

Phase Contrast by Motic

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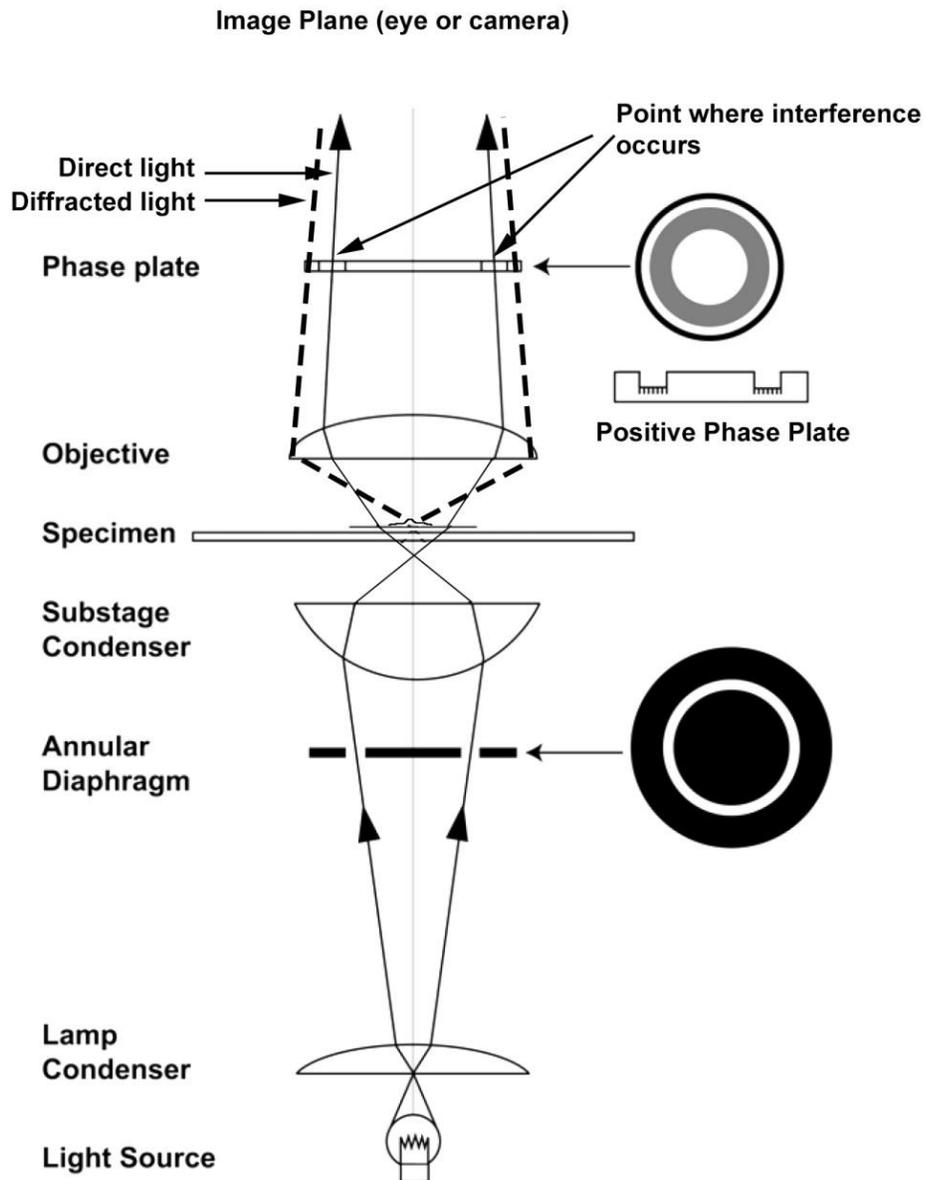
Importance of Phase Contrast

In the history of microscopy one of the challenges microscopists faced was producing enough contrast when observing thin tissue sections and living organisms without needing to kill, fix, dehydrate and then stain the specimens with dyes. Other microscopy techniques like dark field, oblique (relief), polarizing and Rheinberg lighting were also developed and are easy to implement. In the 1930's a Dutch physicist by the name of Fritz Zernike developed a new technique called phase contrast that was so significant that he garnered the Nobel Prize in 1953.

Our eyes are sensitive to changes in movement, wavelength (colour) and amplitude (brightness) but not to changes in phase. A phase contrast microscope converts changes in phase to changes in amplitude which our eyes can see. Most living cells and many aquatic microorganisms appear like translucent "bags of water" when viewed by bright light microscopy. There are some dyes that in low concentration can be used to stain living cells (e.g. iodine, acridine orange, methylene blue, Nile red) but most are toxic at high concentrations and the dyes can alter an organism or cells behavior. Phase contrast is a technique that can be used to view living cells or organisms with a minimum of deleterious effects. One other advantage of phase contrast microscopy is that it is insensitive to polarized light and birefringence and can be used to observe cells cultured in plastic dishes.

Basic Phase Contrast Design

A phase contrast microscope uses several optical techniques to produce contrast within living cells. The first is a circular annulus in or below the condenser that provides a cone of partially coherent light focused onto the specimen. The light passes around and through the specimen. Some of this light is diffracted as it passes through the specimen and the light waves are retarded by $\frac{1}{4}$ of wavelength on average. This diffracted light and the direct unaffected light passing around the specimen enters the objective. Both the direct light and some of the diffracted light passes through a phase ring at the back of the objective. The phase ring has two functions 1) it reduces the overall brightness of the direct light so it doesn't overwhelm the diffracted light and 2) it slows (retards) or speeds up (accelerates) the direct light by $\frac{1}{4}$ wavelength so that the direct light and diffracted light are $\frac{1}{2}$ a wavelength out of phase causing constructive or destructive interference. Constructive interference makes cellular components brighter (negative phase contrast) and destructive interference makes them darker (positive phase contrast). The phase ring is also grey and you can see it by looking through the back of the objective. The resultant changes in amplitude caused by interference between the direct and indirect (diffracted) light become visible as differences in brightness and contrast when living organisms are viewed by phase contrast.



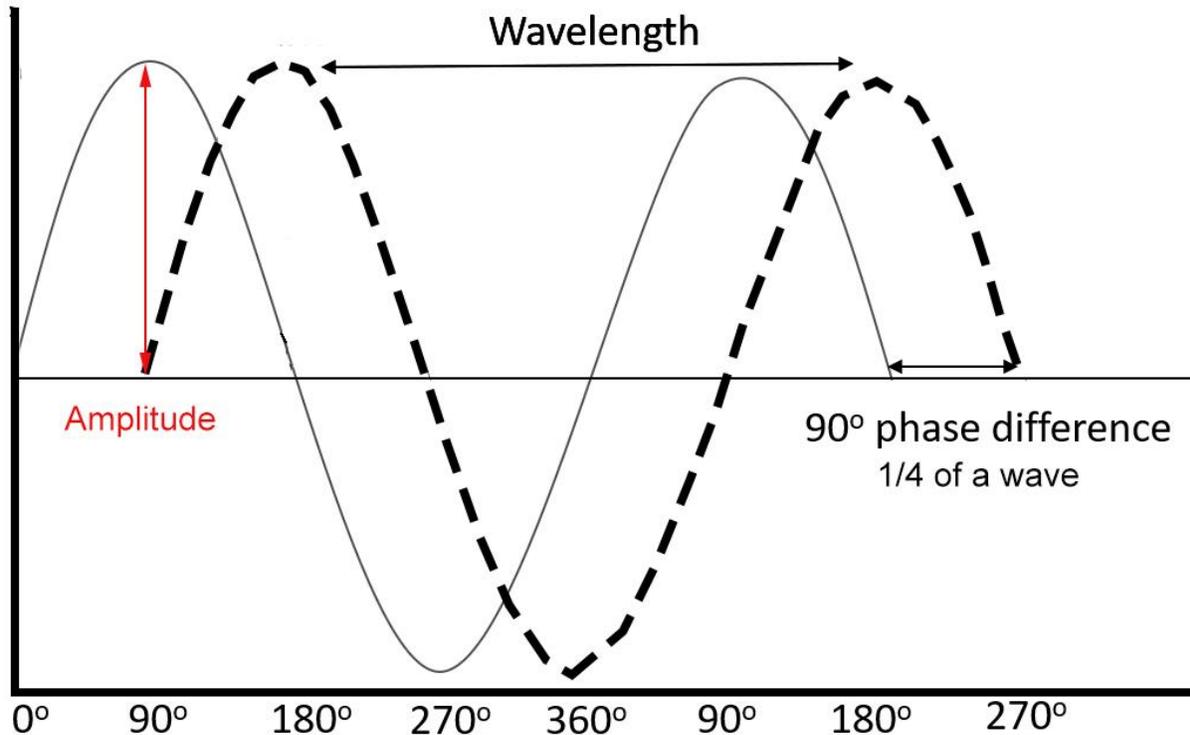
Schematic diagram of a positive phase contrast microscope. The annular diaphragm creates a cone of light focused on the specimen. The specimen causes some of the light to be become diffracted and the light is retarded in phase by $\frac{1}{4}$ of a wavelength. Direct light proceeds to the objective and around the specimen and some of it passes through the phase plate rings where it is attenuated and the phase altered by $\frac{1}{4}$ wavelength. The direct light and diffracted light interfere resulting in interference causing changes in brightness and contrast (diagram modified from the Motic manual).

Phase Annulus (Annular Diaphragm)

The Phase Annulus is a black disk with a clear ring or slit that sits in or under the condenser. Its' purpose is to provide a cone of light that is focused on the specimen. Unlike dark field lighting, light from the phase annulus enters the objective. Different objectives require different sized annuli which match the objectives (in practice one disc may support more than one objective e.g. Motic Phase 2 disc supports both the 20X and 40X objectives). Zernike experimented with different slit patterns (see Fig. 7 Pelc et. al. 2020) but the circular disc is the most common one in use today in part because it was the easiest to align with the phase plate such that halo artifacts are spread in an angular direction. The function of the phase annulus is also to provide partially coherent light. Coherence occurs when the majority of light of a single colour is in phase – i.e. most of the light waves are in sync. Laser light is perfectly coherent but it turns out it is too coherent (Hard et. al. 1972) and creates optical noise by revealing flaws and dust in the optical system. The other function of the phase annulus is to match the phase disc at the back of the objective focal plane so the direct light can be directed and aligned with the phase plate.

Phase Plate

The phase plate is a circular disk usually positioned at the back of the objective focal plane. The disc must be perfectly aligned with the phase annulus in the condenser. It serves two purposes: the first is to attenuate the direct light by about 75% to match the relative intensity of the diffracted light. The attenuation of the direct light in the past was done using a thin layer of soot, today the rings are coated with thin metallic films. The second function of the phase ring is to change the phase of the direct light by a $\frac{1}{4}$ wave relative to the diffracted light. If the direct light is retarded $\frac{1}{4}$ of a wave it then comes in phase with the diffracted light resulting in constructive interference (negative phase) or it advances the phase of the direct wave by $\frac{1}{4}$ wavelength so that the direct and diffracted light waves are now $\frac{1}{2}$ wave out of phase resulting in destructive interference (positive phase contrast).



Schematic diagram showing two light wave forms $\frac{1}{4}$ wave length out of phase. For destructive interference the waves need to be a $\frac{1}{2}$ wave out of phase. Diagram modified based on Teledyne Photometrics website (see link in the reference section).

Positive phase contrast is the most common type found in light microscopes. With positive phase contrast thicker organelles in the cell appear dark against a light background e.g. nucleus and mitochondria. Positive phase contrast is commonly used for examining cells in culture or aquatic microorganisms.

Negative phase contrast makes thicker regions of the cell and organelles appear bright against a dark background. The direct light is retarded by $\frac{1}{4}$ wave producing constructive interference resulting in bright details on a dark background. Negative phase is good for observing some protists e.g. vorticella. Nuclei in cheek cells appear bright white by negative phase contrast (see figure below).

Motic offers both positive and negative phase objectives

There are some microscopes that have the phase plates positioned outside of the objectives such that they can use ordinary bright field objectives to produce phase contrast (e.g. some inverted microscopes). Motic also offers inverted phase contrast microscopes.

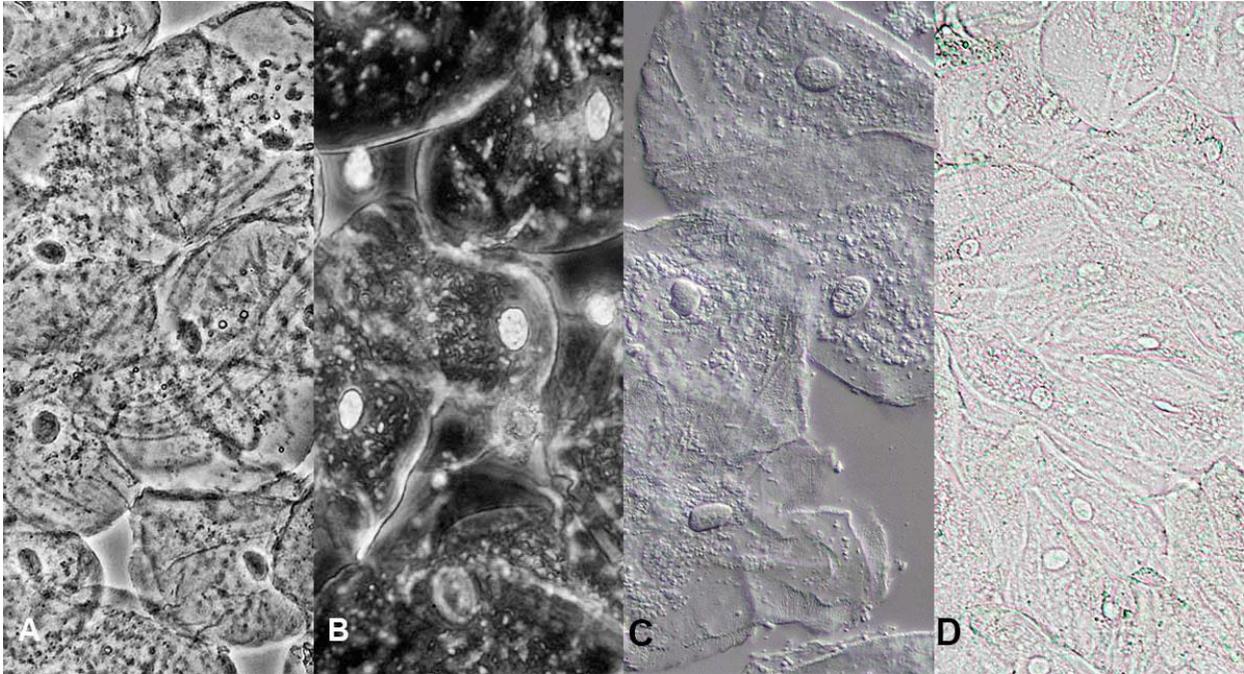
The images in this article were taken using a Motic phase contrast kit added to my Motic BA310 polarizing microscope. Photographs were taken using a 24 megapixel DSLR camera attached to the microscope by a Motic photo-tube, camera T mount and 5X projection eyepiece.



Above is a Motic phase contrast kit consisting of a phase turret containing three phase annuli (PH1, 2, 3), a bright field annuli and dark field annuli. At the top right is a phase or centering telescope used to align the phase rings with the annular diaphragm. The kit includes 4 infinity phase objectives, and immersion oil. This phase contrast kit can be installed on Motic upright microscopes.



Above image shows a Motic BA210 Phase annuli with a 3 position slider for Phase 1 and Phase 2. It supports 2 phase objectives and the center disc is used for bright field microscopy. It is another way to add phase contrast to a Motic upright light microscope equipped with two phase objectives.



Above image shows human cheek cells photographed with 4 different microscopy techniques A) Positive phase contrast - note the cell nuclei are dark B) Negative phase contrast - nuclei are bright C) Differential Interference Contrast (DIC) exhibiting a 3D relief and D) Bright field microscopy where cells are translucent and barely visible - all images at 200X. Motic does not offer DIC optics at this time.

Phase contrast setup

Phase contrast installation is straight forward taking about 10-15 minutes. First, install the condenser turret and phase objectives and then set up the microscope for Köhler illumination to produce even lighting (see reference below). Focus on the specimen, remove one eyepiece and insert the centering telescope (or use a Bertrand lens on a polarizing microscope) and align the condenser annuli with the phase ring, remove the centering telescope, put the eyepiece back in and view the specimen. If there are polarizing filters in the optical path remove them so they don't diminish the light.



Above photographs show human cheek cells on the left by phase contrast and the inset shows the bright annulus aligned with the darker phase ring. On the right the image shows the appearance of the cheek cells when the annulus is not aligned properly and the image contrast and background are uneven. The inset photographs shows the incorrect alignment of the phase plate (dark) and the annular diaphragm (bright) as viewed with a phase telescope or Bertrand lens. I recommend checking the condenser and phase annuli for each objective to assure they are aligned properly before taking pictures.

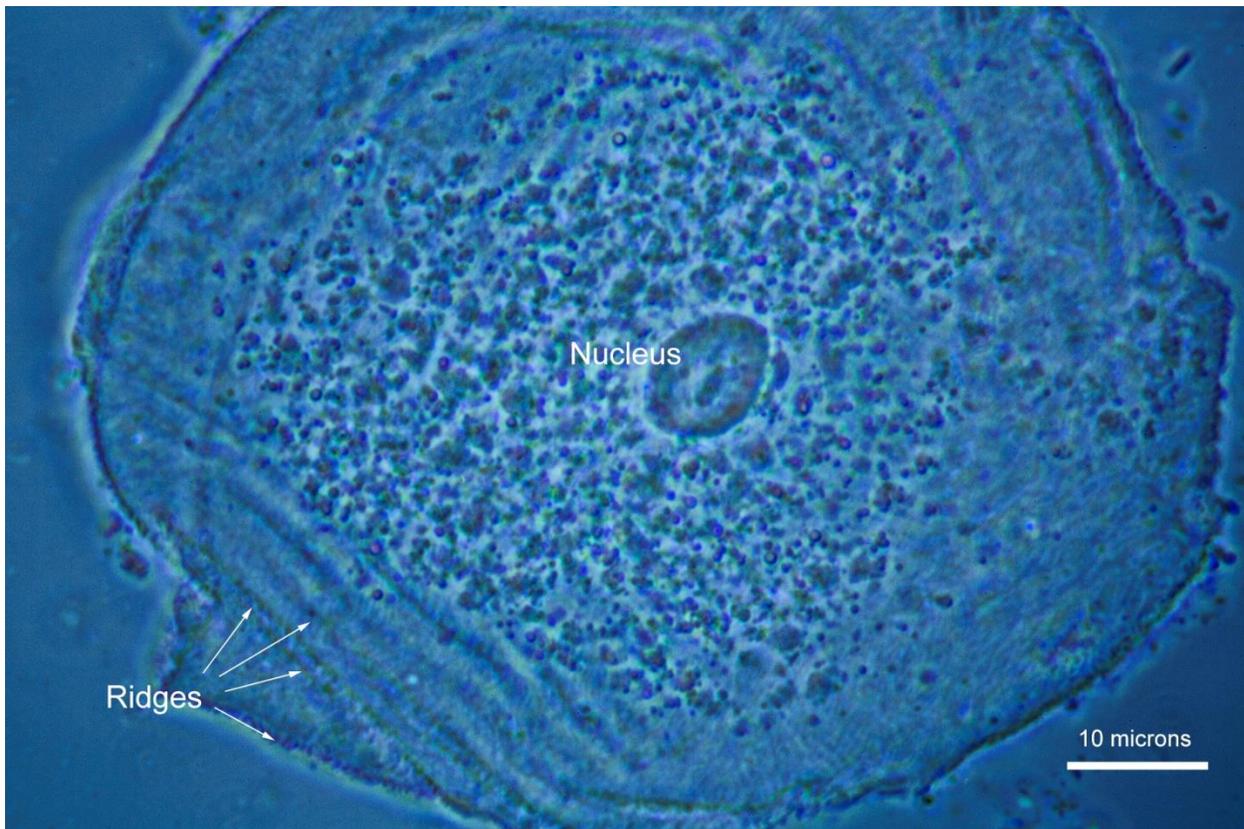


Human cheek cells photographed with the Motic 40X positive phase objective. Note the bright halos that are characteristic of positive phase contrast. Halos occur because the circular phase-retarding neutral density ring in the objective transmits a small amount of the diffracted light from the specimen. There are methods to reduce or eliminate halos (Pelc et. al. 2020). The cheek cell nucleus is about 10 microns in diameter. Although the image appears monochromatic, bright colours can still be seen in some ciliates, algae or aquatic organisms by phase contrast (see images below).

Implementing phase contrast requires dialing the correct condenser annulus (e.g. Phase ring 1 with 10X objective, Phase ring 2 with 20 or 40X objective, Phase ring 3 with 100X objective) and aligning them with the phase annuli. The alignment is done by moving the condenser annuli with alignment tools on the condenser. If the phase contrast image doesn't look good it might be due to using the wrong condenser annulus and phase ring, the rings are not perfectly aligned, or the specimen may be too thick. Sometimes deliberately using the wrong condenser annulus produces a dark field effect and by adjusting the annulus so it only partially overlaps with the phase ring it can produce oblique lighting. Oblique

lighting is created by illuminating the sample by only a portion of the light coming from the condenser. Objectives that use oil immersion fluid require a little extra effort; be careful not to trap air bubbles in the oil and lower the objective into the oil rather than rotate it in from the side. Most oil immersion objectives are spring loaded so they retract if you contact the coverslip. If the contrast is poor try adding a bit more oil immersion fluid. After using the oil objective clean it and the coverslip before you use another objective like the 40X to avoid getting oil on regular dry objective. When you are finished using the oil immersion objective clean the oil off with lens tissue and small amount of xylene (Duke 2003 – describes best practices for cleaning oil immersion objectives including alternative solvents).

Phase contrast is one of the most important methods used to enhance the visibility of live cells and aquatic organisms. You don't have to understand the physics to see the benefits of phase contrast. A simple test subject are human cheek cells smeared on a microscope slide under a coverslip.



Human cheek cell photographed with Motic 100X oil immersion phase objective and a blue filter. Ridges or fold-like extensions of cytoplasm (microplasm) provide more surface area, trap mucous and provide adhesion to other cheek cells. These ridges or membrane folds in the plasma membrane can be seen more clearly using a scanning electron microscope and their size (0.2 microns) is at the limits of resolution for an ordinary light microscope (P. Asikainen et. al. 2015).

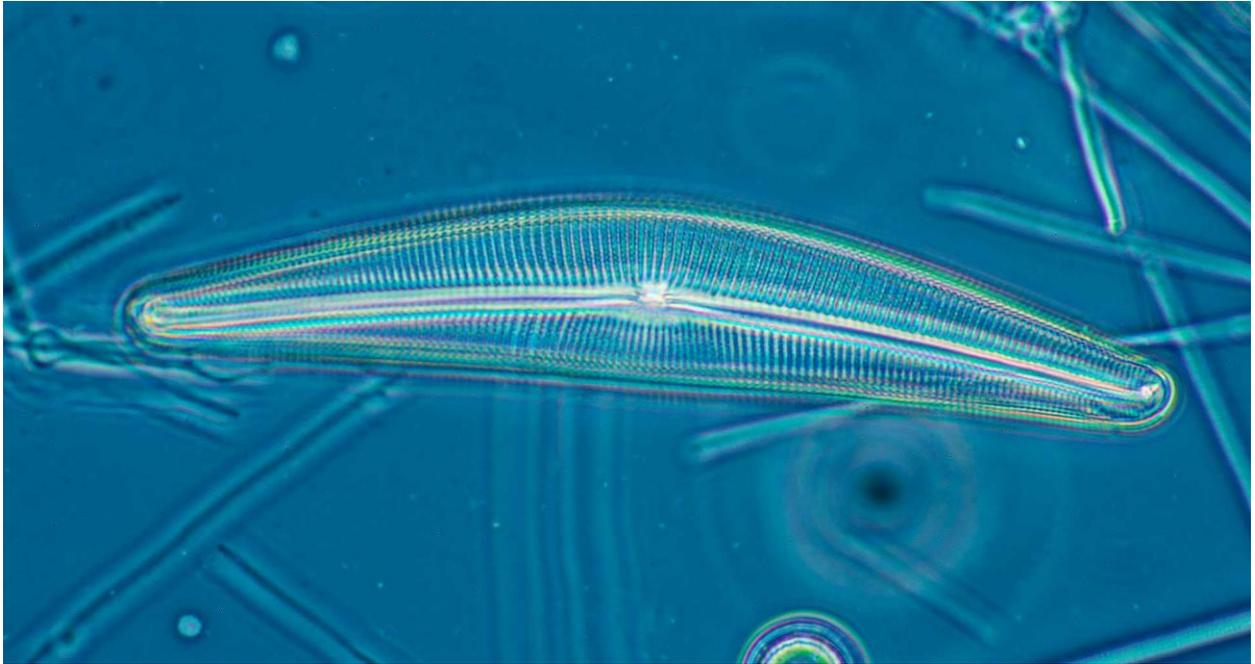
To achieve the maximum resolution with a 100X oil immersion objective requires oil immersion fluid on top of the coverslip and if your microscope has a condenser with a numerical aperture equal to or greater than the 100X objective numerical aperture (NA) condenser (NA 1.25 to 1.4) you should also put

oil between the condenser and microscope slide (J. Cargille, 1985). When I tested oil on the condenser I could not see a significant improvement in resolution so I only used oil on the coverslip after this. The numerical aperture of the Motic phase condenser is 0.9 so putting oil between the condenser and slide will not help improve resolution.

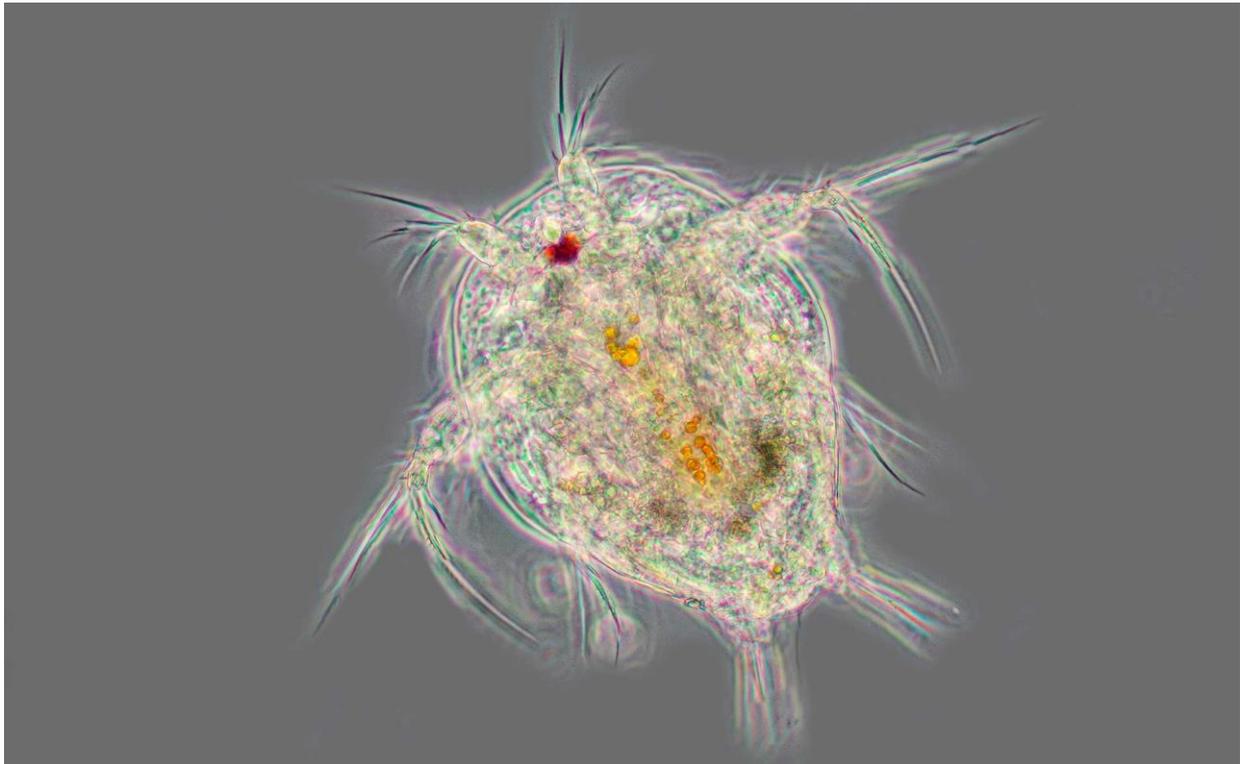
When using a polarizing microscope for phase contrast one should remove the polarizer, analyzer and any compensation plates from the optical path as each polarizer reduces the overall light by about $\frac{1}{2}$ the light intensity. A special tool is required to remove the bottom analyzer in the Motic polarizing microscope. Photos below show some examples of positive phase contrast images using aquatic microorganisms.



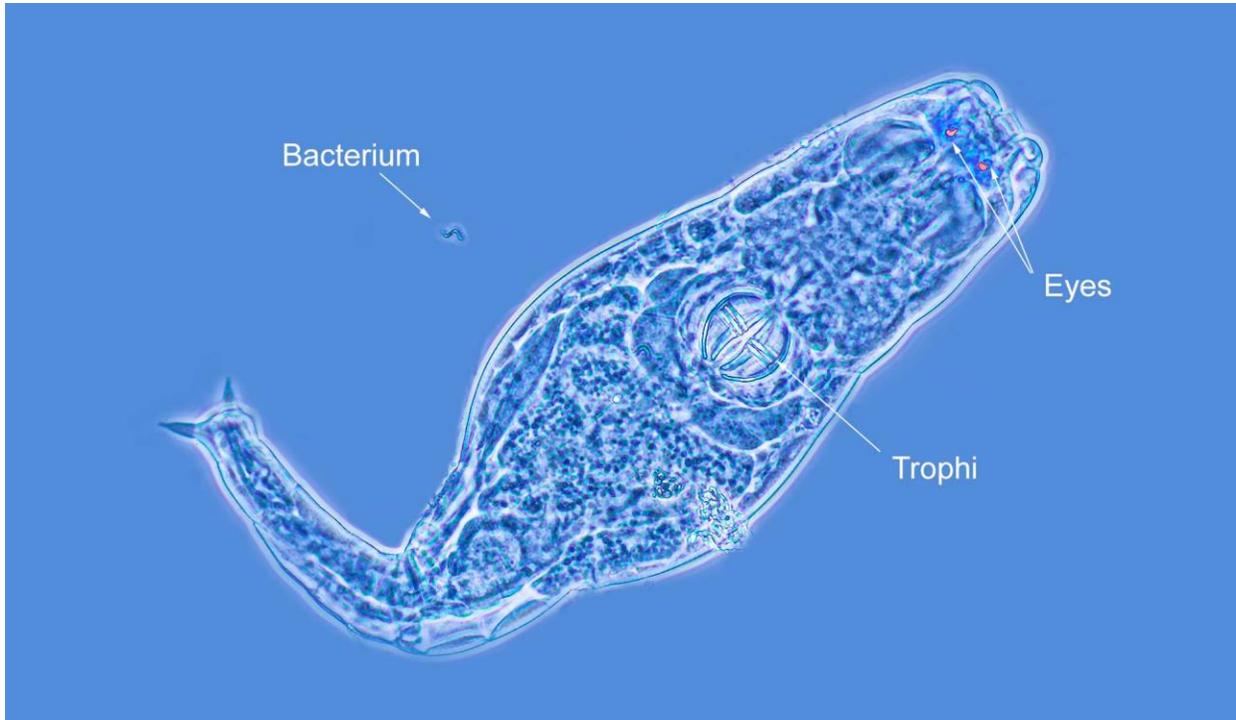
Euglena photographed with a 40X Motic Phase objective. This image was extracted from a video, using Photoshop. The long flagellum is about 0.5 microns (500 nm) thick.



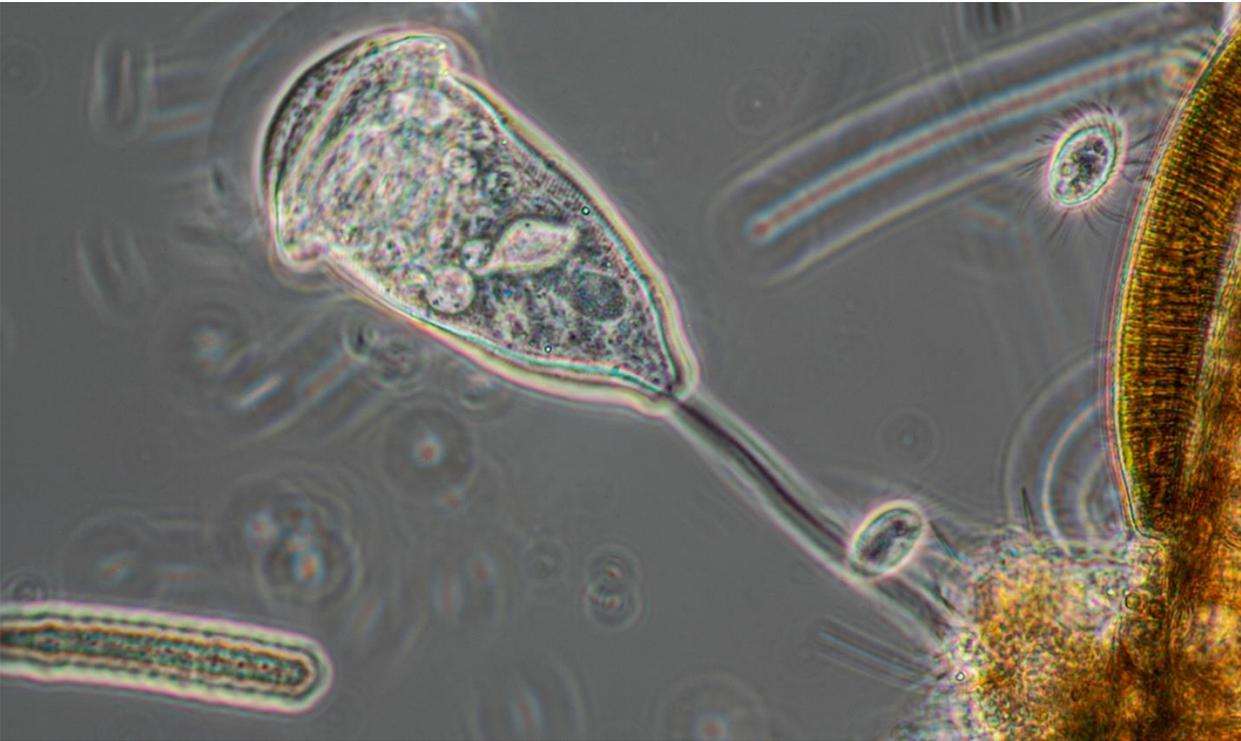
Diatom frustule *Cymbella* sp Motoc 20X phase contrast objective and a blue filter.



Nauplius larva 10X phase objective with no filters. Green or blue filters are sometimes used with phase contrast because monochromatic light can sometimes enhance the contrast and resolution slightly by eliminating some chromatic aberration. Focus stack of several images.



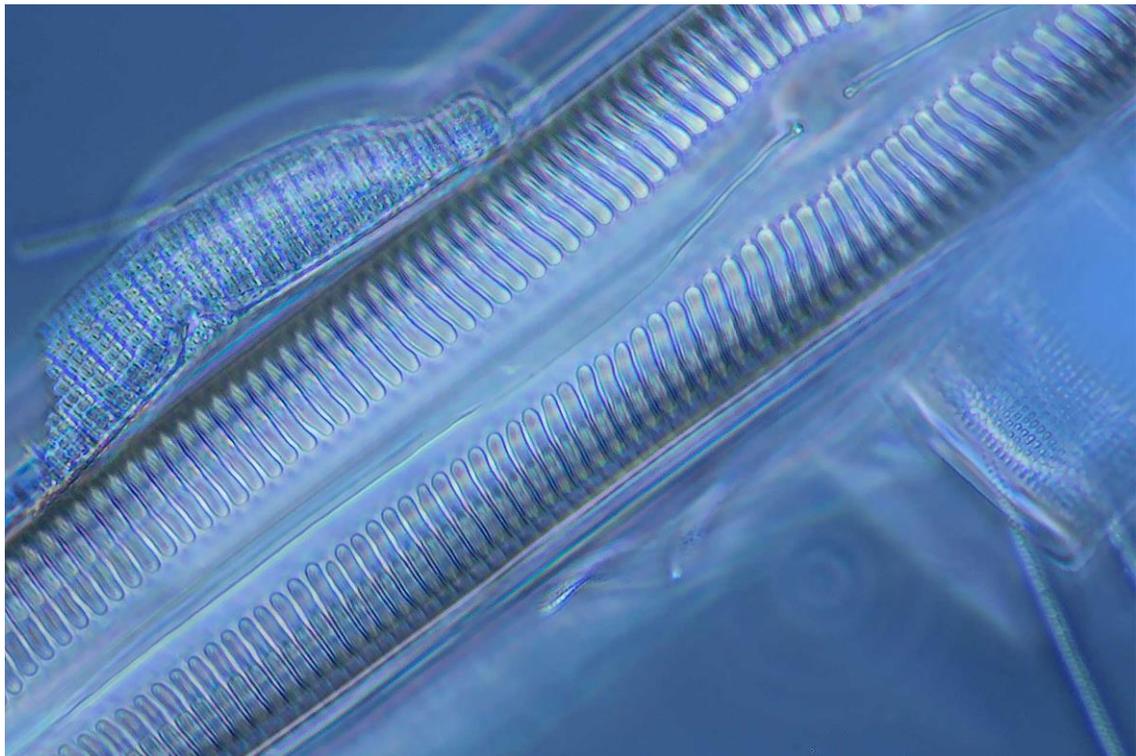
Bdelloid rotifer photographed with a 40X Motic Phase objective. The red eyes can be clearly seen along with the Trophi (jaws) and a single bacterium. Blue filter, background debris was removed digitally.



Peritrich ciliate and two smaller ciliates, a diatom and blue green algae photographed with Motic 40X phase contrast objective, no filters.



Fast moving ciliate *Spirostomum minus* photographed using a Motic phase contrast 10X objective and blue filter.



Diatom frustules, the middle diatom is *Pinnularia sp* taken with 100X Motic phase objective, oil immersion fluid and blue filter.

Adding phase contrast to an existing microscope is one of the best ways to upgrade a light microscope. It is important to note that while some phase condensers and phase objectives from different microscope manufacturers may work together many will not because the phase rings won't align with the condenser annuli. The cost of a phase contrast system varies with the microscope brand, the quality of the objectives purchased (Achromat, Plan Achromat, Fluorite, Apochromat) and type of phase contrast. Adding phase contrast to a microscope is significantly lower in cost than adding fluorescence or differential interference contrast (DIC).

Phase contrast microscopy does have some small disadvantages. The first is that cells often have bright or dark halos around them and their intracellular components that can reduce the resolution, but these halos also serve to enhance contrast. The halos can be reduced or eliminated several ways including computer processing, altering the refractive index of the medium, and using special apodized phase rings in the objectives that reduce edge effects (Pelc et. al. 2020) Using a 100X phase objective requires a reasonably bright light source and oil immersion fluid. Finally, thick specimens can sometimes be difficult to interpret in phase contrast.

Summary

There are many varieties of phase contrast including: positive, negative, anoptral, relief, apodized, variable and color phase contrast (Pelc et. al. 2020; Piper, 2007; van Wezel 2004). A phase kit consists of 10, 20, 40 and 100X objectives with internal phase rings and usually a turret condenser with 3-4 different annular diaphragms. The phase condenser also allows normal bright field viewing and some offer dark field viewing as well. To carefully align the phase annulus and the phase plates you will need a phase telescope or Bertrand lens. Phase contrast is available for both upright and inverted microscopes and it's one of the best ways to view live cells, bacteria, yeast, protists, and other aquatic invertebrates.

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Phase-Contrast Microscopy

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M.W. Davidson Configuring a Microscope for Köhler illumination.
<http://zeiss-campus.magnet.fsu.edu/print/basics/kohler-print.html>

Anoptral phase contrast – images appear brown with reduced halos.
<https://spockssister.com/2014/01/22/diatoms-anoptral-phase-contrast/>