

**Techniques of Materials Characterization**  
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**Lecture – 7**  
**Phase Contrast Optical Microscopy**

Welcome everyone to this NPTEL online certification course on Techniques of Materials Characterization and we are now in module 2 where we are discussing various modes of optical microscopy. And in the last lecture, first lecture in this module we discussed about basically reflected and transmitted light microscope configurations and then about bright-field microscopy and then just started discussing about contrast enhancing modes.

And so we discussed about like what is the basic philosophy of contrast enhancement and we just discussed about one such contrast enhancing mode dark-field microscopy. And today we will be discussing about one more contrast enhancing mode which is known as phase contrast optical microscopy.

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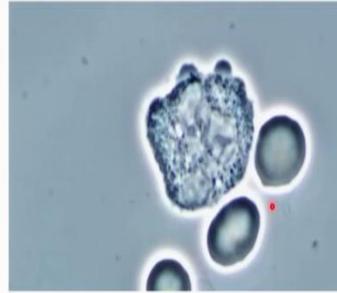


So, we will be covering about basic principle of phase contrast microscopy, then some examples of phase contrast microscopy. What do you require to do phase contrast microscopy and finally certain artifacts of phase contrast microscopy, which will also tell us some about the understanding the images that we are getting from this technique, this contrast enhancement techniques.

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## Phase contrast microscopy

- First described by the Frits Zernike in 1934, phase contrast earned its discoverer the Nobel Prize for physics in 1953 while revolutionizing basic biomedical research on living cells.
- Phase contrast employs an optical mechanism to translate minute variations in phase into corresponding changes in amplitude, which can be visualized as differences in image contrast.
- The technique is ideal for thin unstained specimens (such as culture cells on glass), which are approximately 5 to 10  $\mu\text{m}$  thick above the nucleus, but less than 1  $\mu\text{m}$  thick at the periphery.
- Such specimens barely exhibit any light absorption in the visible portion of the spectrum and the human eye cannot detect them in bright-field and dark-field illumination.



So, phase contrast microscopy, one word is enough to tell about the importance of phase contrast microscopy that is it was first discovered by a Dutchman Frits Zernike in 1934. And he got a Nobel Prize in 1953 because it was so pathbreaking. This entire phase contrast microscopy, the philosophy of it or the technique or the underlying technique mechanism was so pathbreaking.

Particularly for biological field or life science field with people involved with biological research because for a very long time as I was discussing in the last class, for a very long time people dealing with the biological systems they were having this difficulty are very poor contrast if you go for something like bright-field mode. Even in the transmitted mode if you go for bright field or if you go for dark field still they were not very happy with the kind of contrast that they were getting.

Bright field of course the problem is that these biological systems are not very good for changing the intensity of the direct beam. So, they were not very good in absorbing light or scattering light or change the color or do whatever. So, finally if you just capture the direct light and try to produce an image, you are not getting much contrast from these biological systems.

Same thing if you go for a dark field, of course now you reduce the overall intensity of this image you are completely cutting off the background possibly, but still the contrast is not generated because they are as I said not so good in scattering. So, diffracted light also in that there is not much difference really from different regions. So, for them the

contrast enhancing modes has to do something with the material itself to some kind of a signal, some kind of an optical manipulation, this is possibly the right word.

You have to do some optical manipulation with your signal, the signal that is generated after the source of illumination after the illumination signal encounters the specimen, after that there should be some change and that change an optically significant change should happen depending on different regions so that you can use finally that source of detection signal, the detection signal that is generated, you can use that for generating an image with adequate contrast.

That is what people were looking for, the life science people dealing with the biological systems they were looking for. And this phase contrast microscopy was the answer to, in a big time this was the answer for them, and that is why the inventor of it got the Nobel Prize of course. What you do here is basically as I said you do something with an optical manipulation.

You produce a slight phase difference in the light after it encounters the specimen and it comes out, mostly this phase contrast microscopy is done in transmission mode. And after the light comes out from the specimen it will have some amount of phase change, phase shift that is what we call it. So, this phase shift will then be transmitted into a change in amplitude which will form the contrast in the image that we will finally be able to see.

So, we will be discussing about this definitely. But remember one thing that this technique is good for biological samples and for thin specimen, for some obvious reasons is there which we will discuss. So, this is very good for specimens almost like a single cell, so 5 to 10  $\mu\text{m}$  thick above the nucleus but around 1 micron thick in the periphery.

So, if you have this kind of difference in their thickness, almost one single layer of cells with this much of this difference, then the phase contrast microscopy works the best and mostly for thin unstrained specimens. And that is kind of a specimen you can imagine that such a thin specimen of course cannot produce much contrast in either bright field and dark field. So, this is one such example of a phase contrast microscopy.

And as I said this is very good for biological studies and this is something, phase contrast microscopy is possibly something you will be encountering if you visit a pathological lab because this image is our own human blood cells. It is showing human blood cells basically. So, this is the white blood cells and this is the red blood cells, it is taken under this phase contrast microscopy.

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- Before their entry into the specimen, the waves are still in phase (wavefronts beneath the coverslip), but this is no longer the case when they have passed through the various materials having different refractive indices.
- A light wave that has passed through a cell nucleus lags behind the light waves that only had to pass through water. The amount of lag is called a phase shift.
- The amount of the phase shift depends on what media (refractive index) the waves have passed through on their paths, and how long the paths were through these media.
- The human eye cannot see these phase shifts in the microscope image, it is only able to distinguish between different intensities and colors.

Phase Contrast Imaging of Transparent Thin Specimens

Now, how it works? As I said so if you imagine that you have one single cell in your material and usually you have a coverslip which is a glass coverslip, on top of that you generally tend to have an aqueous medium. So, what happens is that this glass coverslip and the aqueous medium on top of them they do not have much of a difference in terms of refractive index.

So, in this entire phase contrast microscopy the difference in refractive index is very important that is what plays the most important most vital role in creating this phase difference. So, you have this coverslip and the aqueous medium somewhere over here which does not have much of a change in the diffraction in the refractive index. Then you have the cell and you have the cell with two differences basically.

First of all, you have a thickness difference. So, wherever you have this possibly the nucleus region that is a thicker region plus wherever you have this some other parts towards the periphery you have the thinner region. So, the thickness goes down plus

there is a big difference in refractive index between these regions known as cytoplasm and this nucleus region.

So, these two regions they are made of different type of protein and they will have some difference in their refractive index, not much basically, but that is good enough for producing a contrast in phase contrast microscopy. Now what happens is that the phase contrast microscopy takes advantage of this very tiny differences in refractive index and that is what where it starts first.

And that is where it basically takes up this small refractive index difference and then finally sort of magnifies this and produce a huge amount of contrast. This entire philosophy of phase contrast is basically this. So, in this case what happens it will produce a phase shift between light traveling at different places. So if you imagine the lights which are entering into this specimen, they all have the same phase. So, they are a very coherent beam with the same amount of same phase.

There is no difference and we will come to that. So, source of this illuminating signal also needs to be specially monitored. So, this also needs to be specially prepared, this this one so that when they enter, there should not be any kind of a phase difference between them. Now when they pass through his specimen, depending on whether they passes through without encountering the specimen.

Encountering only the regions which has cytoplasm and a thinner region or the regions which has a nucleus thicker region with a different refractive index, depending on that they will have different phases. And that is what will make this contrast, we will discuss. Basically what happens is that if the refractive index is higher for any medium, then this velocity of light decreases in that medium.

So if higher refractive index basically in this region, so in the nucleus region, if that nucleus region has a higher refractive index and cytoplasm then obviously the speed of light here will be lesser. So, number one reason is that and here itself in this refractive index difference itself the point of thickness comes in. So thickness if some regions are let us say if you are in the same medium having the same refractive index.

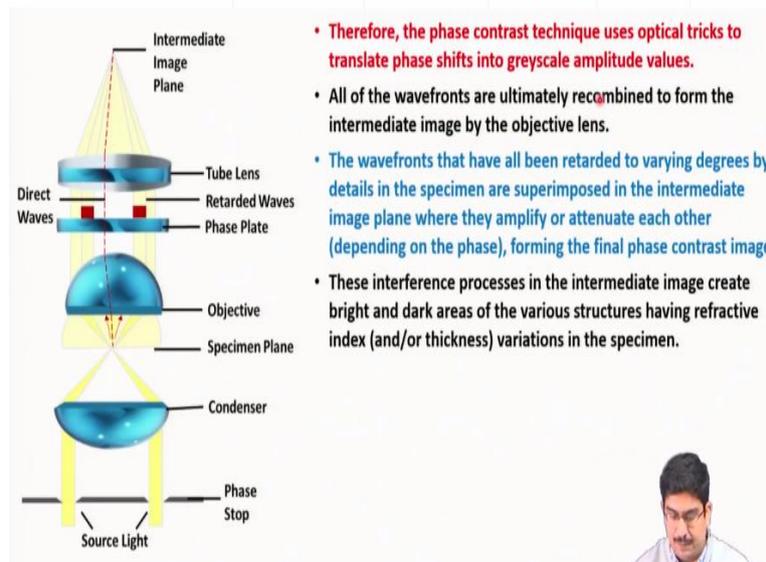
But if the light is traveling through thinner region, it is staying within that region for a lesser time rather than some region which is thicker region. So altogether what happens the light velocity is changing depending on whether it is traveling through this region which is having basically some kind of there is no thickness as such it is just traveling through the coverslip and the aqueous medium compared to that if it is traveling to this region and this region.

All of these regions because of these two factors refractive index and the thickness you will have some amount of difference in the speed of the light and that speed of the light will next produce this shift in the phase. So, as I said before they enter there is no phase change. When they come out of this, they now have a huge amount of or at least they have some amount of change in phase between them.

So, this slide and this slide they have a difference in phase and this slide also they have a differential phase, this is called the phase shift. So, this difference in refractive index and the thickness is finally causing this phase shift. Then after that when they pass through the objective lens this phase shift in these lights coming from different regions that is again they use it to change the amplitude and then what we get is something different.

So, this is basically because human eye is not, we are not sensitive, we cannot differentiate between the phase shifts or we cannot differentiate between the light rays with two different phases. What we can differentiate is an amplitude or rather the intensity that is what So, that is why we ultimately have this phase shift, we have to finally transform this phase shift into some kind of amplitude difference.

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- Therefore, the phase contrast technique uses optical tricks to translate phase shifts into greyscale amplitude values.
- All of the wavefronts are ultimately recombined to form the intermediate image by the objective lens.
- The wavefronts that have all been retarded to varying degrees by details in the specimen are superimposed in the intermediate image plane where they amplify or attenuate each other (depending on the phase), forming the final phase contrast image.
- These interference processes in the intermediate image create bright and dark areas of the various structures having refractive index (and/or thickness) variations in the specimen.

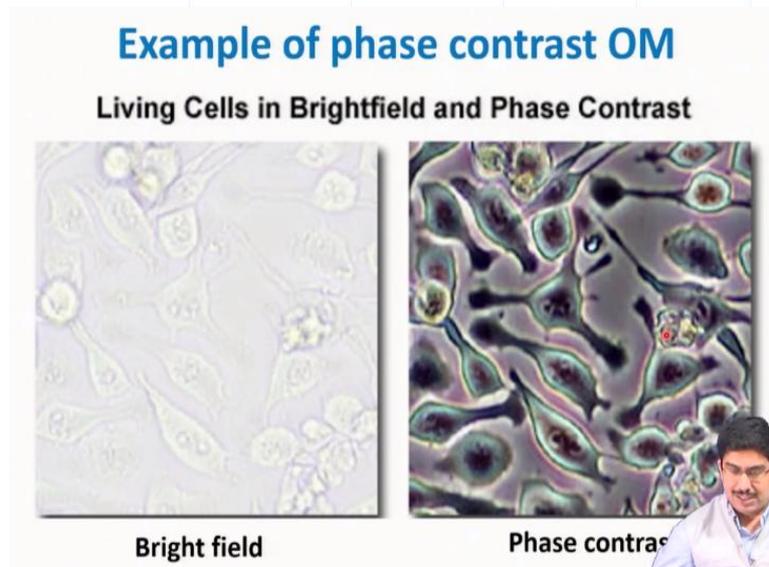
So, that is what we do it through the objective lens. So, in the condenser lens or in this illumination system, there is one requirement that we must produce a light which is having the same phase and then in the objective lens we have another requirement as the phase shifted the lights with the different phases, now they should produce an amplitude difference that is what the purpose of this objective lens.

Now, what the objective lens does is that it basically recombines this all these different and they are all direct lights, now I am talking only about the direct light. So, all the direct beams that are coming from different regions with different phases, the optic objective lens basically recombines them when they recombine this depending on their phase, so this phase shift also is different.

So when that objective lens recombines all of these different direct lights depending on the phase shift basically they are superimposed in the intermediate image plane. And there they have an interference they either amplify or attenuate this each other, these direct lights, different direct lights, different regions whatever the direct lights come they basically interfere and there is a change in their amplitude.

Finally that we can see as a change in contrast. So that is how the phase contrast microscopy basically works in this.

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Now let us deal with this example and basically when we deal with this example, it will become much more clearer. So, we are seeing cells, what kind of cell I do not know, I am not a biologist. So, we are seeing cells. This is the bright field image and this is the phase contrast image. What first thing you can notice is that here of course the light intensity of the background intensity is very high.

Because as I explained that we are using the direct light directly and that direct light basically here the conscious formation mechanism is light absorption by these different phases, which is not so much for these cells. So ultimately what you get is a huge amount of background, then that will produce this overall intensity and the contrast is very low. This is a very bright image but with a very poor contrast.

Here what is happening is that now depending on the phase shift at different regions, the amplitude of the direct beam is getting modified at different regions. So that is why we are getting enhancing that contrast hugely. Not only the contrast between the background, these regions which are background which you can imagine this is the coverslip and the aqueous region here, not only that.

And that is if you notice it that is not showing much of a contrast difference that is pretty much the constant this aqueous region. But the contrast usually varies between this aqueous region and the cell number one and then within the cell itself now you are seeing certain kinds of features, which are not so much visible if you look at this bright

field image. So, in the bright field image within, this is not a resolution problem, this is a problem of contrast generation.

So please understand this. So at times when we were discussing about resolution, magnification, empty magnification, so on and so forth we discuss about this problem of empty magnification where without improving resolution if you just magnify you will not be seeing any new features within that. This is not that kind of a problem. This is a problem with a contrast itself.

So since there is not adequate contrast within this cell or best example within this cell, there is not adequate contrast between different regions, so we are not able to see them in bright field. When we go for phase contrast now we are sort of magnifying this contrast difference between them and which is coming because of the phase shift that is what we just now explained.

So, now you started seeing some other kind of features also within this single cell, right. So not only you are enhancing the contrast between your background and the features, but you are enhancing now contrast within the features within those cells as well. That is why phase contrast microscopy works so good for this kind of biological system.

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### Requirement for phase contrast OM

- The microscope must be equipped with a specialized condenser containing an annulus or a series of annuli matched to a set of objectives containing phase rings (designed as Ph) in the rear focal plane.
- The technique is not useful for thick specimens (such as plant and animal tissue sections) because shifts in phase occur in regions removed from the focal plane that distort image detail.
- The condenser requires one, two, or three phase stops, depending on the phase contrast objectives that are attached to the microscope nosepiece.
- The required ring diameter increases with the numerical aperture (high apertures require the maximum diameter (Ph3), for example 0.9 in air or 1.3 with oil immersion).

The diagram illustrates the optical setup for phase contrast microscopy. It shows a light source at the bottom, passing through an annular ring in the condenser. The light then passes through a specimen, and finally through a phase ring in the objective lens. Labels indicate the 'Phase ring', 'Deflected Light', 'Objective', 'Specimen', 'Condenser', 'Annular ring', and 'Light from Source'. A photograph of a person is shown next to a microscope, with 'Condenser Phase Stops' labeled on the condenser.

Now, what do we require for a phase contrast? In order to do phase contrast optical microscope what do we need to do? What do we need to have? As I said you need to have something in the condenser lens which will produce the light rays with the same

phase. So, here we put something called annular ring in the condenser lens side and then in the objective lens again you have to put another attachment which will basically help to recombine all these direct lights which are coming with different phases.

So, there what we put is called a phase ring. So, these are the two attachments that needs to be put if you want to do a phase contrast microscopy. And so that is basically in the condenser lens side you have to put the annular ring and the objective lens side you have to put this phase ring. And phase ring comes there are different phase rings basically. It is attached to the kind of objective lens that we use.

And as I said there are different objective lens with different numerical aperture, different level of magnification resolution that we use. So, for all of them different objective lenses, I have to use a different phase ring. So, this is pretty much coupled between them what kind of an objective lens I use and what kind of a phase ring I will use. And these names, the phase ring names these are denoted by Ph.

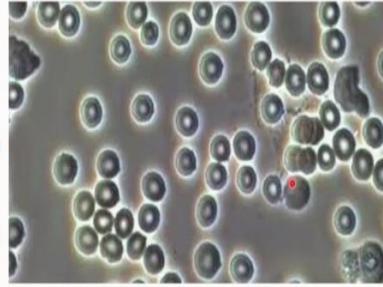
This term Ph, and there are almost 4 to 5 different types of phase rings are available and those phase rings are very much coupled as I said. Now one problem is for this phase contrast microscopy as I was discussing is that they are not so good for thicker specimens. They are very much limited for very thin specimens particularly as I mentioned that biological samples with almost single row of or almost single cells.

The cells should be of some optimum thickness and there should not be very thick specimen used. So, otherwise there will be certain problems and we will discuss about that.

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## Halo and Shade-Off artefacts in phase contrast OM

- Phase contrast is an excellent method to increase contrast when viewing or imaging living cells in culture, but typically results in halos surrounding the outlines of edge features.
- Halos arise because of the configurational parameters of the phase contrast microscope optical system.
- The circular phase-retarding ring located in the objective phase plate also transmits a small degree of diffracted light from the specimen.
- The presence and severity of halos in phase contrast is dependent on the refractive index difference between the specimen and the surrounding medium.
- Areas having a lower refractive index than the surrounding medium exhibit dark halos as opposed to the bright halos that can be observed surrounding higher refractive index features.



So, now we will come to some kind of an artifacts in this phase contrast microscopy. If you have noticed this phase contrast images most often if you recall, this one, this image, this is very much clearer here that around this image you are seeing some kind of a white bright regions and around some other regions you are seeing some kind of a darker region. So, this is not a real contrast, this is coming because of an artifact which we need to understand.

And if you do not understand that you may mislead that this region also has some kind of a feature and this is coming because of some kind of change in their phase and so on. So, this is a not a real thing, it is coming out of an artifact and that artifact is basically is called Halo and shadow of artifacts in the phase contrast microscopy. So, this one is basically coming because the phase ring that you use here in the objective lens.

This phase ring not only allows the direct beam, it will also allow certain amount of the diffracted beam. So, as I said the phase shift the entire mechanism works for direct light, the direct lights have some different type of phase shift, some phase relationship between them depending on which area they are traveling. And ultimately, we use the direct lights to recombine and produce and do interference and produce the contrast that is what.

But problem is that this phase ring basically not only allows the direct beam, they also allow some amount of diffracted beam which again have a completely different phase relationship with the direct beams and that is not very much controlled. So, these

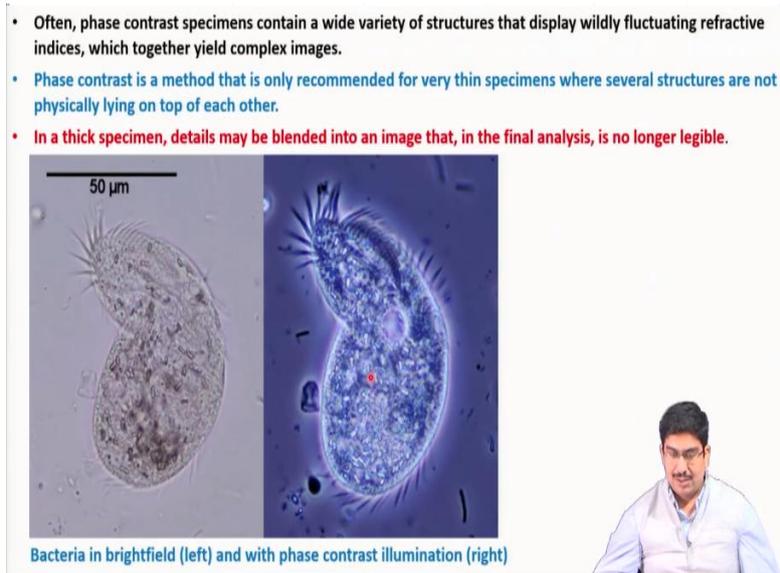
diffracted beams will also go and interfere with the direct beams during the image formation process. And that is how they will generate this kind of artifacts, the halo or shadow artifacts.

They will generate this different contrast which is really not a true contrast and which is just simply an artifact from these diffracted beams that are going and interfering with the direct beam with a different phase relationship, altogether very phase complex phase relationship. They are going and interfering with the direct beam, all the direct beams and they are finally producing these brighter regions around the periphery of the specimen.

So, the way this generally we can think of this is like the areas which are having lower refractive index than the surrounding medium, they generally tend to show a dark halo as opposed to the brighter halos when the surrounding medium have higher refractive index features. So, surrounding medium as I already discussed that this is mostly an aqueous medium. So if the aqueous medium has a higher refractive index, then the cell itself then you will be getting this very bright halo around them.

Whereas again I said that there is another kind of a refractive index difference between cytoplasm and this nucleus. So, if the nucleus again has lower refractive index than the cytoplasm region, then there will be a darker halo around this nucleus within the cell itself. So, both of these can be there depending on the difference in refractive index between these various regions.

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Finally, another artifact that comes out is that again if the samples are too thick, now we are coming to that problem. If the samples are too thick and you have more than one level of cell, then this phase shift mechanism the complex becomes very complex and the contrast generation also becomes very complex mechanism. For example, if you have one single cell and then on top of you have another single cell with the features like.

Now the lights which are traveling in bottom cell, the lights which are traveling in the lower refractive index regions but on the top on the above cell it is traveling through the higher refractive index region, then this phase relationship will be completely that difference maybe or very complex contrast generation will be there. The difference between these rays will be, I mean you will not be able to predict them in the proper way.

So in order to reduce that possibility, this entire phase contrast microscopy is limited to a very thin specimen where you do not have, you are trying to see only a single cell, you do not really want other features or other cells to be there on top of the single cell. So, for this thicker specimen it does not work. So, this is another very nice example that I could find out is a bacteria taken in the bright-field mode here and in the phase contrast illumination here.

So, the bright-field mode as I was saying definitely first thing you can notice is that the contrast between this background and this feature is much higher now in the phase contrast microscopy. So, this is the first thing that this does. So, in the background

without in the dark field what we were doing, we were completely cutting the background that was a complete black background.

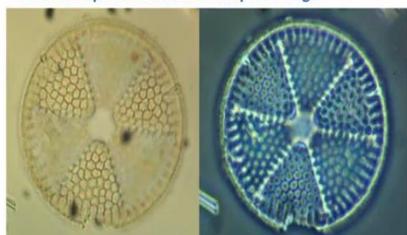
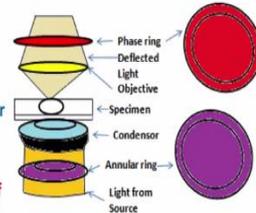
Here, it is not a black background because we still get some amounts of direct lights from here and we use the direct light only, but with a phase difference between them. So, in these regions we still get some amount of intensity and we still can see them, only we are increasing the difference between this region and this region. Then you can notice that there is this artifact, these halos are there.

And then this is one region, this is possibly the nucleus. So, this region you are not at all seeing in the bright-field mode, this now you can see them only in this phase contrast illumination mode because in bright field what was happening this region and this region almost they were producing the same contrast here, they were producing the same amount of absorption or whatever produce the contrast in bright field that was pretty much the same.

Only if you go for this phase contrast microscopy, now you are able to see the nucleus. And also if you notice this nucleus is showing again another kind of a halo because of the difference in refractive index between this region and this nucleus region and same thing you can see for all other features. So you have to live with this artifact, you have to understand this artifact, but the bottom line is that the phase contrast microscopy can give very good contrast in particularly for biological system.

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- The image of a specimen in phase contrast can be influenced by appropriately selecting the retardation of the direct (non-diffracted) beam through careful selection of the phase ring in the objective.
- Depending on the retardation value selected, objects with a higher refractive index than their surroundings appear either brighter or darker than their surroundings.
- This is called either positive or negative phase contrast. In modern microscopes, positive phase contrast is standard, where the darkness of object features increases with their refractive index.
- Diatoms from Carolina Biological on a test slide, first at 40x Ph3, and on the right is a comparison of three different 100x Ph4 objectives, dark low, dark low low and dark medium (DL, DLL and DM), which have different optical densities in the phase rings.



So the final thing that we need to discuss is that again with a phase ring just by playing around with a phase ring you can basically produce different kinds of contrast. Here in this phase ring in the objective lens just by changing exactly what you want to see, which regions you want to make brighter or darker, this refractive index, complete interference phenomena you can change the way you want.

And that way you can change the contrast generation, final contrast that is generated between various features that can be completely modified changing this phase ring or just by changing the way which light it is allowing you can play around with this contrast between different phases. For example, you can see these two images which are created and more importantly this is called positive. There is some mode called positive and negative contrast.

So, what happened is that objects when you have this depends on this makes you like with higher refractive index if a medium is of higher refractive index than the surrounding whether it will appear brighter or darker that is what you do in this positive or negative phase contrast mode. And here positive phase contrast mode as I said where the darkness of the object features that will increase with the refractive index that is what is the positive contrast mode.

So, when the darkness increases as the refractive index if something is of higher refractive index, then it will appear darker that is what is called the positive phase contrast and most of the microscope these days biological microscopes and that is what works best for the biological sample because as I said the biological medium the aqueous medium is usually of higher refractive index.

So, it is better that you keep the aqueous regions darker compared to the features that you see. And aqueous mediums basically make the background for your image. So, if you keep that one as a darker you manipulate your phasing in such a way that you keep the background to be darker, the aqueous medium, and increase the contrast or make this lower refractive index regions within the cell brighter, but you can play around again with that.

So for example, these are the two different both of them are phase contrast images. But what happens is that in one case these regions are not visible at all, and then you play around with your phase, you would basically change the kind of phase ring that you are going to use. So from Ph3 you are going to Ph4 objective and then you are creating much better contrast.

So, the difference between these phasing is which kind of light you are allowing and what is the phase relationship between the light, how the interference is happening and so and that is how you can pretty much imagine that this is kind of a trial and error method. And with changing that you can increase the contrast, you can enhance the contrast for even those regions which you are not able to see here that also is a possibility in phase contrast microscopy.

So, you can further play around with this contrast enhancement the entire optical manipulation process and further increase the contrast of your material. So, with this we are closing this part, the phase contrast microscopy part and in the next lecture we will be discussing about another very important contrast enhancing mode that is called polarized light microscopy. And after that, we will be discussing differential interference contrast and finally we will end up with fluorescence microscopy.