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Optical Microscopy

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Introduction

Over the past decades, the number of applications of optical microscopy has grown enormously. Optical microscopes are now used as routine tools in most fields of science and industry, such as microelectronics, nanoscience, biotechnology and pharmaceutical industry, cell/microbiology etc. The knowledge gained from this lab session could therefore be highly useful and important in your future work.

We will explore the basics of image formation in optical microscopy. Understanding how the microscope works is crucial for interpretation of results and an ability to adjust a microscope correctly is essential in order to make use of its full potential. In addition, we will discuss some different optical imaging techniques.

Literature

As literature we recommend the text "Optical Microscopy" by Davidson and Abramovitz, found at http://www.olympusmicro.com/primer/opticalmicroscopy.html. The basics of image formation, and optical phenomena in general, is discussed at length in the book "Optics" by Hecht. The webpage www.olympusmicro.com can be used as an encyclopaedia of optical microscopy. This web page also contains a virtual microscope and a number of java tutorials.

Theory

Abbe's theory of image formation

The image of a specimen is formed due to diffraction. The specimen is seen by the light as a complex superposition of gratings with varying grating constants. Some of the light will pass through the specimen undeviated (0th order diffraction) and will only give rise to a uniform bright background. The deviated (diffracted) light carries the information about the structures in the specimen.

To simplify things, consider the grating specimen schematically shown in Figure 1. Parallel light (i.e. a plane wave) that enters from below along the optical axis will be diffracted and the different diffraction orders will emerge at different angles. If the distance between the grids decreases (i.e. the grating constant decreases) the diffraction angles will increase.

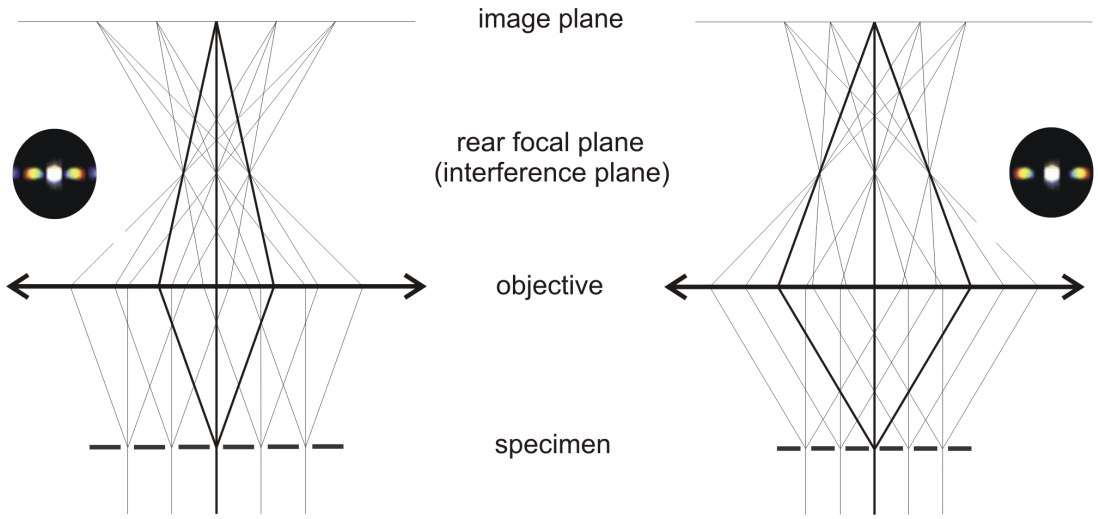

Figure 1 Schematic showing how parallel light pass through two gratings with different periodicity. The diffraction pattern will occur in the rear focal plane of the lens/objective. Note that smaller grating constant results in a wider spread of the diffraction pattern.

In Figure 1, the objective of a microscope is simplified as a single lens that produces an image at a location given by simple geometrical optics (e.g. Gauss lens formula). A (Frauenhofer) diffraction pattern is formed in the diffraction/interference plane, which corresponds to the rear/back focal plane of a real objective. The diffraction pattern is the Fourier transform of the image. The grid will then appear as bright spots on a line. The central spot corresponds to 0th order diffraction. All light that go through the sample undeviated will pass this spot in the back aperture of the objective. The spots next to the zeroth order are the first diffraction orders (+1 and -1) and so on. As illustrated in Figure 2, blocking all spots but the zeroth order will result in a uniform background in the image plane, without any information about the specimen whatsoever ("information" here means information about the grating constant). Blocking all light but the first order spots will result in an image with an intensity variation having the same frequency as the grating. The second and third diffraction order alone gives a false periodicity. However, by adding the four orders (zeroth to third) we get a reasonable image of the specimen.

Ernst Abbé, a German microscopist of the late 19th century employed by Carl Zeizz in Jena, Germany, developed the theory of image formation, which states that an image will be formed only if at least two of the diffraction orders are captured by the objective. The more diffraction orders that can be captured by the objective, the finer the details that can be resolved.

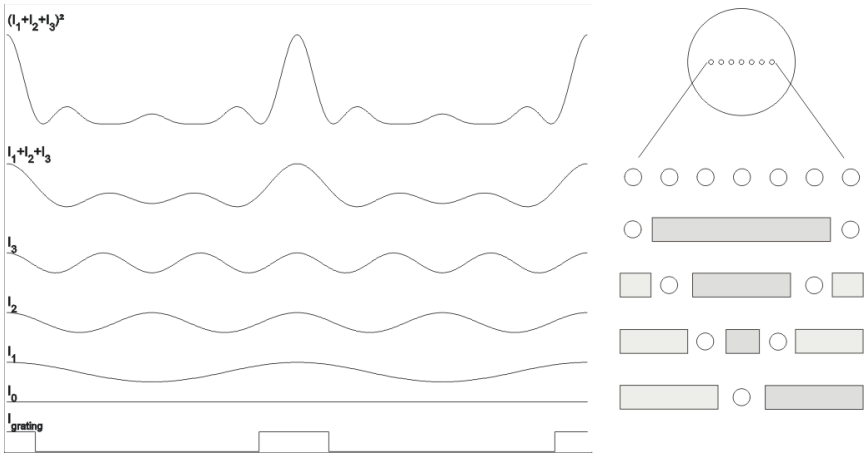

Figure 2 Plot showing the image of a grating when blocking different diffraction orders (as shown to the left). $I_{grating}$ shows what the intensity image of the grating should look like.

The resolution limit

We now want to estimate the resolution of an optical microscope. This problem can be addressed in two ways, both based on so-called Fourier optics theory.

In the first approach, we think of the object as the superposition of an infinite number of gratings with different grating constants, and ask ourselves: what is the smallest grating constant d that can be resolved? This clearly corresponds to the smallest details that can be resolved in the specimen. We thus consider a grating with grating constant d and, to be general, we assume that the grating is embedded in a medium with refractive index n_2 (typically air, n=1, or water, n=1.33). We image the grating using a lens/objective with numerical aperture $NA = n_1 \sin(\alpha)$, where α is half the opening angle of the objective (more or less given by $\sin\alpha \approx \tan\alpha = r/f$, where r is the radius of the lens and f the focal length) and n_1 is the refractive index of the immersion medium (typically air or immersion oil, n≈1.5 ≈n_{glass}). We know that we need at least two diffraction orders m to obtain an image. The best choice is m=1 and m=0, because the angular divergence between those waves are smaller than for any other combination. The smallest grating constant that can be resolved then corresponds to the case when the m=1 and m=0 waves are just able to enter on the opposite sides of the lens, see Figure 3, i.e. when they form the angle α with respect to the optical

axis. This means that the two waves have to emerge from the specimen (the grating in our case) with the same angle β, given by "Snell's Law" $n_2 \sin\beta = n_1 \sin\alpha$. We now apply the grating equation $d(\sin\theta + \sin\gamma) = m\lambda$, where θ is the illumination angle with respect to the grating normal (same as the optical axis), γ is the diffraction angle for diffraction order m (m=1 here) and $\lambda = \lambda_0/n_2$ is the light wavelength in the medium surrounding the grating. From Figure 3, we see that both θ and γ has to equal β. We thus have:

$$d(\sin\beta + \sin\beta) = m\lambda \;\Rightarrow\; d2\frac{n_1}{n_2}\sin\alpha = 1\cdot\frac{\lambda_0}{n_2} \;\Rightarrow\; d2NA = \lambda_0 \;\Rightarrow\; d = \lambda_0 \big/ 2NA \qquad (1)$$

This simple analysis thus shows that the resolution of optical microscopy is roughly half the vacuum wavelength. Specifically, for green light ($\lambda_0 \approx 500$ nm) and a good oil immersion objective (NA = 1.4), we would have a theoretical resolution of the order 180 nm.

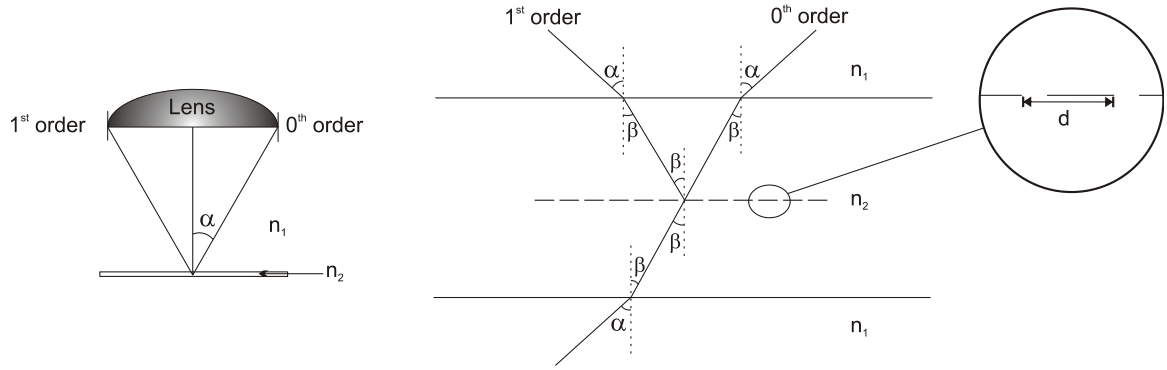

Figure 3 **An illustration of how an objective with an opening angle α is just able to resolve a grating with grating constant d.**

From Figure 3, we see that the illumination wave has to form an angle α with respect to the optical axis, i.e. the numerical aperture of the condenser has to be the same as for the objective. In order to approach the theoretical resolution of 180 nm mentioned above, obtained using an oil immersion objective, one thus also have to use an oil immersion condenser with the same NA as the objective. If the NA of the condenser is smaller than that of the objective (e.g. one use an air condenser together with an oil objective), the theoretical resolution is modified to:

$$d = \frac{\lambda_0}{NA_{condenser} + NA_{objective}}. \qquad (2)$$

However, one cannot increase the resolution by applying a condenser with a higher NA than the objective, i.e. the maximum resolution is given by Eq. (1). As a rule of thumb the resolution limit is said to be approximately $\lambda_0/2$.

In the second approach to the resolution limit, we instead think of the object as a superposition of point sources. We ask ourselves: What is the smallest distance between two point sources that can be resolved by optical microscopy?
First recall that a plane wave that hits a circular aperture diffracts at the edges of the aperture, resulting in a diffraction pattern described by the so-called Airy function (see e.g. "Optics" by Hecht). If a lens completely fills the aperture, one can image the diffraction pattern on a screen. Most of the light (~84%) is confined to the central maximum of the Airy pattern, the so-called Airy disk, and around this disk is a dark ring. Imagine that the aperture has a radius r, the lens a focal length f and the diffraction pattern is formed in a medium with refractive index n_1. The radius R of the dark ring, which defines the size of the Airy disk, is approximately:

$$R \approx 1.22 \frac{f\lambda}{2r} \approx \frac{1.22\lambda_0}{2n_1 \sin\alpha} = \frac{1.22\lambda_0}{2NA} \qquad (3)$$

Eq. (3) applies, for example, to the case when we want to focus a laser beam of wavelength λ_0 to the smallest possible spot using a lens or objective with a certain NA (as in confocal microscopy). However, one can also "reverse" the problem, i.e. one use an objective to collimate light from a point source and a second lens to form an image of this point source. The image will then be an Airy pattern. If one instead has two nearby point sources, their respective Airy patterns will overlap and if they are too close it will be impossible to resolve them. One then defines the minimum resolvable separation distance d as the situation when the first dark ring of one of the Airy patterns overlaps the centre of the second Airy disk, see Figure 4. The resolution is then defined as $d=R$ according to Eq. (3). This is called the Rayleigh criterion.

Figure 4 The intensity distribution point sources. In A, an Airy pattern is formed from a single point. In, B two points are overlapping. In C, according to the Rayleigh criterium, the two points are just resolved (note that dark regions correspond to high light intensity, and vice versa).

Similar to the earlier discussion, one can also take into account the NA of the condenser, which results in the equation:

$$d = \frac{1.22\lambda}{NA_{condenser} + NA_{objective}}. \qquad (4)$$

Köhler illumination

The equipment needed to build an optical microscope is basically a light source to illuminate the specimen and a good lens for magnification. However, depending on the design of the microscope, the illumination and resolution of the microscope can be improved significantly. In 1893 August Köhler, of the Carl Zeiss corporation, introduced the microscope design that is used in all modern microscopes. The principle is shown in Figure 5.

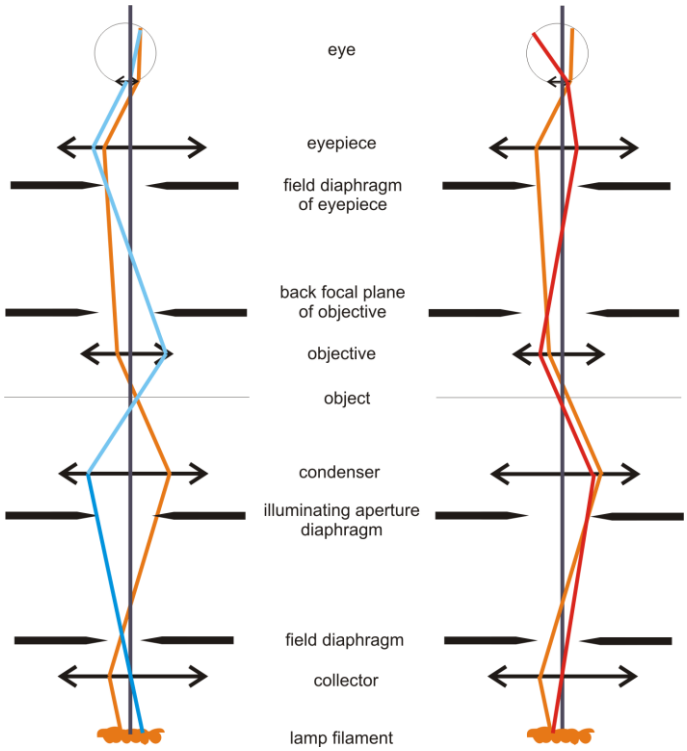

Figure 5 The two illumination pathways of Köhler illumination. Note that the conjugate planes for the two pathways are completely opposite. Hence, in the image planes the lamp filament is totally out of focus. Further, using Köhler illumination the field of view and the NA of the condenser can easily be changed.

In Köhler illumination there are two light pathways, the illumination pathway and the image-forming pathway. The illumination pathway originates from the parallel light from the whole lamp, whereas the light in the image forming pathway originates from each point in the lamp. By placing the lenses at specific distances from each other, the light in the illumination pathway is totally out of focus when the light in the image forming pathway is in focus, and the other way around. By putting the sample in the plane when the light from each point in the lamp is totally out of focus the sample will be perfectly evenly illuminated. This is not the only beauty with Köhler illumination. By putting diaphragms in the all conjugate planes where the light forms a focus, one can also correct for illumination area, using the illumination field diaphragm, and the angle of illumination with the illuminating aperture diaphragm.

Phase contrast

We have discussed how an image is formed due to diffraction. A biological specimen is almost uniformly transparent, and therefore the intensity variation in the image will be poor. However, the light does not go through the specimen unaffected. The light going through the specimen will be shifted in phase. Our eyes can not detect this phase shift. However, using phase contrast microscopy, developed by Frits Zernike in the early 1930s, we can produce an intensity image based on the phase shift.

To illustrate what happens, consider a cubic cell with a thickness d and refractive index n_2 in a medium with refractive index of n_1, as shown in Figure 6.

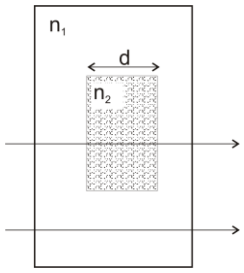

Figure 6 Schematic of a cell with refractive index n_2 and thickness d in a media with refractive index n_1.

The two light rays will experience an optical pathway length difference of $(n_1 - n_2)\, d$ which gives the phase shift of:

$$\theta = 2\pi d \frac{n_1 - n_2}{\lambda}.$$

For a cell this phase shift is approximately $\pi/2$. Frits Zernike understood that if the undeviated light would be speeded up with $\pi/2$, the phase difference would be π and an intensity image could be formed, due to constructive and destructive interference. To speed up the light a phase shifter is placed in the rear focal plane of the objective. The phase shifter is optically thinner in areas where the undeviated light will pass. To know there the phase shifter has to be optically thinner the illuminating light goes through an annulus ring, placed directly in front if the condenser and conjugate to the phase shifter. This is shown in Figure 7.

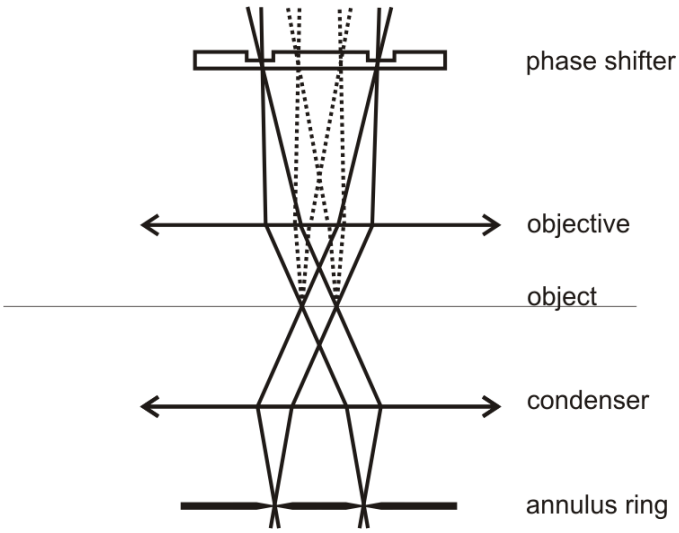

Figure 7 The principle of phase contrast. The diffracted light (shown with dotted lines) will be spread over the entire back aperture of the objective. The underviated lines will pass the back aperture in a circle with a certain diameter. The phase shifter with a ring of the same diameter will shift the deviated light $\pi/2$.

The light going through the specimen undeviated will continue in the same optical path and, with the correct phase shifter, through the optically thinner part of the phase shifter. The deviated light

is spread over the entire rear back aperture and most of the light will therefore go through the optically thicker area of the phase shifter. In this way a phase shift of π, and an intensity image can be formed.

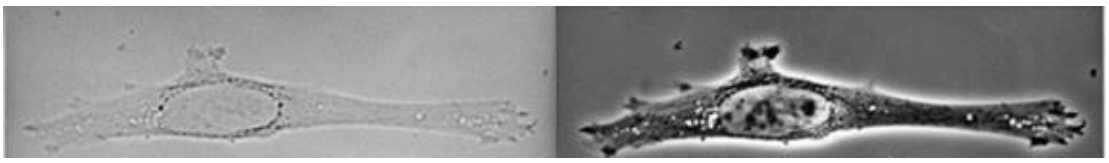

Figure 8 The difference between bright field (left) image and phase contrast (right) image of the same cell.

Dark field microscopy

Objects scatter light strongly can be studied with dark field microscopy. As the name implies the image of the specimen will be bright against a dark background. The concept of dark field microscopy is to let the illuminating light hit the specimen with an angle so high that undeviated light will not be captured by the objective. In other words, the zeroth order is removed. This can be achieved using special dark field condensers or by blocking the central part of the illuminating light, as is shown in Figure 10. Dark field can be used to study submicron particles.

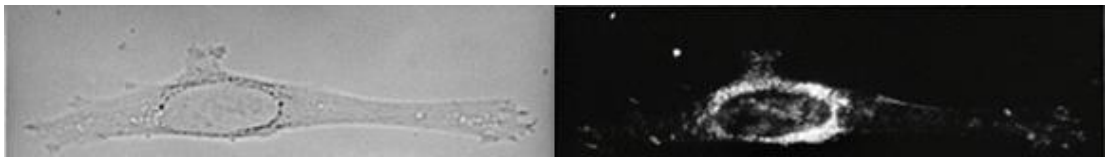

Figure 9 The same cell as in Figure 8, now imaged in bright field (left) and dark field (right).

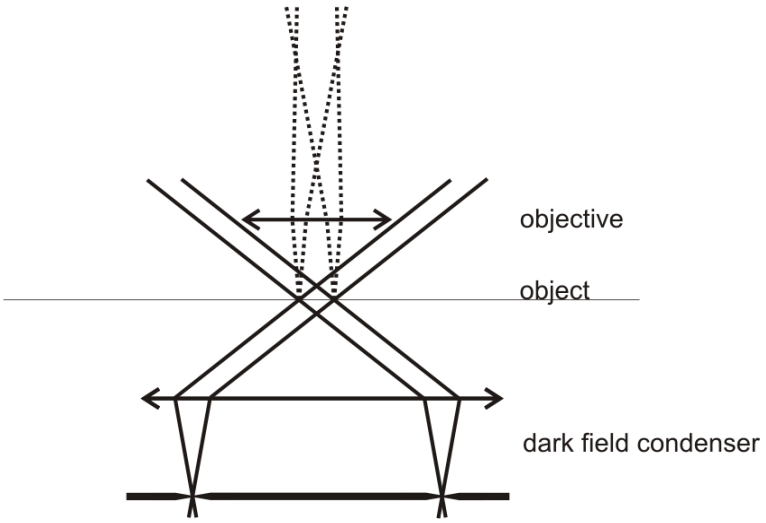

Figure 10 The concept of dark field microscopy. Only the diffracted light (shown as dotted lines) will be captured by the objective.

Home assignments

1. Put up some points on how you would build a microscope according to Köhler.

2. What are the expressions for the resolution limit of a microscope?

3. Explain the concept and design of phase contrast and dark field.

4. Think of a way to test the resolution limit of your microscope.

Lab assignments

1. Building an optical microscope

Equipment: light source, collector, condenser, xy-stage, objective, camera connected to computer.
Object: Human epithelial cells

1.1 Build the microscope according to Köhler in the way you described answering your home assignment or as discussed with the supervisor.

1.2 Test your microscope by imaging epithelial cells, handed to you by the supervisor.

For stability reasons we will now switch to a Nikon microscope.

Before we get started we must adjust the Köhler illumination of the microscope. Ask your supervisor for help and make sure you learn and understand the procedure!

2. Calibration of the objective-ocular combination

Equipment: 100X immersion objective, ocular screw meter.
Object: test plate
It is often important to know distances in a specimen. With a calibration of the microscope distances can easily be measured. By using a test plate with a ruler in the range of interest we can measure how big the distances will appear in the ocular. Using a camera, we can measure how many pixels correspond to the distance in the ruler. That knowledge can then be used to measure distances in the real specimens. Without a camera the distances can be measure using a modified ocular with a build in hair cross which can be moved with a micrometer screw. This is what we will do now!

2.1 Place the test plate "1/100mm" from Jena in the microscope and find the focus. Then change one of the oculars to the modified one and measure how many micrometers on the screw correspond to one micrometer.

3. Measuring the grating constant of a test diatome

Equipment: 100X immersion, ocular screw meter
Object: test diatom

A test diatom is a silica alga, *Pleurosigma angulatum*, which appear as small worms with an internal silica skeleton in the form of a periodic structure with a well defined grating constant.

3.1 Using the calibration in assignment 2 to measure the grating constant.

4. Test of Abbe's resolution limit

Objective: 100X immersion
Object: test diatom

According to Abbe, we need at least two diffraction orders to form an image. This means the pattern in the test diatom will vanish if we block all other orders than the first.

4.1 The diffraction pattern can be observed by removing the ocular. Make a sketch of what you see.

4.2 What happens with the pattern if you change the NA of the objective?

4.3 Adjust the NA so that only the zeroth order is visible. Put back the ocular. What happened to the image of the test diatom?

4.4 Change the NA of the objective back to the highest. What happened to the image now?

4.5 Are you convinced that Abbe is right?

5. Putting the resolution limit to the test

Equipment: 100X immersion, blue filter
Object: test diatom

The resolution equation states that $d = \lambda_0 / (NA_{objective} + NA_{condenser})$. We can assume the wavelength to be 480 nm. Find the NA of the condenser by measuring the angle of the cone of light on the sample.

5.1 Use a small piece of paper to see the cone of light. Now, adjust the NA of the objective so that the grating in the test diatom barely is resolved.

5.2 Calculate d and compare with the result in assignment 3.

6. Bright field, phase contrast, dark field

Objective: 20X
Object: human epithelial cells

Now we will experience what a difference different imaging techniques make. We will look at human epithelial cells using bright field, phase contrast and dark field. It is easy to change between the three modes on the microscope. Just switch the annulus ring in the condenser. Make sure to match the phase contrast ring with the phase shifter of the objective. If the image is poor the optics in the microscope might not be aligned.

6.1 Describe the differences and try to explain them.