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2006-01-20

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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, and is now found in almost any field of science and industry, such as microelectronics, nanophysics, biotechnology, phasmaseutic industry and microbiology. The knowledge (hopefully) gained in this lab could therefore be of outmost importance in future work.
In this lab we will explore basics of image formation in optical microscopy. Understanding how the microscope works is crucial to be able to adjust it correctly and to make use of its full potential. Not adjusting the microscope can result in lost of resolution and poor illumination. In addition, we will discuss some different optical imaging techniques.

Literature

As literature we recommend the text "Optical Microscopy" by Davidson and Abramovitz, which has been handed out on the lectures, and the book "Optics" by Hecht. The webpage www.olympusmicro.com can be used as an encyclopaedia of optical microscopy. The web page also contains a virtual microscope and a number of java tutorials.

Theory

Abbe's theory of image formation

The image of a light absorbing specimen is formed due to diffraction. The specimen is seen by the light as a complex superposition of gratings with varying grating constants and holes. Some of the light will pass through the specimen undeviated and will only give rise to a uniformly bright image. 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) which enters from below along the optical axis, will be diffracted and the different diffraction orders will emerge at different angles. The smaller the distance between the grids, i.e. the smaller the periodicity, the bigger the angles will be.

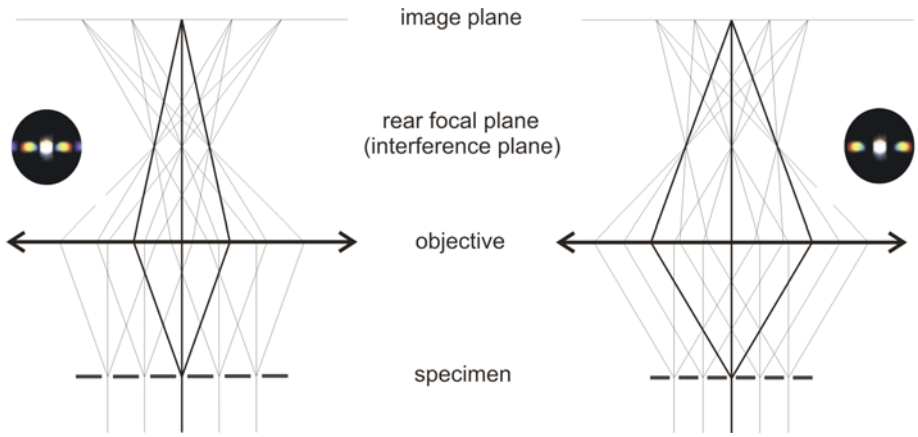

Figure 1 Schematic showing how parallel light pass through two gratings with different periodicity. The diffraction pattern will occur in the rear focal plane of the objective. Note that smaller spatial distances results in a wider spread of the diffraction pattern.

In the rear focal plane of the objective we find the diffraction pattern, which is the Fourier transform of the image. The grid will appear as bright spots on a line. The central spot is the zeroth order. 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 and so on. As illustrated in Figure 2, blocking all spots but the zeroth order will result in an evenly intense image in the image plane. Blocking all light but the first order spots will result in an image with an intensity variation having the same frequency as the grid. The second and third

diffraction order alone gives a false period. However, by adding the four orders (zeroth to third) we get a reasonable image of the specimen.

Ernst Abbé, a German microscopist of the 19th hundred century, stated 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 details can be resolved.

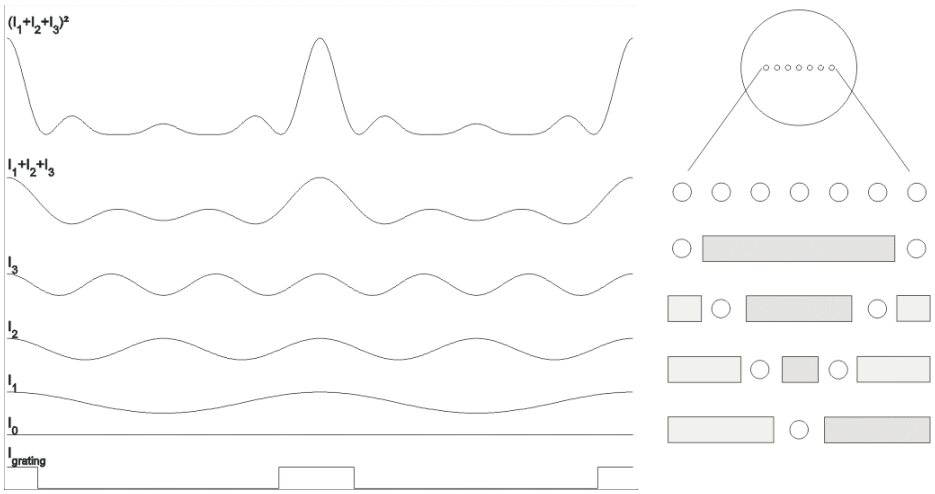

Figure 2 Plot showing the image of a grating when blocking different diffraction orders (as shown to the left). $I_{grating}$ shows what the intensity image of the grating should look like.

The resolution limit

Knowing that at least the first diffraction order is needed to obtain an image we can calculate the resolution limit. Consider a grating with the grating constant d. The grating equation gives:

$$d \sin\theta = m\lambda,$$

where θ is the angle to the optical axis, m is the diffraction order and λ is the wavelength of light. According to this equation the smaller the distance d, the higher the angle θ for the same diffraction order. Hence, the smaller the spatial distances in the specimen, the more the light will bend off. Resolution is therefore dependent on how many of the diffraction orders we can capture with the objective.

For the first order m=1, the grating equation gives

$$d = \frac{\lambda}{\sin\theta}.$$

This would be the smallest distance which can be resolved with the microscope. This limit could be decreased by using an oil or water immersion objective.

If only air is in between the cover slide and the objective the light will bend off in a higher angle due to refraction. See Figure 3. Oil and water have a higher refractive index than air (approximately 1.5 and 1.3 compared to 1). By using oil or water in between the cover slide the light will travel to the objective at a lower angle Hence, the resolution limit will decrease.

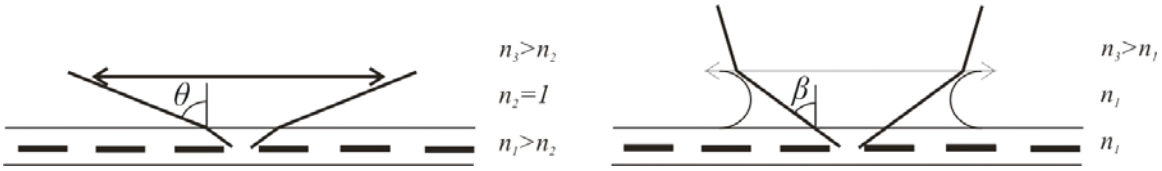

Figure 3 To the left an air objective and to the right an oil immersion objective imaging the same grating. The air objective will not be able to capture the first diffraction order as the light will bend off with a bigger angle, due to refraction. By reducing the refractive index difference using oil, the light will be captured by the objective.

According to Snell's law of refraction

$$n_1 \sin\beta = n_2 \sin\theta$$

where n is the refraction index. If n_2 =1, as for air, we get

$$n_1 \sin\beta = \sin\theta$$

The resolution limit could now be written

$$d = \frac{\lambda}{n_1 \sin\beta} = \frac{\lambda}{NA},$$

where NA is the numerical aperture. The NA is given on all objectives and therefore we could calculate the resolution limit of the microscope. However, this expression is not true unless the light is emerging from one point. Also the NA of the condenser has to be taken into account. Consider the following figure.

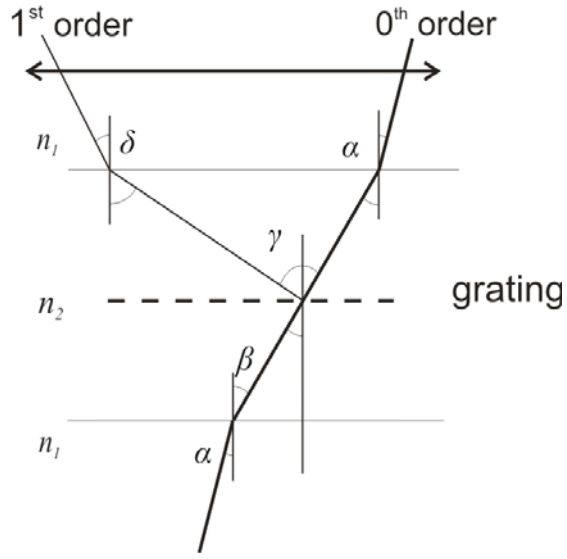

Figure 4 An illustration on how a ray of light with an angle of α will travel from the oil of a condenser with refractive index n_1 through a grating with refractive index n_2 and to the oil of the objective, also with a refraction index of n_1.

Parallel light from the condenser travels from refractive index n_1 and into the specimen, which has a lower refractive index of n_2. Assume $NA_{condenser} = n_1 \sin\alpha$. According to refractive and diffraction laws

$$n_1 \sin\alpha = n_2 \sin\beta,$$

$$d(\sin\beta + \sin\gamma) = m\lambda,$$

$$n_2 \sin\gamma = n_1 \sin\delta.$$

From the equations we can calculate the smallest distance d to be resolved. As we only need the zeroth and first diffraction orders to be captured by the objective we put m=1. The wavelength of light $\lambda=\lambda_0/n_2$ and we find that

$$d = \frac{\lambda_0}{n_2(\sin\beta + \sin\gamma)} = \frac{\lambda_0}{n1(\sin\alpha + \sin\delta)} = \frac{\lambda_0}{NA_{condenser} + NA_{objective}}$$

If we would use a higher NA of the condenser than the NA of the objective the zeroth order would not be captured by the objective. The maximal resolution limit can therefore be written as

$$d = \frac{\lambda_0}{2NA_{objective}}$$

The resolution limit with incoherent light gives another equation of resolution. Also in this case the resolution limit is determined by Fraunhofer diffraction phenomenon and a point object is imaged as an Airy disk which is shown in Figure 5.

Figure 5 The intensity distribution of 5 Airy discs. In A, an Airy disc formed from a single point. In, B two points are overlapping. In C, according to the Rayleigh criteria, the two points are just resolved.

However, in the case of incoherent light the, there will be no interference between two Airy disks overlapping. The resolution can therefore be determined by the Rayleigh criteria: two points are just resolved when the centre of one Airy disk falls on the first minimum of the other Airy pattern. That will give us:

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

This expression is similar to the equation for coherent light. As a rule of thumb the resolution limit is said to be approximately $\lambda/2$.

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 design of a microscope which is used in all modern microscopes today. The principle is shown in Figure 6.

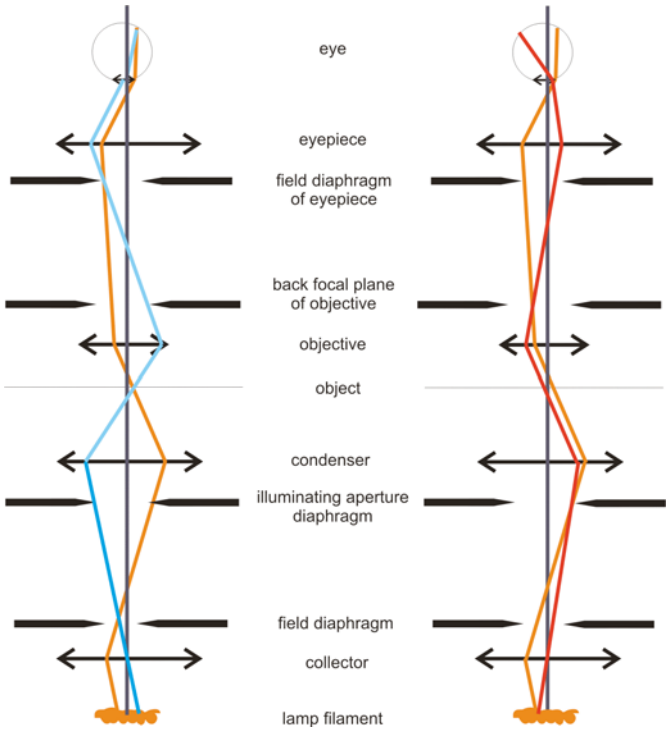

Figure 6 The two illumination pathways of Köhler illumination. Note that the conjugate planes for the two pathways are completely opposite. Hence, in the image planes the lamp filament is totally out of focus. Further, using Köhler illumination the field of view and the NA of the condenser can easily 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, where as 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 is in 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 media with refractive index of n_1, as shown in Figure 7.

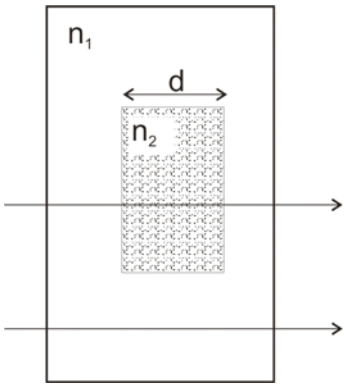

Figure 7 Schematic of a cell with refractive index n_2 and thickness d in a media with refractive index n_1.

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 a annulus ring, placed directly in front if the condenser and conjugate to the phase shifter. This is shown in Figure 8.

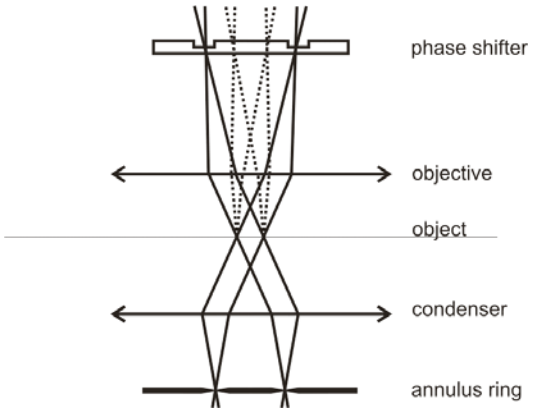

Figure 8 The principle of phase contrast. The diffracted light (shown with dotted lines) will be spread over the entire back aperture of the objective. The underviated lines will pass the back aperture in a circle with a certain diameter. The phase shifter with a ring of the same diameter will shift the deviated light π/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.

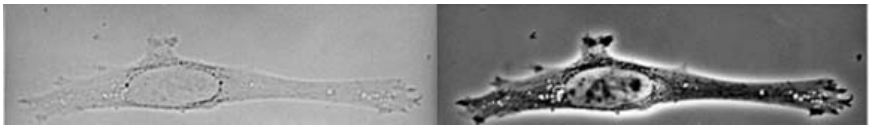

Figure 9 The difference between bright field (left) image and phase contrast (right) image of the same cell.

Dark field microscopy

Objects witch significantly scatter light 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.

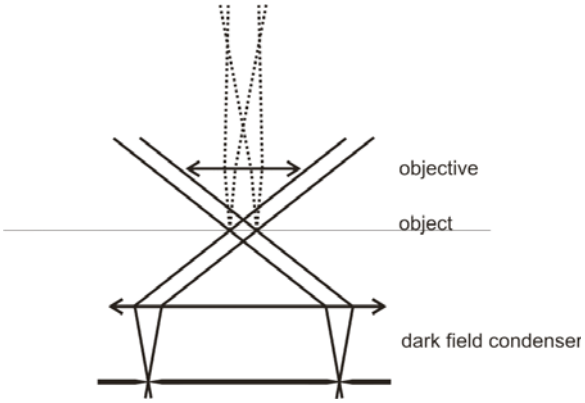

Figure 10 The concept of dark field microscopy. Only the diffracted light (shown as dotted lines) will be captured by the objective.

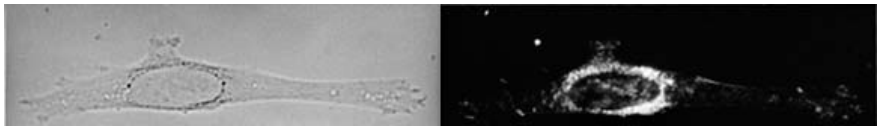

Figure 11 The same cell as in Figure 9 now imaged in bright field (left) and dark field (right).

Home assignments

1. Draw a sketch on a simple microscope. Mark collector, objective, condenser and ocular.
2. Draw an image of Köhler illumination and explain the design.
3. Put up some points on how you would build a microscope according to Köhler.
4. Explain diffraction of light.
5. What is the expression for the resolution limit of a microscope?
6. What is the definition of the numerical aperture of a microscope objective?
7. In what way is the NA of the objective important for resolution?
8. Draw an image and explain the concept of phase contrast.
9. Which types of samples are suited for phase contrast microscopy?
10. 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 with 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 gitterkonstant of a test diatome

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

A test diatom is a kisel alga, pleurosigma angulatum, which appear as small worms with a periodic structure with a fix grating constant.

3.1 Using the calibration in assignment 1, measure the grating constant.

4. Test of Abbe's resolution limit

Objective: 100X immersion
Object: test diatom

According to Abbé 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 Abbé is right?

5. Putting the resolution limit to the test

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

The resolution equation states that $d = \lambda / (NA_{objective} + NA_{condenser})$. 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 2.

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.