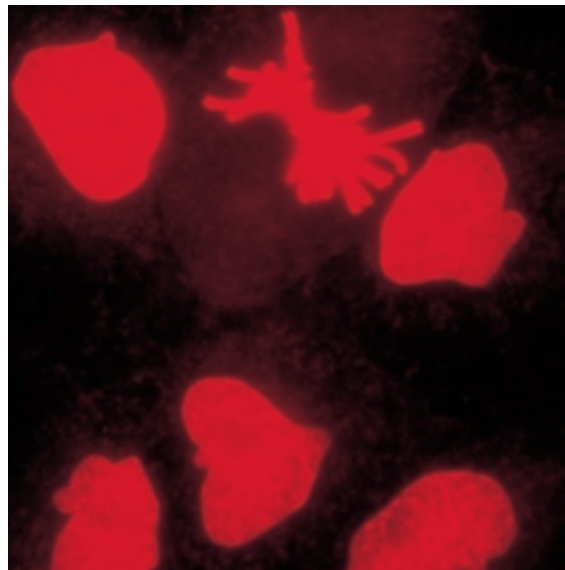
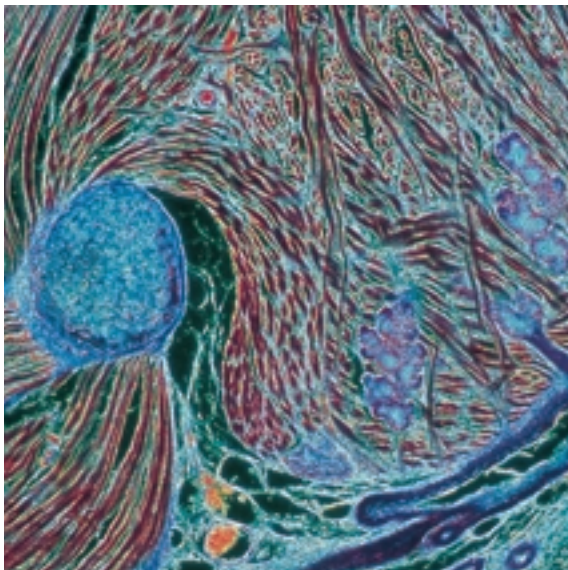
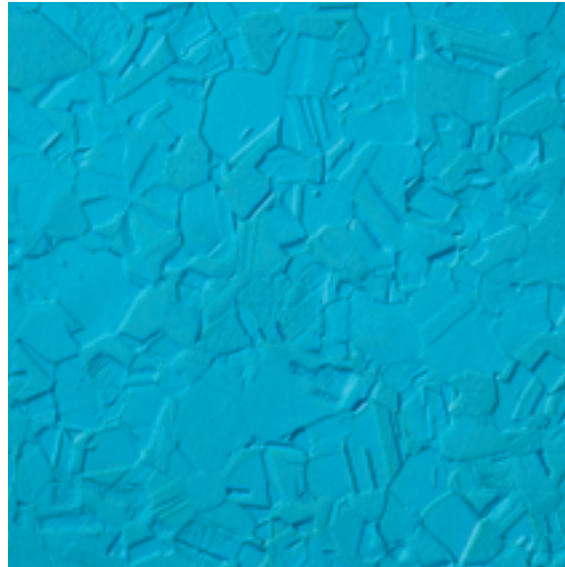


# Microscopy from the very beginning



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## **Microscopy is not difficult.**

There you are with your microscope in front of you and you try taking your first steps into the world of microscopy. To start with, all of the screws, switches, mysterious numbers and color rings on the objectives may be confusing. And, to make it even worse, you will not even see a decent image when you look through the microscope.

Nevertheless, microscopy is easier than you may think.

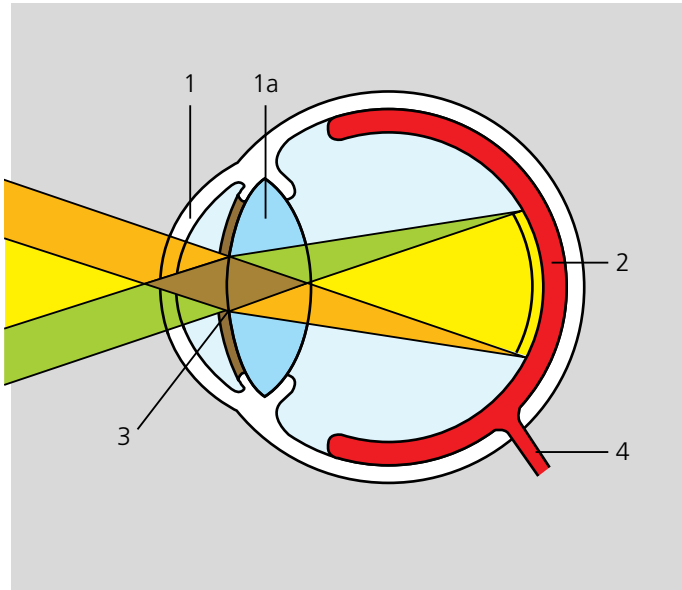
## **Using a microscope is a skill you can learn.**

Everything is based on rules which never change. Once you have understood and used these rules in practice, success is bound to follow. Many years of practice and improvement and making your own creative changes to standard methods may also make you a master craftsman/woman in this field.

This manual may help you to make a successful beginning.



## Microscopy means seeing a large image of something small

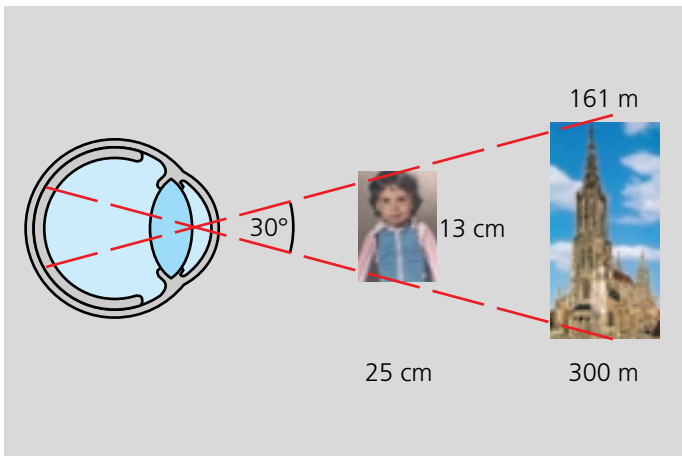


2.1

In spite of all technical progress, the eye as the visual organ – in combination with the brain behind it – is the most efficient image-processing system available to date. All the appliances provided by technology are no match for the eye as regards speed and resolution. The structure of the eye is related to that of a camera. Together with the muscle-adjusted lens (1a), the curved surface of the cornea (1) projects an optical image onto the retina (2). The amount of incident brightness is controlled via the variable diameter of an iris (3). A sharp image is provided by the flexible lens, the focal length of which is changed by muscles in such a way that focusing is possible on any subject at a distance between approx. 20 cm and infinity. The image itself is picked up on the retina by approx. 130 million receptor rods (recognition of grey levels) and 7 million cones (color recognition) and transferred to the brain on the shortest possible path via the optic nerve.

The light rays shown in the following illustration form a viewing angle of  $30^\circ$ . The following objects, for example, are seen under this angle:

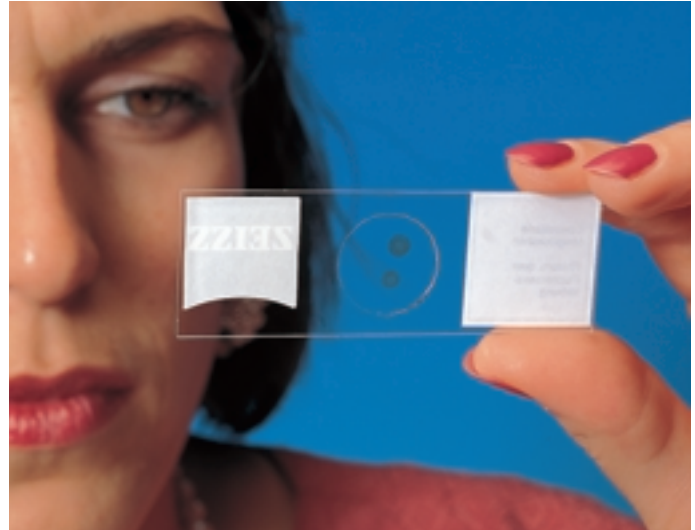
- the spire of the Ulm Minster with a height of 161 m, seen from a distance of 300 m
- a photo with a height of 13 cm seen from a distance of 25 cm.



2.2

## What does "small" mean?

Since we want to have a closer look at the fine capillaries of the stalk of a plant, we cut a wafer-thin slice from the stalk, place it on a microscope slide (glass plate) and protect the sensitive object using a cover slip. When we hold the finished specimen up to the light, we are not able to discover much. We can only see that fine structures are present.

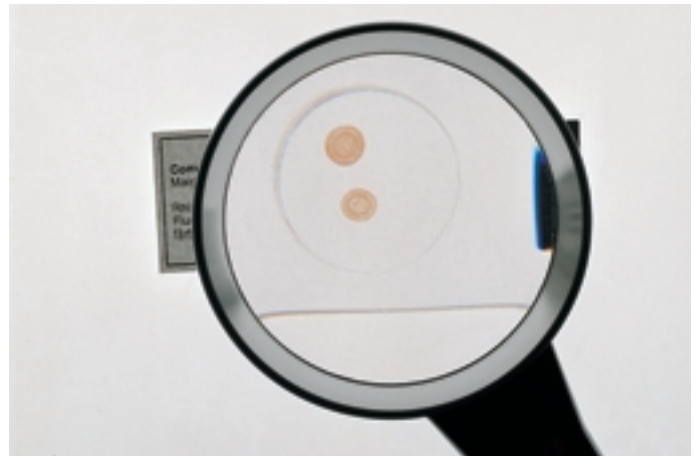


2.3

The reason for these problems is easy to explain. The details we want to see have a diameter of only  $1/100$  or even  $1/1000$  of a millimeter. However, we are unable to go any closer to the object than approx. 20 cm. As a consequence, the viewing angle becomes extremely small, which is why we are unable to recognize any details. A similar situation is experienced if we view the spire already mentioned from a distance of 300 m. The many intricate details created by the stonemasons cannot be recognized from such great a distance because the viewing angles are too small.

## First Aid: The magnifier

A remedy has been known for such cases for centuries: the use of a "magnifying glass" which – when put between the eye and the object – makes everything appear larger. However, there is a limit to this method: a magnification of more than 8-fold or 10-fold is not possible. Anyone who wants to see more must use the "compound" microscope.



2.4

## Microscopes magnify in steps.

If one lens is not sufficient, several lenses can be arranged one behind the other. The magnifying effect is thus multiplied, allowing magnifications of up to 2000x. The classic microscope magnifies in two steps: The objective produces a magnified image of the object in the so-called intermediate image plane, and the eyepiece or ocular (Latin: oculus = eye) magnifies the intermediate image in the same way as a magnifier.

The beam path on the right shows how light is emitted from an object ( $\leftarrow$ ) and processed in the three lenses. We shall only look at the rays originating in the two ends of the object. This will be sufficient to explain the process of magnification.

The illustration shows the ICS principle (ICS=Infinity Color-corrected System) also used with the **Axiolab** microscope. A further step is included in this modern microscope featuring "infinity optics": a tube lens is added to support the objective. The objective projects an image at an "infinite" distance, the tube lens with its focal length  $f = 164.5$  mm then forms the intermediate image from these parallel beams. First, let us assume that nothing decisive for the image formation happens in the space between objective and tube lens. The light rays coming from the focused specimen plane are parallel in this space anyway.

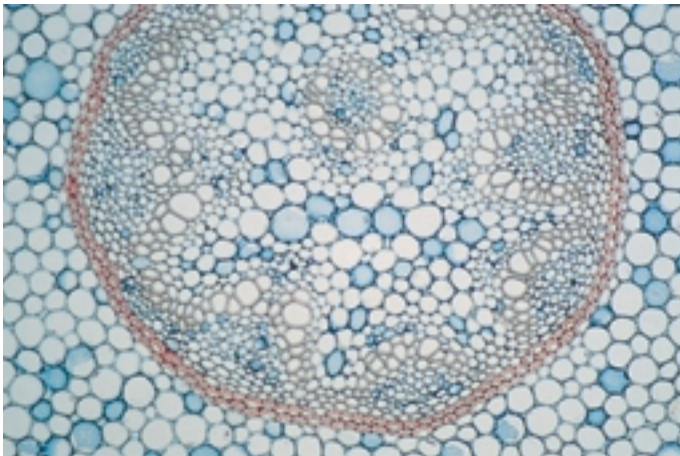
The eyepiece, in turn, serves as a magnifying glass to make this small intermediate image appear even more magnified to the eye.

<p>Total magnification = Objective magnification x Eyepiece magnification</p>
---

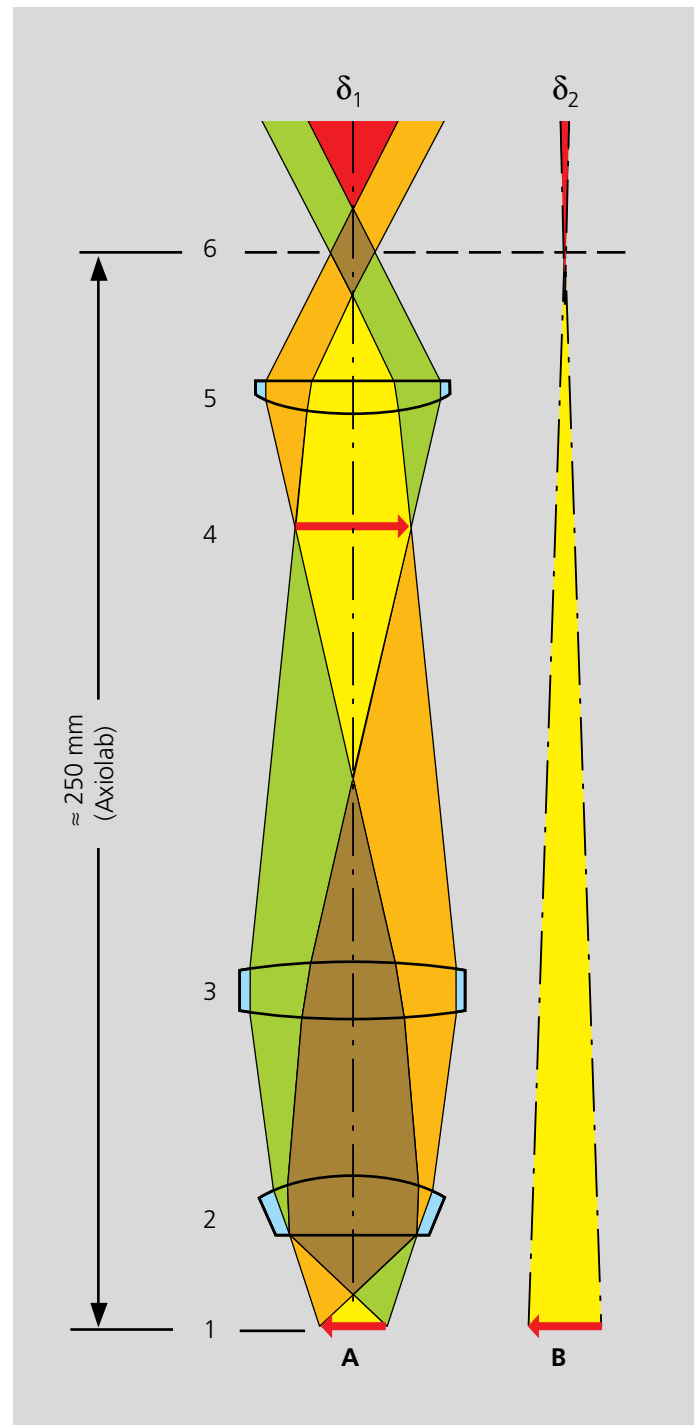
The result:

The section of the plant stalk now exhibits the details which we wanted to see. Here, the overall magnification of the microscope is 100x. (10x objective with 10x eyepiece).

The following photo shows a magnification as high as 100, due to the camera factor and the image magnification.



3.1



3.2

In the microscope beam path (A), the object ( $\leftarrow$ ) (1) is recorded by the objective (2) and is first projected at infinity. Therefore, the light rays originating from one point of the object run parallel behind the objective. The tube lens (3) now functions in a similar way to a camera and produces a magnified intermediate image (4), which is captured by the eyepiece (5) and shown to the eye (6). The resulting viewing angle  $\delta_1$  is much larger than  $\delta_2$  in case B, where the object is seen directly from a distance of approx. 25 cm.

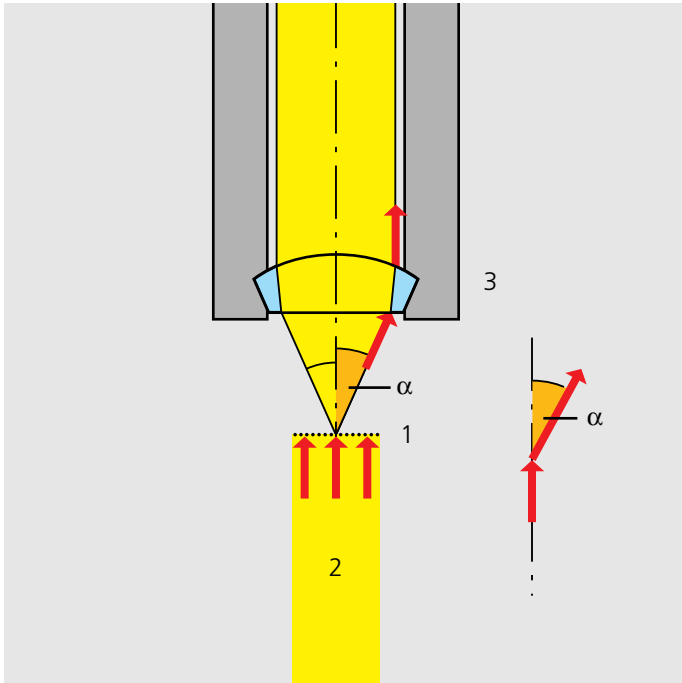
**Magnification alone is not enough:  
The resolution determines what we see.**

White light consists of electromagnetic waves, the period lengths of which total 400 to 700 nm. It is important to know that:

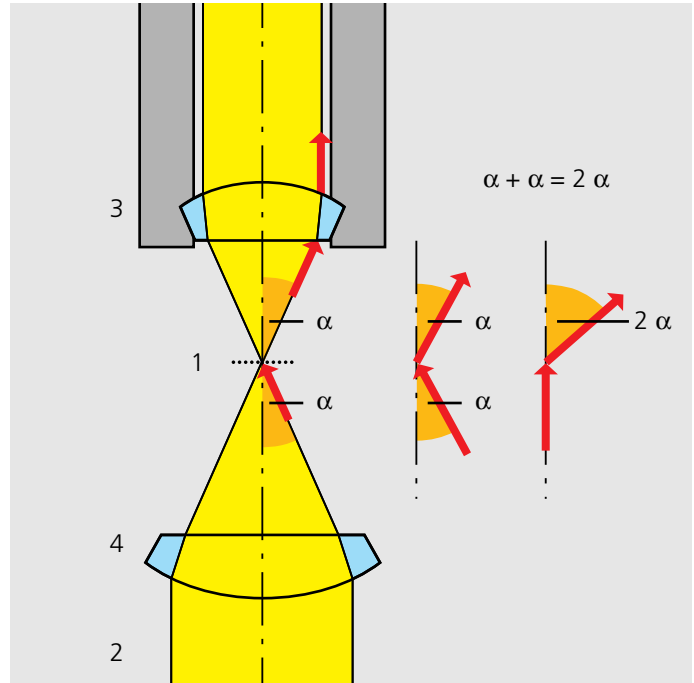
$$1 \text{ mm} = 1000 \mu\text{m}$$

$$1 \mu\text{m} = 1000 \text{ nm}$$

Light of green color has a wavelength of 550 nm which corresponds to 0.55  $\mu\text{m}$ .



4.1



4.2

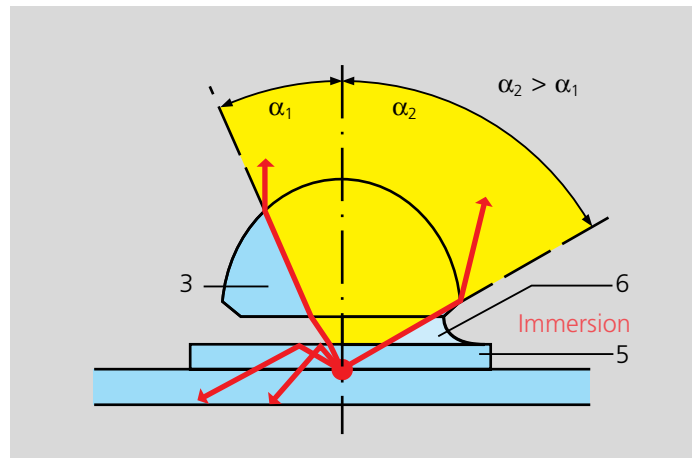
If small objects (1) are viewed through the microscope, the light (2) incident from these objects is deflected (diffracted) from the original direction. The smaller the objects are, the more pronounced the deflection will be. To obtain sharp images of small structures, the objective (3) in the microscope must collect as much of this diffracted light as possible. This works particularly well if the objective covers a large solid angle. The term aperture ("opening") describes this property.

To enable two objectives to be compared, the "numerical aperture" has been defined: this is a measure of the solid angle covered by an objective.

The following definition is made:

**Numerical Aperture = N.A. =  $n \cdot \sin \alpha$**   
 $\alpha$  is half the opening angle of the objective.  
 $n$  is the refractive index of the immersion medium used between the objective and the object.  
 ( $n = 1$  for air;  $n = 1.51$  for oil or glass)

Another possibility is to use immersion liquids (6) between the front lens of the objective (3) and the cover slip (5). Good results have been obtained with a certain oil with the refractive index  $n = 1.51$ , which has been precisely matched to the refractive index of glass. All reflections on the path from the object to the objective are eliminated in this way. If this trick were not used, reflection would always cause a loss of light in the cover slip or on the front lens in the case of large angles (Fig. 4.3 – left half). The useful aperture of the objective and the resolving power would be reduced by this reflection.



4.3

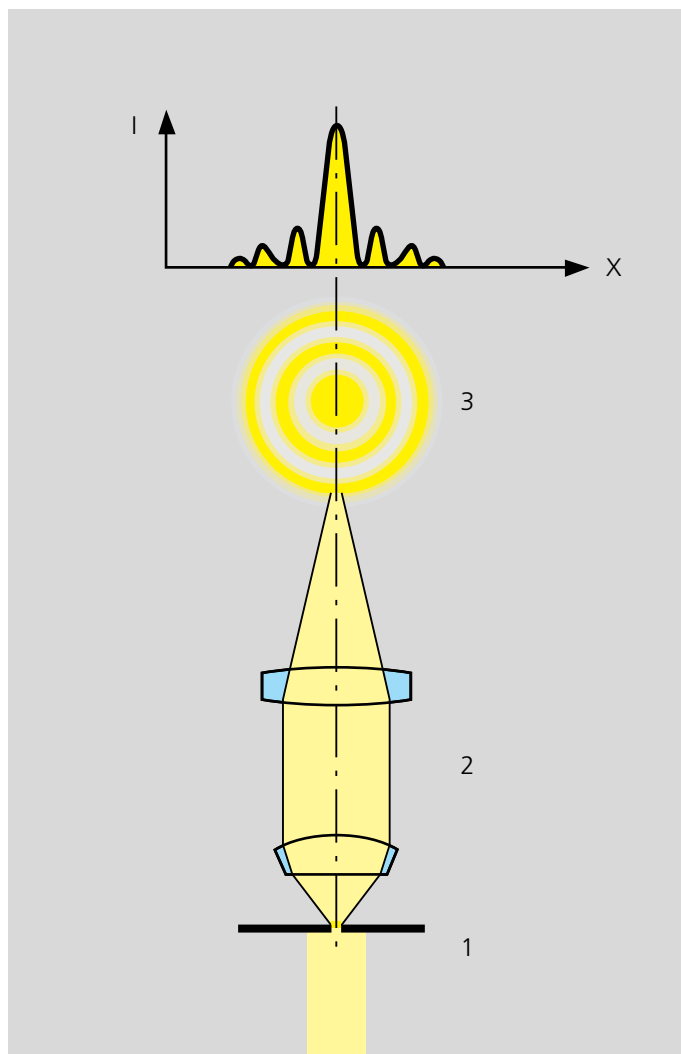
## What does “resolution” actually mean?

The limit up to which two small objects are still seen separately is used as a measure of the resolving power of a microscope. A certain distance  $d_0$  exists where this limit is reached. It can also be calculated theoretically.

For this, please see Fig. 5.1 on the right: First of all, it is important to know that the objective and tube lens (2) do not image a point in the object – for example a minute hole in a metal foil (1) – as a bright disk with sharply defined edges, but as a slightly blurred spot surrounded by diffraction rings (3), called “Airy disks” after their discoverer. The diffraction rings are caused by the limiting function of the objective aperture: the objective acts as a hole, behind which diffraction rings are found. The higher the aperture of the objective (N.A. Obj.) and of the condenser (N.A. cond.), the smaller  $d_0$  will be. A short wavelength is also beneficial for the resolving power:

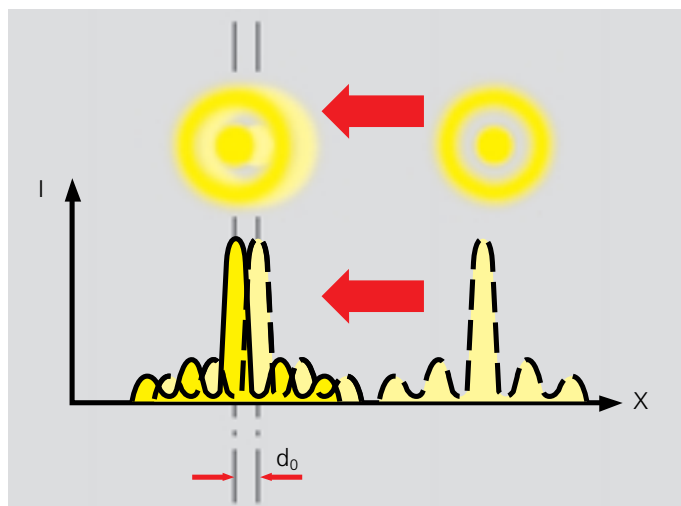
$$d_0 = \frac{1.22\lambda}{N.A._{obj.} + N.A._{Cond}} \quad \text{or more simply } d_0 = \frac{\lambda}{2N.A.}$$

$\lambda$  = wavelength of light, e.g. 550 nm (green)



5.1

The factor “1.22” has been taken from the calculation for the case shown in Fig. 5.2. The intensity profiles of two diffraction disks have been superimposed. If the two image points are far away from each other, they are easy to recognize as separate objects. If the distance is increasingly reduced, the limit point is reached when the principal maximum of object 2 (---) coincides with the first minimum of object 1 (—). The superimposed profiles display two brightness maxima which are separated by a “valley”. The intensity in the “valley” is reduced by approx. 20 % compared with the two maxima. This is just sufficient for the human eye to see two separate points (Rayleigh criterion).



5.2

A comparison may help to make this easier to understand: It is most unlikely that a telephone cable would be used for the electronic transfer of the delicate sound of a violin, since the bandwidth of this medium is very restricted (“small aperture”). Much better results are obtained if high-quality microphones and amplifiers are used, the frequency range of which is identical to the human range of hearing (“high aperture”). In music, information is contained in the medium sound frequencies; *however, the fine nuances of sound are contained in the high overtones*. In the microscope, the subtleties of a structure are “coded” into the diffracted light. If we want to see them again behind the objective, we must make sure that they are first gathered by the objective. This becomes easier, the higher the aperture angle and thus the numerical aperture.

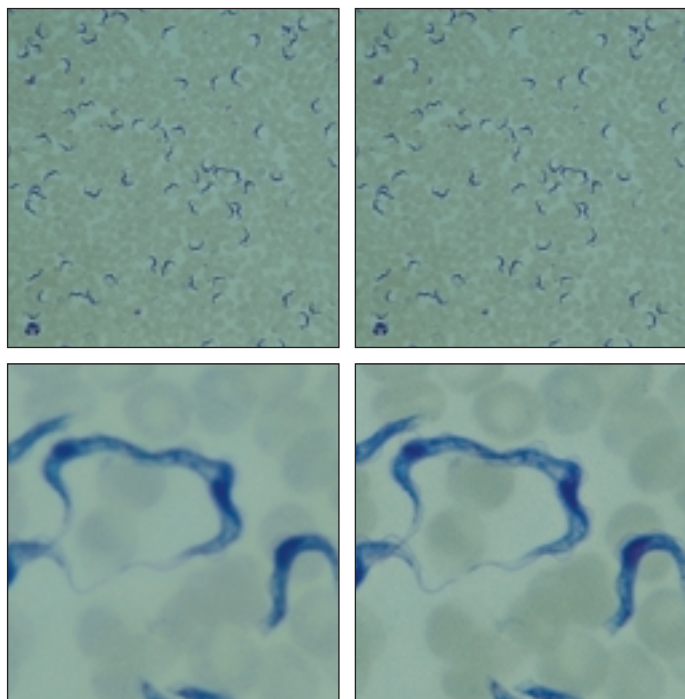
The numerical aperture of objectives increases with the magnification, up to about the 40x objective. For example, the *Plan-Neofluar* objectives with the magnifications 5x, 10x and 20x have the numerical apertures 0.15, 0.30 and 0.50. The theoretical limit in air is a numerical aperture of 1.0. This would be identical to a full 180° aperture angle ( $2\alpha$ ) of the objective. In practice, it is possible to obtain a numerical aperture of 0.95, which corresponds to an angle ( $2\alpha$ ) of more than 140°. Very high apertures at low magnifications are difficult to implement because of the large object fields and lens diameters.

The following table shows the values for the resolution resulting from the calculation for some objectives. The distance  $d_0$  is referred to the specimen and, when multiplied by the magnification, results in the point distance  $D_0$  in the intermediate image (for green light  $\lambda = 550$  nm). Finally, the number  $n$  represents the number of resolved pixels if they are "lined up" along the field diameter of 20 mm ( $20\text{mm} / D_0$ ).

Objective / NA	$d_0$ ( $\mu\text{m}$ )	$D_0$ ( $\mu\text{m}$ )	$n$
5x / 0.15	2.2	11.2	1786
10x / 0.30	1.1	11.2	1786
20x / 0.50	0.7	13.4	1493
40x / 0.75	0.45	17.9	1117
40x / 1.30 Oil	0.26	10.3	1942
63x / 1.40 Oil	0.24	15.1	1325
100x / 1.30 Oil	0.26	25.8	775

(condenser aperture = objective aperture)

The following pictures show the difference in the resolving power between the objectives 40x/0.75 (on the left) and 40x/1.30 Oil (on the right). Scarcely any difference can be recognized in the printed pictures taken with a low magnification (on the top), while the image sections taken with a 8x magnification (below) exhibit marked differences.



## What does "useful magnification" mean?

"A lot helps a lot" – however, this does not apply to the selection of the "useful" magnification. By this we mean that you should not try to increase the overall magnification of a microscope by using eyepieces providing a high additional magnification (e.g. 16x) or other optical "after-burners" if the objective does not supply enough pixels at a low numerical aperture. On the other hand, you will miss fine nuances if the objective (e.g. Planapochromat 10x) projects very fine details onto the intermediate image, and you are using an eyepiece with a low magnification. The following simple rule applies:

- The overall magnification of a microscope should be higher than 500 times, but less than 1000 times the objective aperture. This will then be the range of the useful magnification.

### Resolving power: Practical hints

Modern microscope objectives from Carl Zeiss permit the theoretical resolving power to be also achieved in practice – provided that good specimens are used. However, it is often the small things that prevent success:

#### ● Are the objective and the specimen clean?

A fingerprint on the front lens of an air objective alone may be sufficient to affect the high-contrast reproduction of a specimen since scattered light is produced. The same applies to immersion objectives soiled with residues of resin or emulsions (e.g. oil and water). Such cases require careful cleaning using a soft cloth and pure alcohol.

#### ● Do the cover slips have the correct thickness?

It is very important that cover slips for objectives of a high aperture used without immersion oil have the standard thickness of 0.17 mm, since here the cover slips are already taken into consideration in the complicated computation of objectives. Therefore, if a different thickness than the standard one is used, the quality of the optical image suffers visibly in the case of high apertures. Experience has shown that the following deviations are just acceptable and no more:

$$\begin{aligned} &\pm 0.01 \text{ mm with N.A.} > 0.7 \\ &\pm 0.03 \text{ mm with } 0.3 < \text{N.A.} < 0.7 \end{aligned}$$

#### ● Do you use the correct immersion oil?

Most high-performance objectives from Carl Zeiss are used with immersion oil. The right oil with the refractive index  $n = 1.51$  and a suitable oiler are supplied with the objective. The oil is PCB-free and exhibits scarcely any autofluorescence. The image will be markedly impaired if air bubbles are contained in the immersion layer. To avoid these bubbles, it is vital to apply the oil in the right way. The operating instructions include the relevant hints.



7.1

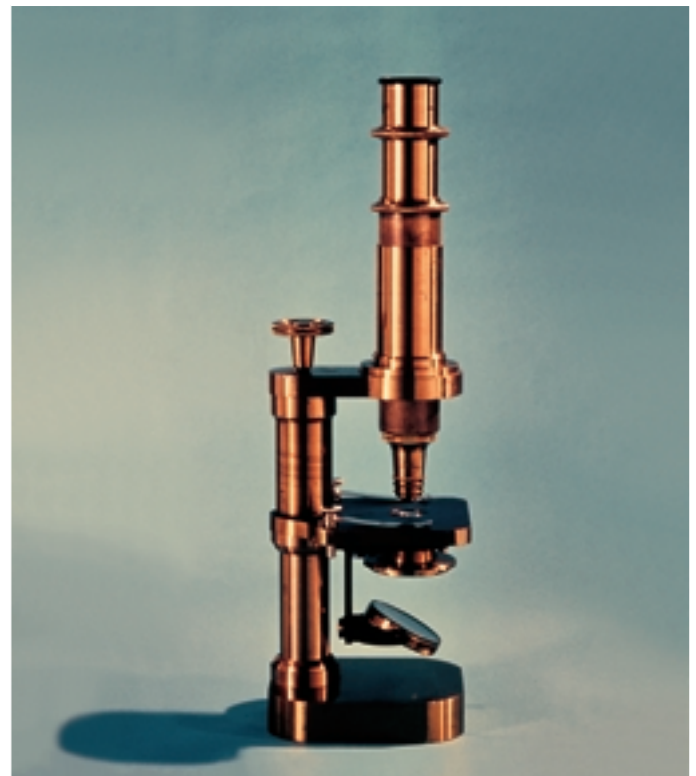
### All show?

It is now taken for granted that everyday microscopy always provides perfect images. However, this has not always been the case. For several centuries, the construction of microscopes and the appropriate optics was purely a matter of craftsmanship. The performance of these early instruments had to rely on the experience of the lens grinders, and the results obtained were often sheer coincidence. Great trouble and effort was invested in the outer appearance of these instruments. In many cases, they were simply show objects intended to increase the prestige of their owners. The dichotomy between appearance and the quality of the images these instruments provided was – by today's standards – striking. Nevertheless, microscopy became a favorite pastime of the rich and beautiful in the 18<sup>th</sup> century. For example, Elizabeth Charlotte of Palatinate, who became famous as the sister-in-law of Louis XIV, owned a valuable microscope and used it regularly; she correctly predicted that this instrument would be of inestimable value for medical applications in the future. This foresight was almost visionary at that time.

### Initial steps.

In the 19<sup>th</sup> century, the precise natural sciences experienced an enormous upswing. Even in the twenties and thirties, the science of light and the theory of optical imaging were placed on a sound foundation. One of the most successful researchers in this field – and not only in theory – was Joseph von Fraunhofer (1787-1826). We have him to thank for the creation of what is now the most common optical lens system with chromatic error correction – known as the Achromat system – and for basic knowledge on the diffraction of light. However, astronomy was the main field of Fraunhofer's activities, and at that time nobody had yet contemplated the direct utilization of knowledge for the production of microscopes.

In this period of innumerable technical advances, a mechanic set up his own business in the university town of Jena in Thuringia in east Germany: Carl Zeiss. This young man set himself the goal of providing researchers with high-quality instruments. Today, we would say he saw a niche in the market and wanted to make use of it. Between 1846 and 1866, microscopes of equally high quality were built in his workshop in accordance with very strict rules of craftsmanship. In the beginning, these were very simple instruments which were used as dissection microscopes, but in 1857 the first "genuine" microscope, i.e. equipped with an eyepiece and an objective, left the Zeiss workshop: the "Stativ 1". This instrument combined practical functionality with the skilled quality provided by a craftsman.



7.2





8.1 Carl Zeiss (1816-1888)



8.2 Ernst Abbe (1840-1905)

After almost 20 years, Carl Zeiss was already employing about 20 qualified staff and took great pleasure in what had become a flourishing business. He had every reason to be satisfied. However, as an entrepreneur, he felt that more was possible and that he could not simply sit back and enjoy what he had achieved. He knew that his instruments were good, but he refused to accept the trial and error method used at the time for the production of optics. And he knew that the competition never sleeps.

His aim was to create reproducible products in his workshop. This made it necessary to base the production procedure on precise rules. His wish: "The working hand should have no other function than to precisely implement the shapes and dimensions of all the design components determined beforehand by computation."



### Technology transfer.

But who should devise these rules? To help him in this endeavor, Carl Zeiss found the 26-year-old, highly talented physicist and mathematician Dr. Ernst Abbe. Then came the investments required. Five or six years of theoretical work on problems of microscopic image formation had to be financed: innovation was expensive even then. But it was the vision of creating better products that made both partners persist in their objective.

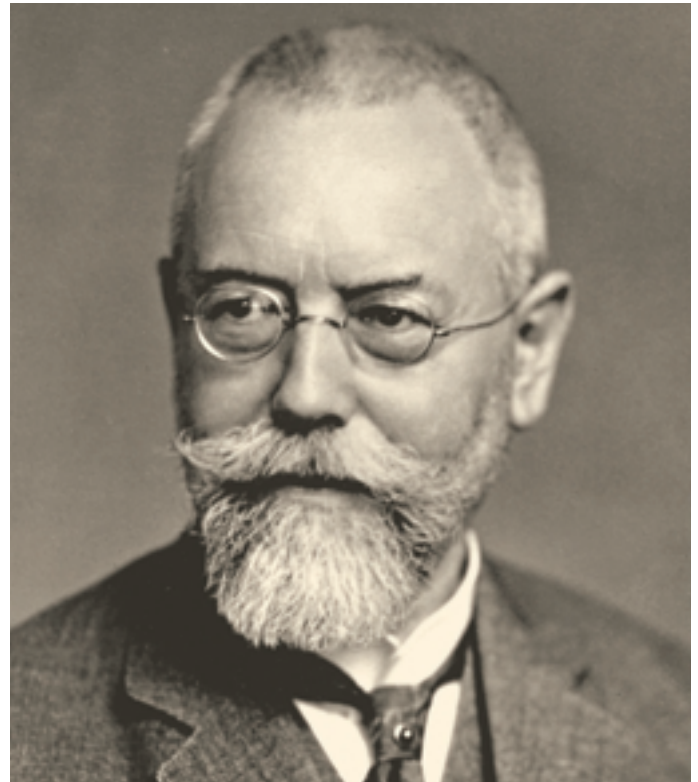
Finally, Abbe had performed such thorough work that, in 1872, it was possible for Carl Zeiss to provide a line of 17 different objectives, including three immersion systems, all featuring a level of image quality unknown until then. The construction of microscopes on a sound theoretical basis was possible at long last. It still is today.

This theoretical basis includes the original formula for the calculation of the theoretically possible resolution of the microscope:

$$d = \frac{\lambda}{2n \sin \alpha}$$



9.1 Otto Schott (1851-1935)



9.2 August Köhler (1866-1948)

### Key technology.

The enterprise continued to evolve. Ernst Abbe became an equal partner of Zeiss: intelligence became the inherent capital of the young company. In his later years, Abbe became equally famous as an entrepreneur of historic importance, as a social reformer and as a scientist. All on the basis of the prosperity that he made possible as a scientist.

The endeavor to innovate was continued with unflagging determination. The theory created by Abbe predicted that even more success would be possible if scientists could comprehend the properties of the types of glass used. In other words: he did not only wish to accept what was available, but wanted to actually create what was needed.

Eventually, Abbe found the third member of the trio: Otto Schott, the glass chemist. Once again, someone who wanted to depart from well-trodden paths – and who was also capable of doing so. His subsequent, large-scale test series to find new glass types and determine their properties cost a fortune. In the end, success was achieved. And it extended far beyond the bounds of microscopy; it also led to the founding of the Jenaer Glaswerk Schott & Genossen.

In the field of microscopy, Abbe's theoretical prediction became reality in 1886. The creation of the Apochromat objectives with and without immersion meant the end of the development work for the time being.

### What is "Köhlering"?

The progress made in the development of objectives led to fields of view larger than anything achieved before. In the course of time it also became evident that more attention would have to be paid to illumination. In microscopy, the era of working on detail was dawning.

Professor August Köhler (1866-1948) became an early member of staff at Carl Zeiss in Jena, and in 1893 he published guidelines for the correct illumination of microscope specimens. He developed a cleverly devised microscope illumination system which made it possible to use the entire resolving power of Abbe's objectives – particularly for microphotography which was later to become a very important application.

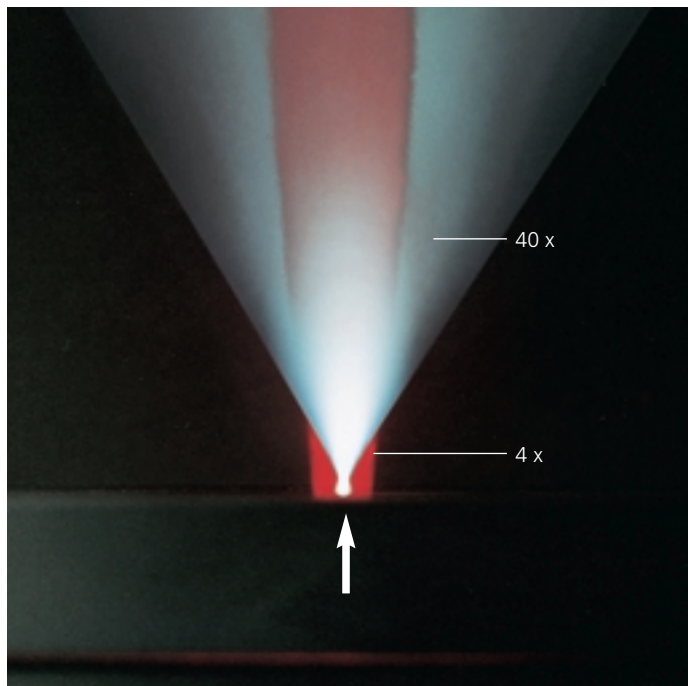
The Köhler illumination system provides homogeneously illuminated images and, at the same time, permits an increase in the resolving power due to the use of a condenser. It was particularly beneficial that the aperture diaphragm in the condenser allowed the image contrast and the resolving power to be balanced against each other without any loss in the homogeneity of the image brightness.

Knowledge and observance of Köhler's rules, and the associated settings of the microscope – whether automatically via PC and motorized functions or manually – are still essential today. That little bit of extra effort is always rewarded with results that are not only useful, but highly satisfying.

Let us now return to the present.

## Everything is under control: the path of the light rays – from the illuminator to the eye

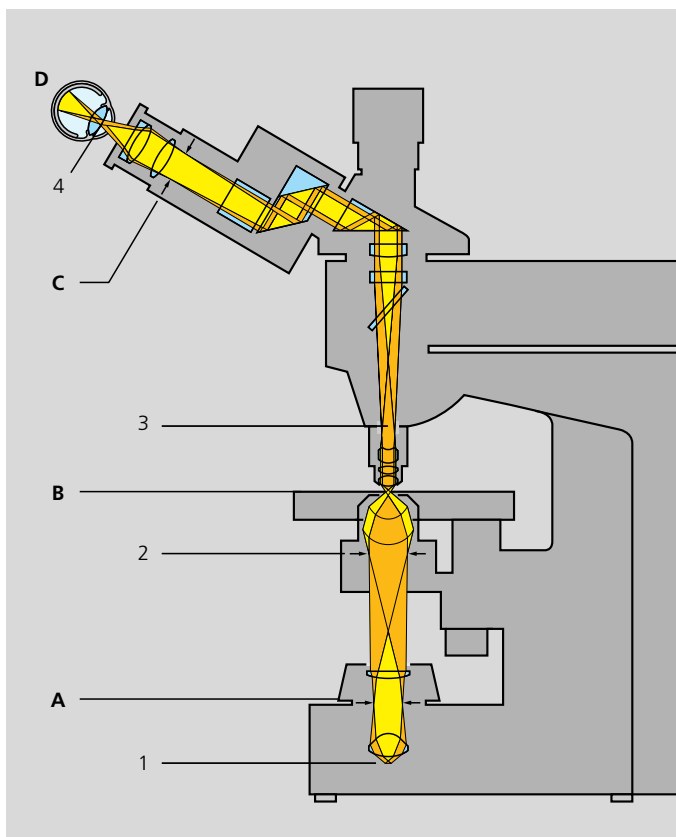
The design of a microscope must ensure that the light rays are precisely guided through the microscope. Only this will make it possible to obtain a bright image even with illuminators of a low wattage. A lack of brightness is no problem in simple brightfield microscopy, but if contrasting techniques such as phase contrast or polarization contrast are used, further optical elements which use up a great portion of the available light flow are inserted into the beam path. This leaves little light for observation, and, as a result, the images become dark.



10.1

A further important reason for the existence of diaphragms and filters in the microscope is that, strictly speaking, the illumination should be reset after every change of objectives for two reasons. Firstly, the size of the specimen section observed changes with every objective change. An objective with a low magnification, e.g. 4x, provides a large field of observation (with a diameter of as high as 5 mm in this case, provided that the eyepiece permits an intermediate image of dia. 20 mm). If a switch is made to the 40x objective, the diameter of the visible field of the specimen shrinks by the factor 10 (to only 0.5 mm). The viewed area becomes as much as 100x smaller. The second reason is that the numerical aperture increases from 0.12 to 0.65 or, expressed as aperture angles, from 15° to 80°. These two cases are illustrated in Fig. 10.1 above.

However, the Köhler guidelines require that nothing more than the viewed field in the specimen is illuminated, since the "excessive" light outside the field of view is disturbing, scattered light. At the same time, however, the light cone of the illumination should always be matched to the angular aperture of the objective to allow the numerical aperture of the optics to be utilized. This is the only way to achieve maximum resolving power.



10.2

The condenser containing the aperture diaphragm (2) and the luminous-field diaphragm (A) normally contained in the stand base are the aids which make all this possible. A closer look reveals that the luminous-field diaphragm is imaged on the specimen by means of the condenser. The luminous-field diaphragm determines which part of the specimen is illuminated. The aperture diaphragm, however, is imaged on the "pupil" of the objective (3) and regulates the illumination of this pupil. The entire optics are computed in such a way that aperture angles of the light cones are correctly set together with the aperture diaphragm.

Therefore, the microscope contains two different groups of optical planes which belong together.

The first group consists of:

- 1 = lamp filament
- 2 = aperture diaphragm
- 3 = objective pupil
- 4 = pupil of the observer's eye

This group defines the beam path of the pupils and determines the resolution of the microscope.

The other group contains:

- A = luminous-field diaphragm
- B = specimen plane
- C = intermediate image in the eyepiece
- D = retina of the observer's eye

A to D are the important optical planes in the image-forming beam path. The image becomes visible here and the image limits are set.

Within a group, the planes are always imaged one on the other. We speak of "conjugate" planes, which means that they are "connected with each other".

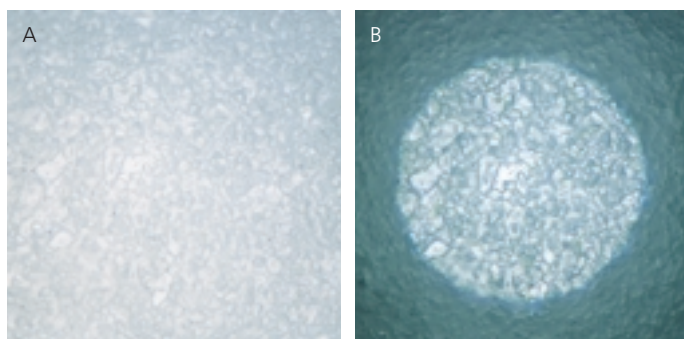
It is no exaggeration to say that almost the entire art of microscopy – if specimen preparation is not taken into account – consists in the correct use of the luminous field and aperture diaphragms. Thankfully, there are simple rules for this. The following pages describe in detail how to correctly set the microscope for Köhler illumination. It will be much easier for you to understand the Köhler principle if you made yourself familiar with the meaning of the special optical planes mentioned before. Their relationship with each other can be simplified as follows:

Planes "1" to "4"	<b>Pupils</b>	For resolving power, contrasting techniques, brightfield.  Components: light filter and contrasting.  Setting is made via the <b>aperture diaphragm</b> .
Planes "A" to "D"	<b>Fields</b>	For fields of view, illumination, intermediate images.  Components: reticles and scales.  Setting is made via the <b>luminous-field diaphragm</b> .

The fact that the beams are superimposed may also have negative consequences. The best example of this are dust particles on a reticle in the eyepiece: these particles will be sharply imaged together with the microscope image and do not exactly embellish the microphoto.

The above makes it clear that the condenser – which concentrates the illuminating light ray on the specimen – plays a vital part in microscopy: it is as important as objectives and eyepieces. The condenser makes the specimen appear in the right light.

If you only want to get optimum intensity in the illumination, select the critical illumination system, where the light source is not imaged on the pupils, but on the object. The homogeneity of the illumination is lost, however. This type of illumination is obtained by the deliberate adjustment of the lamp collector (fluorescence).

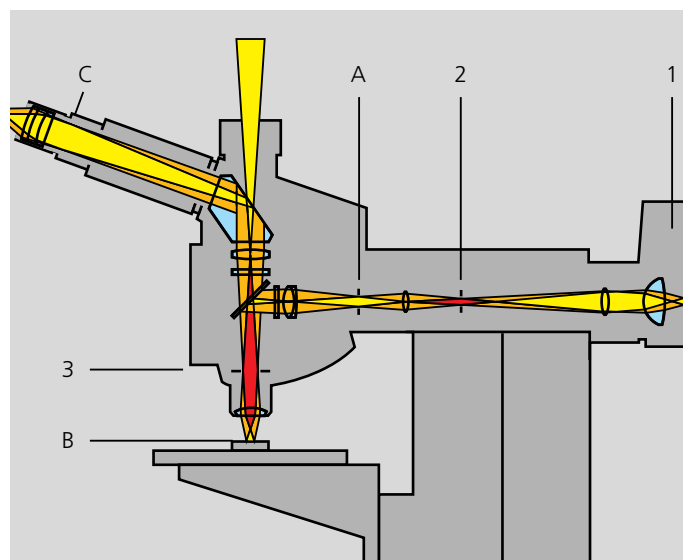


11.1

## Easier to handle: the beam path in reflected light

Anyone working with metal, ceramics or other technical samples will use a reflected-light microscope in most cases, since such samples are opaque to light and normally allow only the surface to be examined.

Together with lenses which are integrated into the stand, the objective of the reflected-light microscope acts as a condenser. This means that the optical axis and the position of the remaining "condenser lenses" cannot be changed. The luminous-field diaphragm also only has to be set once for one objective and is then suitable for all the other objectives.



11.2

The beam path for reflected light shows that the light from light source (1) travels via aperture diaphragm (2) and luminous-field diaphragm (A) to the beam splitter. Here, about half the light is reflected towards the objective pupil (3) and bundled onto the sample surface (B) by the objective. The surface reflects or scatters the light back into the objective. On the return journey, the beam splitter, in turn, lets about half the light pass to the tube lens which produces the intermediate image (C), this then being additionally magnified and observed using the eyepiece.

### Let us give you two tips:

(See the illustrations in the left column)

1. If you view surfaces in reflected light which are very dark and/or strongly scatter light, you will receive a low-contrast image (A). This can be remedied by narrowing the diameter of the luminous-field diaphragm. Although only the image center will then be illuminated, the contrast will visibly increase (B). Another very effective method is to use an immersion medium – for example oil – between the objective and the sample surface, if the sample permits, and a high magnification.

2. In the case of very low magnifications and dark samples, reflection can scarcely be avoided, even with a good anti-reflection coating of the optics. Here, the Antiflex device will be of assistance (more about this on page 32).



12.1

## Microscopy in everyday use Small causes – major repercussions

### ● Relaxed viewing is important

If you are a “beginner” in microscopy, you may tend to tense up when viewing. You think you have to set your eyes for near vision because, after all, you want to view something small. This is not the correct attitude in microscopy and may cause you strain in the long run – and you do not even know the cause. There is an easy remedy: *First, look into the distance with your eyes relaxed* and then into the eyepieces – without changing the setting of your eyes. Only then should you set the interpupillary distance of the eyepieces via the folding bridge until you see only *one* circle instead of two. Remember to consciously use *both* your eyes for viewing.



12.2

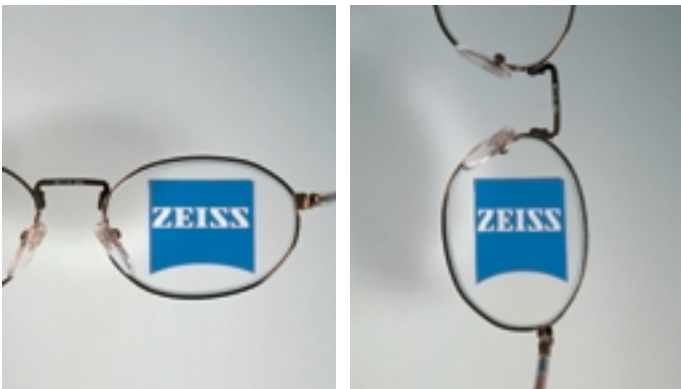
### ● Keep your distance

Microscope eyepieces from Carl Zeiss have usually been designed for eyeglass wearers. Therefore, the exit pupil of the eyepiece is at a considerable distance from the eyepiece. Users who do not wear eyeglasses should also keep this distance to permit the entire light from the microscope to find its way to the iris of the eye. If you slowly move your head to and fro in front of the eyepieces, you will soon find the optimum, relaxed posture allowing you to see the entire circle of the field of view.

If you do not wear eyeglasses, the rubber cups on the eyepieces will be useful to you. They not only protect the eye from ambient light, but also help to keep the correct distance between the eye and the eyepiece.

### ● Exclusively for eyeglass wearers: a little test

Eyeglass wearers requiring simple lenses with a spherical power can use the microscope with or without their glasses, provided that the diopter setting of the (“foc”) eyepiece is sufficient. However, if you require lenses with a toric power, i.e. lenses which diffract light differently in the horizontal and vertical – you had better wear your glasses for microscopy, since your eye then has unsymmetrical aberrations which cannot be compensated by the diopter setting alone. This is how you test your glasses (see the pictures on the left): view a simple geometric figure – a circle or a square – through the glasses you have removed from your eyes. First you hold the glasses horizontally, then vertically. If the figure appears compressed or expanded by this rotation about 90°, you will know that you are wearing lenses with a toric or another non-spherical power.



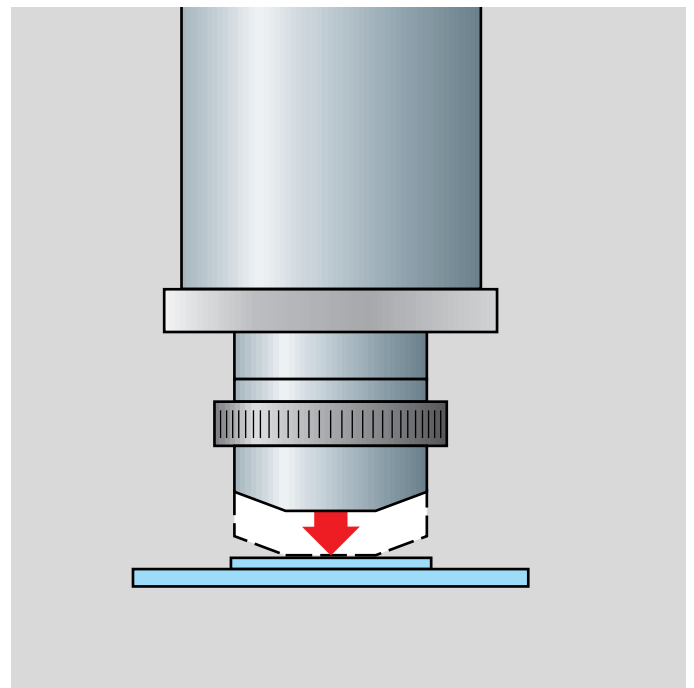
12.3

### ● Avoid the use of force

The sturdy design of our microscopes will even excuse incorrect treatment. However, the front tip of the objective with the sensitive front lens is one particularly critical area. The following precautions have been taken to protect the objective.

- The entire front optics of objectives with a high magnification are contained in a resilient mount. When touched, this mount “backs away” a little. However, the distance available for this movement is only small. Therefore, please make sure that you do not move the stage too much upwards during focusing. In such cases, the sample will press against the objective tip, and once the front lens cannot retreat any further, the outcome may be an expensive breakage of glass.
- Immersion objectives have an additional protection facility to the one described above: they can be locked in the topmost position by carefully turning the front group. The objectives can thus be “parked” at a safe distance. On the other hand, however, the escape route described above is then no longer available.

Should you not be satisfied with the function of your immersion objective, please check whether it is in the “parked position”. This is the case if, after a change of objectives, the focus is far away from the one set before.



13.1

### ● Protect your investment: the dust cover

Microscopes are used for an average of 15 years or longer. Just like rust attacks the car, the problem in microscopes is dust which is present almost everywhere.

Therefore: please switch off the illuminator(s) and put the supplied dust cover on the instrument when you have finished your work.



13.2

### ● Please avoid “do-it-yourself” work on the microscope

Even if you are good with your hands, we must strongly advise you against performing your own repairs on the microscope. Please do not try to take your microscope to pieces if you do not have the necessary training for it or if you do not have any special tools. (It is when you have to resort to using a torch or magnet to find tiny ball bearings or screws under the table that you'll really regret it!). Some of our optics can only be aligned here by us in the factory.

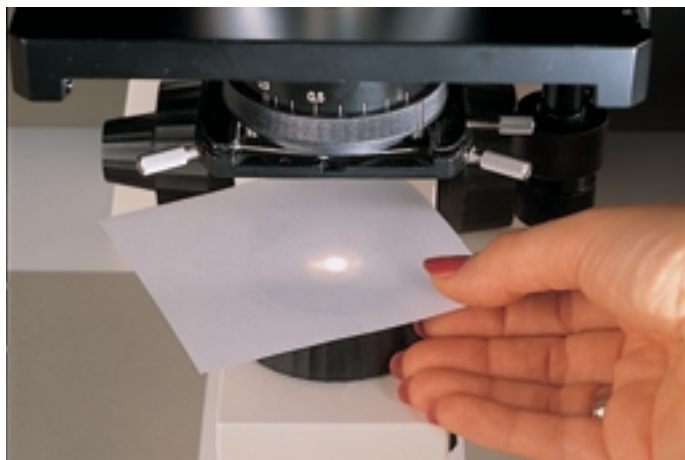
If a repair is required, please contact our service organization which is available to you throughout the world.

## How do I set my microscope correctly?

On the next four pages we shall work together to set the **Axiolab** microscope from Carl Zeiss for transmitted-light illumination in brightfield.

The procedure we choose will always lead to success. What we have just learned in theory will now be put into practice – following Köhler's principles. Everything that applies to the **Axiolab** microscope is also applicable to any other microscope model, provided it is fully equipped for Köhler illumination.

If you unfold the two fold-up pages of this brochure, you will find the description of the microscope components on the left and the beam path on the right.



14.1



14.2



14.3

## How to prepare the microscope for adjustment

- The microscope configuration is complete. If the condenser features a contrasting turret, the position is on "HF" for brightfield.
- You need a sample to perform the setting. Thin, stained sections are ideal (cross sections of a plant stalk or stained tissue section). A small piece of a 35 mm photo slide (approx. 10 x 10 mm), laid on a microscope slide, flattened with a cover slip and sealed, for example with all-purpose glue, is also very useful for the purpose.
- Use the 10x objective, or 20x, if the need arises. Swing this objective into the beam path and make sure that it is tightly screwed and that the nosepiece engages correctly – since otherwise the optical axis will not be correct. Use the focusing drive to create a distance of approx. 10 mm between stage surface and objective.
- Please also have a strip of thin white paper (approx. 3 x 10 cm) ready for use.

### Ready to start

1. Switch on the light source and hold the paper strip above the luminous-field diaphragm in the stand base to check whether light becomes visible.

If everything remains dark, the line plug, the illuminator and maybe also the fuse of the power unit must be checked and the necessary changes made. Normally, however, you will see a spot of light on the paper.

2. Open the luminous-field diaphragm as far as it will go. The spot of light on the paper is now at its maximum diameter.

3. Now hold the paper strip between the sample and the objective. Fully open the aperture diaphragm of the condenser. The small light spot on the paper will then show its maximum brightness. If you use a condenser with a swing-in front lens, the latter must be swung into the light path until the stop is reached.

4. The height of the condenser can be adjusted via the condenser drive. Set the condenser height in such a way that its front lens is approx. 1-3 mm away from the sample from below. However, do not touch the sample with the front lens and be careful not to lift it together with the condenser. If this happened, the result may be that the spring clip of the object guide will suddenly hurl the sample off the stage.

Modern instruments such as the **Axiolab** microscope shown here feature an adjustable stop screw allowing the top position of the condenser to be fixed. The annoying effect described above can thus be avoided.



15.1

5. Light should now already be discernable in the eyepieces. If it is very bright, reduce the brightness until you find it comfortable to work with. Then set the interpupillary distance via the folding bridge of the binocular tube. The correct setting is reached when you see one light circle instead of two. Now check whether you are using focusing eyepieces (labelled "foc", also recognizable by a white scale). If this is the case, turn the eyepiece to "0". Eyeglass wearers can leave their glasses on, since eyepieces from Carl Zeiss (type "Br") are specially designed for use with glasses. For more information – especially for eyeglass wearers – please see page 12.

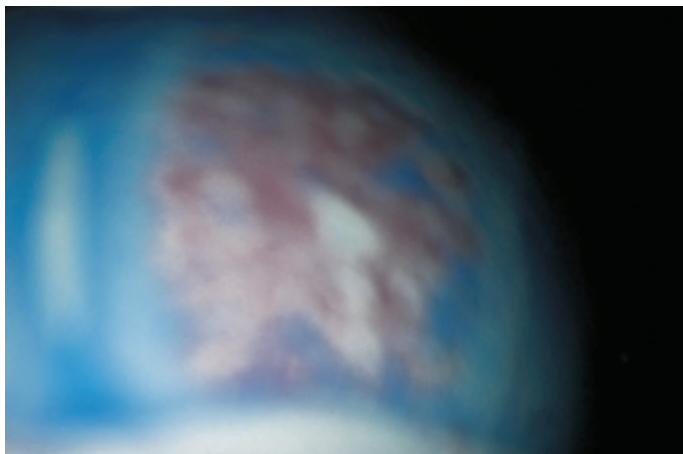


15.2

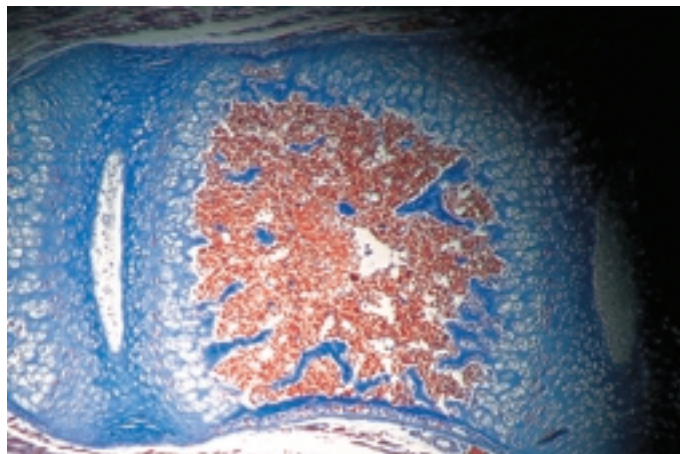
6. Now look into the microscope and carefully move the stage, including the sample, up and down until you see the details as sharply as possible. It is very possible that you will not see a perfect image because the illumination is not correct.



15.3



15.4



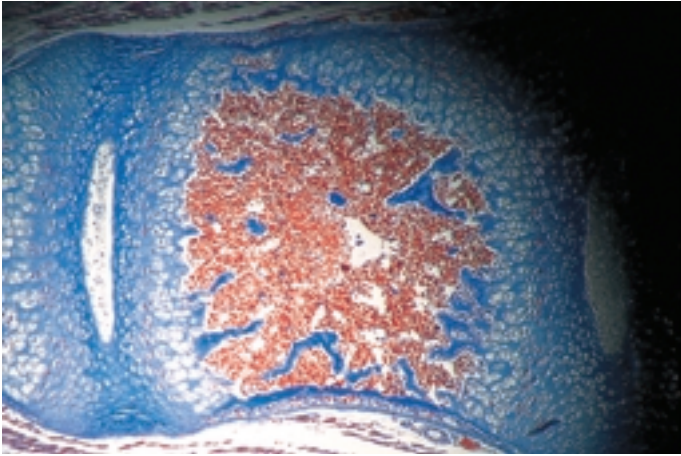
15.5



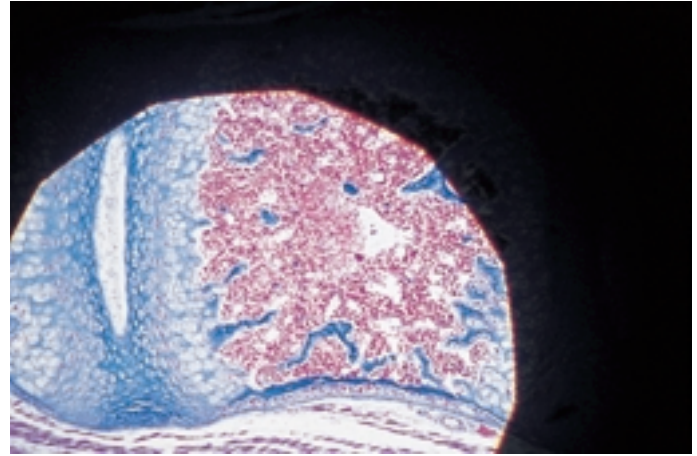


16.1

7. This is where Köhler illumination really begins: we narrow the luminous-field diaphragm and move the condenser carefully up and down via the condenser drive until we see a sharp image of the luminous-field diaphragm, or at least a piece of it in the edge. If this does not work at once, we set different diameters of the luminous-field diaphragm and move the condenser again until a piece of the edge of the diaphragm appears in focus somewhere (16.3). In most cases, this search will take some time.



16.2

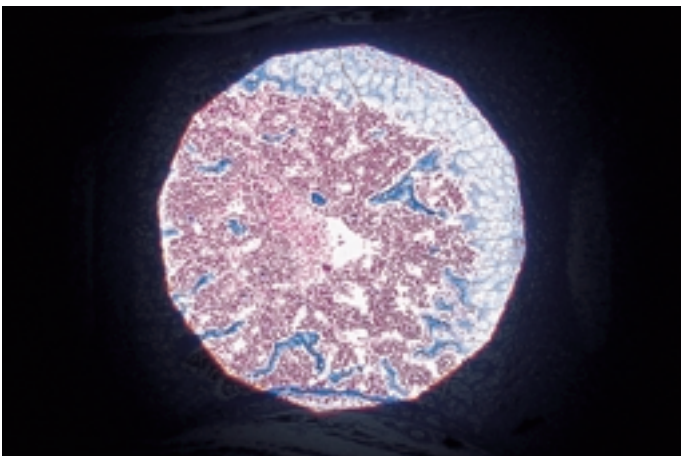


16.3

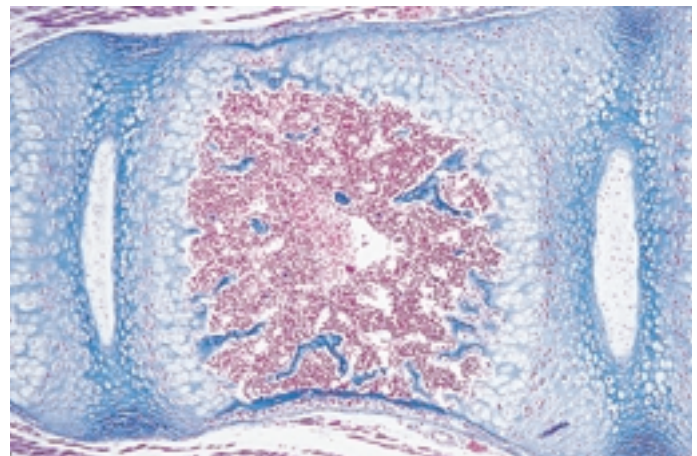


16.4

8. Our microscope image is now slowly taking shape. You can see the image of the luminous-field diaphragm in sharp focus, although it is not yet centered. The centering screws on the condenser are used for this. If you have moved the image of the luminous-field diaphragm almost to the center, continue as follows: open the luminous-field diaphragm until it fills almost the entire image field. If you now view the image edge, you will see that a narrow, dark edge remains. When this black edge has the same all-round distance to the edge of the field of view, open the luminous-field diaphragm until its edge moves out of the field of view.



16.5



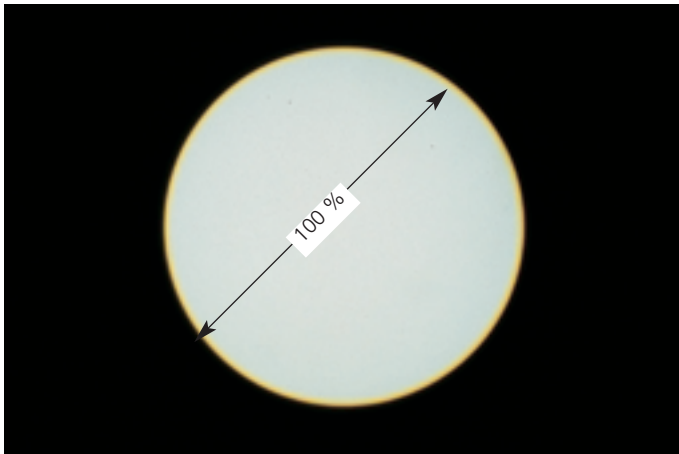
16.6

9. Now you almost have a good microscope image. Only the contrast remains to be improved. You will remember that we first opened the aperture diaphragm completely in order to see more light. We must correct this now in order to obtain more contrast. On the other hand, however, the aperture diaphragm must not be closed too much, since this would reduce the resolution of image details. You can see the aperture diaphragm in the microscope if you remove an eyepiece from the mount and look into the tube directly. Your eye must be about 10 to 20 cm away from the tube. Now widen and narrow the aperture diaphragm in the condenser until you can clearly recognize its image in the pupil of the objective. Finally, set the diameter of the aperture diaphragm in such a way that it illuminates about 2/3 (66 %) to 4/5 (80 %) of the pupil diameter. This setting provides almost full resolution and optimum contrast. This compromise is usually made in the resolution, since only the contrast makes the image acceptable to the eye.

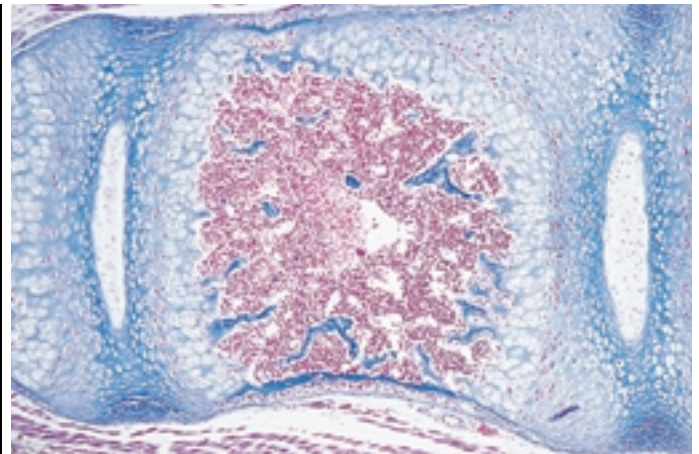
10. Insert the eyepiece again and look into the microscope. You have now done what you set out to do. Your microscope is set to Köhler illumination – at first for the objective just used. If you change over to another objective, you should always adapt the aperture and luminous-field diaphragm. However, you do not have to pull out the eyepiece every time you set the aperture diaphragm. Once the aperture diaphragm has been centered for one objective, it is sufficient to adapt them for other objectives "by instinct": first open the aperture diaphragm until the stop is reached and then close it slowly until the image starts to get slightly darker and the contrast increases at the same time.



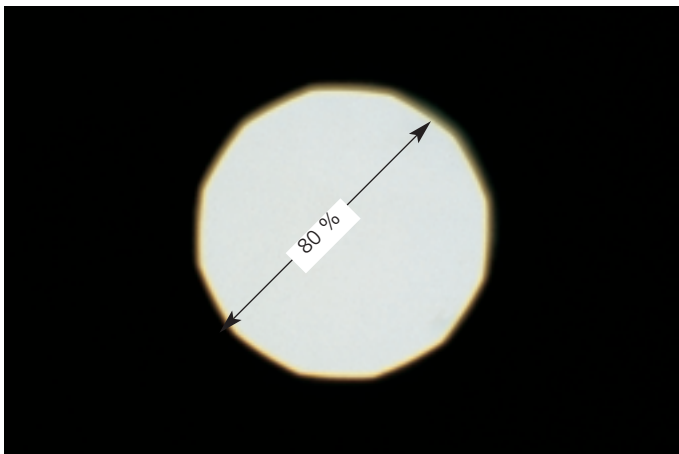
17.1



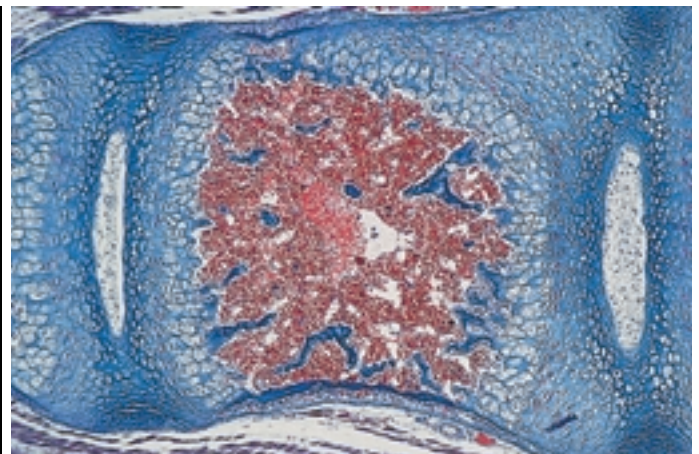
17.2



17.3



17.4



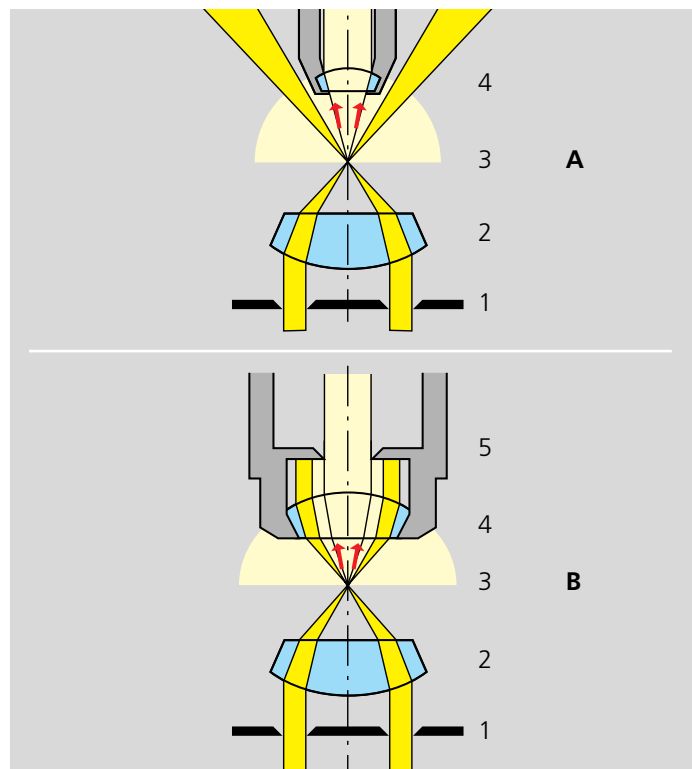
17.5

**See the difference:  
Contrasting techniques in microscopy**

In practical microscopy, you do not always have nicely stained samples which are easy to view in simple brightfield. Unstained samples, such as bacteria or living cell cultures, absorb practically no light and are barely or not at all visible in brightfield, even in a well-aligned microscope. The poor light absorption results in extremely small differences in the intensity distribution in the image. With a bright background, the human eye requires local intensity fluctuations of at least 10 to 20% to be able to recognize objects. This "modulation" of light intensity is far from reached by many microscope objects in brightfield. The contrasting techniques described in the following are tricks which allow optical effects in the sample – not visible to the eye – to be translated into intensity changes which can be recognized by the eye.

**Darkfield in transmitted light**

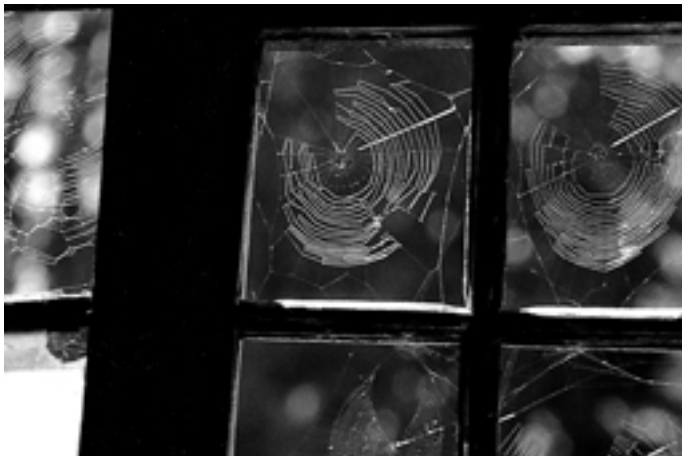
Fine structures can often not be seen in front of a bright background. This situation changes if the structures are illuminated from the side and viewed in front of as dark a background as possible. The structures then really seem to light up.



18.2

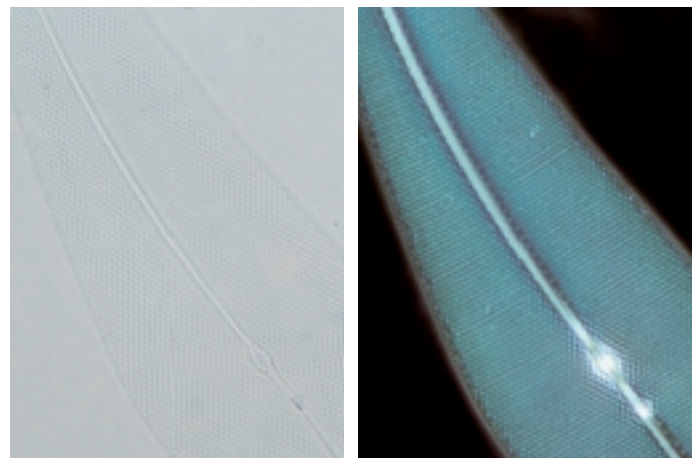
An artificial dark background is created in the microscope using an annular stop (1) in the condenser. Although the condenser optics (2) then illuminates the sample (3), it does so with a hollow cone of light. The light does not hit the objective (4), but passes it by on the outside. If there is no sample, the image seen in the eyepieces remains completely dark. However, if objects, e.g. small particles of bacteria, are in the object plane, light is laterally diffracted away from the straight path. Provided that this light hits the aperture cone of the objective, it is gathered by the objective and fused to form an image. The object becomes brightly visible in front of a dark background.

For this, it is necessary for the objective aperture to be smaller than the inner aperture of the illuminating light cone (case A). However, objectives with an integrated variable iris diaphragm (5) are also available to shutter out the indirect light even if it falls into the aperture cone of the objective (case B). This permits the use of very high apertures for darkfield.



18.1

Fine structures become visible: The spider webs (above) are barely recognizable in front of the bright background – a light green field in this case. The picture completely changes when a dark background – a dark forest – is chosen. The structure seems to light up (below). In both cases, the object is illuminated at an angle from above by the bright afternoon sun. However, the sunlight did not directly hit the camera; this direct illumination would have swamped everything else.

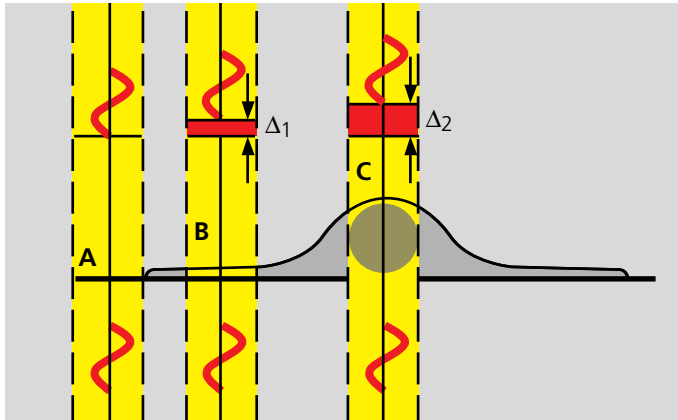


18.3

The diatoms are barely visible in brightfield (left). This is quite different in darkfield (right) where they seem to light up.

## Phase contrast in transmitted light

This method described by the Dutchman Frits Zernike in 1934 not only earned its discoverer the Nobel prize for physics in 1953, but also revolutionized biomedical basic research of living – i.e. unstained – cells. Phase contrast is ideal for thin unstained objects, for example culture cells on glass, which are approx. 5 bis 10  $\mu\text{m}$  “thick” above the cell nucleus, but less than 1  $\mu\text{m}$  “thick” at the periphery, and which barely exhibit any light absorption in the visible part of the spectrum. The eye can scarcely see them in brightfield and darkfield. However, very small differences exist between the refractive indices of the cells and the surrounding aqueous solutions (A) and within the cells between the cytoplasm (B) and the cell nucleus (C).

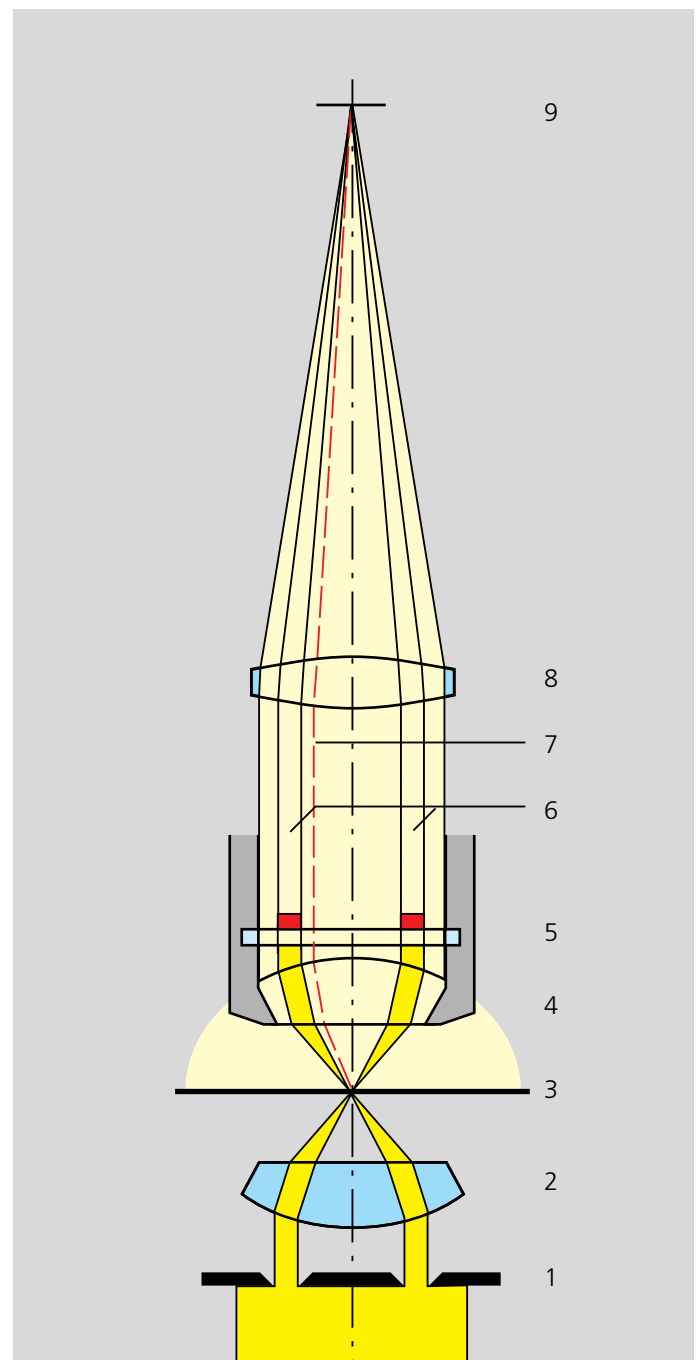


19.1

Phase contrast makes these tiny differences visible by the use of optical devices – i.e. it translates them into differences in intensity. The optical effect used consists of a shift of phase in the light ray. During their journey through cell nuclei, cytoplasm or water, the light waves are shifted by small degrees, since these media have slightly different refractive indices. The higher the refractive index of a medium, the smaller the speed or velocity of light in the medium. As a result, a light wave which has passed through a cell nucleus, lags behind the light waves which only had to pass through water. The amount of “lag” is called phase shift. Before their entry into the sample, the waves are still “in phase”, but this is no longer the case when they have passed through the various materials. The amount of the phase shift behind the sample depends on what media (refractive indices) the waves had to pass through on their paths and how long the paths were in these media.

The human eye cannot see these phase shifts in the microscope image. It can only distinguish between different intensities and colors. Therefore, the phase contrast technique uses optical tricks to translate phase shifts into “grey values”.

Much like darkfield, the aperture diaphragm is replaced by a phase stop (1) which illuminates the sample (3) via the condenser optics (2). However, here the entire light bundle enters the objective (4) and an image of the phase stop (1) is created in the objective pupil (5). A “phase ring” is attached to the objective pupil (5) which does two things: firstly, it attenuates – like a grey filter – the pronounced bright light coming from the phase stop of the condenser, and secondly, it adds a constant phase shift to this light. If the specimen contains objects such as cells and their nuclei, they guide the light from the



19.2

direct ray to new paths (7). This light will not pass through the phase ring in the objective, i.e. it will neither be attenuated nor will it be “retarded”. All the partial rays are fused to form the intermediate image (9) by the tube lens (8).

The partial rays which have all been “retarded” to varying degrees are superimposed in the intermediate image, where they amplify or attenuate each other, depending on the phase position. Since the direct ray was strongly attenuated by the phase ring in the objective, the much weaker, diffracted light can become effective. The result of these interference processes in the intermediate image are bright and dark spots without which the cell to be examined would not be visible to the eye. Optimum contrast is created by selecting the right retardation and attenuation for the light waves in the phase ring of the objective.

A concomitant of phase contrast is the haloes of light which appear on the structure borders. They are caused by the optical principle and may result – especially in the case of thick specimens – in “illegibility” of the image, since the haloes are superimposed many times over. Therefore, phase contrast is a method which is only recommended for very thin objects where several structures are not physically lying on top of each other. In a thick specimen, details may be blended into an image which, in the final analysis, is then no longer “legible”.

### The microscope equipment for phase contrast

Phase contrast requires special objectives which are equipped with a phase ring near the pupil. They are easy to recognize by the green inscription “Ph1”, “Ph2” or “Ph3”. If you hold such an objective against the light and look into the pupil from the screw-on surface, you will be able to see the grey/transparent phase ring.

The condenser requires one, two or three phase stops, depending on the phase contrast objectives you have chosen. The required ring diameter increases with the numerical aperture, i.e. high apertures require the maximum diameter (e.g. 0.9 in air or 1.3 with oil immersion). Three sizes are available and are sufficient for all objectives. If you very rarely use phase contrast and with only one ring size, an easily attachable and removable plug-on stop for the condenser will suffice. A turret disk with several mounts (“eyes”) is more convenient, since it contains all three phase stops and allows fast changeover. Additional openings are available for the aperture iris for brightfield and, for example, for the darkfield diaphragm.

The phase stops must be centered once after they have been inserted in the condenser so that the image of the phase stop in the objective pupil corresponds exactly with the position of the phase ring in the beam path. Centering is performed using two small wrenches on the turret disk of the condenser. Again, look into the objective pupil and bring the bright image of the condenser phase stop into coincidence with the phase ring of the objective. This is clearly shown in the figure below: On the left side, the phase stop of the condenser (bright) is not aligned, while it is in perfect congruence with the phase ring of the objective on the right.

If you want to be particularly precise, use a centering telescope for the setting. This small accessory looks like an eyepiece and is also inserted into the tube instead of an eyepiece. When it is focused on the pupil of the objective, the aperture diaphragm and the phase stops can be conveniently controlled.

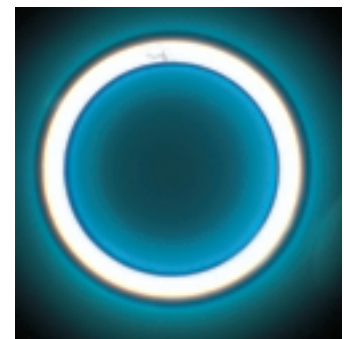


20.1

The image of an object in phase contrast can be influenced by appropriately selecting the retardation of the main beam through the phase ring in the objective. Depending on the retardation selected, objects with a higher refractive index than their surroundings appear either brighter or darker than their surroundings. This is also called “positive” or “negative” phase contrast. Today, “positive” phase contrast is standard, where the darkness of objects increases with their refractive index. This simulates absorption to the observer’s eye in areas where a higher refractive index becomes locally effective. This impression is considered “correct” in particular with cells and tissue in aqueous media because cell nuclei and organella, for example, appear darker than the cytoplasm of a cell.



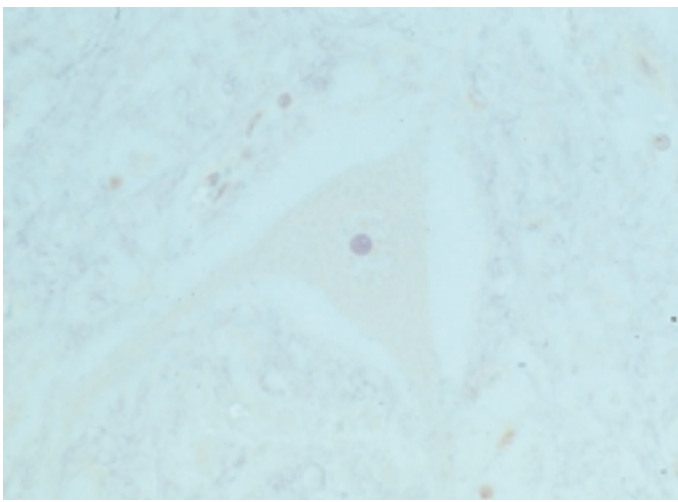
20.2a



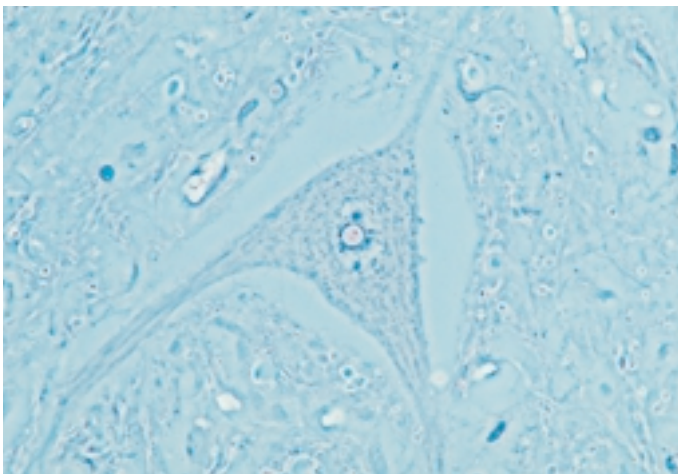
20.2b



20.3



21.1a



21.1b

The cells (above) are almost invisible in brightfield, but can be seen clearly in phase contrast (below).

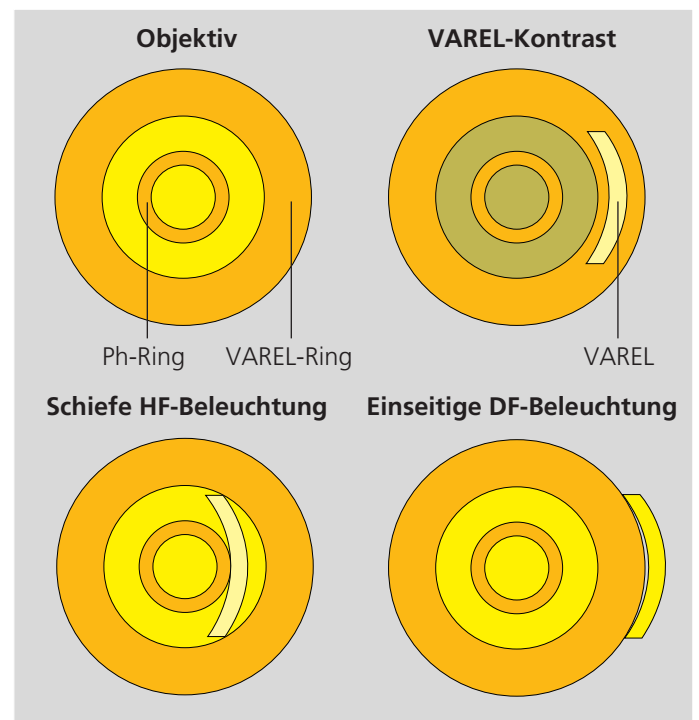
### VAREL contrast

A new contrasting technique, in which phase contrast and inclined unilateral illumination are mixed, has been developed for the examination of living cells in culture vessels (21.2). An additional stop shaped like a ring sector is used on the illumination side, permitting unilateral inclined illumination. Unilateral darkfield, VAREL contrast superimposed on phase contrast and inclined brightfield are set by shifting the stop in the radial direction from the outside to the inside (21.3). This is a very low-price method of imaging cells in culture vessels. The image shows pseudo-relief (21.4). Even vessels with a curved bottom allow a useful image to be produced. Phase contrast alone sometimes fails in such cases because a curved chamber base acts like a lens and impairs the superimposition of phase rings. The method is successfully used for the examination of living objects (micromanipulation).

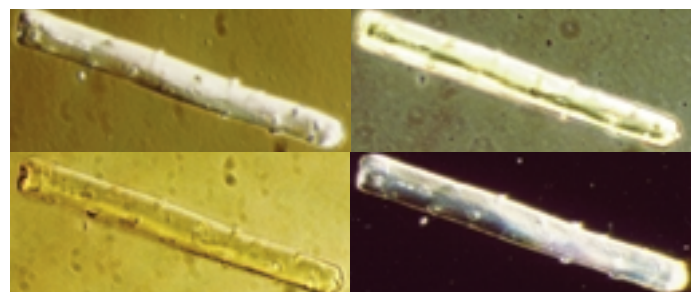
A slider contains two of the mentioned sectors to allow illumination to be performed from the left or right, as required. This makes it possible to contrast cells even in the "holes" of microtiter plates in the vicinity of the "hole" edges.



21.2



21.3



21.4

## Polarization contrast in transmitted light

In this method, polarized light is used; it consists of light waves which all feature the same direction of vibration, i.e. which are linearly polarized. This very "ordered" light is generated by polarizers which filter out a privileged plane from the statistical confusion of vibration directions prevailing in natural light.

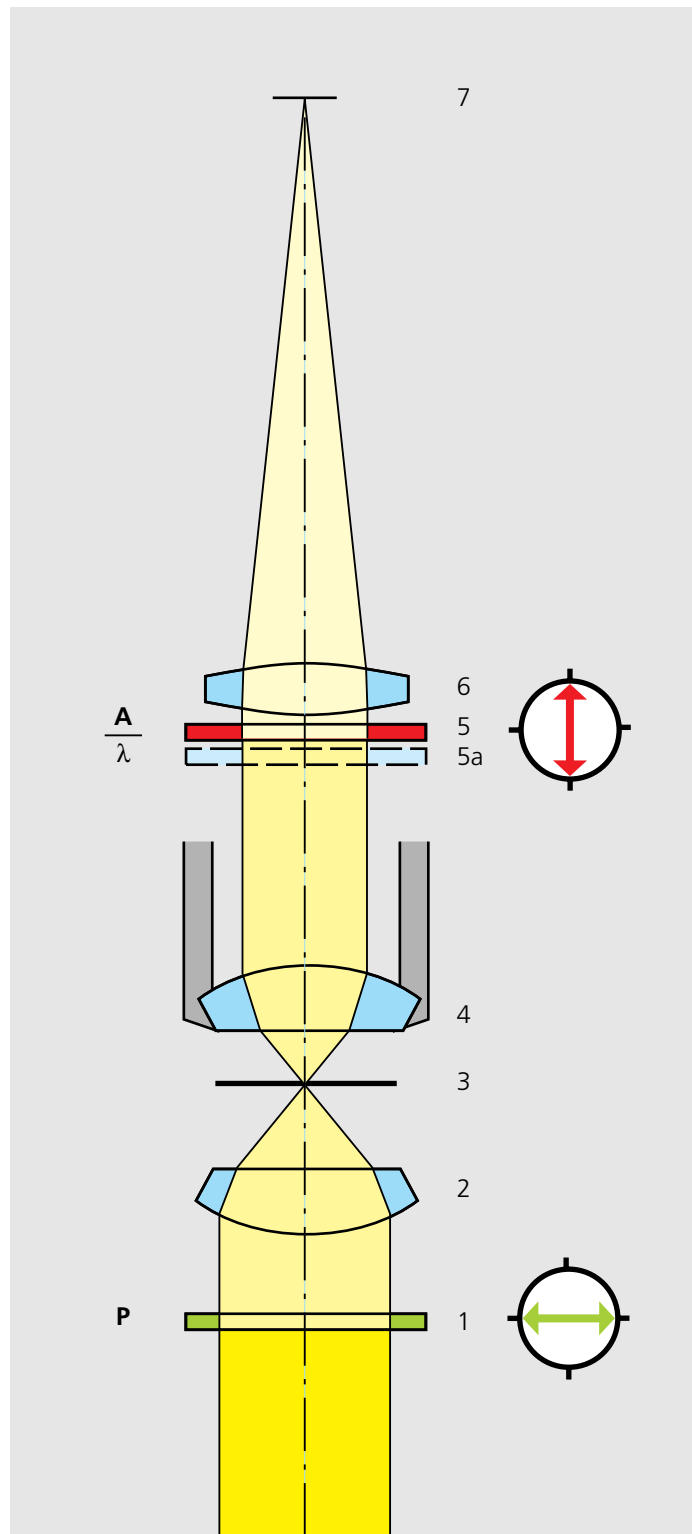
It is an important fact that two filters of this type do not let any light pass when they are arranged one behind the other in the beam path at an angle of  $90^\circ$  to each other. The first filter sorts out the vibration directions in such a way that the second filter cannot let pass this very selection. The second filter is called "analyzer", since it allows the privileged direction of the first filter – called "polarizer" – to be checked.

The appropriate arrangement is relatively easy to implement in the microscope. The polarizer (1) on the condenser – near the aperture diaphragm – ensures that the specimen (3) is illuminated with linearly polarized light via the condenser. The analyzer (5), arranged at an angle of  $90^\circ$  to the polarizer (1), is located behind the objective. The tube lens (6) forms the intermediate image (7).

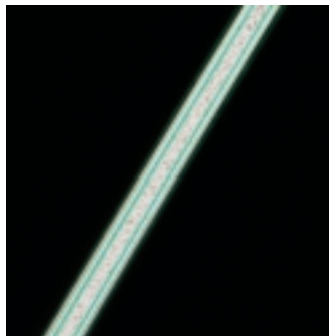
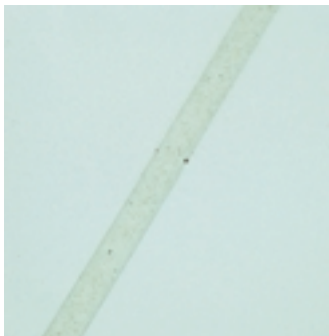
If no specimen is on the microscope stage – or only an empty, clean microscope slide – the image will remain completely dark. When illuminated, many specimens turn the vibration direction of the polarized light out of the plane produced by the polarizer. Such specimens are mainly birefringent materials, in which the refractive index depends on the vibration direction of the incident light. This is mainly the case with crystals, such as starch or minerals, but also with polymers. If such materials are viewed under the polarization microscope, bright areas can be seen in the image because light is partially transmitted by the analyzer.

The drawing of the beam path also includes a so-called *auxiliary object* (5a), also termed *lambda plate*. In polarized light, this lambda plate converts contrast to colors. As in phase contrast, path differences are used for this purpose, although this time with polarized light and birefringent material in the auxiliary object. The path differences generated lead to an extinction of certain wavelengths in the light, i.e. only certain colors remain from the white light and create beautiful, colored pictures.

Mechanical stress in the glass results in so-called stress-induced birefringence, which – in turn – influences the polarized light. Therefore, Pol examinations in the microscope require condensers and objectives which are free of such internal stress. Such objectives can be recognized by the "Pol" marking inscribed in red.



22.2



The unstained polymer fiber is only indistinctly visible in brightfield (left) but can be seen in detail in polarized light (center). The lambda plate (right) converts contrast to colors.

22.1

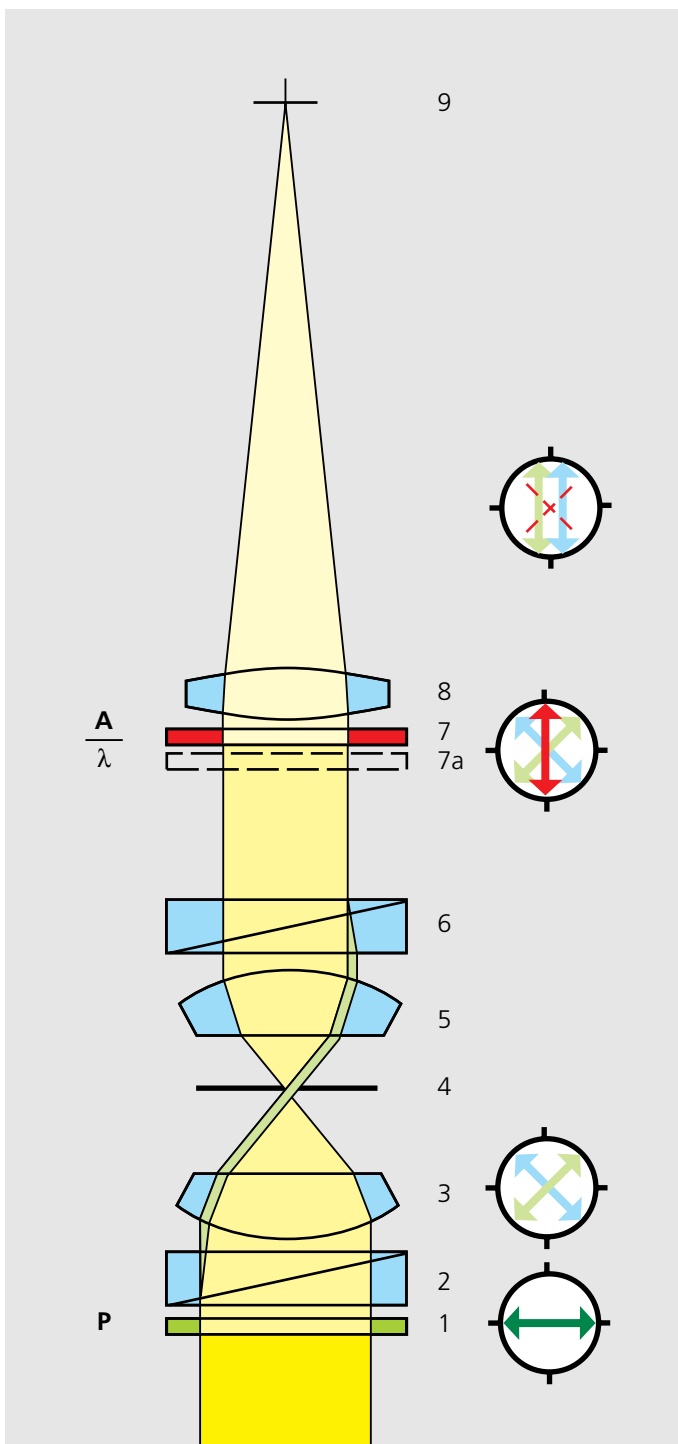
## The ultimate in sophistication: Differential Interference Contrast (DIC) in transmitted light

This highly efficient contrasting technique is based on the Pol contrast technique (page 22) as far as the components used are concerned. In its function, it is indeed related to DIC in reflected light (page 32). However, DIC in transmitted light is slightly more complicated than in reflected light because, firstly, two birefringent prisms are used, and, secondly, the path difference in the object is created in a different way.

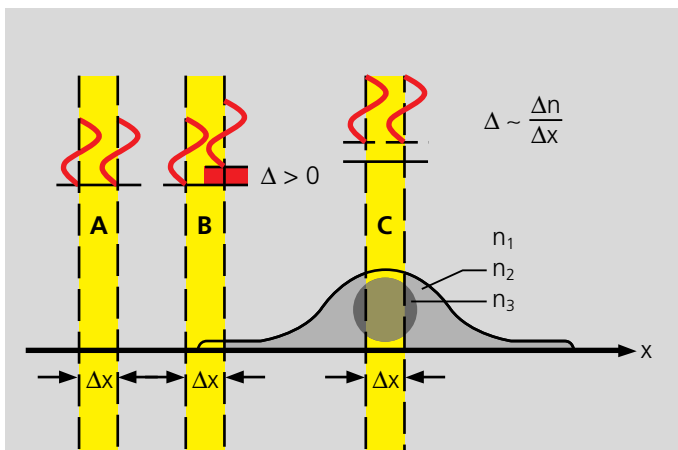
Fig. 23.1 shows the beam path which, initially, is identical to that of polarized transmitted light. Additionally, the two birefringent prisms (2) are inserted in the condenser and near the objective pupil (6). The condenser prism (2) performs a vectorial decomposition of the previously linearly polarized light into two vibration directions which are perpendicular to each other, and laterally shifts these partial beams in such a way that a lateral displacement of  $\Delta x = k \cdot \lambda$  occurs in the specimen.  $\lambda$  is the wavelength of the light used and  $k$  is a number which normally is smaller than 1.

If the two partial beams now pass through exactly the same structures, no further path difference will occur in the specimen (cases A and C in Fig. 23.2). However, if the two partial beams "see" slightly different conditions, each of them will "experience" its own path difference which accompanies it on the remaining path to the intermediate image (case B in Fig. 23.2). The second prism (6) cancels the splitting process again behind the objective, and analyzer (7) selects those components from the now phase-shifted wave trains which lie in its vibration direction.

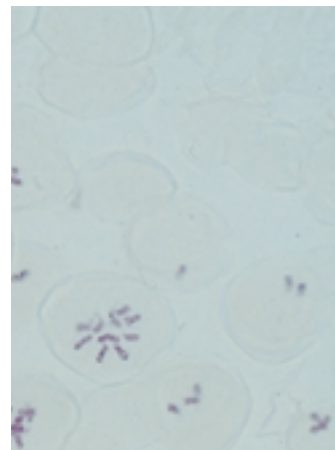
It is only now – with a *common vibration plane* – that the two partial beams can interfere with each other and therefore convert path differences to intensity differences which can be seen by the eye. A  $\lambda$ -plate (7a,  $\lambda$ ) permits additional color contrast to be produced. The resulting images look like reliefs because this method displays only "lateral" changes. DIC is therefore also ideal for the optical sectioning of unstained, thick objects.



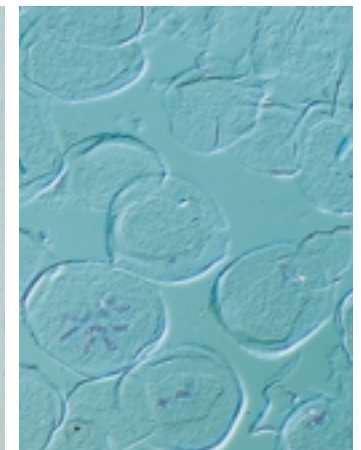
23.1



23.2

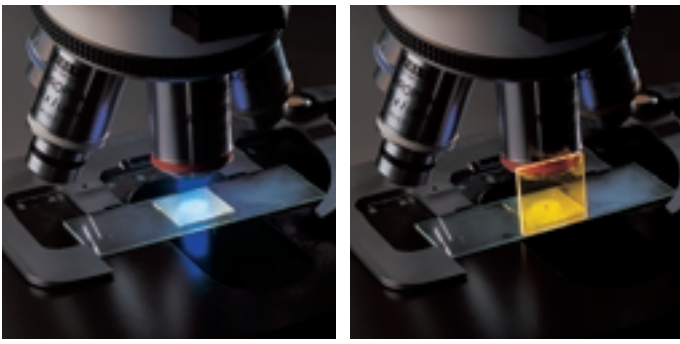


23.3a



23.3b

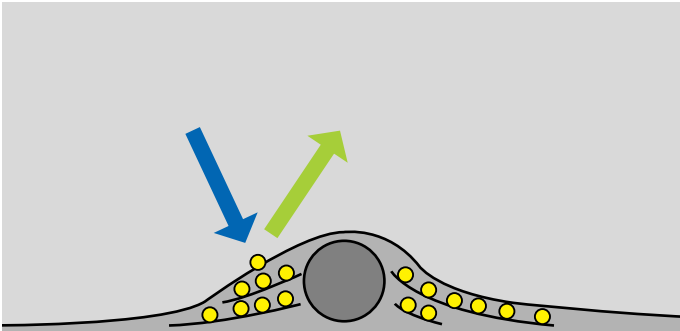




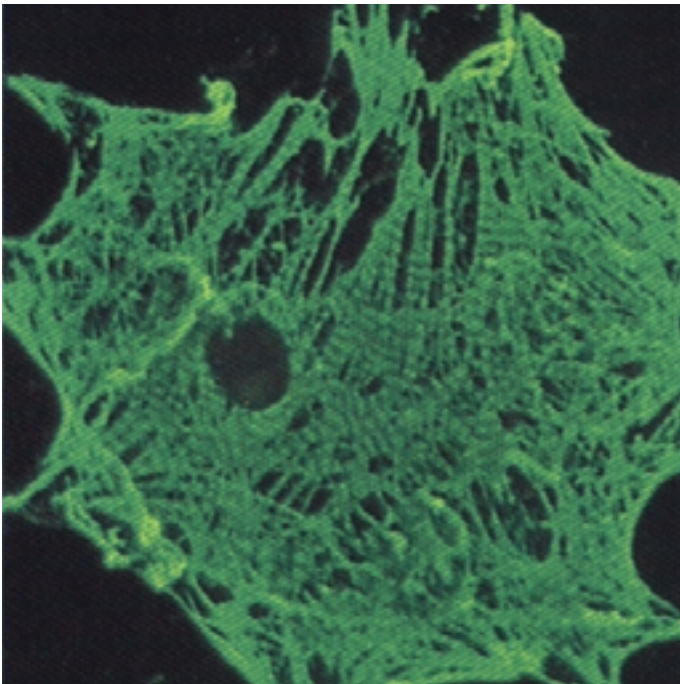
24.1

Left: A specimen marked with a fluorescence dye is illuminated in reflected light with intensive, blue light.

Right: If the specimen is viewed through a barrier filter, the blue excitation light can no longer be seen. Instead, the yellow fluorescence light is clearly visible on the specimen.



24.2a



24.2b

## Fluorescence microscopy

In fluorescence microscopy, the specimens are treated with special reagents. Their individual molecules are able to absorb light for an extremely short time – usually billionths of a second – and then to emit it again. However, the emitted light features a wavelength which is slightly shifted “towards red”. If, for example, blue light is absorbed, green light will be emitted immediately afterwards. Green is changed to yellow, yellow to reddish orange and invisible UV light to visible light. This shift is termed Stokes shift after its discoverer. In fluorescence, the wavelength of the emitted light is about 20 to 50 nanometers longer than absorbed exciting light.

Fluorescence molecules can only absorb light of a certain wavelength. Each of the various fluorochromes exhibits its own, very specific absorption spectrum, depending on the internal structure of the fluorescence molecules and sometimes also on their surroundings. Furthermore, not every photon is absorbed, but only a part of their radiating light. The absorbed photons are not emitted again in their entirety either. Good fluorescence markers feature a high “quantum yield” – a term describing the ratio of the emitted to the absorbed photons.

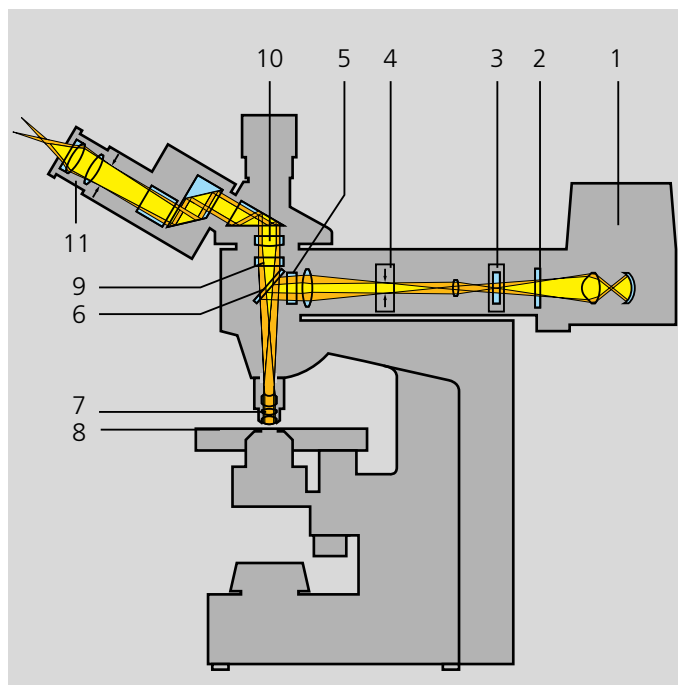
This effect is very useful for microscopy: a specimen marked in this way is illuminated with pure, filtered blue light and viewed using a barrier filter which is completely opaque to blue light, but which transmits long-wave green, yellow and red light. The structures marked with fluorescence molecules – e.g. parts of a cytoskeleton – then light up green against a black background.

When microfluorescence was initially introduced, the specimens were usually dyed non-specifically with fluorochromes. This type of marking usually looks bright, since many fluorescence molecules are bonded everywhere. Nowadays, however, fluorescence methods are much more specific. This has been made possible in particular by the permanent coupling of the fluorescence molecules with biological substances, e.g. antibodies (in this case, it is no longer the dye which determines the bonding position, but the biologically active molecule). Normally, this results in weak fluorescence images in the microscope because much less dye is bonded. However, the information obtained, e.g. in the diagnosis of illnesses, is becoming much more exact.

If cells are treated with fluorescence markers, these are deposited on certain structures. When illuminated with excitation light, the fluorescence markers light up and make the structures clearly visible by their emission light.

## Beam path of the fluorescence microscope

The illustration on the right shows the beam path in the **Axio-lab** microscope with fluorescence equipment. The light travels from the powerful additional light source (1) to the exciter filter (5) via the heat-protection filter (2), the red-attenuation filter/barrier slider (3) and the luminous-field diaphragm (4). The exciter filter is integrated into the reflector slider which also contains the beam splitter (6). The dichroic beam splitter *reflects* the *short-wave* exciting light into the specimen (8) via the objective (7). The resulting emission is gathered by the objective (7) and *transmitted* by the dichroic beam splitter (6) because it exhibits *longer wavelengths* than the excitation light. The rays then also pass through the emission filter (9), where the remaining excitation light is filtered out. Therefore, this filter is also called barrier filter. As usual, the tube lens (10) and the eyepiece (11) form the microscope image, which now only consists of fluorescence light.



25.1

### The essentials for success in fluorescence microscopy:

#### ● Optimum quality of the fluorescence filters:

The fluorescence light generated must be treated with care. The filters must transmit the required wavelengths, and block the unrequired ones as completely as possible. This is very difficult to do, since the intensity of the excitation light is many times higher than that of the emission light. The entire exciting light must be kept away from the microscope image, while the emission light, however, must "arrive" in its entirety.

#### ● High-intensity excitation:

The light source must provide a large amount of excitation energy in very narrow ranges of the spectrum – typically 10 to 50 nm. For this purpose, so-called line emitters, usually high-pressure mercury lamps, are used.

#### ● High light transmission of the objectives:

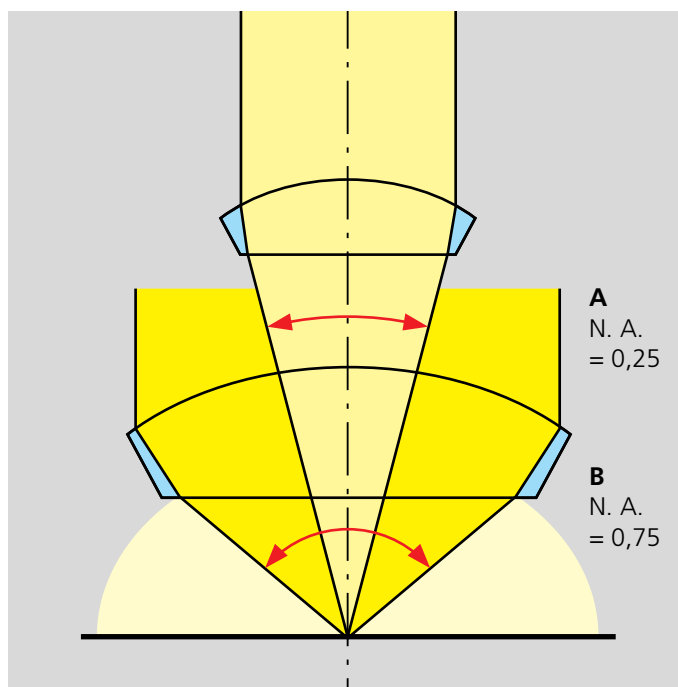
The objectives of the microscope – particularly those offering optimum imaging quality – often consist of many individual lenses. Nevertheless, objectives which are suitable for fluorescence feature high light transmission values into the UV range.

#### ● No autofluorescence of the microscope optics:

If lenses and filters – or immersion liquids – of the microscope exhibit autofluorescence, this disturbing light will be mixed inseparably in the fluorescence image. The result is a brightened background which reduces the contrast available.

#### ● High numerical aperture of the objective:

As with a radio transmitter, the fluorescence excited in the specimen is radiated in all directions. The objective must gather as much of this radiation as possible (25.2). The output will be low with a small numerical aperture (A). A higher aperture angle (B) will be much more effective. If the objective aperture is doubled in size, approximately four times the fluorescence light can be gathered. Immersion, particularly with oil – also eliminates the loss of light caused by light reflection on the surfaces. The image will become even brighter.

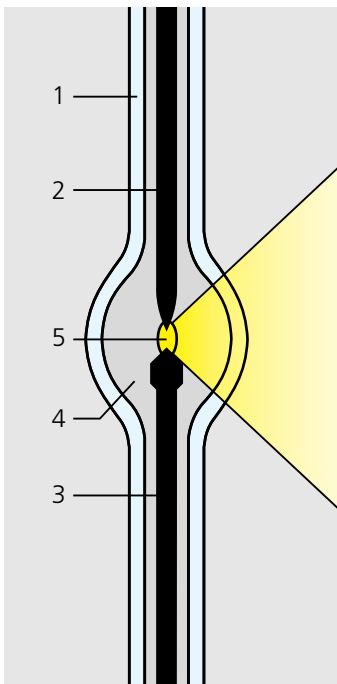


25.2

## More light: fluorescence becomes visible

Filament lamps are not suitable as light sources in fluorescence microscopy. The glowing metal filaments convert most of the electrical energy utilized into red or even invisible infrared light. Fluorescence, however, requires intensive, short-wave light. The table on the right gives you a simplified overview of the colours we perceive at the various wavelengths.

	Wavelength	Color
1	340 - 400 nm	near ultraviolet (UV) – invisible
2	400 - 430 nm	violet
3	430 - 500 nm	blue
4	500 - 560 nm	green
5	560 - 620 nm	yellow to orange
6	620 - 700 nm	orange to red
7	over 700 nm	near infrared (IR) – invisible

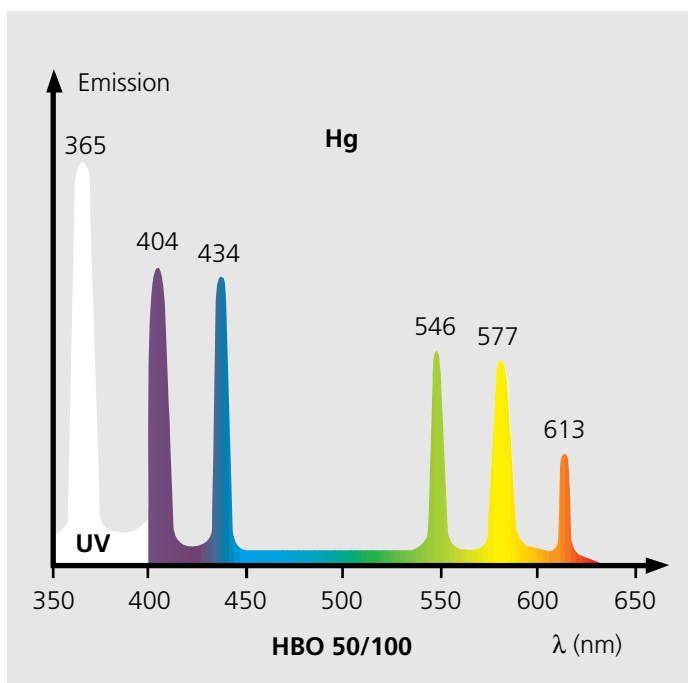


The high-pressure mercury lamp is a time-tried light source for fluorescence. Depending on its wattage, it is either called HBO 50 or HBO 100. Unlike the filament lamp, it uses the gas discharge principle and does not feature a continuous, but a discrete light spectrum.

This is how these light sources function: Two electrodes (cathode 2 and anode 3) are fused into a quartz glass bulb of a high pressure resistance (1). The burning chamber (4) contains some mercury. A light arc (5) is ignited between the electrodes by bursts of high-voltage surges and is kept "burning" via the power supply. The resultant heat vaporizes the mercury into gas and an enormous overpressure is created in the lamp. The lamp gets very hot and radiates extremely bright light with a high UV-portion. The radiated light energy is concentrated in certain wavelengths, the so-called "mercury lines" (see below: emission spectrum).

The ability to change between intensive lines and weak spectral ranges is a major benefit of this line emitter and is very helpful in fluorescence. Excitation is made with a single "line" and – because of Stokes shift – the fluorescence is viewed at a wavelength at which the light of the illuminator causes practically no disturbance.

26.1



Some safety regulations, mentioned in the operating instructions, must be observed when using these illuminators. These are the most important:

- Operate the lamp in the housing only.
- Never look into the intensive light.
- Never expose the skin directly to radiation. Intensive radiation may lead to burns and cause skin cancer in the long term.
- Change lamps when cold only: danger of explosion because of the high internal pressure in warm lamps.

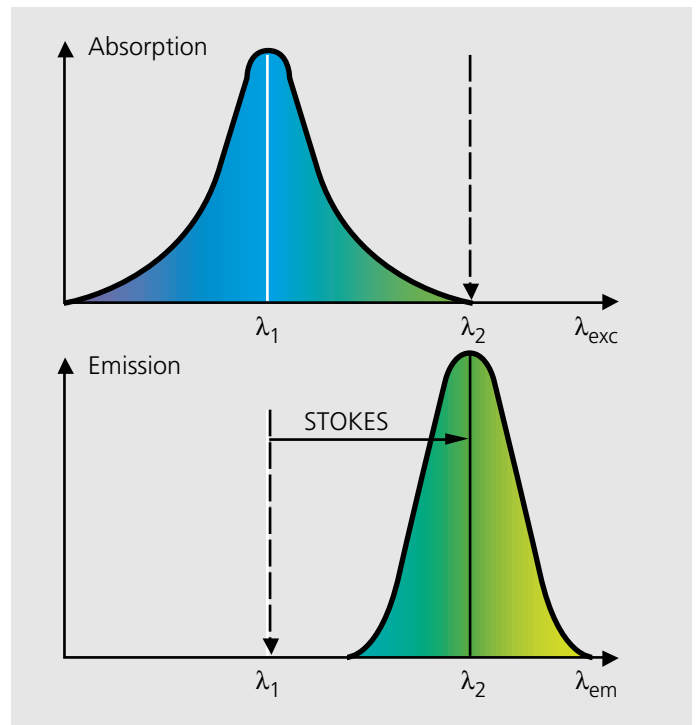
## Light is assorted: filters and filter sets

The graph on the right shows the spectral properties in (single) fluorescence. The fluorochromes absorb light ( $\lambda_1$ ) in a narrow spectral range and emit it into a range of a longer wavelength ( $\lambda_2$ ). The Stokes shift lies between the two.

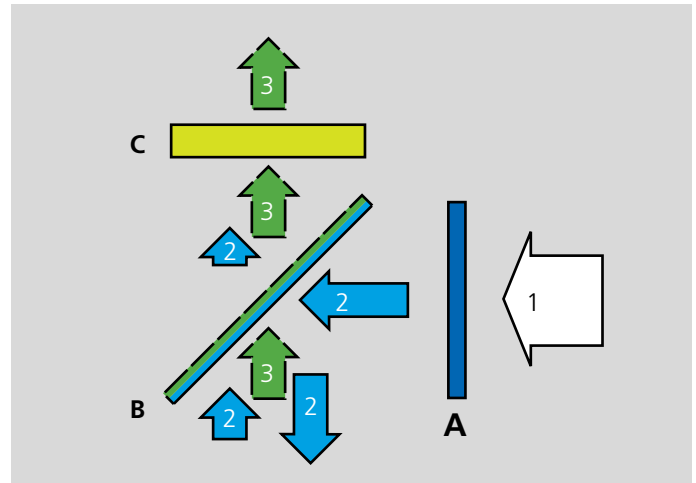
In fluorescence microscopy, filter combinations are arranged in the reflector area between the objective and the tube lens. These combinations determine the beam path of the excitation and emission light.

The exciter filter (A) (Fig. 27.2) filters almost monochromatic light (2) out of the light source radiation (1). The properties of the dichroic beam splitter (B) are fascinating: it reflects the short-wave excitation light to the objective almost without any loss, but allows the fluorescence light (3) returning from the specimen via the objective to pass through almost completely. At the same time, most of the excitation light is reflected again and can therefore no longer affect the formation of the intermediate image. Above the beam splitter, the emission light and the remainder of the excitation light hit the barrier filter (C). Only the fluorescence light can pass the filter almost unhindered because its wavelengths are longer than those of the excitation light.

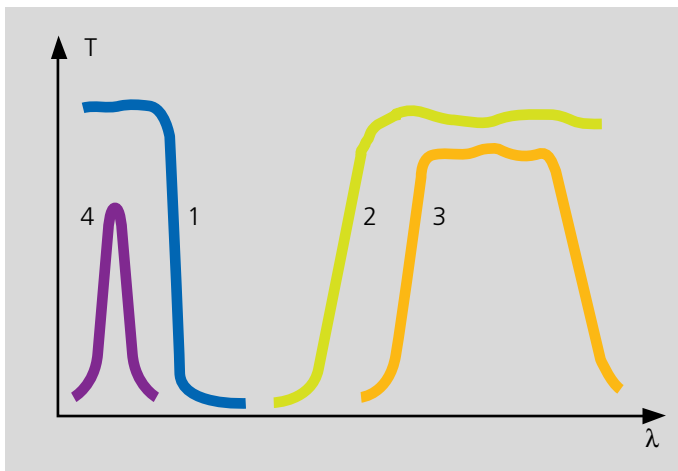
In most cases, modern fluorescence filters are combinations of color lenses and interference filters – with the exception of some classical longpass filters. The interference filters have been given coatings precisely adapted to the wavelengths of the light. Such filters can be “tailor-made” and exactly meet the spectral requirements made on a fluorescence dye.



27.1



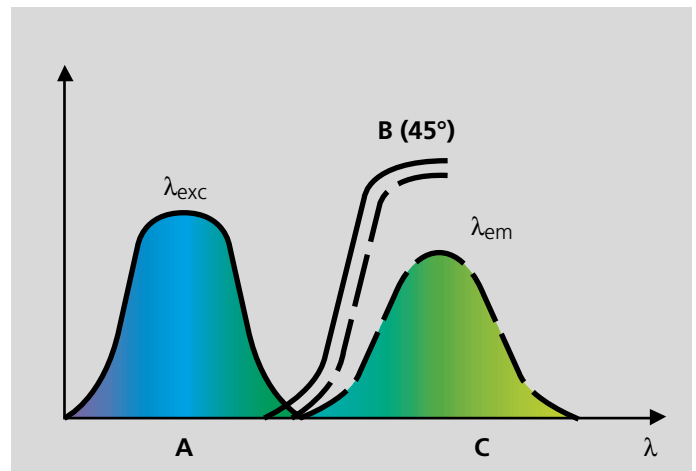
27.2



27.4

Typical spectral curves of the transmission  $T$  of filters for fluorescence microscopy:

- 1 Shortpass filter
- 2 Longpass filter
- 3 Bandpass filter
- 4 Narrowband filter



27.3

27.1 Spectral position of excitation (left) and emission (right) in fluorescence.

27.2 Function of the filter combinations (see the text)

27.3 Typical, individual spectral curves for the components of the filter combination:

A: Exciter filter B: Beam splitter C: Emission filter.

## Accessories for fluorescence microscopy

The components of the fluorescence microscope described until now are available as modules and can be easily integrated into the **Axiolab** microscope or any other model. The main components are the HBO 50 or HBO 100 illuminators (1), the reflected-light illuminator (2) and the reflector slider (3). The reflected-light illuminator contains a barrier and filter slider (2a) and the variable iris for the setting of the illuminated field (2b).

The main difference between the epi-fluorescence illuminator and the reflected-light illuminator is the omission of the diffusion disk in fluorescence because it would unnecessarily reduce the excitation intensity. In "proper" reflected-light microscopy, however, this disk is required for homogeneous illumination. In fluorescence, the red-attenuation filter is used to eliminate the sometimes disturbing red and infrared light portions before they reach the specimen.



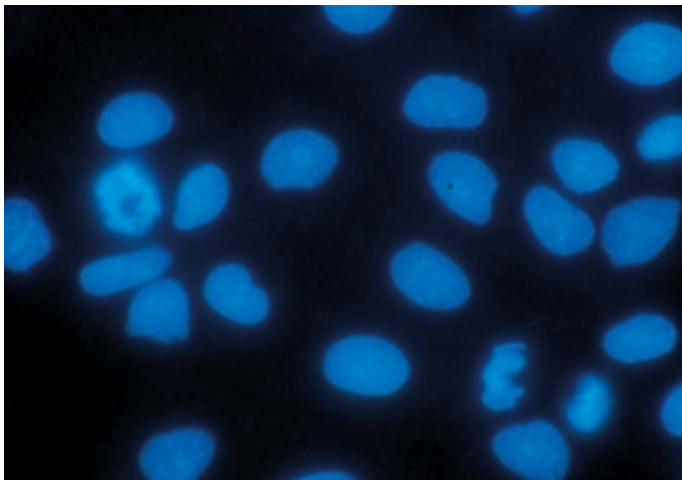
28.1

The reflector slider (see Fig. 28.2) permits up to three filter sets to be mounted for different fluorescence markers. This makes changeover very easy.

Multifluorescence is now being increasingly used for the marking of specimens. This method makes different structures light up in different colors which can be viewed separately – each on its own. The reflector slider can be used to change between the images. However, it is easier to use special filter sets which permit the simultaneous viewing of two or three markers *in one image*. (Fig. 29.1).

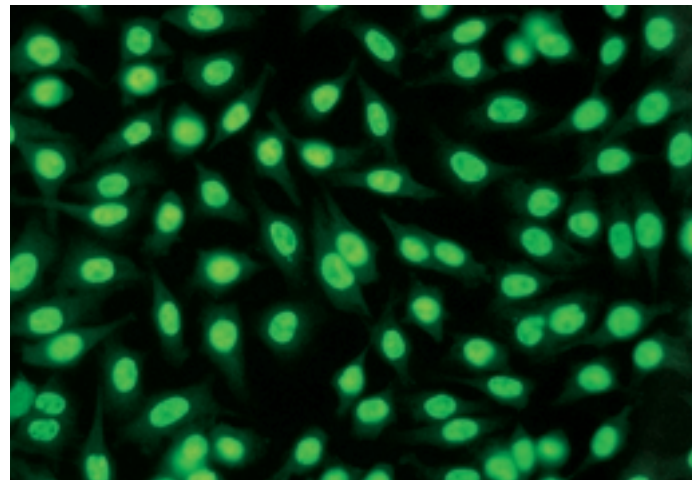


28.2



28.3

A type of simple fluorescence:  
DAPI is excited in near UV with 365 nm and emitted in the violet-blue spectral range. The marking reveals cell nuclei and particularly the chromosomes (Planapo 63x/1.40 oil)



28.4

Specific marking for medical diagnosis:  
Fluorescein Isothiocyanate (FITC) linked with the antinuclear factor HEP 2).  
(Plan-Neofluar 40x/1.30 oil)

## Practical hints on fluorescence microscopy:

### ● The work environment:

If fluorescence is weak, you had better work in a dark environment. Make sure that you do not have to enter a bright area during work or even have to look at lamps or bright windows.

### ● Bleaching of specimens:

When the specimen is not being viewed or photographed, block out the excitation light using the filter slider in the fluorescence illuminator in order to avoid undesired bleaching by the excitation light.

### ● Fluorescence-free immersion oil:

If autofluorescent immersion media are used, the background brightens and the image contrast is reduced.

### ● The heat-protection filter:

Fluorescence filters are sensitive to the heat emitted by the illuminator. Please never remove the integrated heat-protection filter.

### ● Adjustment of the lamp:

The illuminator must be readjusted occasionally – in all cases after lamp replacement. Please see the operating instructions for further information and remember to observe the safety regulations (see page 26).

### ● Specimen preparation:

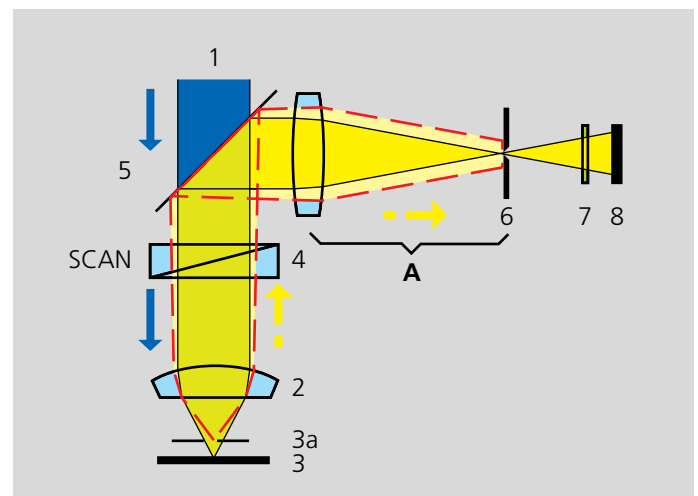
The unbonded fluorochromes should be removed from the specimen, e.g. by washing them out. The “contrast” in the fluorescence image is generated by the dark background only, which – in turn – is unnecessarily brightened by excess fluorochromes.

## For a new insight:

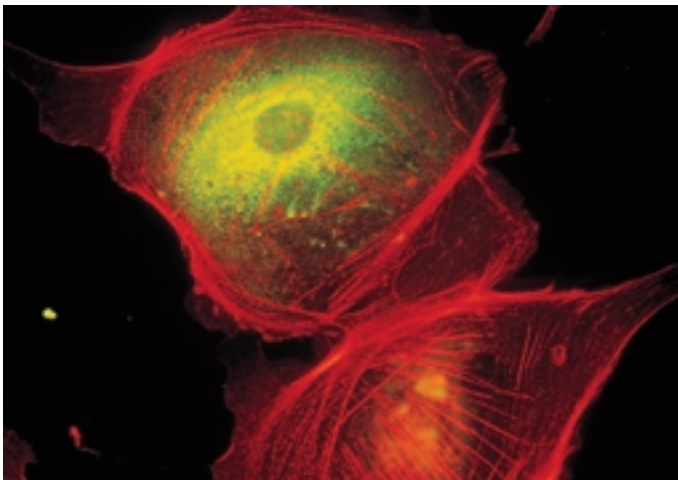
### Confocal fluorescence microscopy

This method is only possible with very special microscopes, i.e. the confocal microscopes. This method will be mentioned briefly here because it considerably enriches microscopy.

In the confocal microscope, a beam of intensive light (1) is transferred to the specimen (3) via the objective (2). The scanners (4), with which the light ray scans the specimen (3), are included in the beam path. The fluorescence light generated there travels back via the scanners – i.e. the ray motion is neutralized again – passes through the beam splitter (5) and is focused on the pinhole (6). Behind the pinhole, the fluorescent light is separated from the excitation light by barrier filters (7) and is continuously measured by the light detector (8). The computer composes an electronic image from the measured values. The decisive factor is that only *fluorescent light from the focal plane of the objective* (3) can pass through the pinhole (6) in the spatial filter (A). Light from other planes of the specimen (e.g. 3a) is blocked out very effectively by the pinhole (6). This permits individual planes of the specimen to be viewed. 3D images are generated with computer support.

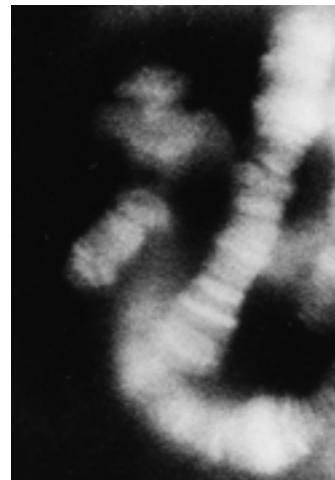


29.2



29.1

Highly specific multiple fluorescence: Various fluorochromes mark exactly defined structures in the cytoskeleton of individual cells.



29.3



The regular fluorescence image (left) is swamped and does not permit the recognition of any details within the object. This is completely different in the confocal image (right): an individual plane from the inside becomes clearly visible.

## A must in materials research and technology: Reflected-light microscopy.

A transmitted light microscope will normally not be very useful to anyone wanting to examine the structure of metal samples, the surface of ceramics or printed paper documents. The reflected-light microscope has been developed for these purposes. Much like the fluorescence microscope, the sample is illuminated from above through the objective. On the whole, the Köhler principle applies if the objective with its pupil plane is seen as the front lens of the condenser.

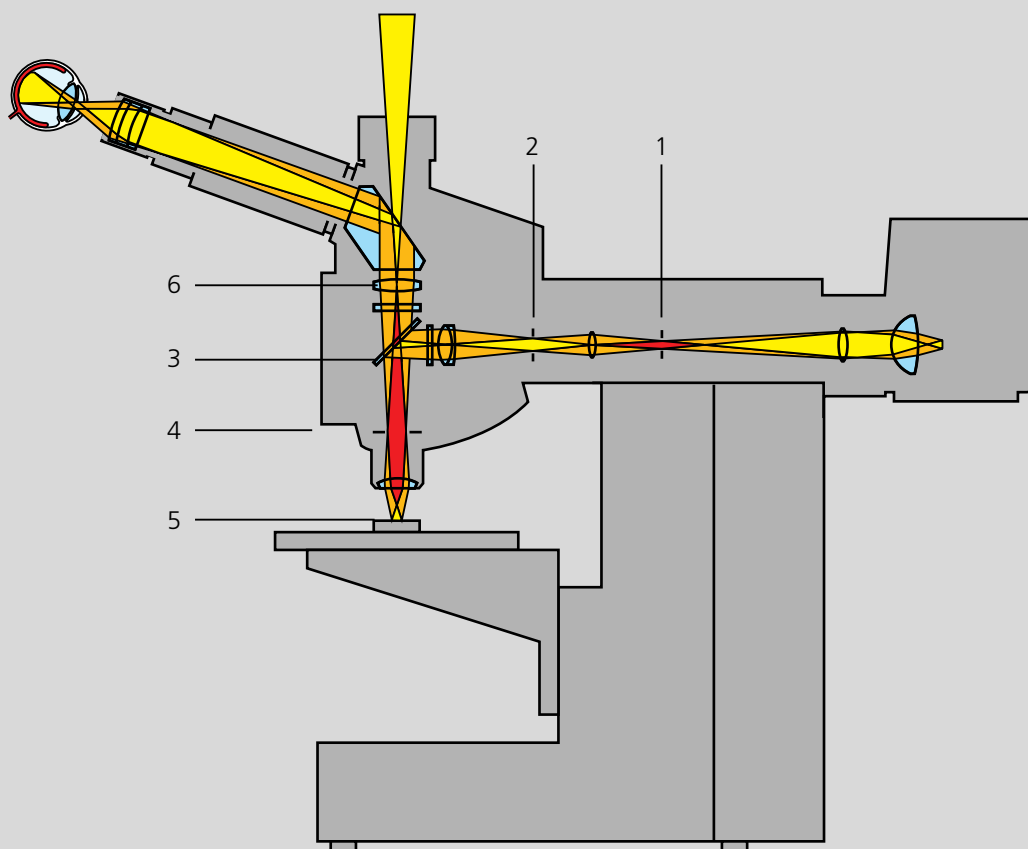
Reflected-light microscopes like the **Axiotech** from Carl Zeiss shown on the right – the **Axiotech<sup>vario</sup>** model for large specimens behind – feature an integrated reflected-light illuminator. The beam path (30.1) for the *brightfield technique* again includes the aperture diaphragm for reflected light (1) and the luminous-field diaphragm (2). The aperture diaphragm (1) is imaged on the objective pupil (4) via the reflector (3), and the luminous-field diaphragm (2) is imaged on the sample surface (5). Unlike reflected-light fluorescence, the reflector (3) is equipped with a neutral beam splitter which reflects or transmits white light equally well in all wavelengths. The illuminating light hits the sample surface and is reflected or scattered. The objective gathers these rays and the tube lens (6) projects the intermediate image.

The resolving power in reflected light is based on the same relationship between the wavelength of light and numerical aperture as in transmitted light. Köhler illumination in reflected light normally covers only the setting of the aperture iris, since – once set – the luminous-field diaphragm is correct for all objectives.

Reflected-light microscopes also permit the use of objectives for observation in transmitted light because the parfocal length of 45 mm is maintained in all objectives. As shown in Fig. 30.2, simple transmitted-light systems are also available for such applications, in addition to the classical transmitted-light condensers. These simple systems do not comply with the Köhler principle, but provide good results, particularly with small magnifications and large fields of view.



30.2



## Microscope objectives for reflected-light techniques:

### The optical system with a difference.

Objectives for reflected light can be recognized by the “Epi” inscription. They differ from objectives for transmitted light in two main points: their lens surfaces are particularly well anti-reflection coated to prevent the light coming from the illuminator being reflected towards the eyepiece. Such reflections would be superimposed on the image and have a disturbing effect. The second difference is that reflected-light objectives are designed for “uncovered” samples. Surface samples are usually viewed without a cover slip. Therefore, higher apertures require a different optical computation than for transmitted-light objectives.

Reflected-light objectives are also suitable for examinations in transmitted light without any restrictions. However, if objectives with a high aperture (from approx. 0.3) are used, the objects viewed must not be covered by cover slips or the like, since this would make the optical correction invalid. Furthermore, cover slips may produce undesired reflections.

A special design of the objectives permits darkfield in reflected light (Fig. 31.1). The light from the illuminator is guided on the outside around the actual objective in a second sleeve and hits the sample surface from the side. The objectives are therefore “thicker” than usual.

### Fast change:

### The contrasting techniques in reflected light.

In the reflected-light microscope, the condenser function is mainly performed by the objective. All the components required for optical contrasting can therefore be arranged in a very small area in the “infinity space” above the objective.

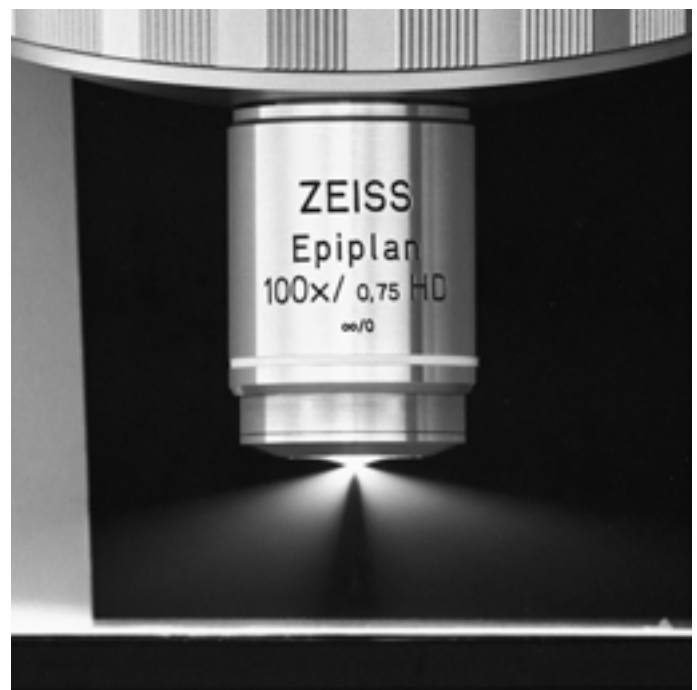
The arrangement of the contrasting components is shown in figure 31.2.

- A: Contrasting element of the illumination side
- B: Contrasting element of the observation side
- C: Additional space for components for both paths

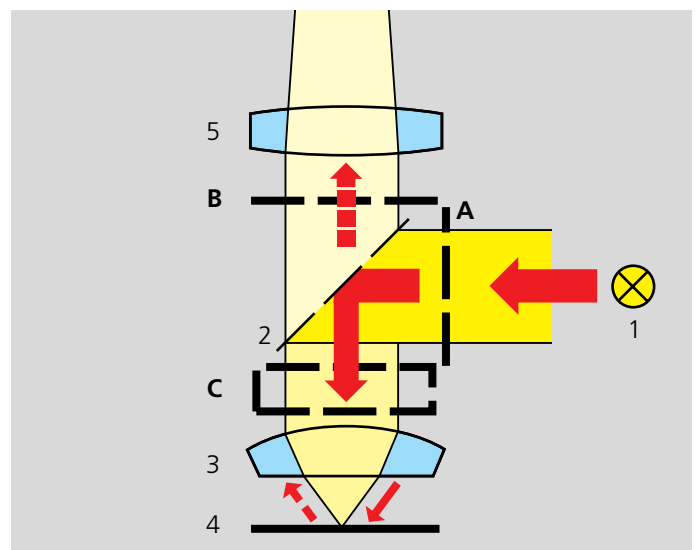
The other optical components are:

- 1 Light source
- 2 Color-neutral beam splitter
- 3 Reflected-light objective
- 4 Sample surface
- 5 Tube lens

A fast change of techniques is possible because the reflector slider contains permanently integrated contrasting components – like the light filters of the fluorescence microscope. If the reflector slider features three stop positions, the technique can be quickly changed by adjusting the position of the reflector slider – equipped, for example, for brightfield, darkfield and polarization.



31.1



31.2



31.3

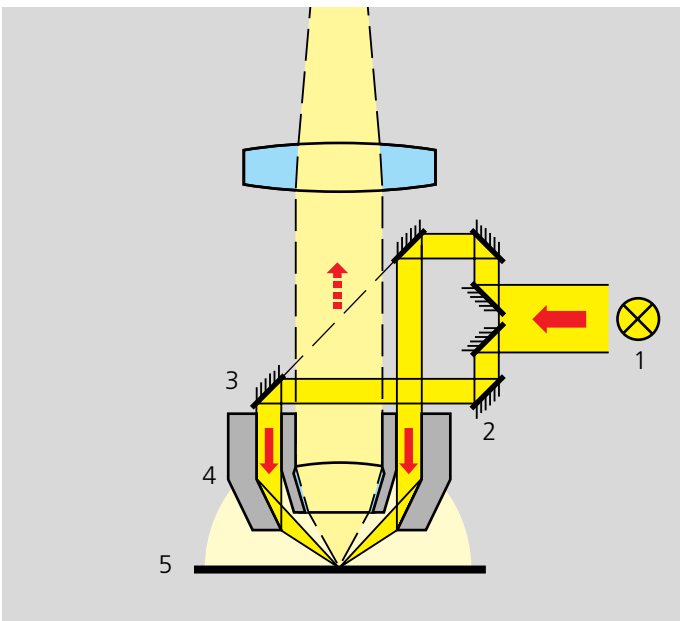


## Contrasting methods in reflected light:

### Darkfield in reflected light (DF)

This method is ideal for the inspection of surfaces. The light coming from the reflected-light illuminator is directed downwards towards the objective (4) via a mirror step assembly (2) and a mirror with an oval hole (3). Having passed the outer sleeve of the objective, it hits a ring-shaped concave mirror which directs the light rays for grazing incidence on the sample surface (5). If the object were a perfect mirror, no light would be reflected into the objective and the image would remain dark. Existing structures, however, direct light towards the objective and become brightly visible against a dark background.

**Important:** open the luminous-field and aperture diaphragms so that the light beam will illuminate the mirror assembly (2)!

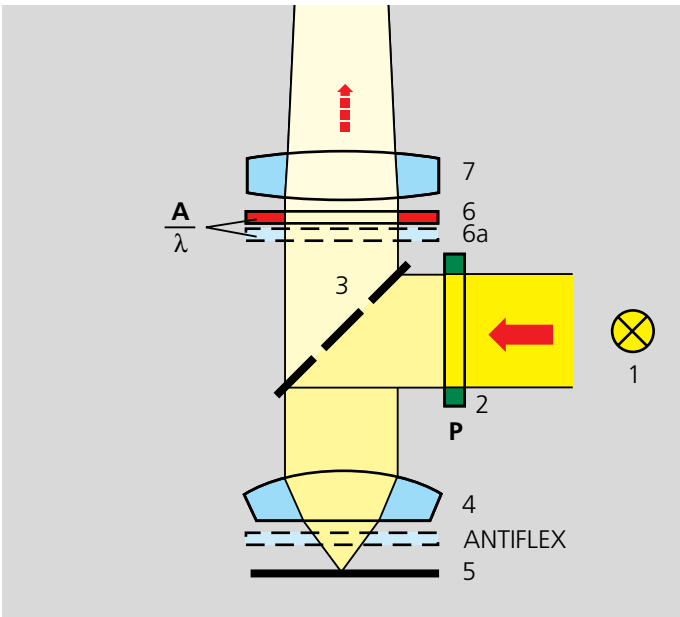


32.1

### Polarization contrast in reflected light (POL)

Suitable for surfaces with structures which change the state of polarization during reflection, e.g. structure grains in samples of ore. The illuminating light hits a polarizer (2,P) first and is imaged, linearly polarized, on the sample surface (5). Behind the beam splitter, it hits the analyzer (6,A), which allows only the depolarized portion of the light to reach the tube lens (7). As in transmitted light, an optional lambda plate (6a, $\lambda$ ) enables the changeover from grey contrast to color contrast.

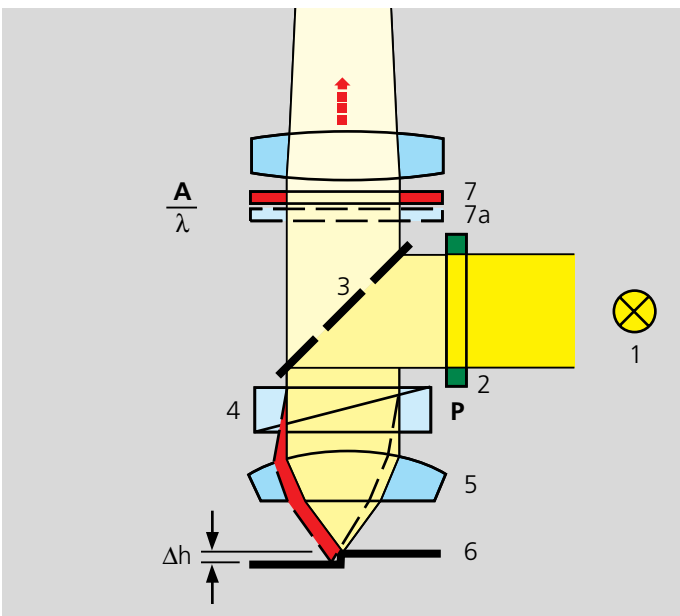
When objectives of a very low magnification are used, a so-called Antiflex cap (a rotatable  $\lambda/4$ -Platte in front of the objective) permits otherwise unavoidable reflection to be also eliminated from "dark" sample surfaces.



32.2

### Differential Interference Contrast in reflected light (DIC)

As an extension of polarization contrast, this method also allows the visualization of minute elevation differences in surfaces. A birefringent prism (4) is used, which splits the polarized light beam into two partial beams on its way to the sample. These partial beams hit the sample (6) with lateral displacement from each other. If the surface is completely flat, nothing will happen. However, if there is a small step between the two partial beams, one of the two beams must travel a path which is  $2\Delta h$  longer and is assigned this path difference. Once the partial beams have returned via the DIC prism (4) and the analyzer (7), they feature the same vibration direction again – due to the analyzer – and can interfere with each other in the intermediate image. The path difference assigned on the surface then changes into grey values which can be seen by the eye: steps become visible as a relief. As an auxiliary object, the lambda plate (7a) changes the grey values into colors again.



32.3



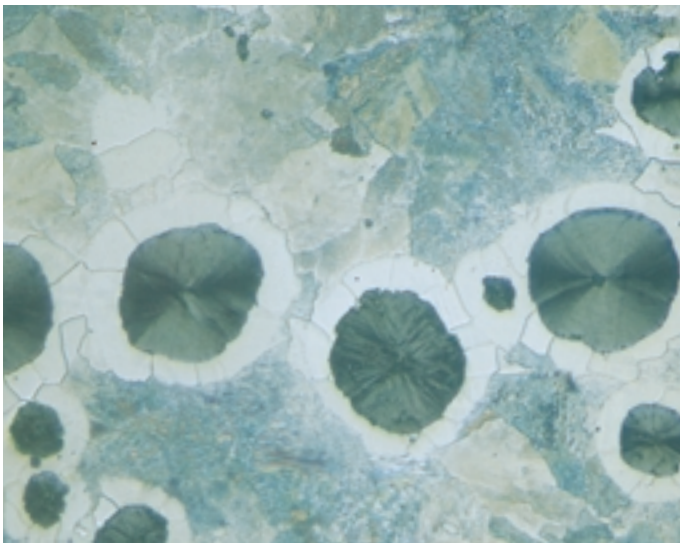
33.1

In reflected-light brightfield, the fine structures in the iron casting are difficult to see.



33.2

Only in reflected-light darkfield they are clearly visible in the surface.



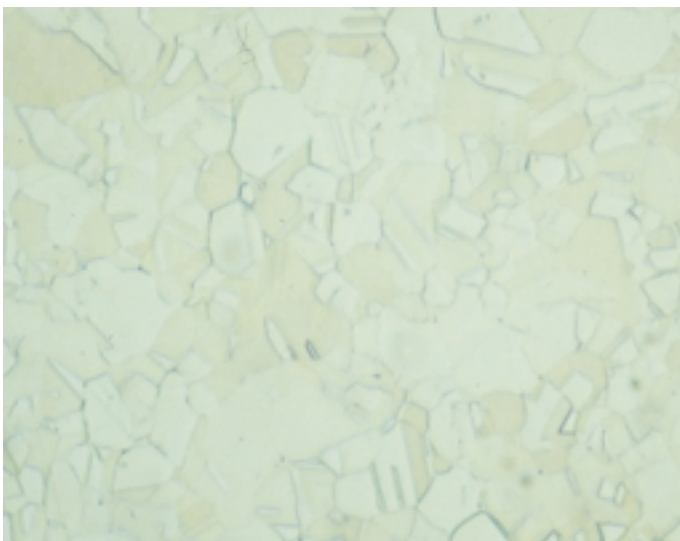
33.3

The inner structure of these spherulites is barely visible in reflected-light brightfield.



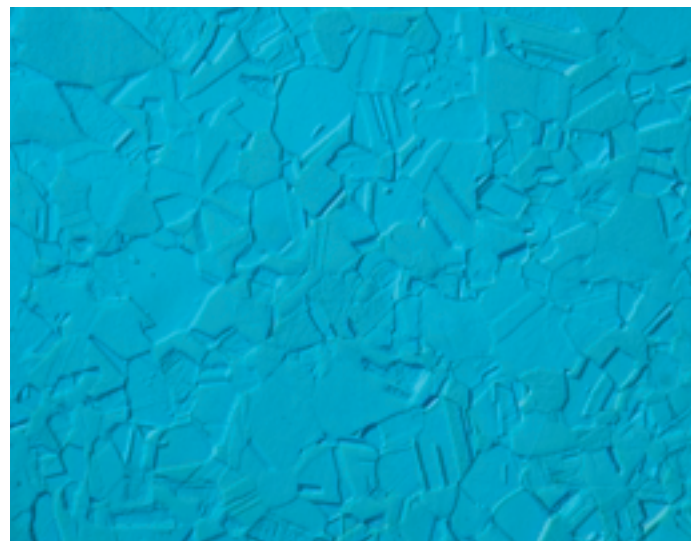
33.4

The structure in the polished iron sample becomes clearly visible in polarization contrast.



33.5

The structure of this brass sample can be seen only very faintly in reflected-light brightfield.



33.6

The same sample surface looks like a three-dimensional relief image in Differential Interference Contrast (DIC).

## Ready for all eventualities: “Separable” optics.

There are hundreds of problems which can be solved by using the light microscope. In addition, the microscope also has to meet a number of demands made by the users regarding performance and purchase prices. As a result, a wide variety of separable optics is available, mainly consisting of condensers (1), objectives (2) and eyepieces (3).



34.1

One criterion in the purchase of a microscope is the required field of application, another is the state of correction of the optical components, in particular the objectives. Single lenses have very poor image quality. Pronounced aberrations will occur which can only be corrected by the intelligent use of several lenses. The design and production of optics for perfect color rendition and with large, flat fields of view at the same time is very difficult and time-consuming.

## Putting the specimen in the right light: the condenser

Let us follow the path of the light in the microscope and begin with the condenser. As mentioned in detail before, the condenser illuminates the sample and images the luminous-field diaphragm on the specimen and the aperture diaphragm on the objective pupil. Without condensers, the microscope cannot achieve its maximum resolving power, and homogeneous illumination of the images is barely possible either.

The standard version of the condenser for the Axiolab microscope is designed as an “Abbe condenser”. This condenser type displays good quality in the imaging of the diaphragms and has one major benefit: one fixed front lens suffices to supply the objectives 4x to 100x with light – in compliance with the Köhler principle. Even with the simple version, the homogeneously illuminated fields are large enough to allow the use of eyepieces for fields of view with diameter 20 mm in the intermediate image. For objectives with a magnification of 2.5x or lower, an auxiliary lens can be used below the condenser which helps to illuminate the then very large fields in the specimen.



34.2

This condenser, for its part, is available in two versions; the simple brightfield version is shown on the left of Fig. 34.2, the version with turret disk for phase contrast and darkfield diaphragms on the right. The rotatable turret disk features two precise stops to enable fast changeover between the contrasting techniques.

The dual condenser – also called pathology condenser – is not shown in the illustration; it features a sturdy switchover lever to allow the fast changeover between low-power observation and the viewing of details. However, the dual condenser has only been designed for use in brightfield.

Optimum quality is achieved with the aplanatic-achromatic condenser systems which are used mainly in research.

The traditional term “aplanatic” is often mistaken as a description of the image flatness (“plane”). In fact, however, the ancient Greek verb *πλανειν*, meaning “to wander around”, is its root. The negating prefix “a” expresses exactly the opposite. The aplanatic condenser makes the light beams intersect exactly in the object plane. (The light beams do not “wander around”). An image of the luminous-field diaphragm is thus produced with edge-to-edge sharpness.

Achromatic means “free from color” (from *χρῶμα*, Greek for color) and indicates the state of correction which is high for a condenser.

## Perfection in miniature: Microscope objectives for every application.

While there is a limited number of condensers available, the very great number of objectives reveals the great diversity of microscopy. Here, we try to classify the objectives according to their state of optical correction. The two main criteria are the elimination of chromatic errors and the flatness of the intermediate image – which, when perfect, provides an image with edge-to-edge sharpness, even with large fields of view. Furthermore, the objectives can be classified into transmitted-light and reflected-light versions. The first type is always designed for use with cover slips (0.17mm). Reflected-light objectives – with the prefix “*Epi-*” – feature specially coated glass surfaces to avoid reflection in the optics (“anti-reflection coating”).



35.1

All the objectives mentioned here are members of the family of *ICS-Optics* (ICS: Infinity Color-corrected System). These objectives project their images to “infinity” first. Only the tube lens produces an intermediate image – to be more precise, at a distance of approx. 164.5 mm behind the tube lens. This distance was chosen to comply with the classical tube length.

### These are the main benefits of the ICS optics:

- The light rays in these “*infinity optics*” run parallel between the objective and the tube lens. Filters, reflectors, plane-parallel components can be used without the need for additional optics, which would only be disturbing.
- Objectives and tube lens together produce the final – fully-corrected – intermediate image. Classical microscopes with “*finite optics*” require the eyepieces to perform a lot of additional compensation work. Hence the name “compensating eyepieces”.

## Identical features of all ICS objectives:

The **parfocal length**, i.e. the distance between the specimen plane and the screw-on surface of the objective nosepiece, is always 45 mm. All ICS objectives can be arranged side by side in a nosepiece. The **screw-on threads** are standardized. Normally, the so-called **W 0.8 x 1/36** thread is available, while **M 27 x 0.75** is used for “thick” objectives, such as used for reflected-light darkfield.

## ...and their differences:

### 1. Achromat:

Good color correction – exactly for two wavelengths. Field flatness in the image center, refocusing also covers the peripheral areas. Designed for fields of view up to dia. 18 mm. Versions for phase contrast. Budget-priced objectives. Names: **CP-Achromat** (CP: Clinical Plan) and **Achrostigmat**.

### 2. Plan and Epiplan:

Improved Achromat objectives with good image flatness for fields of view with dia. 20 or even 23 mm. Therefore ideal for photomicrography. Names: **Achroplan** for transmitted light and **Achroplan Ph** for phase contrast. **Epiplan**, for reflected light, suitable for DIC, also as **Epiplan HD** darkfield objective. The reflected-light objectives feature good image contrast and safe working distances.

### 3. Plan-Neofluar and Epiplan-Neofluar:

Modern all-round objectives with excellent color correction for at least three wavelengths. Field flattening for the field of view with dia. 25 mm. Due to special glass types, highly transmitting for the important UV excitation at 365 nm (HBO illuminator) in fluorescence. All methods possible, special high-quality variants are available for Pol and DIC. Research class.

### 4. Planapochromat and Epiplan-Apochromat:

Absolutely top of the class: Perfect color rendition (correction for four wavelengths!) and, simultaneously, flawless image flatness for fields of view with dia. 25 mm. Highest numerical apertures for a resolving power at the very limits of the physically possible. For professional photomicrography. In a word: the Stradivaris of microscope optics.

### 5. Special objectives – only a small selection:

Fig. 35.1: 5a water immersion (electrophysiology)  
5b multi-immersion oil, water, glycerin  
5c transmitted-light darkfield with aperture iris  
5d UV-excited fluorescence: Fluor lenses

## For images of size: the eyepieces

Eyepieces (or oculars, from the Latin "oculus" = the eye) are the magnifiers with which you view the intermediate image in the microscope, produced by the objective and the tube lens. In the **Axiolab** microscope, the intermediate image has a useful diameter of 20 mm. Eyepieces are not just simple lenses, but are corrected optical systems consisting of several lenses. It would be a pity if the intermediate image produced with such sophisticated optics were to be impaired just before it reaches the eye.

Normally, the additional magnification provided by the eyepiece is 10x. The intermediate image then has a diameter of 20 cm at a reading distance of 25 cm to the eye. A comparison: this diameter is about as large as the width of this page.

The overall magnification of the microscope is easy to calculate:

$$M_{\text{Microscope}} = M_{\text{Objective}} \times M_{\text{eyepiece}}$$

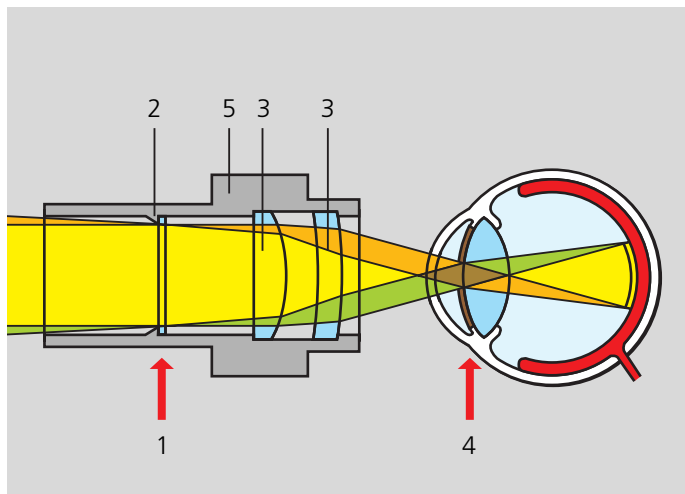
**M = magnification**

Example: *Plan-Neofluar* objective 10x/0.30  
PL eyepiece 10x/20 Br foc

The overall magnification is  $10 \times 10 = 100$ -fold

The schematic section through an eyepiece reveals the following:

- 1 position of the intermediate image – also position of a reticle
- 2 limit of the field of view: the "black edge" of the microscope image is produced here
- 3 eyepiece optics
- 4 position of the eyepiece pupil = pupil of the observer's eye
- 5 focusing ring for the diopter compensation



ICS eyepieces from Carl Zeiss have a good interpupillary distance (from the eye to the last lens surface). Therefore, eyeglass wearers can perform microscopy *with their glasses on*. The inscription "Br" (from the German "Brille = glasses) on the eyepiece informs them of this fact. Nevertheless, it is standard practice to use one of the two eyepieces for focusing, i.e. it is adjusted, since small focusing differences between the two eyes can then be compensated for. The eyepieces are labelled with a diopter scale (" + 0 - ") and "foc". If an *integrated reticle* is used, the red dot represents the zero mark because of the image shift which then occurs.

There are also different performance categories for the eyepieces. However, their differences can only be recognized with large fields of view – and particularly at the image edges. Even the standard PL eyepiece is very good: its name (PL = plane) indicates the good image flatness which it provides. E-PL eyepieces for perfect images up to the edges are outstanding in quality. Special S-PL eyepieces for a camera port pointing upwards are available for photomicrography (we shall return to this on page 38).

## Length measurements using eyepiece reticles

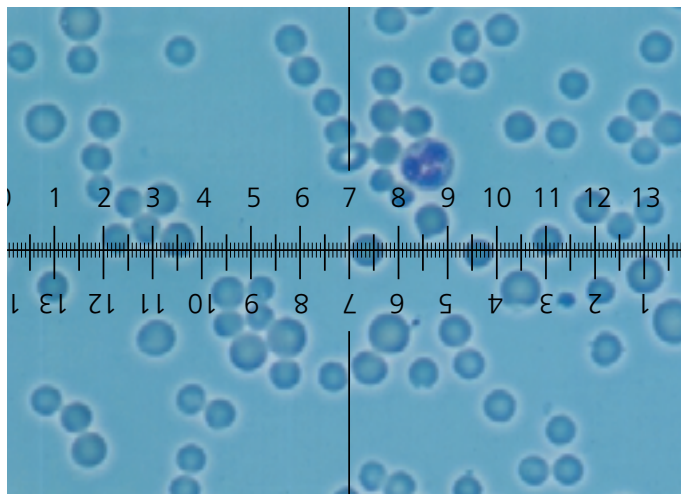
As you have seen, eyepieces have been designed in such a way that the intermediate image of the microscope is located *inside* them. Therefore, so-called reticles can be attached which contain scales, grids, or comparison patterns. The reticles are made of thin glass plates on which scales are etched. These scales are aligned exactly with the intermediate image plane and then become visible in the microscope image.

If the size of an object in the specimen is to be determined, the eyepiece reticle is used as the comparison scale. The very precise scale divisions have a fixed spacing, for example 1/10 mm. To determine the size of an object, size D appearing in the intermediate image – here 7/10 mm – must be divided by the magnification of the objective. The magnification of the eyepiece, however, is of no relevance, since it only becomes effective after the intermediate image.

The result of the division is the object size d:

$$d = D:M = 0.7\text{mm}:100 = 0.007\text{mm} = 7 \mu\text{m}$$

M is the magnification of the objective used.



### Accurate: calibration using a stage micrometer

If you want to have more exact information on the magnification, place a stage micrometer with an accurate scale – e.g. a reticle with 1/10 mm (100  $\mu$ m) increments – under the objective.

Microscope objectives deviate slightly from their specified magnification, and the stage micrometer permits correction factors to be measured for the objectives. All parameters measured with the eyepiece micrometer are then multiplied by the correction factors – which only have to be determined once – making them more precise.

In general, however, the reading error is larger than the inaccuracy of the magnification, particularly in the estimation of intermediate values.



37.1

### Convenient: eyepieces with digital micrometer screw

This accessory for the demanding user contains two reticles which are laterally displaced with respect to each other by a sensitive micrometer screw. Miscounting – which occurs every now and then when counting lines – is thus impossible. Furthermore, *any size between the lines* can be exactly determined. Also, a digital micrometer screw can be “zeroed” at the push of a button, eliminating the troublesome calculation of the difference between two readout values. The direct transfer of the measured values into a computer is also possible.

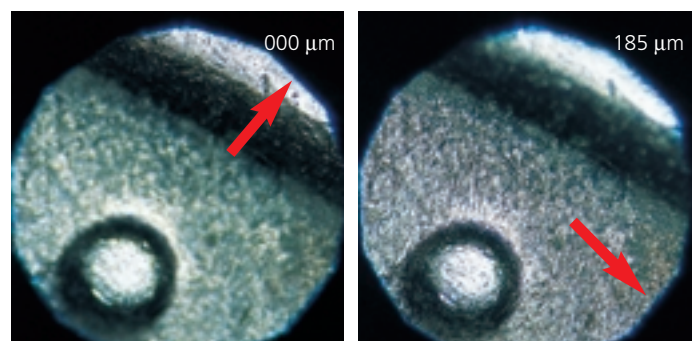


37.2

### Simple: measuring height differences in reflected light

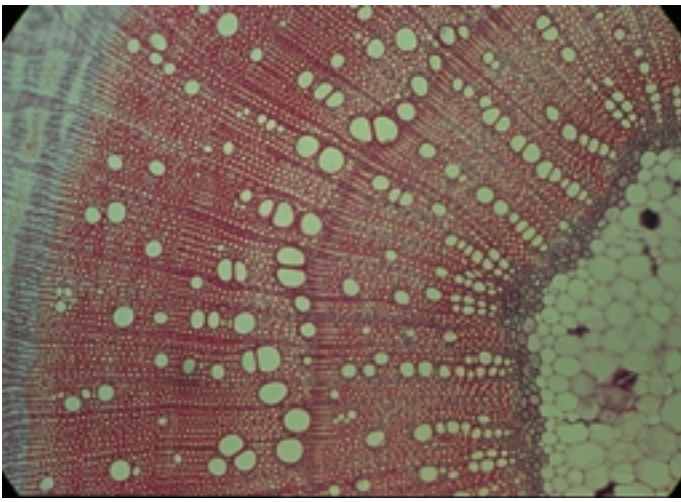
The third dimension of a microscope object is easy to determine in reflected light (usually brightfield), if it totals only a few  $\mu$ m. Objectives with a high numerical aperture are ideal for this purpose, since their “light cone” is opened widely and therefore permits very precise focusing. And this is how it is done: Close the luminous-field diaphragm to a small circle and focus the edge clearly visible in the image center on the reference plane. A dial gauge – or travel pickup – connected to the microscope stage is now zeroed. Focusing is then made on another plane – do not change the position of the stage and the luminous-field diaphragm – and the height difference can be read directly as a parameter. Fig. 37.3 shows a simple example of this.

In principle, this procedure is also possible in transmitted light, though without the use of the luminous-field diaphragm because the condenser moves with the stage. However, it is also possible to focus on object details one after the other. You'll be surprised that a cover slip seems to be 113  $\mu$ m thick with an air objective, while it is in fact 170  $\mu$ m thick! The explanation lies in the glass-to-air transition where the refractive index 1.51 of glass becomes effective:  $113 \mu\text{m} \times 1.51 = 170 \mu\text{m}$ !



37.3

The height of the rim of a coin (Dime, USA) is measured in reflected light by double focusing using the luminous-field diaphragm.



38.1

**Pictures to keep: Photomicrography**

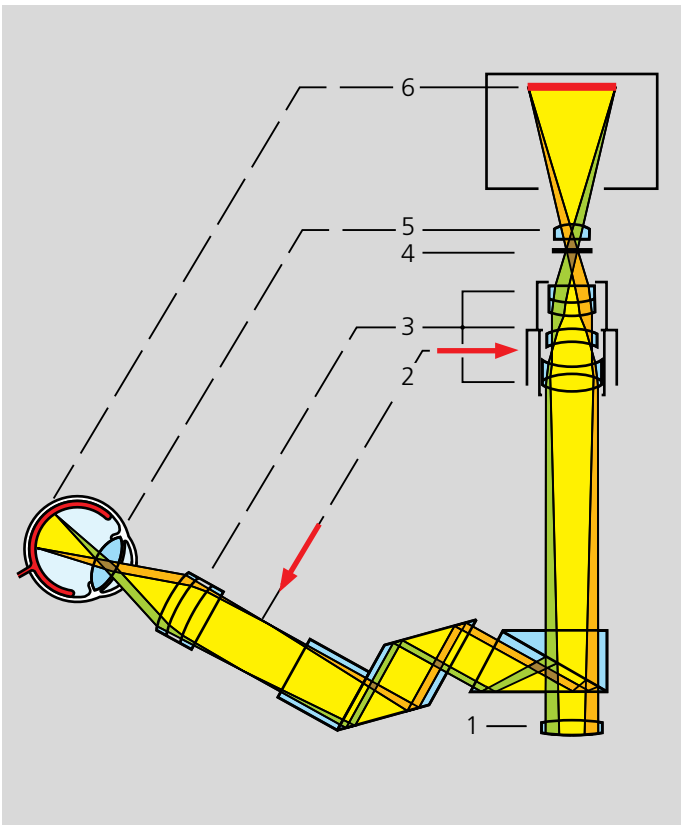
If you want to or have to document microscope images, the easiest way is to hold a standard camera to an eyepiece and to release the exposure. This will actually work, since the design of the camera is identical to that of the eye. The objective is the lens, and the film plane the retina. However, photos taken in this way will exhibit poor quality (see Fig. 38.1) because the optical alignment is not correct. In particular, the pupil of the camera lens is not illuminated, which results in unsharp images. The lateral magnification is much too small, i.e. the frame is not filled by the image; furthermore, the lens stop can also be a hindrance in the light path, not to mention the correct exposure.



38.2

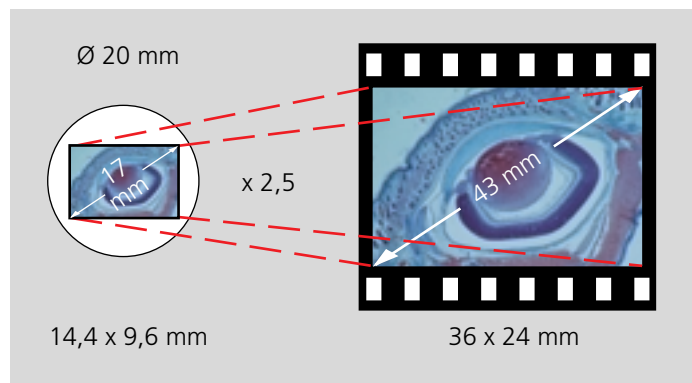
Therefore, the camera on the microscope has its own camera port. Fig. 38.2 shows the typical binocular photo/TV tube. In addition to the normal eyepieces, the tube contains a switchable beam splitter – a prism in most cases – which allows the entire light, or parts of it, from the tube lens to travel straight to the top to the camera/TV adapter. Here, the familiar intermediate image is formed.

The beam path for photomicrography in Fig. 38.3 shows the path of the light from the tube lens up to the film plane. The intermediate image (2) formed by the tube lens (1) and lenses of the photo eyepiece (3) is magnified by the photo eyepiece (3). For the camera lens (5), the light rays then look as if they have come from far away. If the central shutter (4) is opened for exposure, the objective produces a further – now much larger – intermediate image (6) in the film plane.

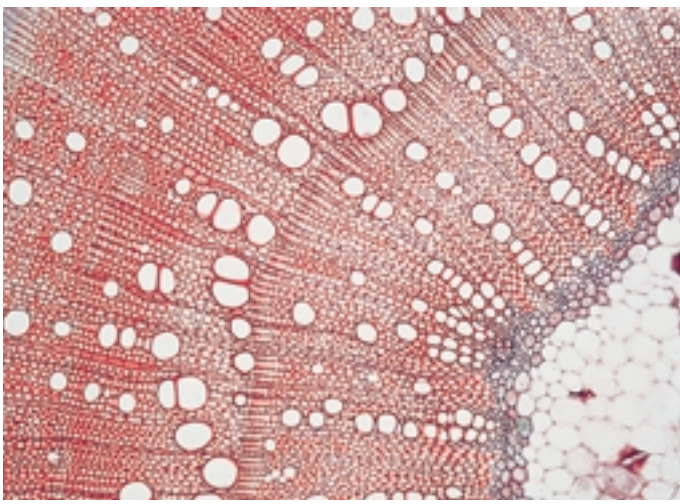


38.3

The new intermediate image produced by the photo eyepiece is now adapted to the film size. It is additionally magnified for 35 mm films with an overall factor of 2.5x. While the original intermediate image had a diameter of 20 mm, the diameter in the film plane now is  $20 \text{ mm} \times 2.5 = 50 \text{ mm}$ . The image diagonal in 35 mm films ( $36 \text{ mm} \times 24 \text{ mm}$  edge length) is approx. 43 mm, which is less than 50 mm.



38.4



39.1

The photomicrography equipment in Fig. 39.2 consists of a special camera connector with a photo eyepiece. This basic design is used with all camera equipment. As regards the choice of the camera, the following options exist:

### Simple and low-priced: the SLR camera

An SLR camera can be attached to the camera connector mentioned above via an adapter including a built-in objective. Such a camera contains everything that is needed for exposure metering and film transport. A variable shutter is available, the viewfinder can be used for image monitoring. Unfortunately, however, this simple version also has its drawbacks: The mirror "swings back" with every exposure and can transfer vibrations to the microscope. Critical images with very fine details might thus become blurred. The shutter of such cameras can cause similar interference, since it is moved in front of the film plane. An SLR camera should be released by remote control in order to avoid vibrations by manual operation. For the same reason, the film should be transported by a winder.

### For demanding photos and high operating convenience: Microscope cameras with changeable cassette

The problems just mentioned will not occur with microscope cameras in the MC series from Carl Zeiss. The entire control is performed via a separate panel with an integrated microprocessor. Large, illuminated displays and clearly arranged keys facilitate your work, particularly if you have to take a lot of photos. The camera features a completely vibration-free shutter, and the film cassette can be changed quickly. This is very useful if different users want to have a cassette of their own. Furthermore, the remaining exposure time, the frame number, the film type, and other information are displayed.



39.2



39.3



39.4

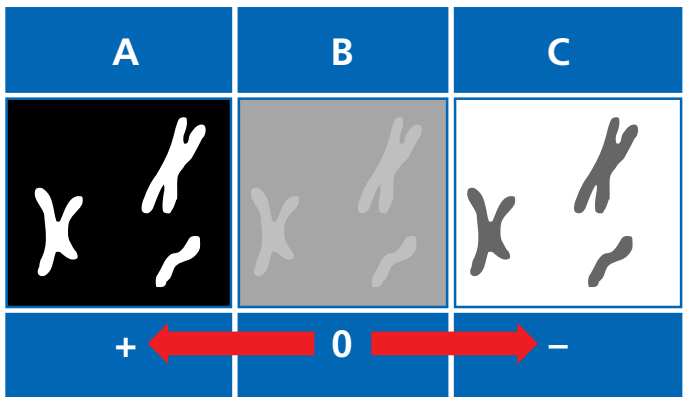




40.1



40.2



40.3

ISO-number	Sensitivity	Grain	Resolution	Exposure tolerance
25 - 50	low	fine	high	wide
100 - 200	medium	medium	medium	medium
400 +	high	coarse	low	low

## Photomicrography in practice

- If you do not need glasses or wear your eyeglasses for microscopy, always use one of the eyepieces without focusing (or a foc. eyepiece in the "0" position). This ensures that both the microscope camera and your eye see a sharp image.

- If you want to photograph very fine structures with a low or medium magnification, but cannot see the ideal setting of the focus, an aid is available: the auxiliary telescope. It is placed between the eye and the eyepiece and provides an additional magnification which makes the details appear greater. This considerably facilitates focusing for photomicrography. The auxiliary telescope must be focused to "the distance" before use, with your eyes being relaxed.

- Make sure you choose the correct color temperature when using color films. There are two types of both films for slides and for prints: daylight films and artificial light films. Daylight as the "natural" illumination includes many more blue components than artificial light, which is mainly produced by glowing metal filaments and therefore tends towards yellow-red. Film manufacturers provide suitable films for both types. Modern light microscopes, such as the **Axiolab**, feature the so-called 3200 K setting for the illuminator. This setting permits images on artificial light films to be produced without color distortion. However, if you use a daylight film, the yellowish light from the microscope illuminator must be changed to bluish light. This is done by a color conversion filter, which prevents a yellow tinge in the photo. You can simply place the filter on the opening above the luminous-field diaphragm (see the figure on the left).

- Exposure correction: exposure metering only provides correct values if the image is well mixed with grey values, as is the case in phase contrast, for example. In some extreme cases, metering provides the wrong values and "manual" correction is necessary. The illustrations on the left show a standard situation (B) and the extreme situations A and C:

Case A: Small, bright objects against a black background, typical of darkfield and fluorescence

Case C: Small, dark objects against a bright background; typical in some cases in brightfield

Find the optimum setting for such cases by performing various exposure series during which you prolong or reduce the exposure time in steps.

- Use the appropriate film material. The most important criterion is the ISO number which indicates the film speed. Other properties of the film material also change when the speed changes. The table on the left gives you a rough overview.

## More than just television: videomicroscopy

About 30 years ago, researchers tried to replace the heavy, slightly whirring 16 mm cine cameras by TV cameras – then also rather heavy. The movement of tiny organisms could now be shown “live” to many spectators. Moving pictures could be recorded on magnetic tape, which made the handling of photo documents much more easier. Only the so-called “C-adapter” has remained from the days of the cine cameras. The first TV cameras had one thing in common with the cine cameras: The pick-up tubes had an outer diameter of one inch, but an active target for image recording with a 16 mm image diagonal – identical to the film. TV cameras could therefore be attached to a microscope via the same adapter as cine cameras, namely via the C-adapter.

The tube technology is now a thing of the past, although it has survived in the names of the modern CCD sensors. Nobody could say that any part of the 1/2” CCD sensor actually is 1/2” in size. This all becomes clearer, however, when we remember the tube with the outer diameter of 1/2”, the target of which was only 8 mm in the diagonal. There is one thing, however, which the new semiconductor sensors have in common: their active areas are very small (1/3” CCD: image diagonal of only 5.3 mm). And that is where the problem starts: If these sensors are inserted directly in the intermediate image, they record only a fraction of the image visible in the eyepiece, i.e. only just about 4% in the case of the 1/3” sensor! Things were much easier with the good old 1” tube camera, since the image diagonal of 16 mm permits a good 37% of the area to be picked up in an intermediate image.

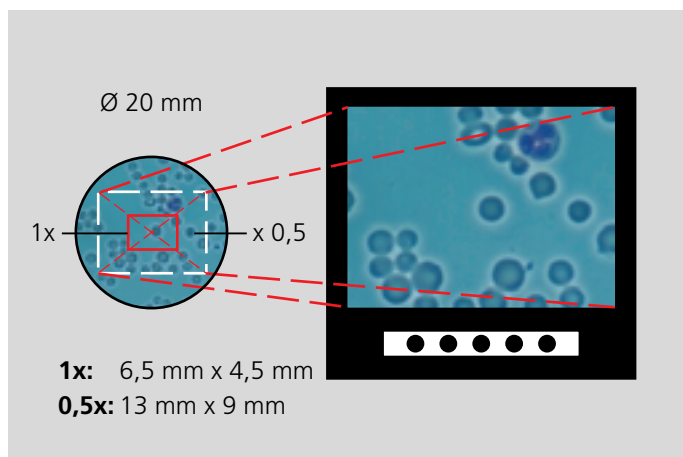
This means that the trend is the very opposite to that of photomicrography, where the image had to be additionally magnified by the factor 2.5x on account of the film size (diagonal: 43 mm).



41.2

Today, optical adapters are required if we want to see object sections which are bigger than a postage stamp. Absurdly, what has to be done is to reduce the size of the intermediate image which we have just magnified with such difficulty. Of course, color fidelity and resolution of the image should be retained, which means complex optics in the *TV adapters*. On the whole, the inevitably large adapter and the wonderfully small CCD camera sometimes combine to form units of a considerable size.

Meanwhile, some changes have taken place in videomicroscopy. Although TV microscopy is still used in lecture rooms for simultaneous observation by hundreds of viewers, CCD cameras, connected to video printers, are now much more widely used because this combination provides a fast and neat alternative to instant photography. Even the digitization of TV images for further processing and storage in computers has become very budget-priced. The trend regarding electronic image sensors in microscopy is moving away from the (low-price) TV camera towards the “Slow-Scan CCD sensor” which is more like a digital photo camera. The reason is evident: Why read out 25 or 30 images every second if *still picture videography* is of the essence? A somewhat slower scan, but with higher precision, with less noise and better resolution is then to be preferred.



41.1



42.1

### Reliable connection: adapters for TV cameras

For videomicroscopy, adapters with a fixed (reducing) factor are normally used. They contain several lenses, the surfaces of which are coated to prevent reflection. For the **Axiolab** microscope, an adapter with factor 0.5x is recommended for the 1/2" CCD camera in order to obtain a sufficiently large image section on the monitor or the video print (see Fig. 41.1). Another benefit of the reducing TV adapters is that they concentrate the light on the sensor: the 0.5x adapter produces a luminous intensity on the sensor which is almost four times greater than that achieved by the purely mechanical alignment with factor 1x.

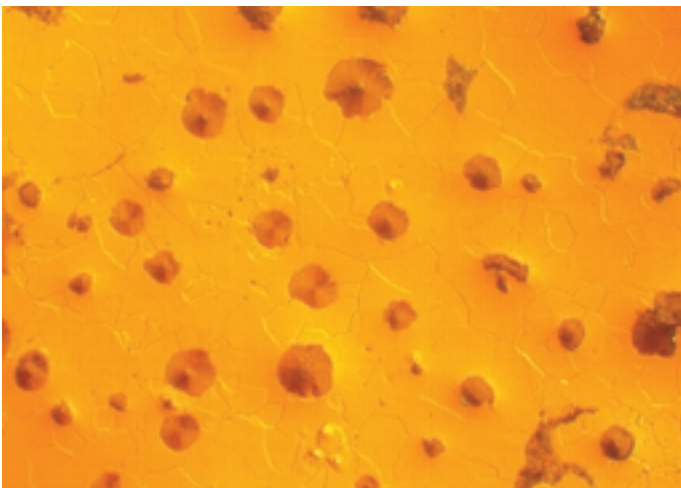


42.2

### Adaptable: the TV zoom adapters

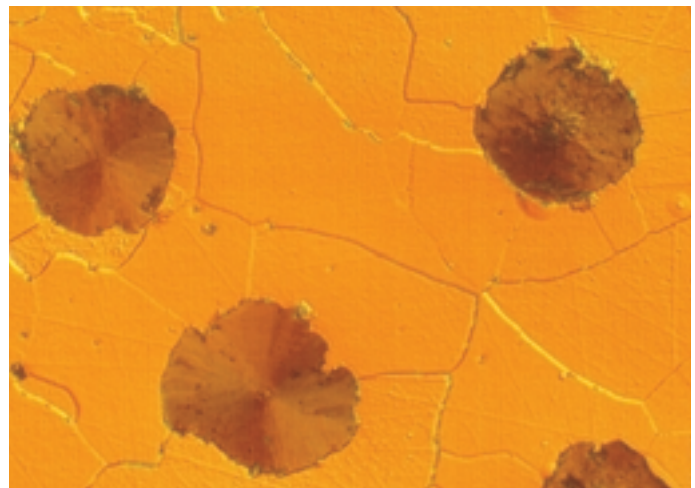
If you want to vary the image section on the monitor or the video print, the TV zoom adapter is the method to choose. For example, you can quickly and continuously vary the factor from 0.4x (for an overall view) to 2.0x (for the details). TV zoom adapters are available with the classic C-mount thread for 1-chip cameras or with ENG bayonet mounts (ENG = Electronic News Gathering, a term from the world of TV reporters who use only these cameras).

These variable adapters also have another benefit: If the format of the camera sensor or the video print is changed, the correct magnification factor can be reset. This applies to the standard magnification in metallography in particular.



Zoom 0.5x

42.3a



42.3b

Zoom 2x

## What does "magnification" actually mean?

TV images from the microscope sometimes appear to be enormous on the monitor. The total magnification is normally very high indeed and can be calculated very easily: it is the product of the optical and "electronic" magnification.

A. The optical magnification is:

$$M_{\text{Optical}} = M_{\text{Objective}} \times M_{\text{Adapter}}$$

B. The electronic magnification is easy to calculate from the ratio of the monitor diagonal to the diagonal of the active area of the image sensor.

$$M_{\text{electronic}} = \text{diagonal}_{\text{monitor}} / \text{diagonal}_{\text{sensor}}$$

C. The overall magnification then is:

$$M_{\text{overall}} = M_{\text{optical}} \times M_{\text{electronic}}$$

Example: an erythrocyte ( $\varnothing$  8  $\mu\text{m}$ ) is photographed using the **Achroplan** objective 100x. The TV adapter has the factor 0.5x:

$$M_{\text{optical}} = 100 \times 0.5 = 50 \times$$

The active sensor diagonal of the 1/3"-chip CCD-camera is 5.3 mm. The monitor in the lecture room has a useful image diagonal of 61 cm (= 610 mm).

$$M_{\text{electronic}} = 610 \text{ mm} / 5.3 \text{ mm} = 115 \times$$

The overall magnification then is:

$$M_{\text{overall}} = M_{\text{optical}} \times M_{\text{electronic}} = 50 \times 115 \\ = 5750 \times$$

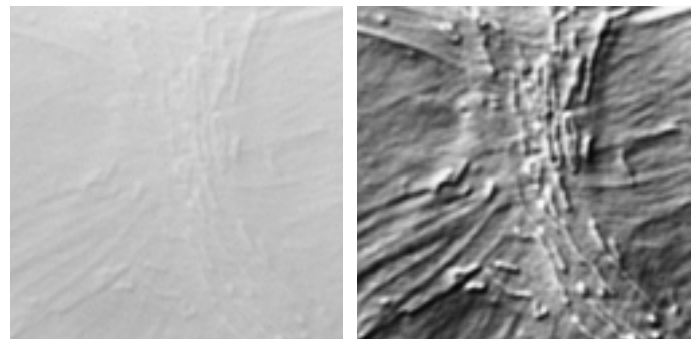
The erythrocyte appears on the screen with 8  $\mu\text{m}$  x 5750 = 46000  $\mu\text{m}$

or 46 mm =  $\varnothing$  4.6 cm

## Videomicroscopy for special applications

### Electronic contrast enhancement

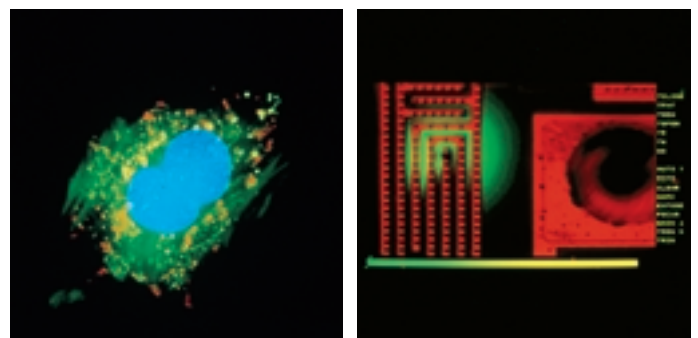
In TV cameras, the brightness information of an image is changed to an electric signal which can be influenced by means of amplifiers. If an image exhibits very poor contrast – e.g. a transparent single cell in a buffer solution – the contrast can be enhanced electronically. The first step is to subtract the DC component which corresponds to the homogeneously bright background. The weak residual signal then only contains the image modulation, which is amplified, resulting in many cases in a high-contrast image on the monitor (see the illustration on the right).



43.1

### Residual light microscopy

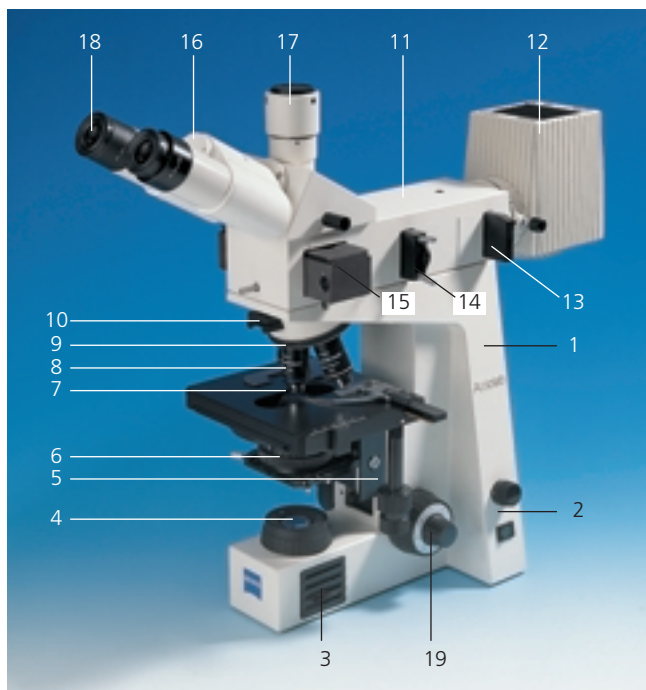
The marking techniques in modern fluorescence microscopy are becoming increasingly specific. On the other hand, however, the fluorescence images are becoming increasingly weak because sometimes only very small quantities of the fluorochromes are bound. So-called *image amplifiers* or, even better, cooled *integrated CCD sensors* are then the remedy; their sensitivity can exceed that of the human eye. The images on the right are examples from biology (fluorescence on a cytoskeleton, left) and from the semiconductor technology (ultraweak emission from active chip structures, right).



43.2

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- 1 Microscope stand
- 2 Power switch and light control
- 3 Built-in illuminator
- 4 Variable luminous-field diaphragm
- 5 Condenser mount with height adjustment
- 6 Transmitted-light condenser with variable aperture diaphragm
- 7 Specimen stage with object guide
- 8 Objective
- 9 Nosepiece
- 10 Filter mounts, in transmitted light on the observation side
- 11 Illumination equipment for epi-fluorescence
- 12 Lamp for epi-fluorescence
- 13 Filter slider for reflected-light fluorescence
- 14 Luminous-field diaphragm for epi-fluorescence
- 15 Reflector slider for epi-fluorescence
- 16 Binocular tube
- 17 Camera/TV port, switchable
- 18 Eyepieces
- 19 Focusing drive

**Color of the inscription:**

Contrasting method – see color chart above the objective. (A)

**Description of the objective:**

Objective category.  
Explanation on page 31 plus special descriptions, such as "LD" for "Long (working) Distance", and others.

**Magnification / Numerical Aperture:**

plus: additional information on  
– immersion medium ("W", "Oil", "Glyc")  
– Adjustable cover slip correction ("Korr")

**Tube length / Cover slip thickness (mm):**

ICS-Optics: "∞"  
Classical optics: "160"

Standard cover slip: "0.17"  
Without cover slip: "0"

**Color coding for the magnification:**

See the color chart below the objective (B)

**Mechanical focusing ring: only for special objectives**

Allows the adaptation of the optical correction for different immersion media and / or the thickness of cover slip / chamber bottom ("Korr"). Also with objectives with variable aperture iris, e.g. for darkfield.

**Type of immersion liquid:**

See the bottom color chart (C)

Other information on objectives *not* included in the inscription:

**Connector thread:**

W 0.8" or M 27

**Parfocal length:**

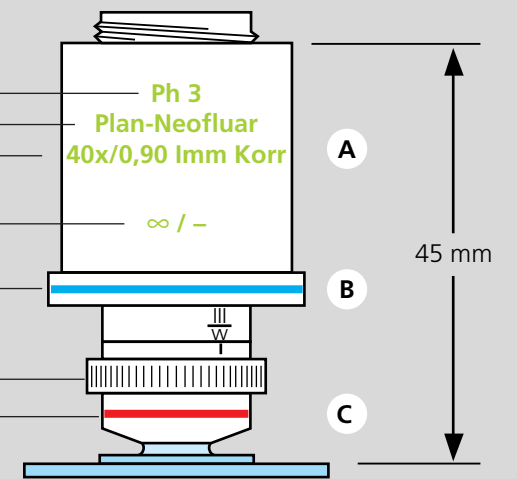
45 mm for all objectives  
Covers the distance from the screw-on surface to the object plane including cover slips.









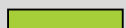








**Free working distances (AA):**

are indicated in mm and describe the distance from the tip of the objective to the specimen surface – or the cover slip surface, where applicable.

**Information by colors:  
The labelling of the microscope objectives**

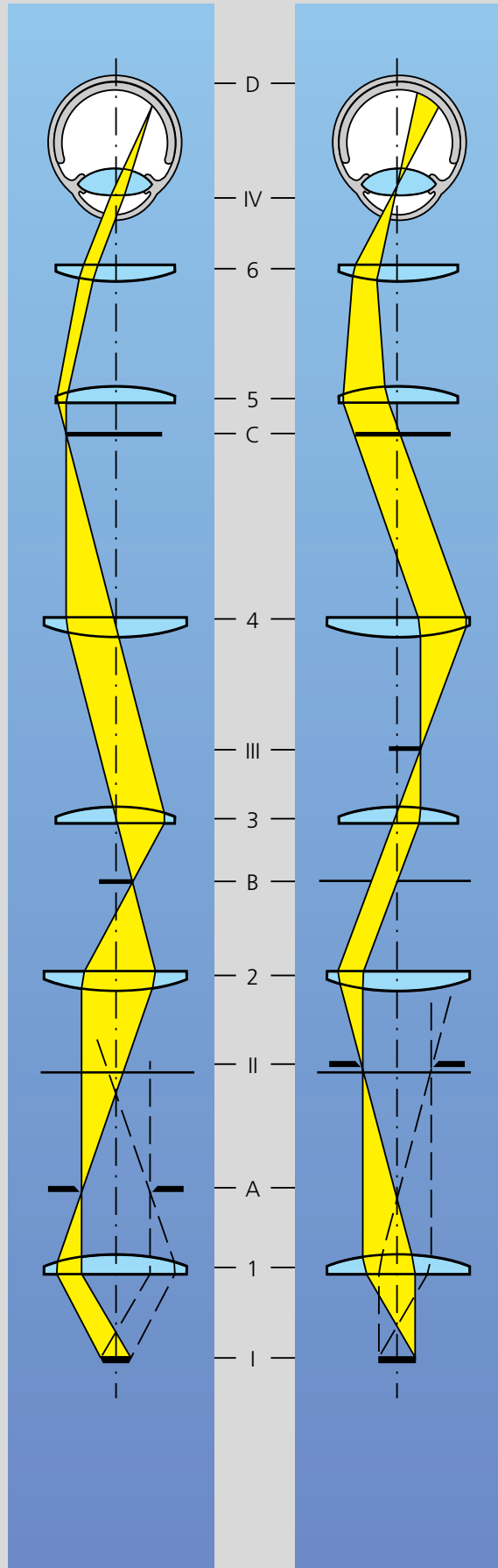
Standard		} A
Pol / DIC		
Ph 1.2.3		



1,25		} B
2,5		
3,2		
4		
5		
6,3		
10		
16		
20		
25		
32		
40		
50		
63		
100		
150		
200		

Oil		} C
Water		
Glycerine		
Multi		

**Schematic beam paths  
in a microscope with ICS optics  
(Infinity Color-corrected System)**



**Left: The image-forming beam path**

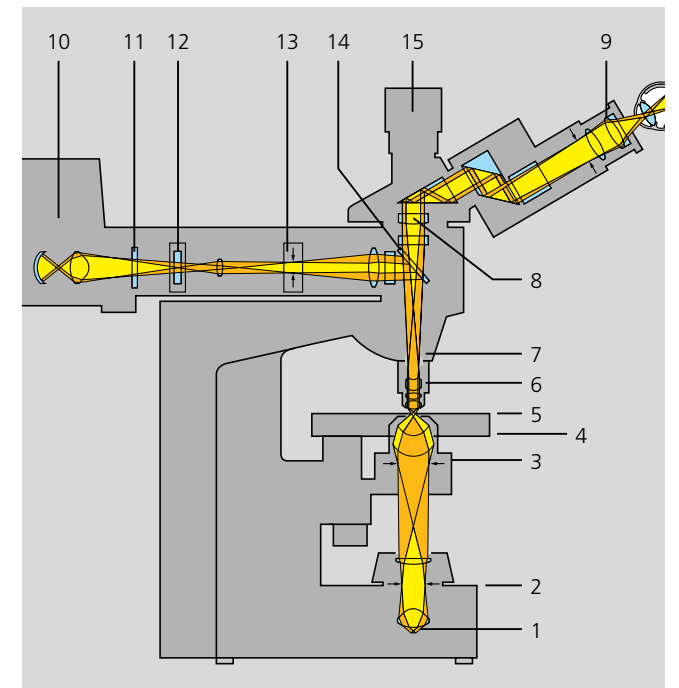
- A = Luminous-field diaphragm
- B = Specimen plane
- C = Intermediate image
- D = Retina of the observer's eye

**Right: The pupil beam path**

- I = Lamp filament
- II = Aperture diaphragm
- III = Objective pupil
- IV = Pupil of the observer's eye

**The main imaging components are:**

- 1 Collector
- 2 Condenser
- 3 Objective
- 4 Tube lens
- 5 Eyepiece



- 1 Built-in illuminator with collector
- 2 Luminous-field diaphragm for transmitted light
- 3 Aperture diaphragm in the transmitted-light condenser
- 4 Transmitted-light condenser (front optics)
- 5 Specimen plane (focal plane)
- 6 Objective
- 7 Pupil plane of the objective
- 8 Tube lens
- 9 Eyepiece
- 10 High-pressure lamp with collector
- 11 Heat-protection filter
- 12 Filter slider / Beam stop
- 13 Luminous-field diaphragm for epi-fluorescence
- 14 Filter combination with beam splitter for epi-fluorescence
- 15 Camera / TV adapter

We have now reached the end of this little handbook. Many subjects have only been touched upon briefly: photomicrography, polarization microscopy, complex microfluorescence and many other subjects would also have been worth looking at.

Inverted microscopes as modern workstations in cell biology or the stereomicroscopes, needed everywhere for three-dimensional vision, would be worthy of mention.

Nevertheless, we hope that the selection we have made has helped you to familiarize yourself with the world of microscopy. Should you have any further questions, please do not hesitate to contact us.

We from Carl Zeiss would be pleased to be of further assistance.

Further copies of this brochure can be ordered.  
Price: DM 19.80 DM per copy

Persons who are active in the microscopy training can order a set of 48 slides (35 mm). The set – without text – contains the most interesting pictures from the technical point of view, in particular the beam paths and illustrations of the optical principles.  
Price on request to:

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