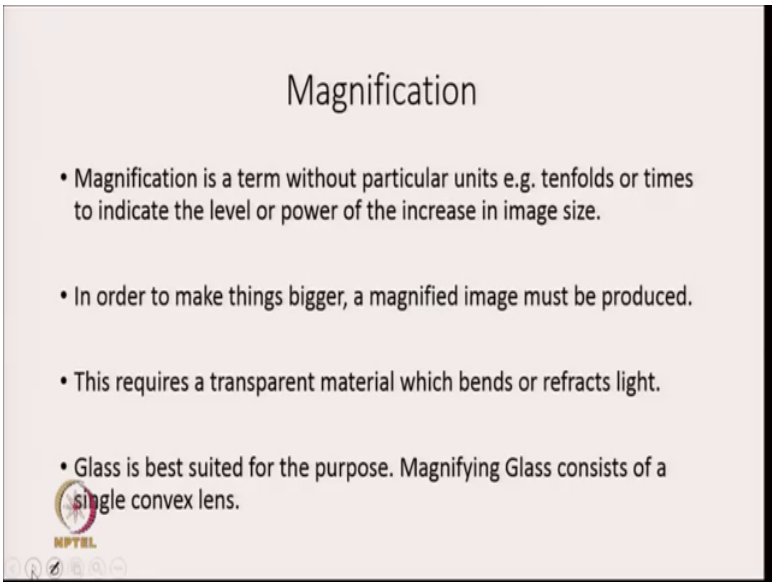
The most common units used for microscopic dimensions are micrometers or microns. So, the objects, which we look from microscope is few of them are given here. Size of virus is from 30 to 300 nanometer, size of bacteria is about 1 micrometer, and size of cells are between 15 to 30 micrometer. These microscopic objects we cannot see through naked eye and so we need a microscope if you want to get more information about these particles.

(Refer Slide Time: 2:49)



So, if we want to look at, if we want to enable our eye to see detail beyond its normal range, we need to provide our eye a magnified image with high resolution. The second thing we need is, we need to illuminate the object, so we can actually visualize it, and then we also need to provide sufficient contrast to pick out separate parts within that image. So, these are the three requirements for a microscope. I will go one by one and discuss how we can achieve it.

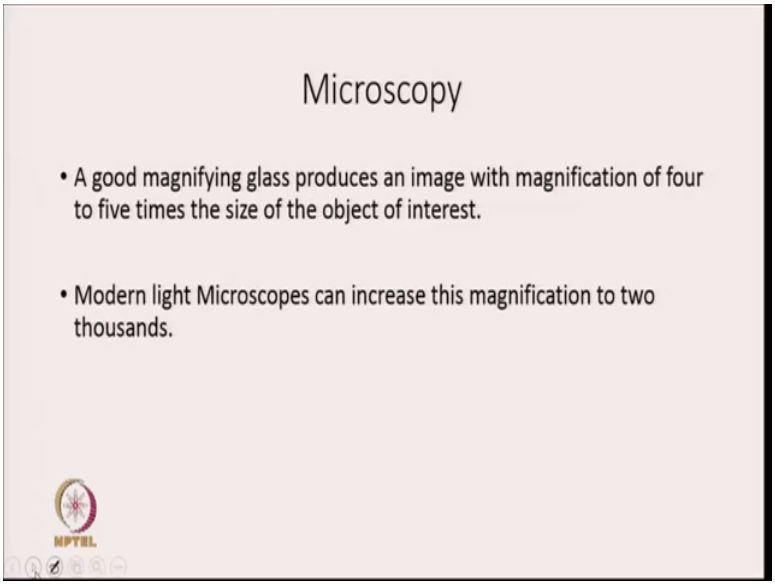
(Refer Slide Time: 3:28)



So, first thing, which need to be discussed is magnification. Magnification is a term without particular unit, for example, we say 10 folds or 10 times to indicate the level or power of the increase in image size. So, basically magnification tells you about the size of image with

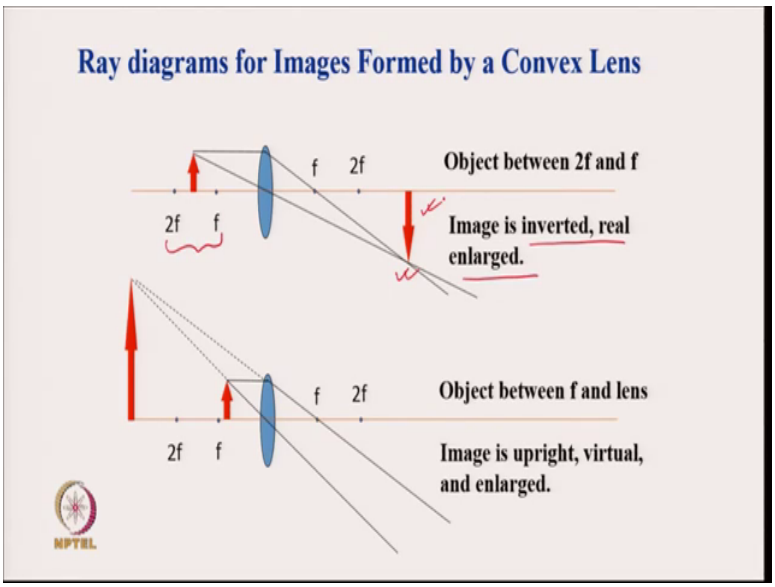
respect to object size. If we want to make image bigger or microscopic objects bigger, a magnified image must be produced and for production of magnified image we require a transparent material, which bends or refracts light. Glass is best suited for this purpose and a magnifying glass consists of single convex lens, so magnifying glass consists of single convex lens.

(Refer Slide Time: 4:46)



A good magnifying glass produces an image with magnification of four to five times the size of the object of interest. So, certainly it is not enough and so we need microscope. Modern light microscope can increase this magnification to two thousands. And that is why microscopy is very important.

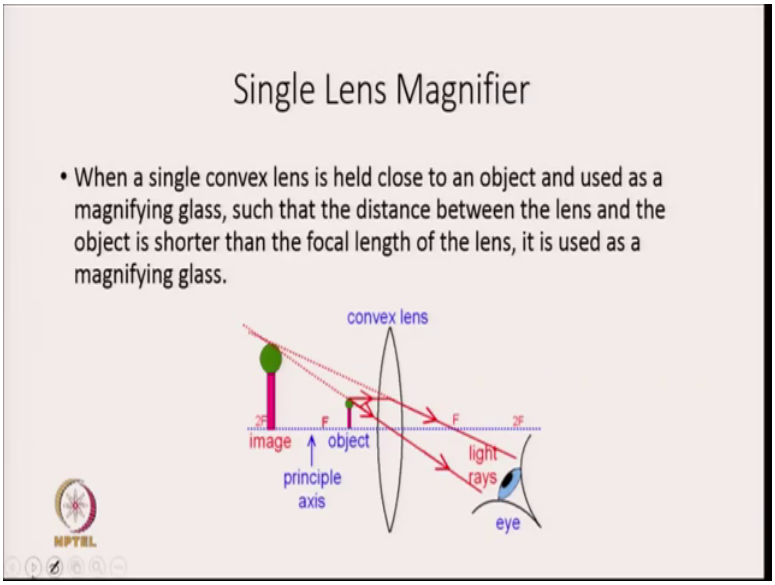
(Refer Slide Time: 5:20)



Now, let us try to understand first about magnifying glass. What I told you that magnifying glass is basically a convex lens? So, if you remember your class tenth optics chapter, if I place an object between 2F and F, where F is focal length of the lens, then we expect image to be form beyond 2F, which is inverted real and enlarged. That is what we studied when we were in our class 10, 11, 12.

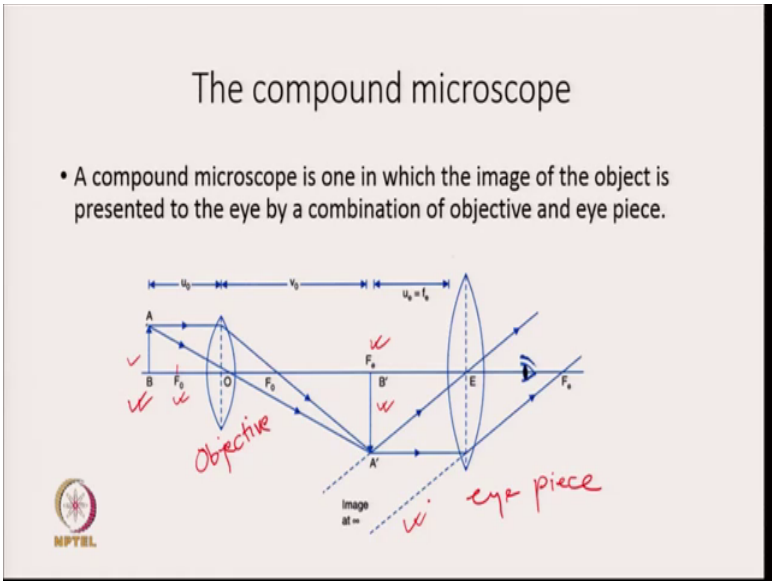
So, here you can see that a magnified image is formed, magnified image is formed. We need magnification, so this is one of the way. The second way is, if I put my object before the focal length, before the focal length, then again I can get an enlarged image, but this image is upright, virtual and enlarged. So, if I place my object between F, which is focal length and lens, then I can get an image, which is upright, virtual and enlarged. So, these are the two ways in which a lens can magnify an object.

(Refer Slide Time: 7:07)



So, if you go for single lens magnifier, what we generally do is, we put object between lens and your F, and what we get is an image, which is virtual, magnified and upright. So, when a single convex lens is held close to an object, and used as a magnifying glass such that distance between lens and object is shorter than the focal length of the lens, shorter than F, then it is used as magnifying glass.

(Refer Slide Time: 7:44)

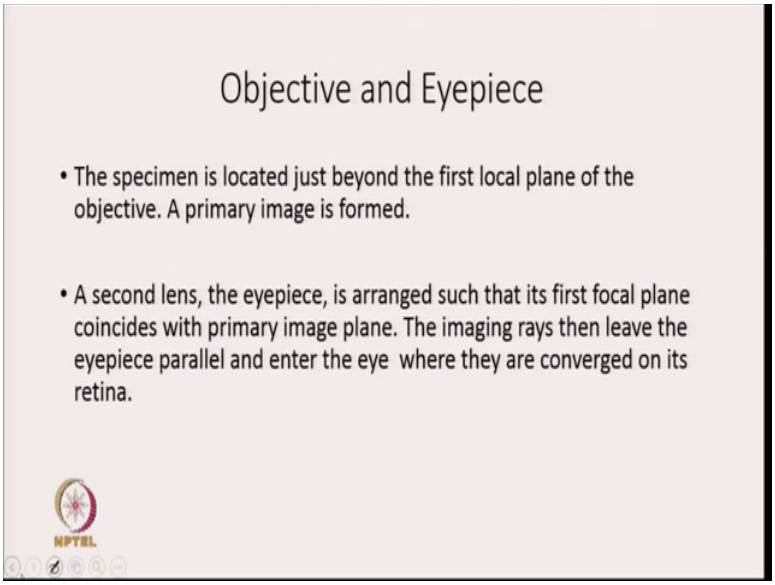


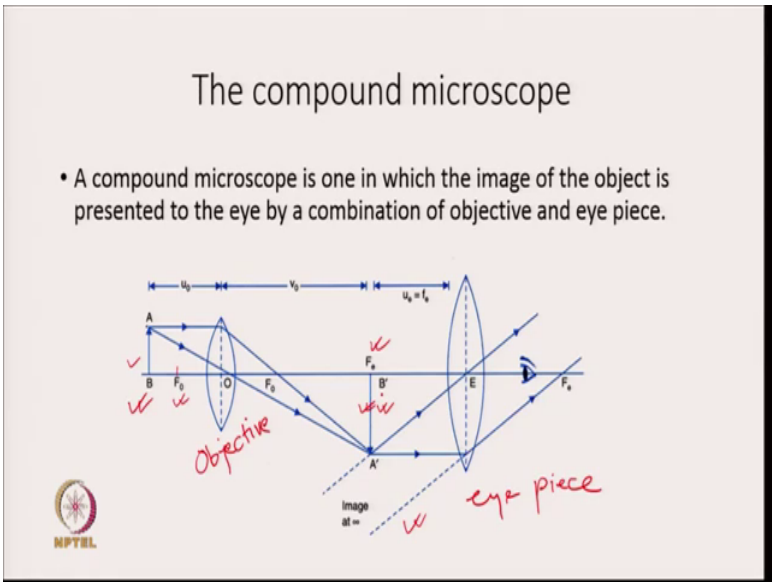
Now, let us go for the second kind of microscope which is known as compound microscope. A compound microscope is one in which the image of the object is presented to the eye by a combination of objective and eyepiece. Both are lens, this is objective and this is your eyepiece. Eyepiece is closer to eye, and A B is your object, A B is object.

So, in this what we do is we use the property that if I put object between two F and F, then I will get an enlarged image, which is inverted, enlarged image which is inverted. So, first what we do is we are placing object between F naught and 2f naught, here Fo, it is basically Fo which tells you that focal length of objective, then there are two lenses. So, now, we are denoting the focal length of object by Fo and focal length of eyepiece by Fe.

So, if I put object between Fo and to 2 Fo we are going to get your image at this position, and then again we take another convex lens which is known as eyepiece and we arrange such that this image, this inverted image is on the Fe of this lens, focal length of eyepiece. When we do that, then we expect image to form at infinity, effect of lens that if we put object at focal length, we expect that image will be formed at infinity.

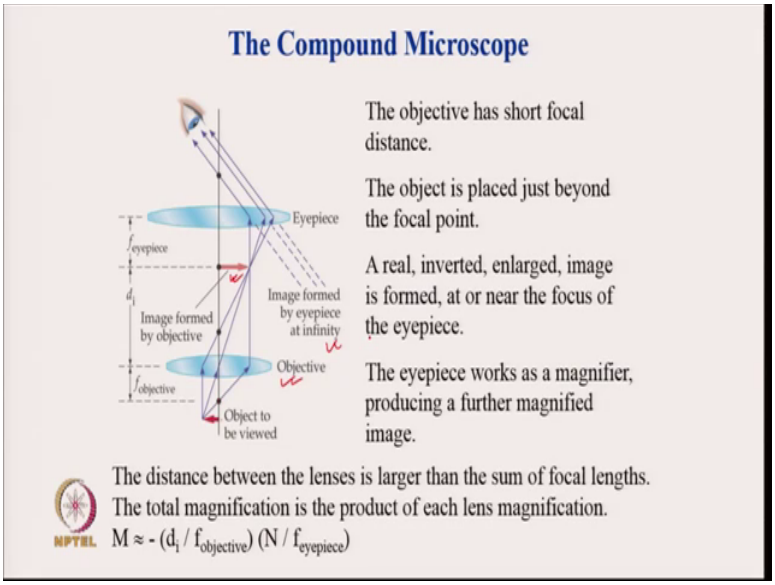
(Refer Slide Time: 10:07)





So, what we did in this? We placed a specimen just to beyond the first focal plane of the objective and a primary image is formed, and this is your primary image. And then, what we did? A second lens, the eyepiece is arranged such that its first focal plane coincides with primary image plane, so that is very important. So, what we did is, what we did is we arranged this eyepiece such that your image, primary image formed is basically at the Fe focal length of eyepiece. The imaging rays then leave the eyepiece parallel and enter the eye, where they are converged on its retina.

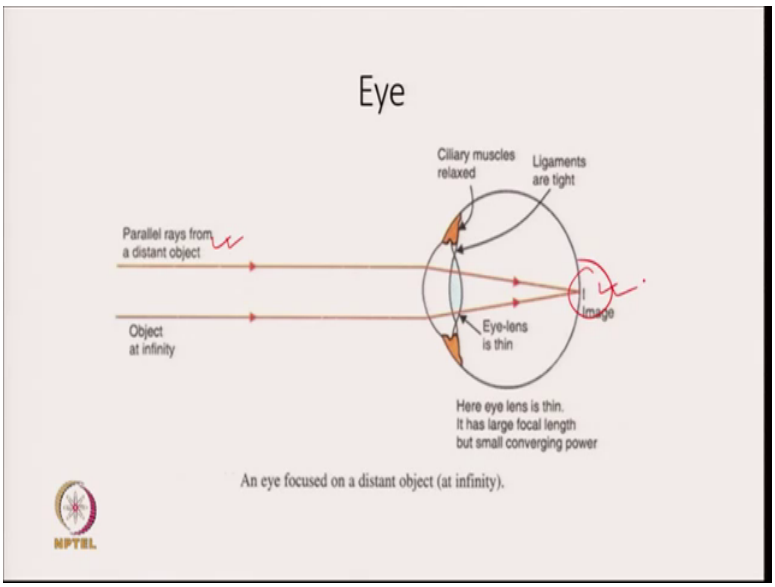
(Refer Slide Time: 11:09)

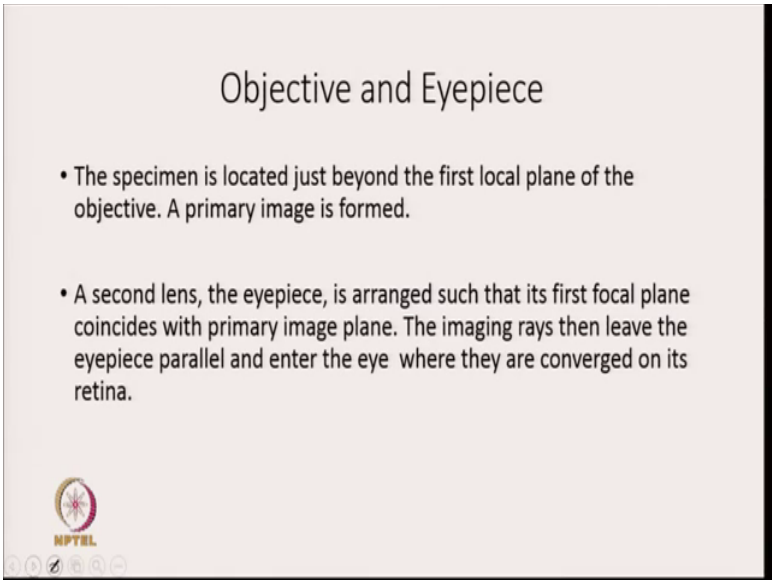


So, these are the few things which is important for compound microscope. Objective has short focal distance, so you can see that focal distance is very small of this objective. Object is placed just beyond the focal point that is what we discussed, so you can see this is a focal point and object is kept here. A real inverted enlarged image form at or near the focus of the eyepiece, so this is the inverted image which is formed and this is at focal point eyepiece.

And when it is at focal point eyepiece, then eyepiece works as a magnifier producing a further magnified image, further magnified image at infinity. The distance between lens is larger than sum of focal length, and then magnification will be multiplication of magnification due to objective multiplied by magnification due to eyepiece, due to eyepiece.

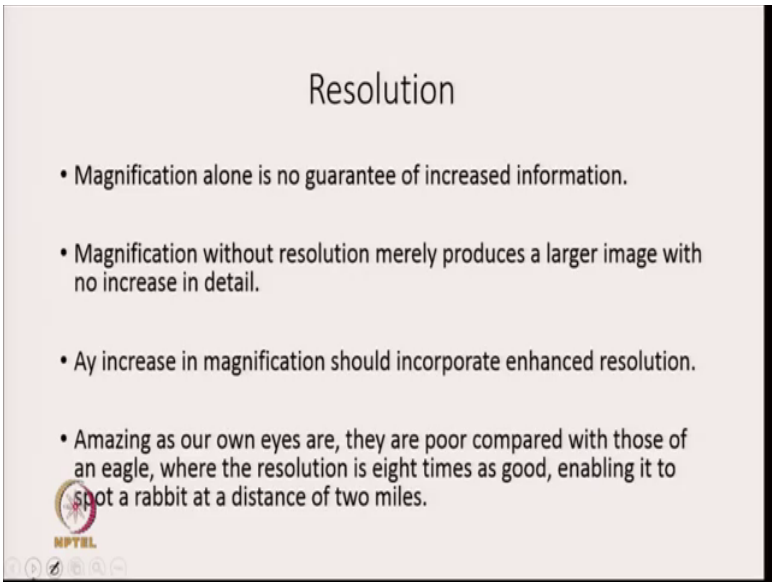
(Refer Slide Time: 12:18)





Okay, so now, when image, image is formed at infinity and when parallel rays from distant object comes to eye and if they are parallel, they form image at retina, then leave the eyepiece parallel and enter the eye where they are converged on its retina. So, here you can see that it is converging on the retina, so this is the principle of compound microscopy. Till now, I discussed about magnification, but the second important thing is resolution.

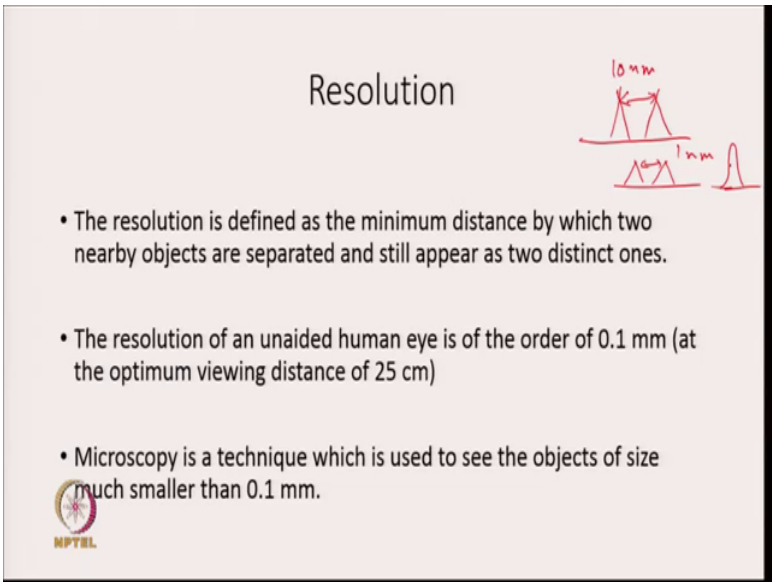
(Refer Slide Time: 13:04)



Magnification alone is no guarantee of increased information, magnification without resolution nearly produces a larger image with no increase in detail. So, if we want more information about a macroscopic of object, magnification is not enough. We need to do magnification with increased resolution. So an increase in magnification must incorporate enhanced resolution.

Our eyes are amazing, but still they are poor compared to those of eagle, eagle's eye, because the resolution is 8 times as good. Enabling it to a spot a rabbit at a distance of 2 miles. So, eyes of different animals have different resolution. So, what now, we need is an increase in magnification must incorporate enhanced resolution.

(Refer Slide Time: 14:18)



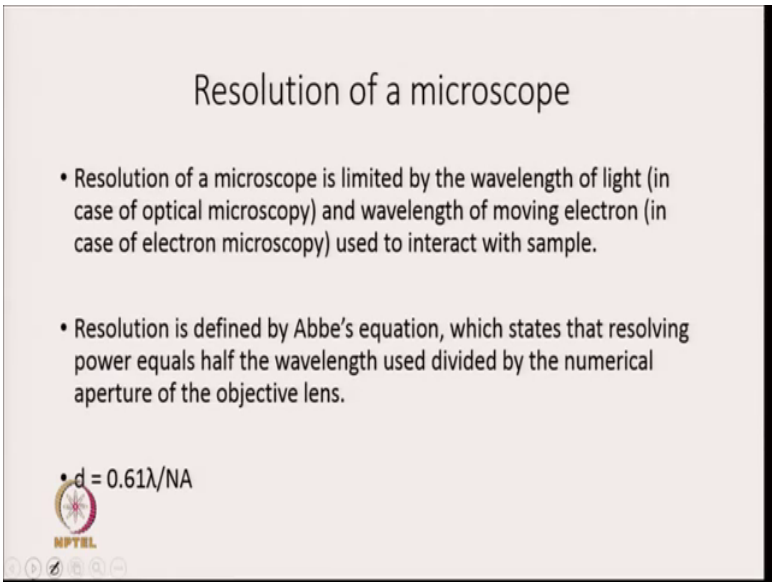
Now, first question is, what is resolution and second question is, how we can increase resolution? Resolution is defined as minimum distance by which two nearby objects are separated and still appear as two distinct ones. So, if there is two objects, so minimum distance, this is basically minimum distance by which two nearby objects are separated and still appear as two distinct ones, that is called resolution.

It means that if suppose, it goes from 10 nanometer, this distance is 10 nanometer, and if we can see this by using suppose a microscope that these are two different objects, then resolution of that microscope will be 10 nanometer. Then suppose, I decrease this distance by 1 nanometer, but now it comes as blurred, it comes as 1. It means we are not able to see these two objects, which is quite close to each other and that is the limit of our resolution.

That is limit of our resolution, so relation is defined as minimum distance by which two nearby objects are separated and still appear as two distinct ones. Resolution of an unaided human eye is of the order of 0.1 millimeter, 0.1 millimeter, at the optimum viewing distance of 25 centimeter, so suppose the distance between, the minimum distance between these two objects is 0.1 millimeter. We can see that as two objects from a distance of 25 centimeter, but suppose it goes to 0.1 micrometer, we cannot see, that with unadded human eye.

It will look like one single object and that is what we mean by resolution. So, if the distance is around 0.1 millimeter between two objects at a distance of 25 centimeter, and our eye can resolve it or our eye can see that has two distinct object. And once we decrease the distance between those two objects much closer, then we, our eye cannot see it as or see them as two distinct ones. Microscopy is a technique which is used to see the objects of the much smaller than 0.1 millimeter, 0.1 millimeter.

(Refer Slide Time: 17:34)

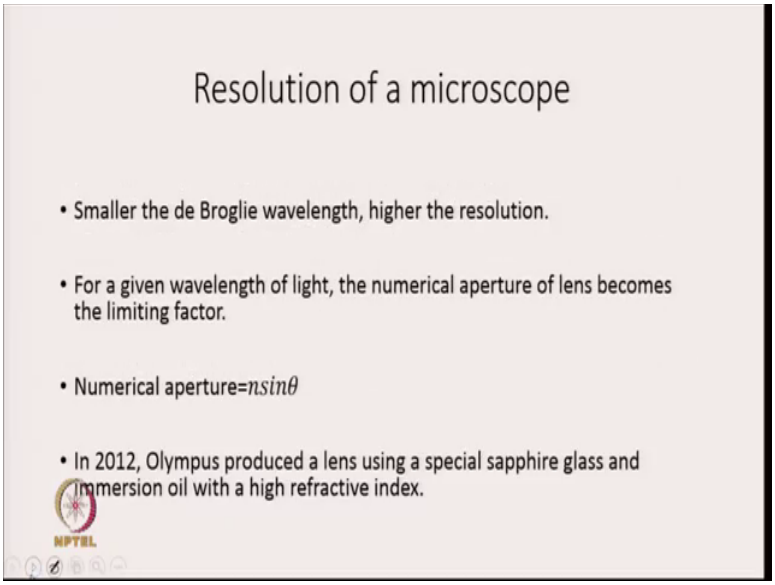


Now, what I am going to do? I am going to discuss about what are the factors, which affect the resolution of a microscope. A resolution of a microscope is limited by wavelength of light in case of optical microscopy and wavelength of moving electron in case of electron microscopy, which is used to interact with samples.

So, in optical microscopy it is the light which is interacting with sample, so resolution is dependent on wavelength of light, whereas in case of electron microscopy, it is electron, which is going to interact with sample, so resolution of electron microscope will depend on wavelength of moving electron. Resolution is defined by Abbe's equation, which tells that resolving power equals half the wavelength used divided by numerical aperture of the objective lens.

And more correct formula is d is equal to 0.61 into lambda divided by NA, where NA is numerical aperture, NA is numerical aperture. So, smaller the d, better the resolution, what does that mean is, smaller the lambda better the resolution, higher the NA better the resolution, higher the NA better the resolution, that is what is written here.

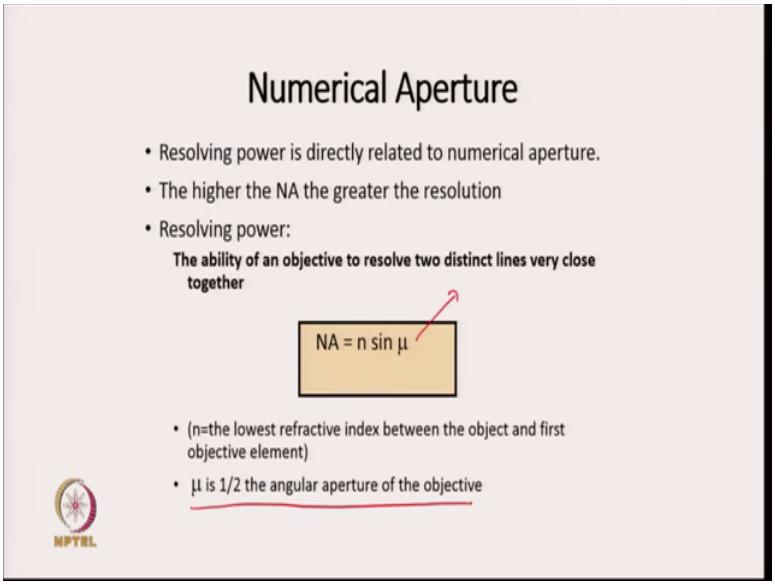
(Refer Slide Time: 19:18)



Smaller the de Broglie wavelength higher the resolution for a given wavelength of light, if suppose, wavelength of light is similar, then the numerical aperture of lens becomes the limiting factor and numerical aperture is given by n sin theta, n sin theta I will discuss in the next slide. Here, you can see that numerical aperture is dependent on n, which is refractive index, so higher the value of n, numerical aperture will be higher.

And in 2012, Olympus produced a lens using a special sapphire glass and immersion oil with high refractive index, numerical aperture is given by n sin theta, so numerical aperture is dependent on n and sin theta, n is refractive index of the material. In 2012 Olympus produced a lens using a special sapphire glass and immersion oil with a high refractive index. So, if there is high refractive index then numerical aperture is going to be high, and if numerical aperture is high then D is going to be low it means there will be high resolution.

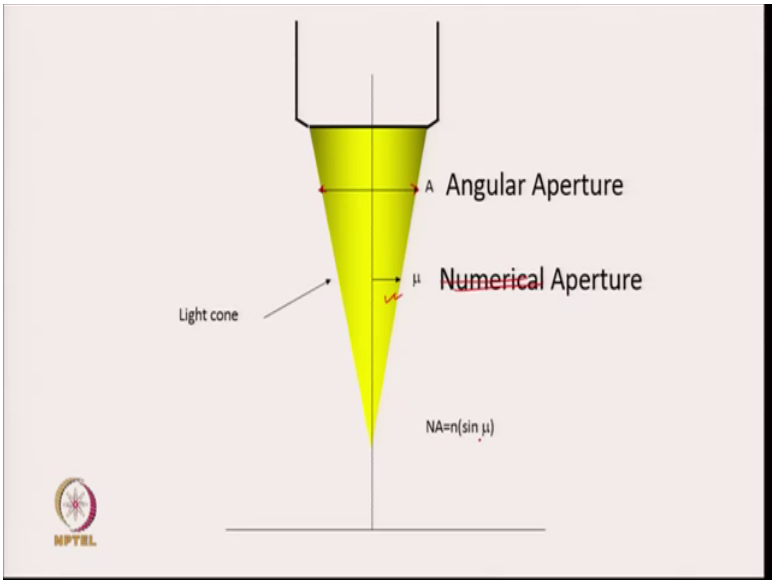
(Refer Slide Time: 20:54)



So, resolving power is directly related to numerical aperture, the higher the NA, the greater the resolution. Resolving power is basically the ability of an objective to resort two distinct lines very close together, that is what we discussed. So, NA is given by n sin mu or n sin theta you can say. N is lowest refractive index between the object and the first objective elements.

So, n is basically your refractive index that is what I talked about. Mu is half the angular aperture of the objective, so mu is basically or theta in the last slide is basically half the angular aperture of the objective. Now, let us see what I mean by this mu value.

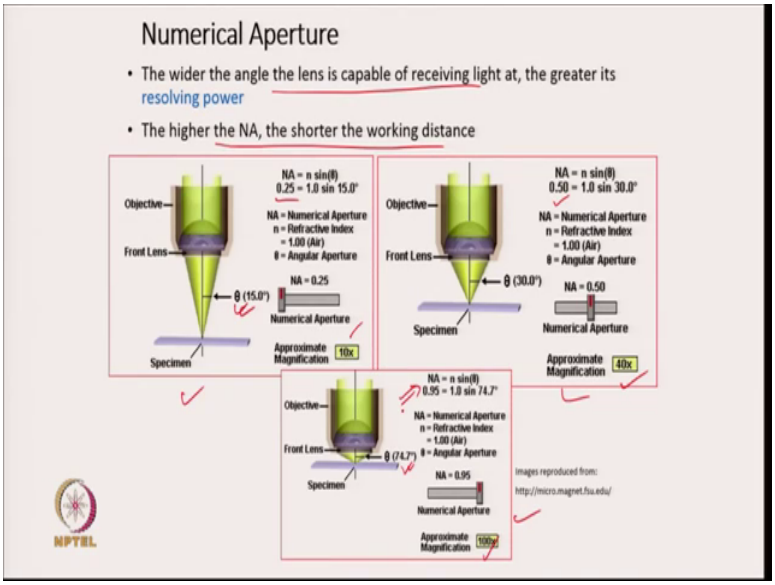
(Refer Slide Time: 21:40)



So, if this is your light cone, if this is the light cone, then this angle is from here to here tells you about angular aperture, angular aperture, and half of this, half of this will give you

numerical aperture, and that is equal to mu. So, NA is not only dependent on n, it is also dependent on sin of the mu, so mu is aperture and NA is equal to n sin mu.

(Refer Slide Time: 22:23)

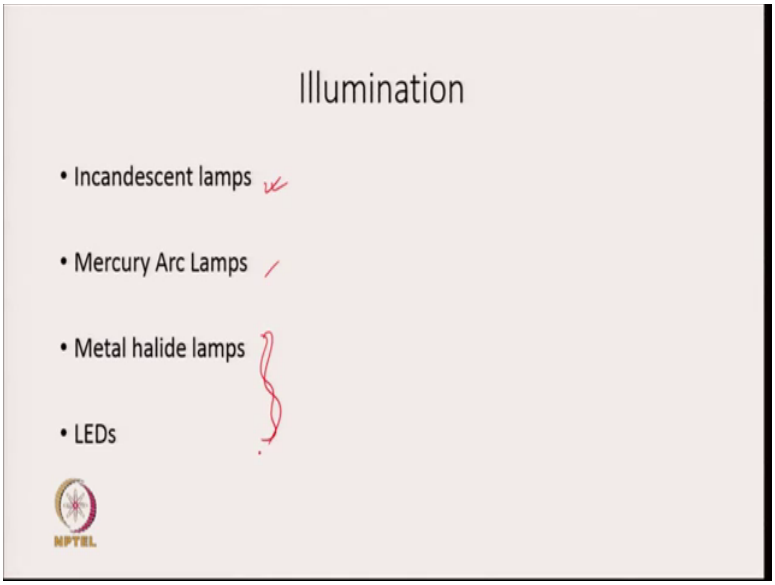


The wider the angle the lens is capable of receiving light at a greater is resolving power. So, if the angle which is mu or your this aperture angular aperture is high, numerical aperture is going to be high, and the resolving power of microscope is going to be greater, resolving power of microscope is going to be greater. Higher the NA, the shorter the working distance, shorter the working distance.

The wider the angle the lens is capable of receiving light at, the greater its resolving power and higher the NA, the shorter the working distance. So, if NA is higher, then working distance is shorter. Here is the three pictures given of your objective with different objective with different numerical aperture. Here you can see this theta is 15 degree that mu is 15 degree and if it is 15 degree then NA will be equal to 0.25 and the approximate magnification is 10x.

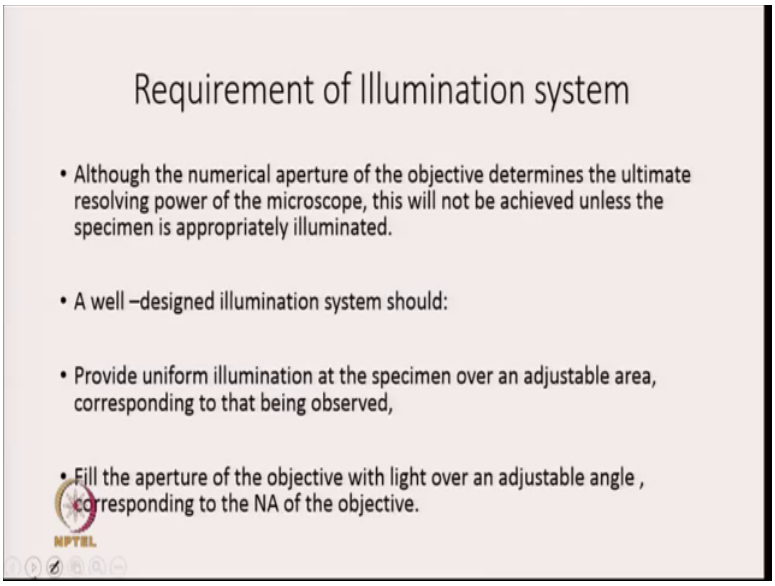
When, if you increase that theta from 15 to 30, then NA is 0.5 and approximate magnification is 40 X, and if I increase the theta to 74.7 degree, then we can get approximate magnification 100x where NA will be 0.95, if we take n is equal to 1. So, for n is equal to 1 and theta is equal to 15 degree, NA is 0.25. When NA is 1 theta is 30 degree your NA is going to be 0.5. When n is equal to 1 theta is 74.7 degree, then NA is going to be 0.95, 0.95. And you can increase the magnification from 10 X to 100 X, 10 X to 100, that is how numerical aperture affect the resolution of the microscope.

(Refer Slide Time: 25:05)



Now, after magnification resolution the third important thing is illumination. And the way we illuminate is by using light source, by using incandescent lamps as light source, mercury arc lamps and recently we started using metal halide lamps and LEDs, metal halides lamps and LEDs.

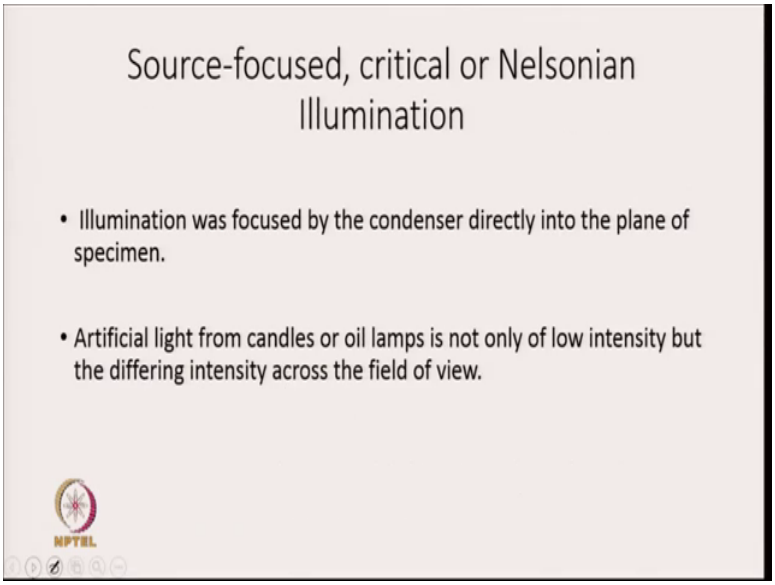
(Refer Slide Time: 25:33)



So, what a requirement of illumination system, we need a very good illumination system to see a much clear picture of the object, so illumination is also a must. So, although numerical aperture of the object determines the ultimate resolving power of microscope, this will not be achieved unless the specimen is appropriately illuminated. So, illumination must be good.

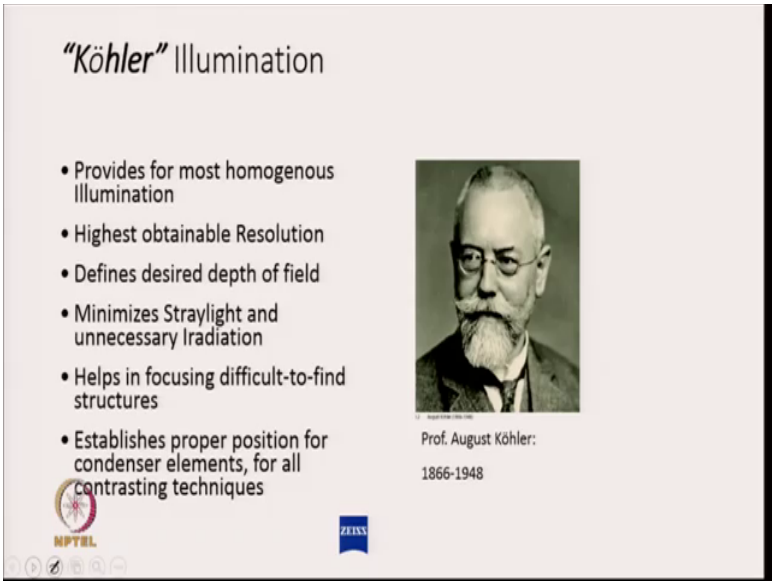
A well-designed illumination system should provide uniform illumination at the specimen over an adjustable area corresponding to that being observed. It must fill the aperture of the objective with light over an adjustable angle corresponding to numerical aperture of the objective. So these are the few very important factors which is important when you are designing an illumination system.

(Refer Slide Time: 26:35)



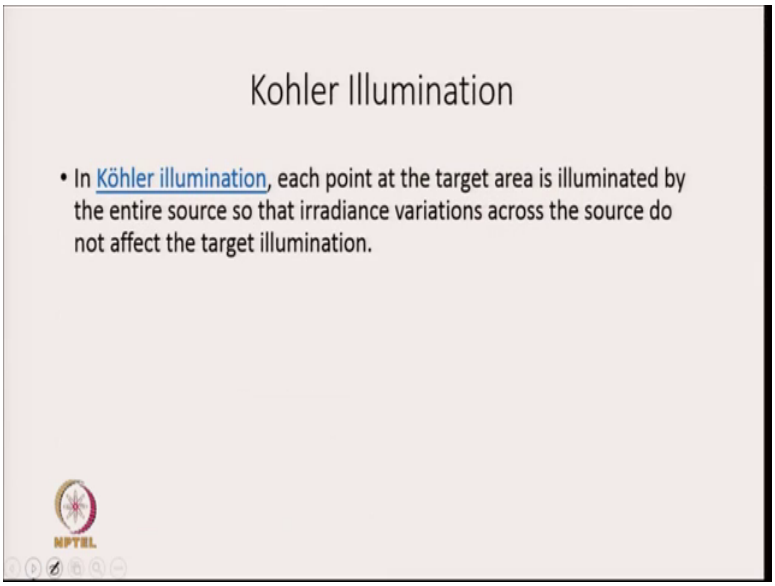
So, there are two important illumination system, one is source focused, critical or Nelsonian illumination in which illumination was focused by condenser directly into the plane of a specimen, directly into the plane of a specimen. Problem with this is that artificial light from candle or oil lamps is not only of low intensity, but the differing intensity across the field of view. So, your illumination is not homogeneous, illumination is not homogeneous which can create problem.

(Refer Slide Time: 27:21)



Then came the Kohler illumination. Kohler provided a method for most homogeneous illumination and if homogenous illumination can be achieved, we can obtain a very high resolution. He also defines desired depth of field. It is the system he devised minimizes the stray light and unnecessary irradiation helps in focusing difficult to find a structure and establishes proper position of condenser elements for all contrasting technique, so that is one of the most often used illumination system.

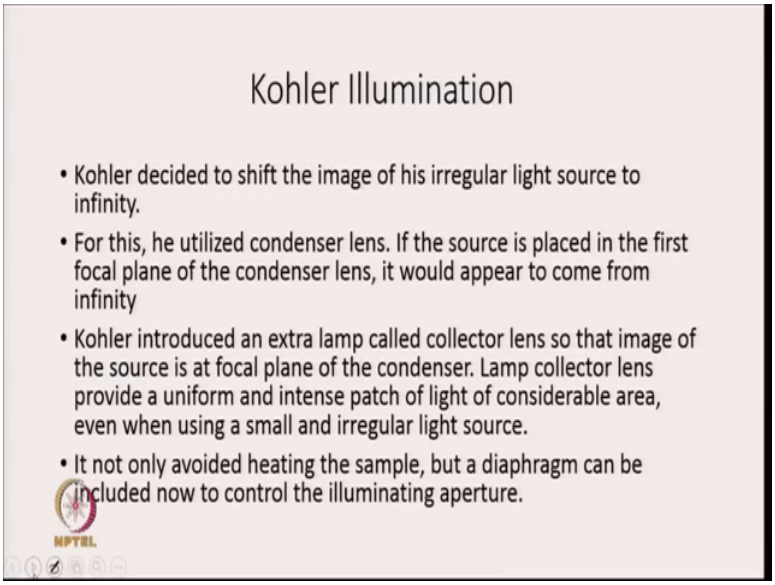
(Refer Slide Time: 28:15)



In Kohler illumination each point and the target area is illuminated by the entire source and so that irradiance variation across the source do not affect the target illumination. So, even if

source as you know variation in its illumination, different point of the source as variation in illumination, it should not affect the image of an object.

(Refer Slide Time: 28:57)

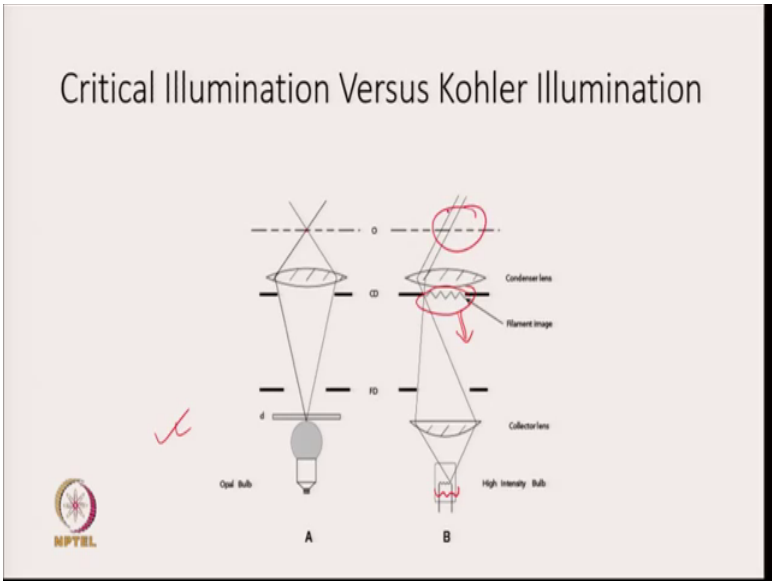


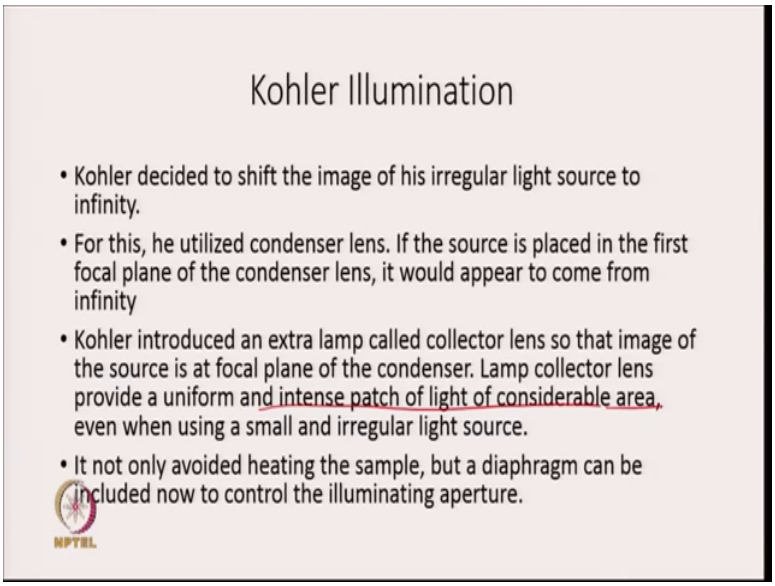
So, what Kohler did is he decided to shift the image of his irregular light source to infinity. So, he decided, ok why not shift the image of irregular light source to infinity and that is how he can focus the light to its focal point. For this he utilized condenser lens. If the source is placed in the first focal plane of condenser lens, it would appear to come from infinity. So it would appear to come from infinity. So, what he did if you place suppose source in focal plane of the condenser lens then what it will show, that it will look like that from the infinity the light is coming.

Kohler again introduced an extra lamp called collector lamps, so that image of source is at the focal plane of the condenser. So, what he told that, “Do not put source at the focal plane, what we can do is you utilize another lens, which is known as collector lens and that can be used to make an image of the source at the focal plane of the condenser, focal plane of the condenser.”

Lamp collector lens provide a uniform intense patch of light of considerable area even when using a small and irregular light source, so by using lamp collector what you can do is, you can get a uniform and intense patch of light of considerable area. So, there are the two things which happened because of that, one is since we have not placed the our light source near the sample and so we can avoid hitting the sample, and also we can use diaphragm in between condenser and lamp collector to control the illuminating aperture, to control the illuminating aperture.

(Refer Slide Time: 31:26)



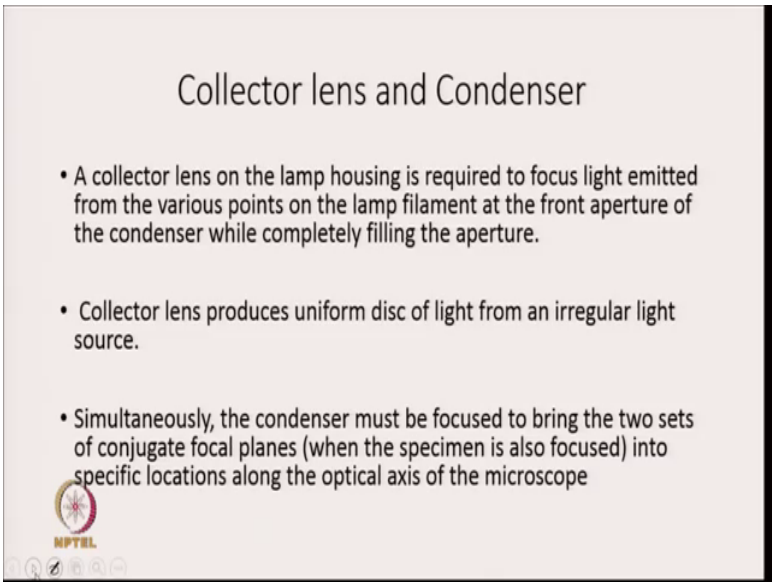


So, critical illumination I talked about two different method critical illumination versus Kohler illumination, this is the schematic which gives you the difference between critical illumination versus Kohler illumination. So, in critical illumination you can see their source of light, this light goes to a convex lens and then here is the object and light reaches this object that is how you can illuminate the object.

In Kohler method what happens that there is a collector lamp and it provides, you see there is a image formation of the filament here, light from the source does not reach condenser, what it does, it first produces a filament image and you see here filament is small, here the image form of filament is big. What you got is uniform intense patch of light of considerable area even when using a small or irregular light source.

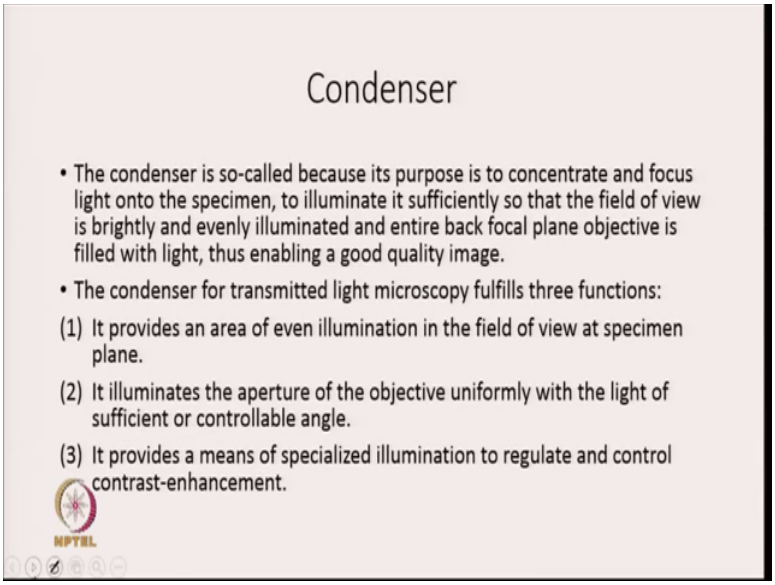
So, although light source is small what you get a filament image of bigger size, filament image of bigger size. Now, you adjusted this image such that it is on the focal length of the condenser lens and if you do that, then what it will look like that your object is receiving, object which is here is receiving light from the infinity, light from the infinity.

(Refer Slide Time: 33:23)



So a collector lens on the lamp housing is required to focus light emitted from the various points on the lamp filament at the front aperture of the condenser while completely filling the aperture, I will show you in the next schematic. Collector lens produces uniform disc of light from an irregular light source, this is very important and what condenser does, it focuses to bring the two set of conjugate focal planes into a specific location along the optical axis of the microscope.

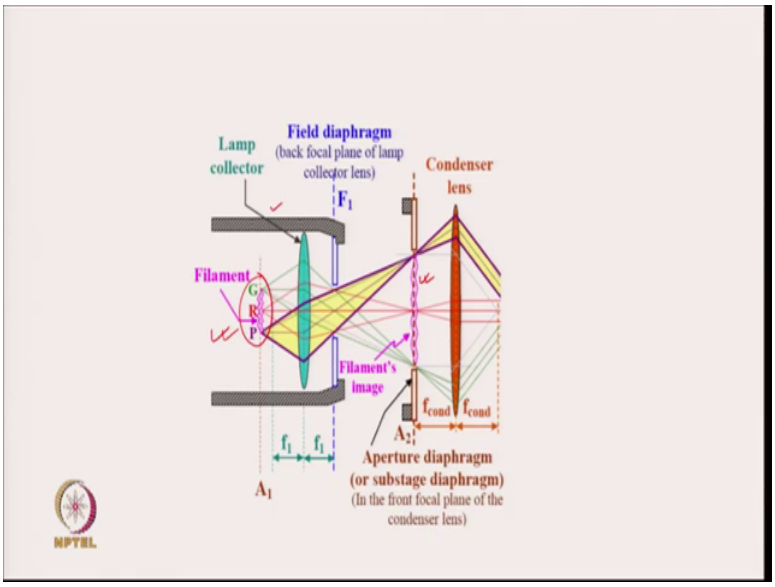
(Refer Slide Time: 34:05)



Let us know more about condenser. The condenser is so-called because it, its purpose is to concentrate and focus light onto the specimen to illuminate it sufficiently so that field of view is brightly and evenly illuminated and entire back focal plane objective is filled with light, thus enabling a good quality image.

So, there are three different roles of condenser, first it provides an area of even illumination in the field of view at a specimen plane. It illuminates the aperture of the objective uniformly with the light of sufficient or controllable angles. And it provides a means of a specialized illumination to regulate and control contrast enhancement.

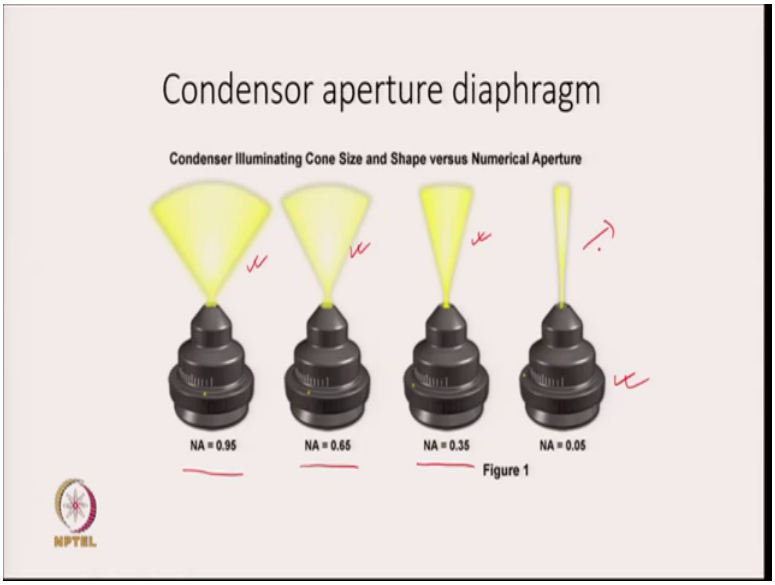
(Refer Slide Time: 35:02)



Okay, so here is a picture, here is a scheme how things happen in Kohler illumination. So, this is your source of light which a filament and these are the three different points, the three different points GRP, here is your lamp collector lens, lamp collector lens. What it does? It makes an image of this filament at this point, filament at this point and you can see this point is here and this point at this place and what does that mean is you get an inverted image of the source, but bigger image, bigger image of the source.

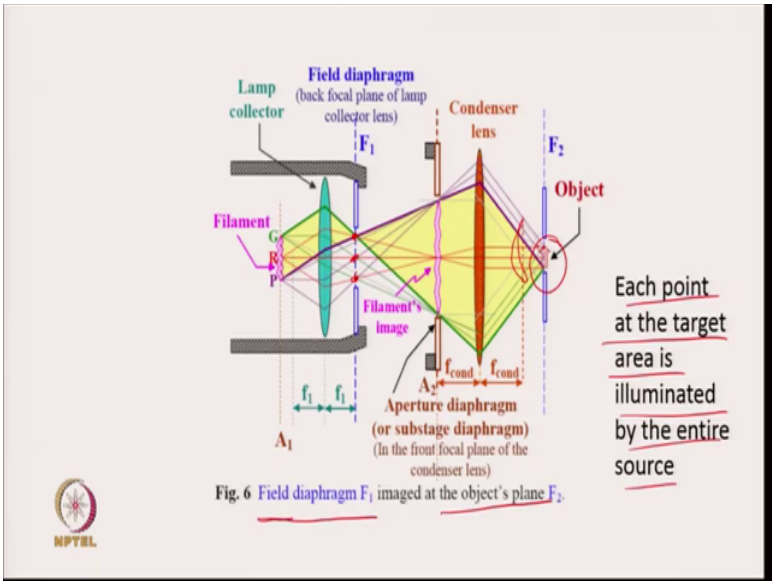
Now, second thing, which you can see is that this condenser lens is for such that your image of the filament form is at focal length of the condenser lens. And so what you see is if it goes here and it comes as a parallel light and it looks like it is coming from infinity, it is coming from infinity, it is coming from the infinity. So, what this condenser aperture does? If you look at this point this is your aperture diaphragm.

(Refer Slide Time: 36:39)



And what it does is, it controls the cone size and that will tell you about numerical aperture, that basically you are controlling the numerical aperture. If light is like this you see numerical aperture is 0.95, if numerical aperture is 0.65 then you have illuminating cone size is like this, if NA is equal to 0.35 then illuminating cone size and shape will be like this, if NA is 0.05 this will be your numerical aperture, this will be the numerical aperture. So, your condenser aperture diaphragm basically controls the illuminating cone size and shape, illuminating cone size and shape.

(Refer Slide Time: 37:35)



So, now, you can see much clearly here that this will control your light and so this is your numerical aperture, this is your numerical aperture, so this angle, this angle basically controlled by your aperture diaphragm, aperture diaphragm. Now, the second thing is that if I put a condenser lens such that image of source is at the focal length of the condenser lens and you expect that a parallel ray will come like this, parallel ray will come like this.

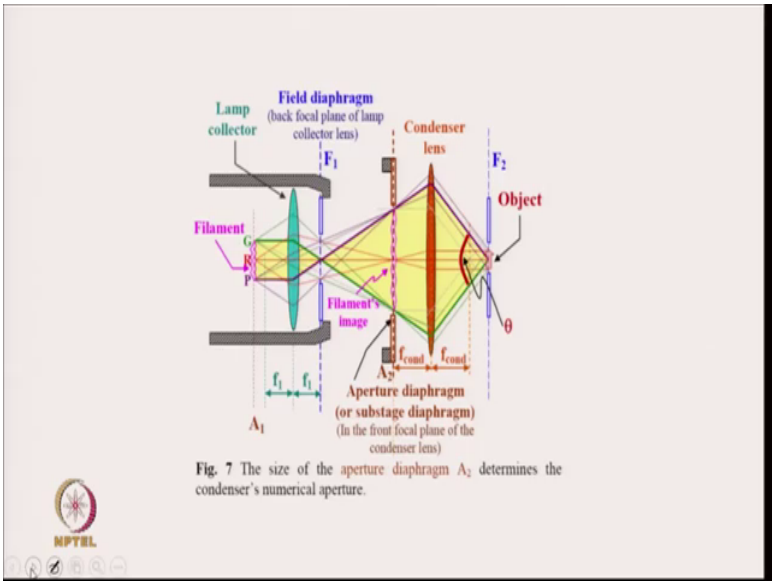
And another thing which you will notice is, another thing which you will notice is that this diaphragm which is field diaphragm. What it does? At this point what you can see here is your, so you see G, R and P, so there are three rays G, R and P, they may be of different intensity, they may be of different intensity and they all are mixing at this point. Similarly, it is mixing at this point, it is similarly mixing at this point.

So, here light is no longer, light is no longer, only G or only R or only P, the mixture of all three, G, R and P. So, here it is G a mixture of GRP, here you can see mixture of GRP and this is mixture of GRP. What does that mean is now you have got homogeneous light, you have got a homogeneous light. And same thing you can see here at this position, this is basically what you are doing at this place is, you are imaging field diaphragm from F1 at the object plane F2.

So, this arrangement also ensures that field diaphragm F1 is imaged at the object plane F2. What does that mean is that every point, at each point at the target area, each point at the target area is illuminated by the entire source. Source has three point and all three, at every point you are receiving light due to each point on the source. So, every point of the object is receiving light from every point of the source. And that is what we mean by saying, each

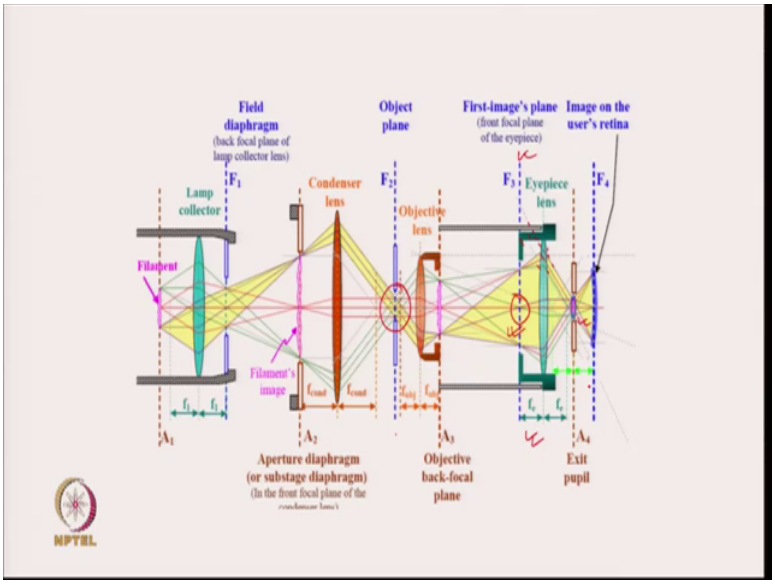
point at the target area is illuminated by the entire source, illuminated by the entire source, and that is the beauty of this arrangement.

(Refer Slide Time: 40:53)



Now, I already discussed with you that the size of diaphragm A2, this diaphragm A2 will determine the condenser numerical aperture, so if you make it small then your numerical aperture is going to be small. So, basically by varying the size or size of the aperture diaphragm A2, you can vary this whole angular aperture and if your angular aperture is high, the resolution is high, if the angular aperture is low then resolution is low, resolution is low.

(Refer Slide Time: 41:46)



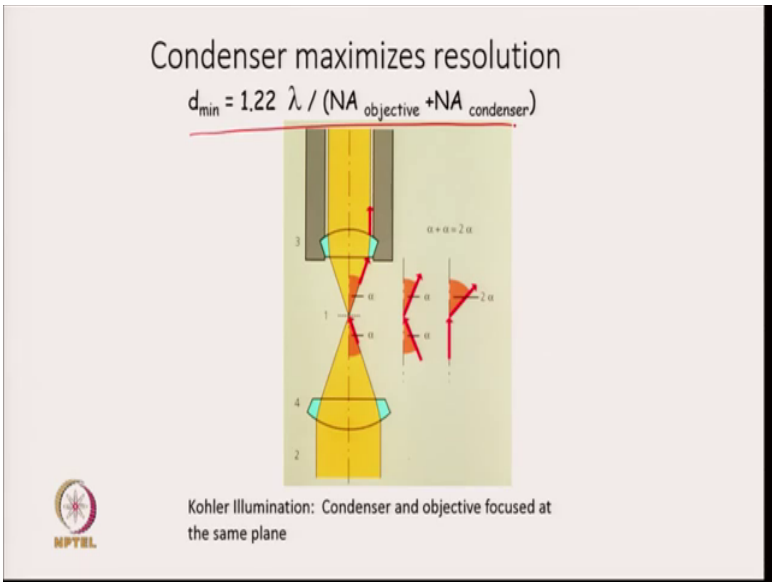
Okay. So, you can see that now this object has got illuminated, now what you do, you put an objective, so as we did in the case of your compound microscope. What now is done is the,

you put objective lens such that, you put objective lens such that your, the object is, now object is basically just after focal length of, focal length of objective lens. So, it is between F and 2 F of the objective lens, and so what you expect is it will give a image, it will give an image which is inverted but magnified, inverted and magnified.

And that is what you get here, at this place you get an inverted and magnified image and that is your line at F3. And now eye lens is kept, eye lens is kept such that you see this is at the focal length of the, this image form, the first image formed by the objective lens is now at the focal length of eyepiece lens, focal length of eyepiece lens. And, so what will happen is that if you do that, then it will look like it is coming from the infinity.

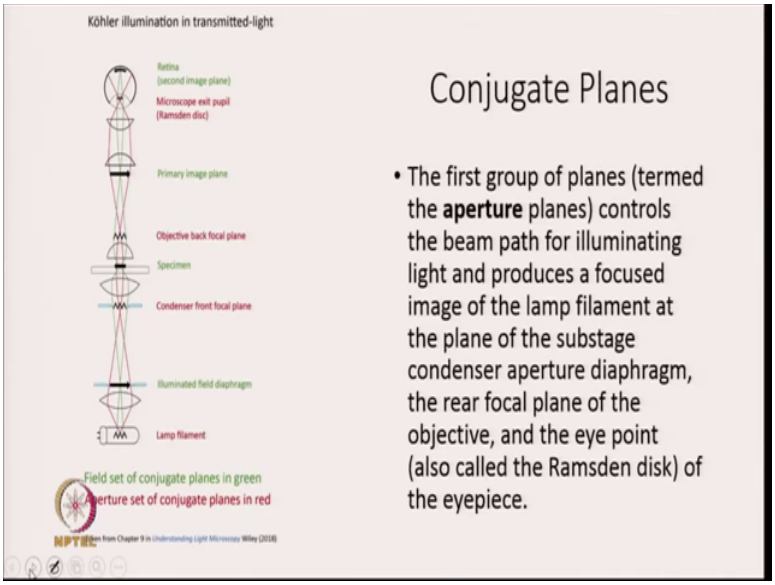
So, it will be, image will be formed at, it will be like at infinity, so rays are coming from infinity to our eye and as we discussed in the compound microscope, now image will be formed at retina, image will formed at retina. So, what has happened? You have adjusted your optics such that, now your image has formed that retina, but with higher illumination, with higher illumination.

(Refer Slide Time: 44:20)



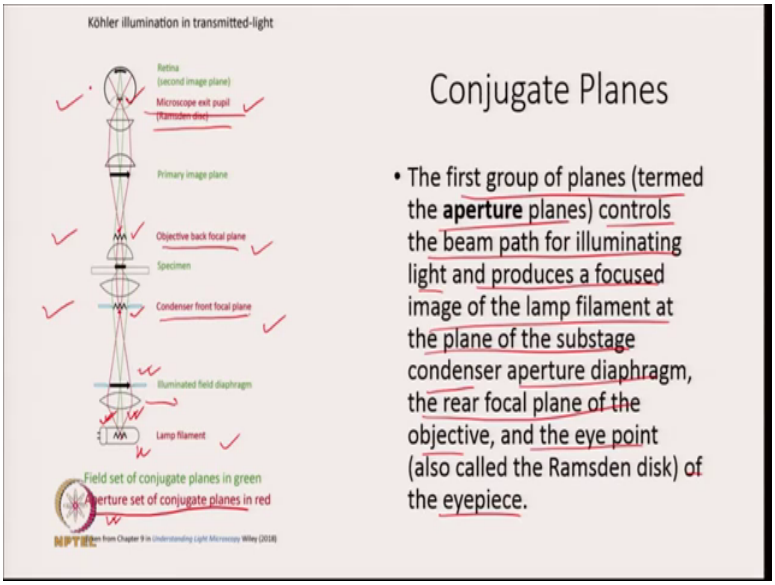
Condenser maximizes resolution and now resolution will depend on this formula, d minimum is equal to 1.22 multiplied by lambda divided by NA objective plus NA condenser. Now, it is no longer only dependent on NA of objective, it is dependent on NA of objective plus NA of condenser. So, if you increase both NA objective or NA condenser, you are going to get maximum resolution.

(Refer Slide Time: 44:57)



Okay, so in this whole thing what is the advantage, in the this Kholer illumination system I talked about several advantages, but let us again look at this from a different perspective then you probably understand it much better.

(Refer Slide Time: 45:27)



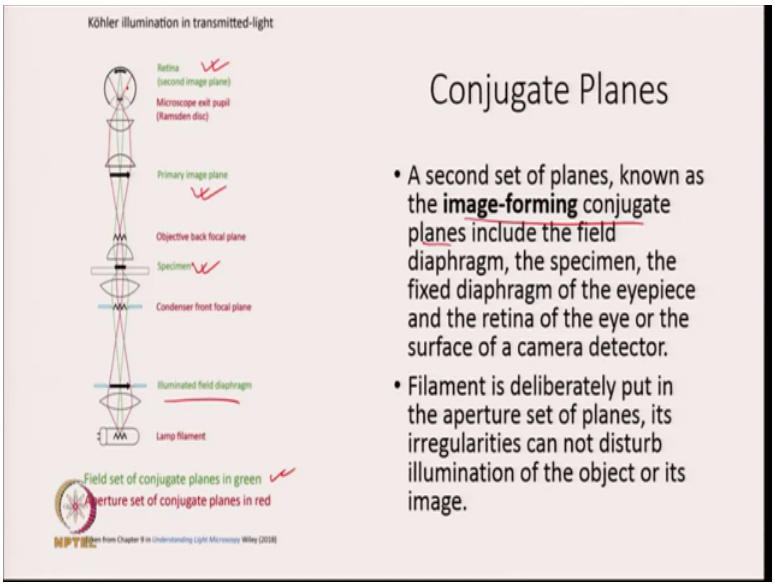
See in Kholer illumination and transmitted light, basically there are two planes, one group of planes is termed as aperture planes, which controls the beam path of illuminating light and produces a focused image of the lamp filament at the plane of substage condenser, aperture diaphragm the real focal plane of the objective and the eye point of the eyepiece. So, first thing, which basically controls the beam path for illuminating light.

What is that thing, let us see here in the diagram. Just look at the red light and that will tell you the aperture set of conjugate planes in the red. Here is the filament and here this red light is meeting, so this is the condenser front focal plane, then where red light is emitting, you see at this point again there is the filament, this objective back focal plane and then you see you here, at this place your plane is meeting, this is called Ramsden disc, Ramsden disc.

So, microscopic disc, so this 1, 2, 3, 4 constitute the aperture set of conjugate lens. Aperture set of conjugate planes, okay aperture set of conjugate planes and what it does is, it controls the beam path for illuminating light and produces a focused image of lamp filaments, you see at this lamp filament is there, this is image of lamp filament at this point.

There is image of lamp filament at this point, this image of lamp filament at this point, there is image of lamp filament at this point and there is image of lamp filament at this point. So, image of, there is a image of lamp filament at this point, image of lamp filament at this point and image of lamp filament and this constitute one conjugate plane known as aperture set of conjugate planes.

(Refer Slide Time: 47:41)



Now, the second conjugate plane is called image forming conjugate planes, which is shown by your green, green font. This includes illuminated field diaphragm, this is a specimen, then primary, you see a primary image plane and the retina where secondary image plane is formed. So, first here you get homogeneous, here homogeneous light is obtained, and then your here, your specimen is illuminated, then primary image plane and this is your, and this constitute your second set of plane.

Filament is delivery put in the aperture set of planes, so that its irregular, irregularities cannot disturb the illumination of the object or its image. So, there are two planes one which controls the illumination and second which works on the image, which works on the image and both plane are different, filament is put in the aperture set of planes where is object is put on, object and its images are put on the, in the image forming conjugate planes. And so, if there is an irregularity in the source of light it will not disturb the illumination of the object or its image.

(Refer Slide Time: 49:24)

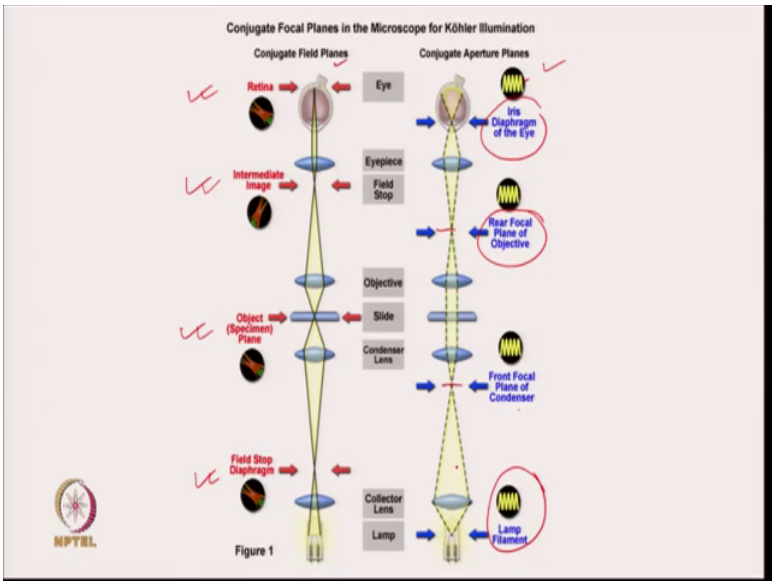
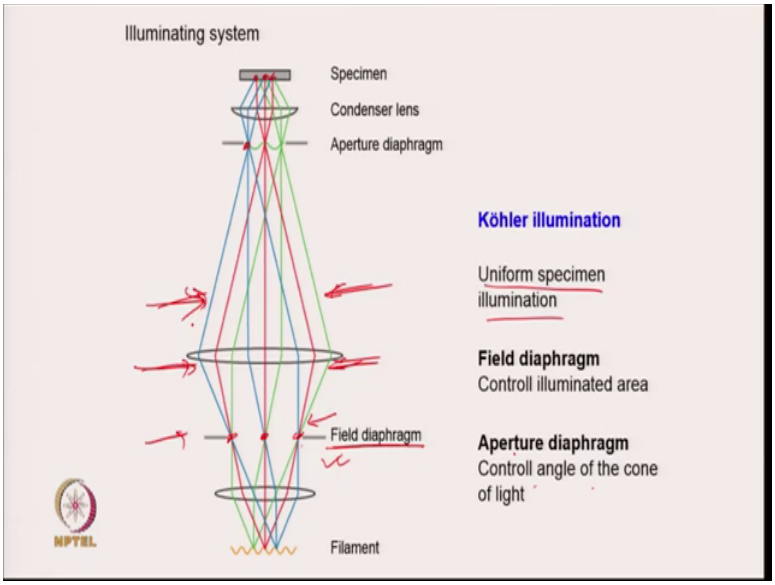


Figure 1

So, these are the two planes, again I will explain this, this is a conjugate field plane, this is conjugate aperture plane, you see lamp here, collector lamps at this position and at this position this lamp filament and this is your part of conjugate aperture plane. And then at this point again it forms, you see again this meets and this is your front focal plane of condenser, condenser and then again here it meets, this is your real focal plane of objective.

And then it meets at iris and there is a, so this four constitute, conjugate aperture plane whereas filled stop diaphragm, object specimen plane, intermediate stage and retina will form conjugate field planes, this controls your illumination while this controls your image formation.

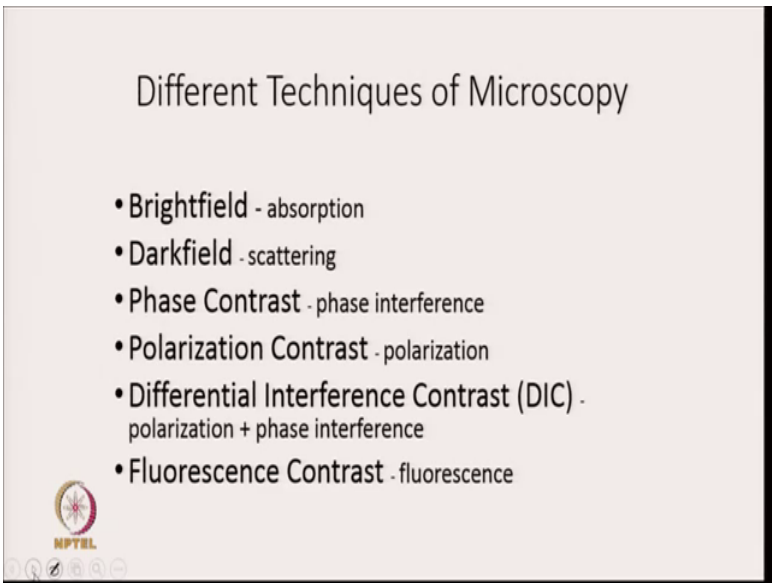
(Refer Slide Time: 50:30)



Now you can see here in this illuminating system, here is the filament and all three types of, you know, light is meeting here, at this point all three are meeting here. All three are meeting here, so you get uniform illumination, so Kohler illumination ensures uniform specimen illumination, so you can see all three red, blue and green meets at every point and so you get uniform specimen illumination.

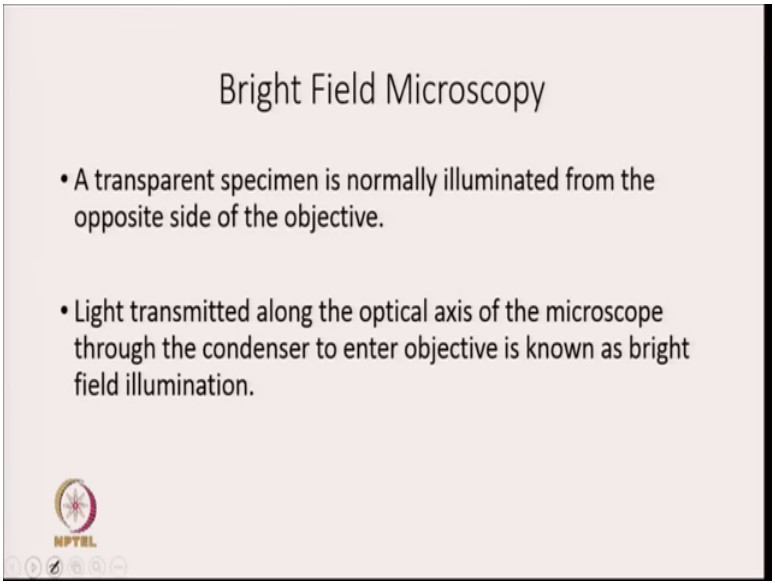
Field diaphragm controls illuminated area, so it controls, basically here you can control this side and this side you can control, field diaphragm controls illuminated area, at the aperture diaphragm controls angle of the cone of the light, so these are angle of the cone of the light, angle of the cone of the light. So, this is about the general hypothesis of a microscopy.

(Refer Slide Time: 51:28)



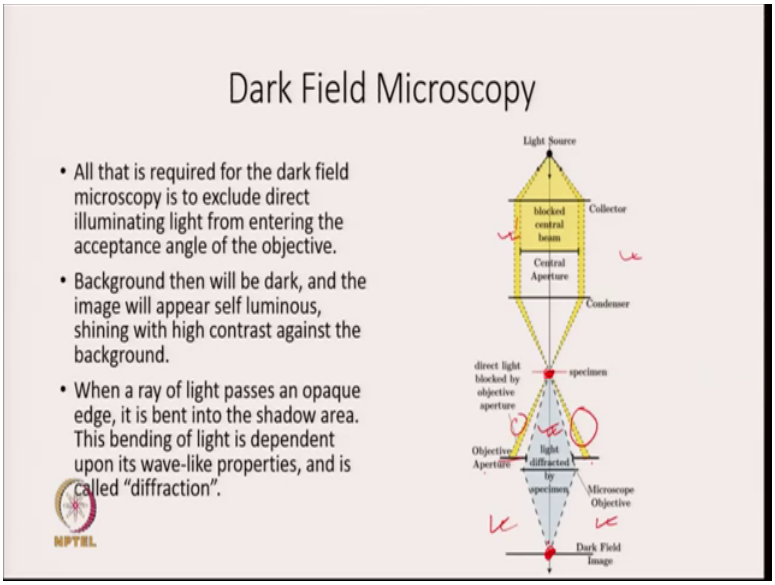
There are different techniques of microscopy, bright field absorption, which is based on absorption technique, dark field which is based on a scattering technique, phase contrast which is based on phase interference, polarization contrast which is based on polarization, and differential interference contrast which is based on polarization plus phase interference, and fluorescence contrast which is based on fluorescence.

(Refer Slide Time: 52:05)



So first is bright field microscopy. This is the normal microscopy in which a transparent specimen is normally illuminated from the opposite side of the objective, opposite side of the objective, so transparent specimen is normally illuminated from the opposite side of the objective, and light transmitted along the optical axis of the microscope through the condenser to enter objective is known as bright field illumination

(Refer Slide Time: 52:38)



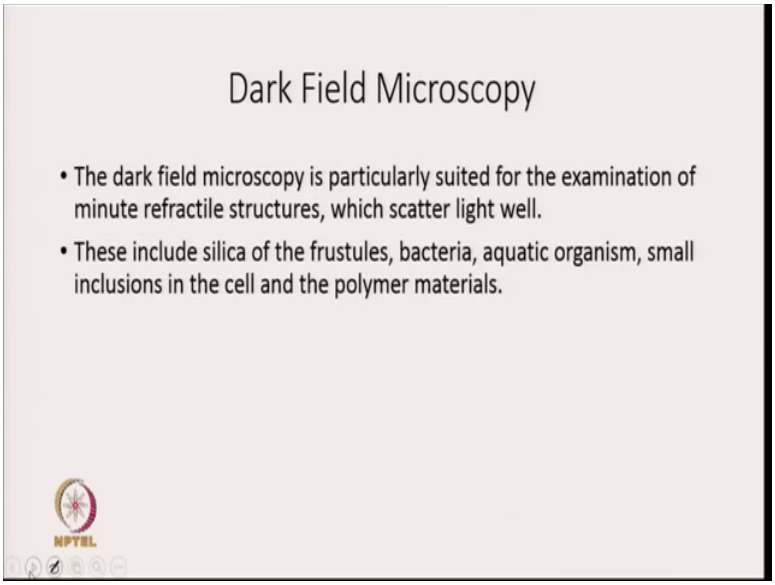
In the dark field microscopy all which is required is to exclude direct illuminating light from entering the acceptance angle of the objective and you can see here what you have done is, you have blocked here, so that only few light comes, but then there is objective aperture and blocked all these lights. So, you are, so direct light, these are direct light, light coming from this side and these are the direct light.

Now, what has been done is that if this light comes it has been focused such that it the light which is coming from this side, basically when it falls on the specimen and it will go like this, if you know, it transmits but that is blocked by here. But the light which are diffracted by this specimen comes here and what will happen that your light, so this is light diffracted by a specimen and then its image is formed at this place.

So, it is diffracted light which basically, diffracted light by a specimen which gives you picture of the object and you can see that this direct light is not coming in this region, and so what you are getting is, what you are getting is a bright image on the dark background. So, background then will be dark and the image will appear self-luminous signing with high contrast against the background.

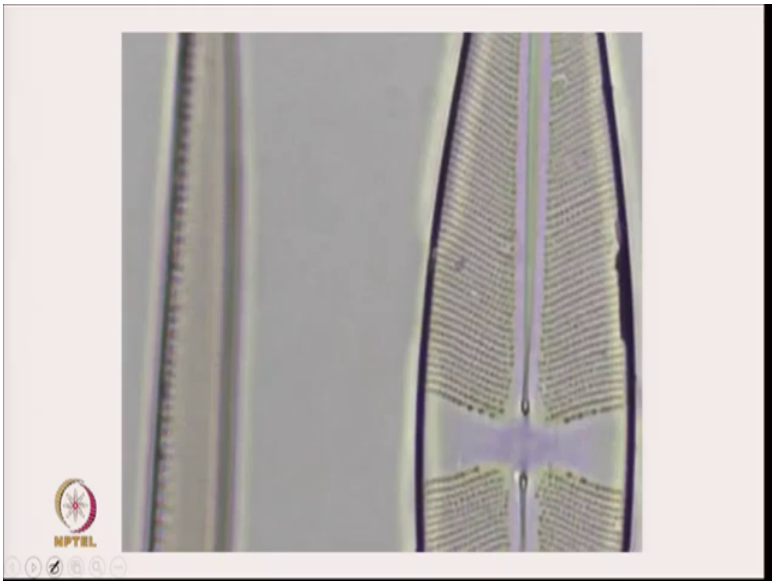
When a ray of light passes an opaque edge, it is bent into the shadow area and that is what you are seeing shadow area, the bending of light is dependent upon its wave-like property and is called diffraction. So, this is basically a diffractive light, which gives you an image against the dark background.

(Refer Slide Time: 54:56)



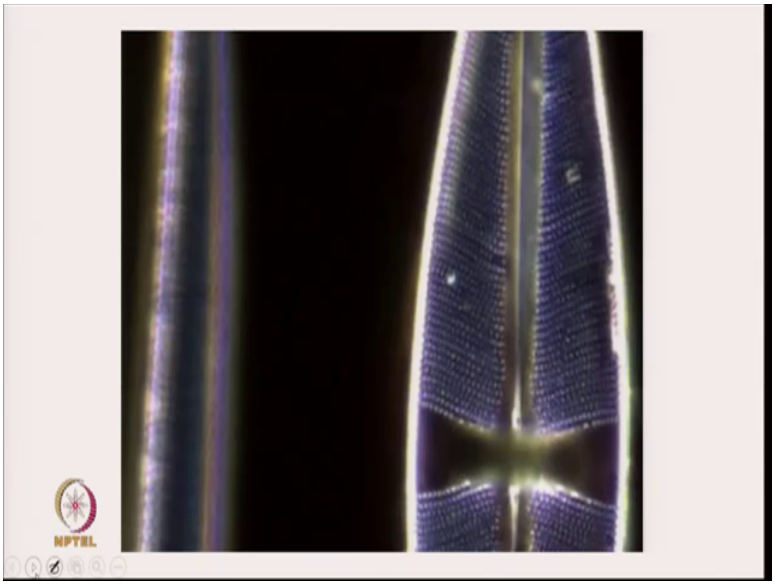
Dark field microscopy is particularly suited for the examination of minute refractile structure, which is scatter light well. This includes silica of the frustules, bacteria, aquatic organisms, small inclusions in the cell and the polymeric materials.

(Refer Slide Time: 55:18)



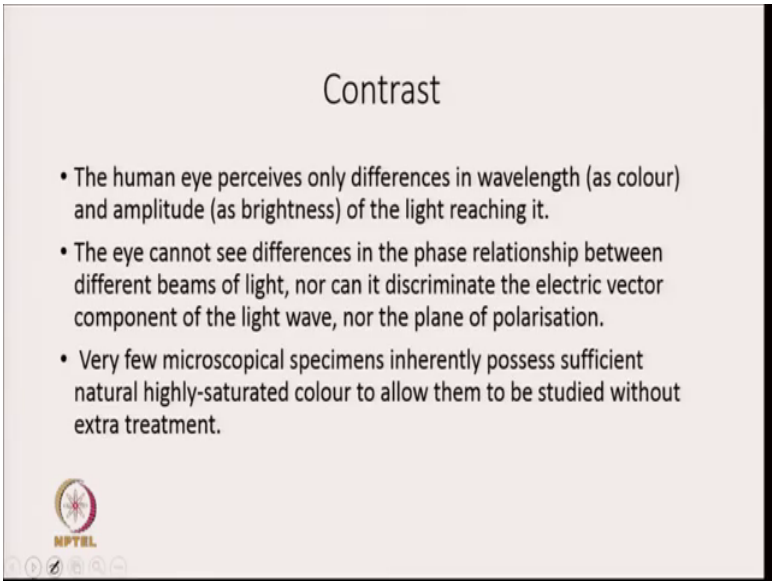
And that you can see this is the picture obtained in bright field microscopy, this is the picture obtained in bright field microscopy.

(Refer Slide Time: 55:32)



And you can see that if you obtain this by dark field microscopy it will look much better, dark field microscopy it will look much better.

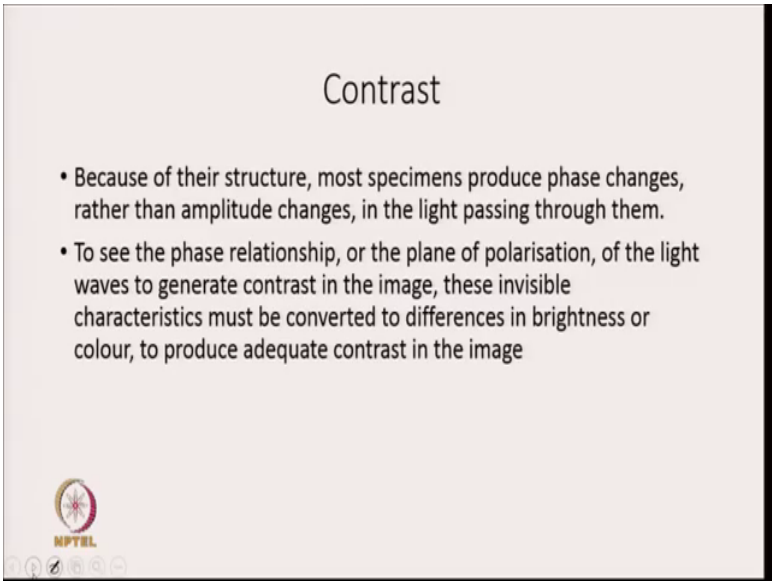
(Refer Slide Time: 55:51)



The next very important factor is contrast. The human eye perceives only difference in wavelength and amplitude of the light reaching our eye. So, difference in wavelength is perceived as color change and difference in amplitude we perceived a brightness change, if I cannot see differences in phase relation between different beams of light nor can you discriminate the electrical field component of light wave nor the plane of polarization. So, that is the limitation with our eye that it cannot see the phase relationship between different

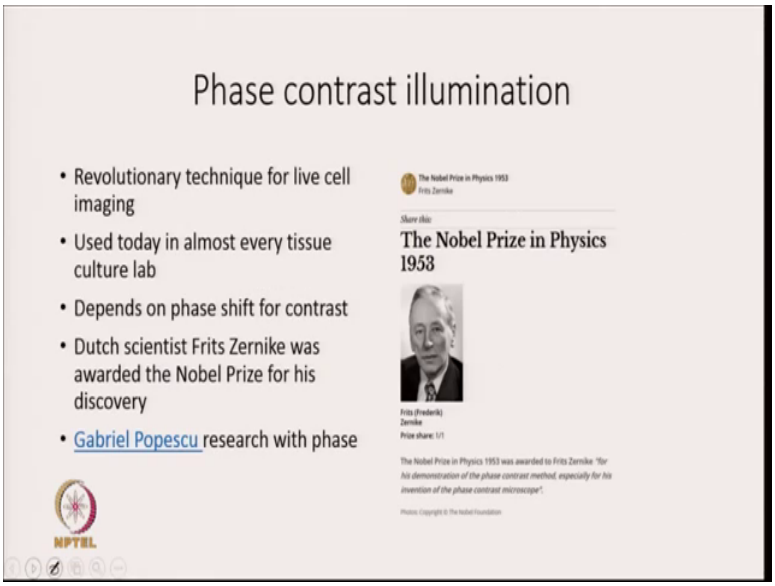
beams of light and very few microscopical specimen inheritantly possess sufficient natural highly saturated color to allow them to be studied without extra treatment.

(Refer Slide Time: 56:46)



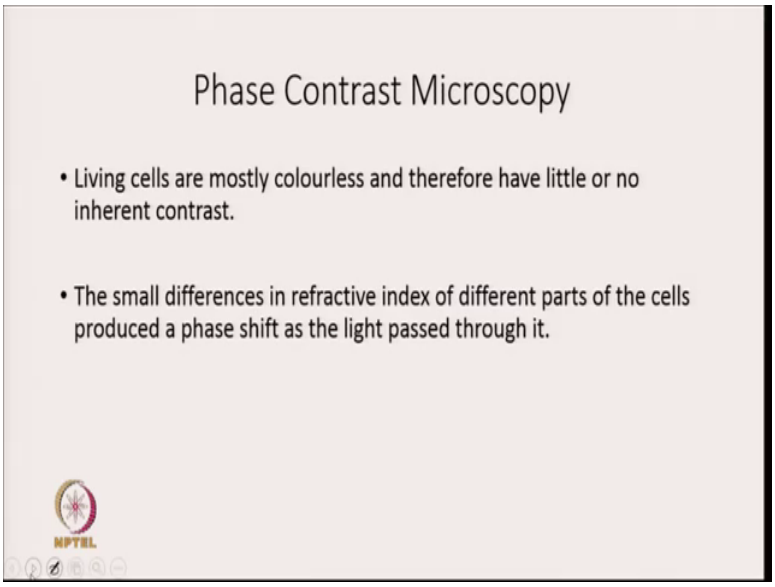
Because of their structure, most specimen produce phase changes, rather than amplitude change, in the light passing through them, so phase changes is normal and we need to find out how to look at the phase relationship. To see the phase relationship or the plane of polarization, of the light waves to generate contrast in the image, these invisible characteristic must be converted to differences in brightness or color, to produce adequate contrast in the image.

(Refer Slide Time: 57:29)



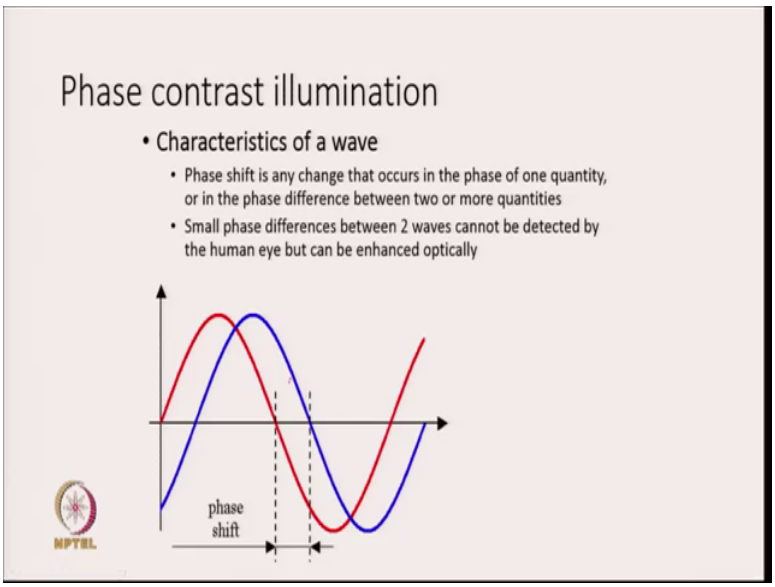
So, keeping that in mind phase contrast illumination was discovered by Frits Zerneki, who got Nobel prize in physics in year 1953. It was a revolutionary, it is a revolutionary technique for live cell imaging used today in almost every tissue culture lab and as I discussed with you, it depends on phase shift for contrast, phase shift for contrast.

(Refer Slide Time: 58:02)



As I told you that this is very important when you are dealing with living cells living, cells are mostly colorless and therefore, have little or no inherent contrast. The small difference in refractive index of different part of the cells produce a phase shift as the light passes through it. So, different part of the cells at different refractive index, it is very small but it is produce a small phase shift.

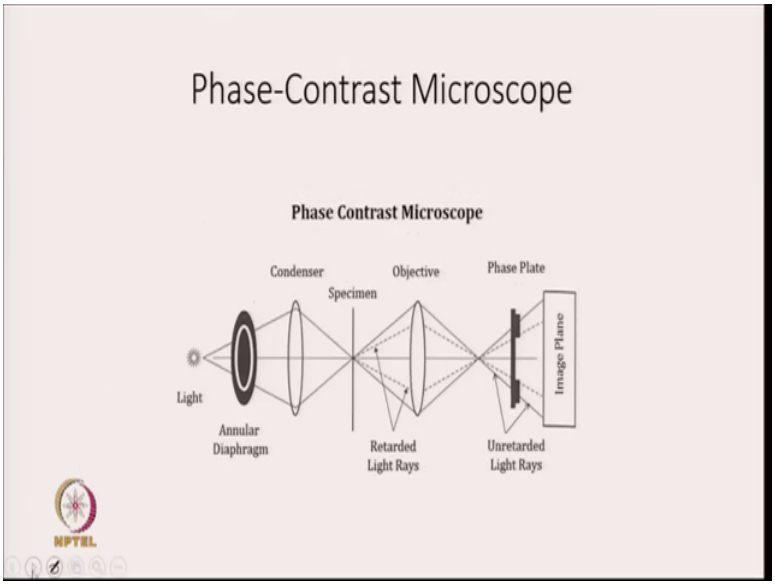
(Refer Slide Time: 58:44)



And I have already discussed about characteristics of wave, and you can see that these are the two waves, which is basically passed through, suppose two different component, then what will happen, there will be phase shift from the different part of the cells because different part of the cells at different refractive index, and you can see it here there is a shift from this point to this point and that is why it is known as phase shift. The phase shift is any change that occur in the phase of one quantity or phase difference between two or more quantity.

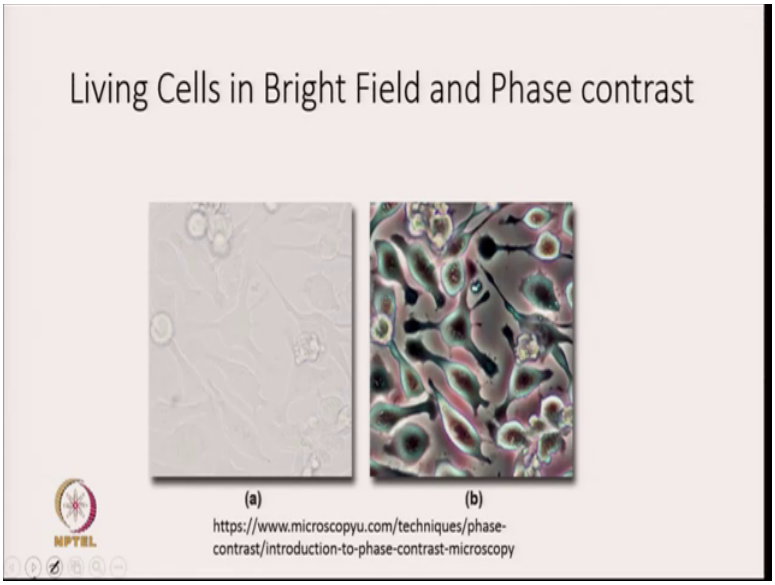
As I told you phase shift is invisible to the human eye, if however, this change is increased to half a wavelength by use of a transparent phase plate, it will destructively interfere with the light that has not passed through the specimen and produce a significant difference in the contrast, that is what was used in contrast microscopy. The optical system requires two phase plate in the form of a new life, one in the condenser, another in the objective itself. And these phase plates are produced by etching of the glass surface, which produces a phase change without affecting the optics.

(Refer Slide Time: 60:14)



And this is your phase contrast microscope and you can see that here two rays are coming, this one is a retarded, this is un-retarded and that is what we look at the image plane, so this is working scheme of a phase contrast microscope.

(Refer Slide Time: 60:39)



And now look at one example, living cell in bright field and phase contrast, this is your living cell in bright field microscopy, and this a living cell in phase contrast and you can see there is a very wide difference in the quality of the image obtained, quality of the image obtained and this can tell you about the details of this living cell.

(Refer Slide Time: 61:10)

So, today, I will stop here, in the next class I will talk about other methods for contrast and then I will go to fluorescence microscopy. So, thank you, thank you very much. These are the four books and lecture notes which I, you know, gone through, I have gone through for making notes and these are very good article and so please go through it and thank you, bye.