# Beyond Brightfield: “Forgotten” Microscopic Modalities

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# Chapter 8

# Beyond Brightfield: "Forgotten" Microscopic Modalities

Radek Pelc

## Abstract

"Forgotten" microscopic modalities, devices, and accessories derived from or complementary to brightfield microscopy are briefly surveyed. These include off-axis illumination, schlieren contrast, Abbe diffraction apparatus, Rheinberg illumination, darkfield and phase-contrast microscopy combined, incident-illumination microscopy, *camera lucida*, and comparison microscopy. Examples of their use are shown. While most of them are no longer or only rarely available or used, they are still important for proper understanding of image formation, contrast generation, and data interpretation in microscopy. In some cases, they are superior to their more modern counterparts.

**Key words** Comparison microscope, Condenser, Conjugate planes, Darkfield, Incident illumination, Köhler illumination, Objective, Rheinberg illumination, Schlieren contrast

## 1 Introduction

The late professor David John Hugh Cockayne (1942–2010), former president of the International Federation of Societies for Microscopy, claimed[1] that it is vital to read the very *original* papers in the field. Ernst Abbe (1840–1905) is, of course, a classical example, as his main paper, although translated to English [1] shortly after the German original [2] was published in 1873, is very rarely quoted (forty times less often than the original according to Google Scholar [earliest citation dated 1996]). A possible explanation may be that over 98% of the English-speaking population can also read German with confidence . . . Whatever is the case, Colin J. R. Sheppard has recently done justice to Abbe in this sense [3]. It is worth noting that a number of previously popular microscopic modalities are either extinct or repeatedly "rediscovered," as exemplified in Subheading 2.

August Köhler suggested a new way of illuminating microscopic specimens [4, 5] some time after the introduction by Ernst

[1] 16th International Microscopy Congress (IMC16), Sapporo, Japan (3-8 Sept 2006)

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Abbe of a better condenser [6, 7]. Both of them worked jointly with Carl Zeiss in the newly founded company, Carl Zeiss AG in Jena, Germany. Köhler's main aim was to facilitate photomicrography so that homogeneous distribution of light intensity in the viewing field is achieved. Previously, the so-called "critical" (Nelson) illumination was more popular, and a ribbon filament lamp was the preferred light source [8].

The chief advantage of the Köhler illumination method is that the light source does not have to be homogeneous for the viewing field to be evenly bright, so that an ordinary light bulb can be used; the tungsten wire coil is not seriously degrading the image (a diffuser is sufficient to mitigate that). This can be achieved by positioning the light source in the condenser front focal plane. However, as this would result in exposing the specimen to excessive heat, it is more practical to place the light source further away, in an optically conjugate plane (Fig. 1); the collector lens (Fig. 2A, B) serves this purpose. The light source in the microscope is typically fixed, and optimal condenser position must be adjusted for each objective lens. This adjustment when changing objectives was easier in the so-called pancratic ("all-mighty") condenser that used to be part of Zeiss microscopes (e.g., NfpK or Amplival); the condenser position did not need to be vertically changed, and an internal zooming system was used to achieve Köhler illumination.

## 2 Off-Axis Illumination and Schlieren (Modulation) Contrast

An important implication of the Köhler illumination principle is that the condenser can be partly obstructed at its front focal plane (aperture diaphragm level), without introducing luminance inhomogeneity into the image. In a microscope properly adjusted for Köhler illumination, and its condenser diaphragm fully open (ideally matching the numerical aperture [NA] of the objective), an axially symmetrical light beam illuminates the specimen. Nonabsorbing objects such as unstained living cells are rendered in minimal contrast, and very thin ones such as filopodia or lamellipodia are hardly visible.

Decentering the condenser diaphragm, or asymmetrically obstructing it results in contrast enhancement. An example is shown in Fig. 2C, D obtained in a slightly different way, in that an accessory lens (rather than the diaphragm) of the condenser is offset. The contrast enhancement is greater in optical thicker objects. Condensers in microscopes made till ~1960s by Carl Zeiss (e.g., NfpK from 1960s) and Meopta (e.g., C36Bi) were equipped with a laterally shifting diaphragm (Fig. 2E) to achieve this effect [9, 10]. In author's experience, however, a shifting *straight-edge* diaphragm (Fig. 2F) is more efficient, and available

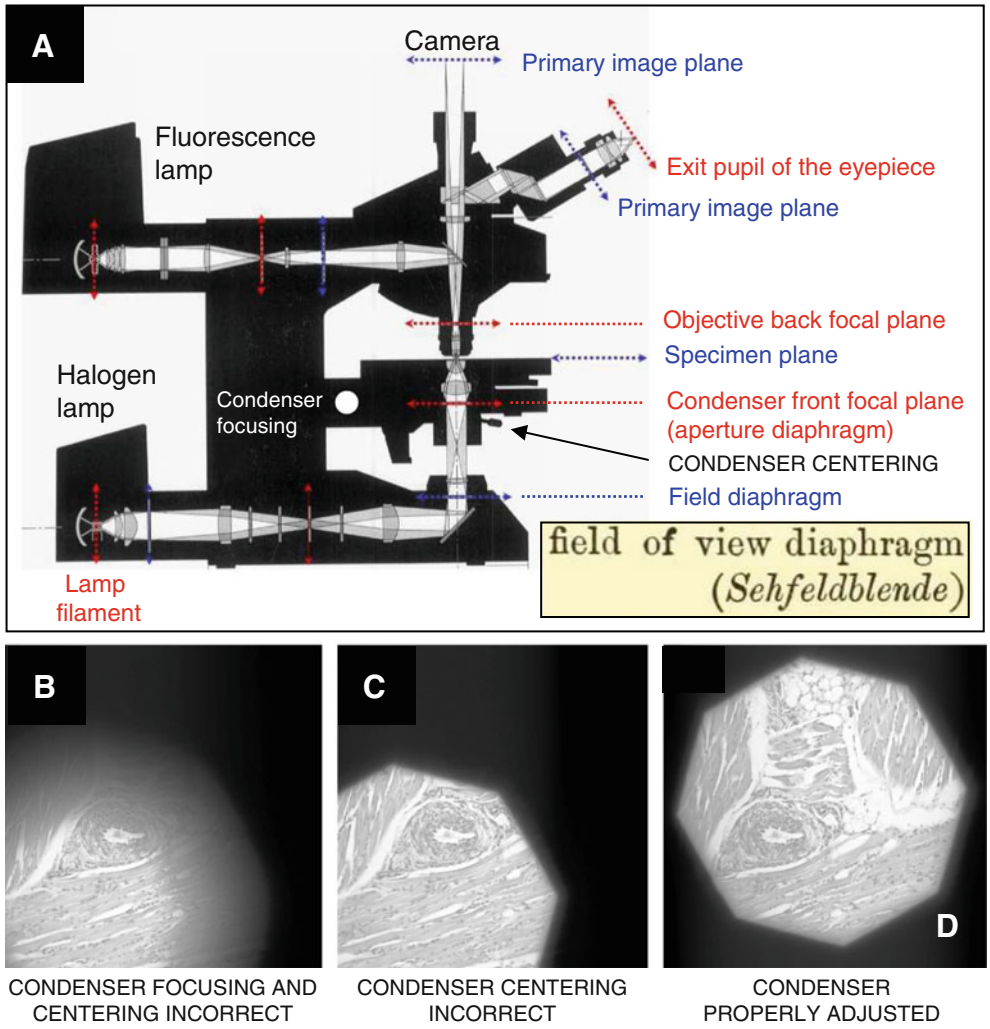


**Fig. 1** Optically conjugate planes of an upright microscope. (**A**) Imaging and aperture planes are marked in blue and red, respectively. Adapted from Ref. [39] by permission of © Elsevier. Note the original designation of the "field diaphragm" was "field-*of-view* diaphragm" (*Sehfeldblende* in German). The framed text is reproduced from Ref. [5]. (**B–D**) Field diaphragm as viewed through the eyepiece while centering the condenser (images by Dr. Lisa Cameron); see Appendix 2 for details

in the RCH condenser[2] made by Lambda Praha (originally Meopta, Czechoslovakia). Examples of its use may be found elsewhere [11, 12].

Image contrast can be further improved by employing another asymmetric diaphragm in the objective back focal plane (optically conjugate with the condenser diaphragm), the so-called schlieren diaphragm or modulator [13]. As surveyed elsewhere [11] this

[2] Relief contrast after Hostounský. Dr. Zdeněk Hostounský (1925-2014) was a protozoologist and insect pathologist at Czechoslovak Academy of Sciences in Prague, and a founding member of The Stentor Institute.

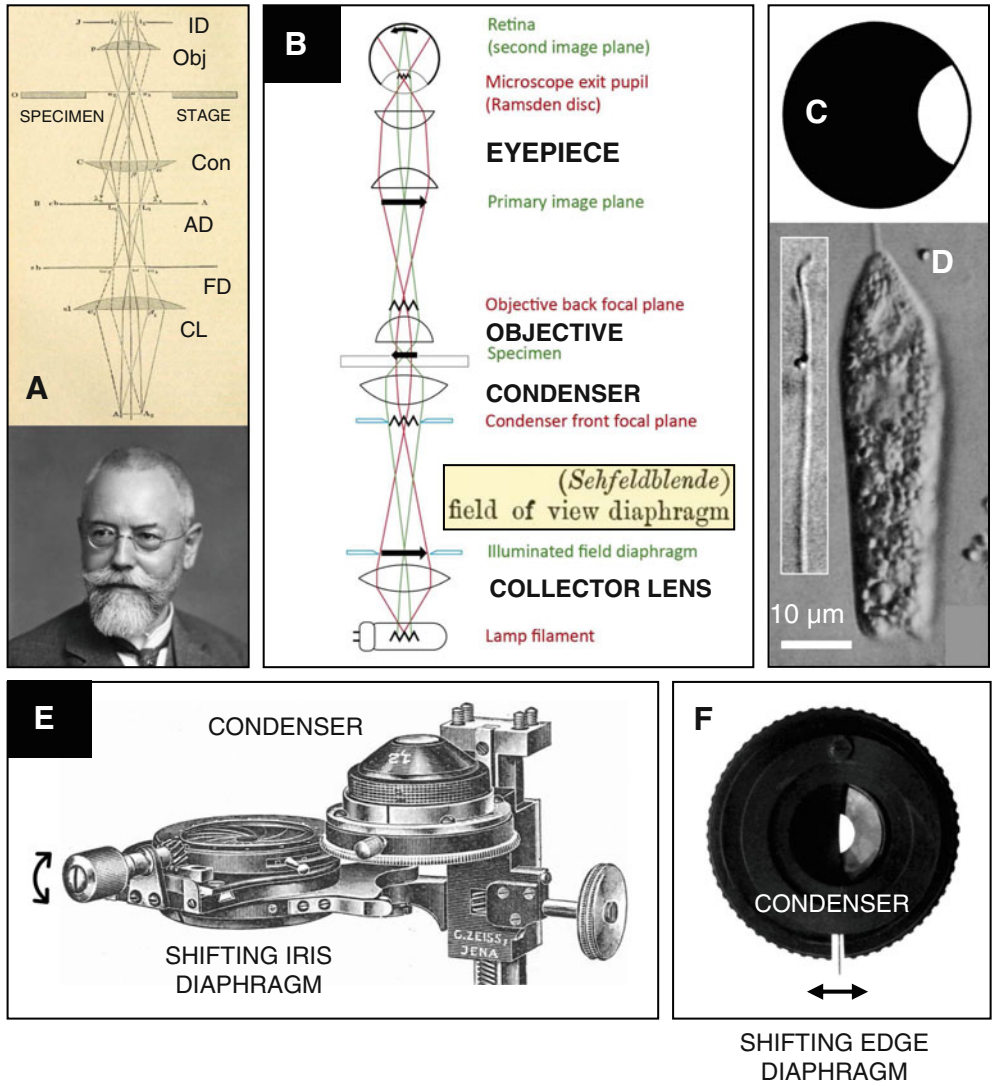


**Fig. 2** Adjustment of a microscope for Köhler and off-axis illumination. (**A**) Original diagram adapted from Refs. [4], [5]; copyright expired 70 years after author's death (August Köhler, 1866–1948). Köhler's portrait is as published by Zeiss Microscopy (Jena, Germany). *AD* Aperture diaphragm (in condenser), *CL* Collector lens, *Con* Condenser, *FD* Field diaphragm, *ID* Iris diaphragm (inside objective), *Obj* Objective lens. (**B**) Field (imaging) and aperture planes. Adapted from Ref. [40]; the origin of this drawing (recently shown in Ref. [41]) may be traced to Carl Zeiss materials at least 75 years old [10]. (**C**, **D**) Off-axis (oblique) illumination obtained by offsetting a condenser accessory lens. Reproduced from Ref. [42]. *C* Condenser aperture diaphragm viewed through a centering telescope. *D* Unstained protozoon (*Peranema trichophorum*) and its separately contrast-optimized flagellum, otherwise hardly visible in axially symmetrical illumination. (**E**, **F**) Off-axis (oblique) illumination setups utilizing a shifting diaphragm. *E* When needed, the shifting-iris diaphragm is swung into a working position under the condenser. Reproduced from Ref. [9]. *F* Dedicated off-axis illumination condenser with a shifting-edge diaphragm. Reproduced from Ref. [12]

simple modality was "rediscovered" approx. every 25 years since it was first published [14], most likely because the papers describing it usually lacked micrographs. Eventually, it matured into Hoffman modulation contrast [15], a direct competitor of a noticeably more costly differential interference contrast (DIC Nomarski) invented more than 20 years earlier [16]. Single-sideband edge-enhancement microscopy represents yet another variant [17, 18].

At this point, it should be emphasized that in images of greater optical thickness such as cell clusters, bigger cells or tissue replicas the setup employing no modulator (i.e., off-axis illumination) is more suitable (data not shown). In that case the objective aperture fulfills the role of a modulator to some extent [19] when the condenser diaphragm is open a bit more than required by the numerical aperture of the objective. This represents a slight departure from the optimal Köhler illumination (i.e., condenser diaphragm setting exactly matching the numerical aperture of the objective) as stray light may start contributing to image formation. However, this is usually more than compensated for by improved image contrast. It should be noted that the improvement is not very significant as the objective aperture is circular rather than straight (cf. the text above on the condenser shifting diaphragms (Fig. 2E, F)).

## 3 Abbe Diffraction Apparatus

The abovementioned modulation (schlieren) contrast relies on selectively filtering certain diffraction maxima (orders) at the objective back focal plane, with the aim to improve image contrast. In brightfield microscopy, there is no filtering. Darkfield microscopy, on the other hand, represents the other extreme, in that all direct (undiffracted) light, also referred to as the 0th order diffraction component, is blocked. Examples are shown in Subheadings 4 and 5.

A classical (the simplest) example of the importance of diffractive phenomena in microscopic image formation is shown in Figs. 3 and 4. Preventing the 1st and higher diffractive orders (maxima) from contributing to image formation results in complete disappearance from images of the structures (periodically spaced dots) represented by them (Fig. 3B). Likewise, merely increasing the illumination wavelength λ (e.g., by switching from blue to red light) renders previously visible structures invisible (Fig. 3C, D) as resolution is inversely proportional to wavelength (resolution $limit = \lambda/2\mathrm{NA}$ [1, 2]).

The first of these phenomena can be demonstrated with the aid of an objective with a built-in iris diaphragm. For example, the Nikon ×100/0.50–1.30 objective offers the option to gradually

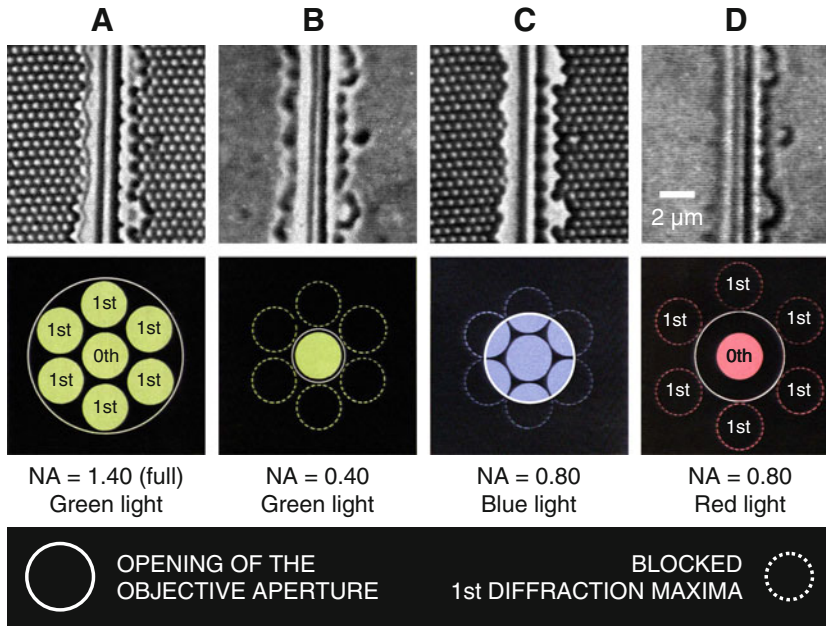


**Fig. 3** Diffractive nature of microscopic image formation. Diatom (*Pleurosigma angulatum*) viewed with a high-resolution, oil-immersion objective (×60/0.40 – 1.40) fitted with an iris diaphragm making it possible to adjust its numerical aperture anywhere between maximum (1.40 in A) and minimum (0.40 in B); an intermediate value (0.80) is shown in C and D. (**A**, **C**) Inclusion of the 1st order diffraction maxima makes the periodic structure visible. (**B**, **D**) Exclusion of the 1st order diffraction maxima renders the periodic structure invisible. (**C**, **D**) Dependence of resolution on wavelength: blue light resolves the structure, red light does not. Adapted from Ref. [43]

reduce its numerical aperture from 1.30 down to 0.50 (resolution worsens 1.30/0.50 = 2.6-fold).

In order to convince the scientific community that the diffraction theory of image formation [1, 2] is indeed valid [20] the so-called Abbe Diffraction Apparatus was designed, and later also made commercially available by Carl Zeiss [8, 21]. Nowadays, it may be occasionally found on eBay. Its components are shown in Figs. 4 and 5, including the effects on images of passing or blocking specific diffraction maxima (Fig. 4). False structures (lines nonexistent in the specimen) appear in the image if only the 1st diffraction maxima are blocked because the spatial frequency (line density) seemingly increases. One may say that the 0th and 2nd diffraction order rays "do not know" the 1st diffraction order rays are missing, and interfere with each other as usual, but this time forming a nonrealistic image.

Similar demonstrations have been described [22] and illustrated [23] in greater detail elsewhere, most extensively by Kurt Michel [24]. They would be surely of great benefit in microscopy training courses. Earlier, the equipment was referred to as the "Abbe Demonstration Microscope" [8] or the "Pulfrich-Abbe

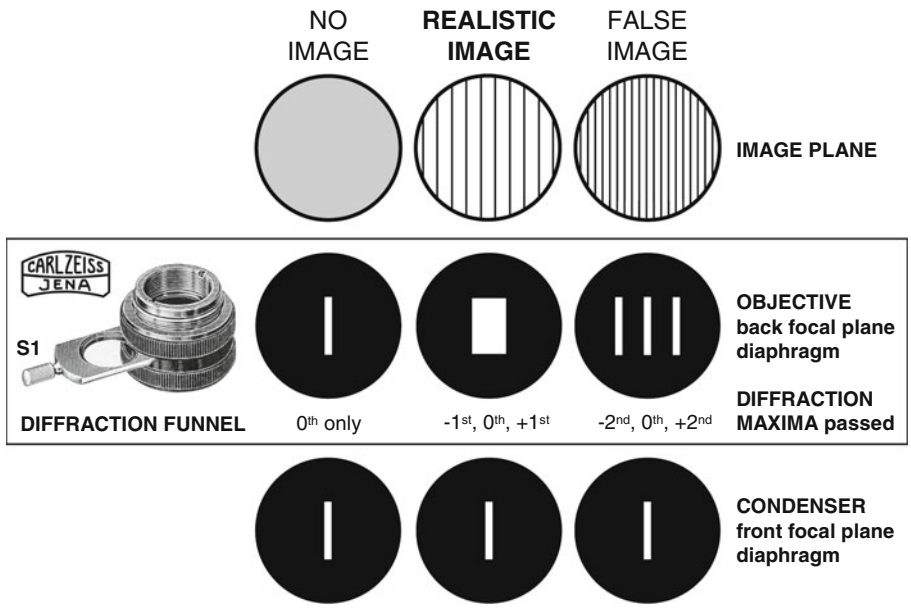


**Fig. 4** Abbe diffraction apparatus in action. This simple device used to be commercially available from Carl Zeiss at least since 1937. The objective back focal plane diaphragms are inserted into a slider (S1) in the "diffraction funnel" fitted above the objective of an upright microscope. Depending on which diffraction maxima are allowed to pass the image looks different (0th order maximum yields no image at all, passing only the second order maxima (i.e., blocking the first order one) results in twice higher density of the lines in the image (i.e., every other line in the image is an artifact). A hypothetical object consisting of vertical parallel lines is considered here, similar to those engraved in the "Diffraction Plate" that is part of the original equipment (Fig. 5). The diffraction funnel is reprinted from Carl Zeiss catalogue, "Der Diffraktions-Apparat nach Abbe" (Druckschrift "Mikro 11-432-1" dated 1940)

Demonstration Microscope" [25]. Please note that the phase plates shown in Fig. 5 were not included in the early models as phase-contrast microscopy was only invented in early 1930s.

## 4 Rheinberg Illumination

Until the invention of phase-contrast microscopy [26] darkfield and schlieren microscopy were dominating the realm of imaging unstained (nonabsorbing) objects. A modality combining darkfield and brightfield microscopy became known as Rheinberg illumination [27]. It can render objects under investigation in a color of choice, against a background of another color. This is achieved by using concentric color filters (Fig. 6) inserted into the condenser filter holder. If the central disc is black and sufficiently large the brightfield component is not present, and a darkfield image in a color of choice is obtained (Fig. 6C) [28].

A dedicated condenser called "Mikropolychromar" (Fig. 6A) used to be manufactured by Carl Zeiss since 1933 [8], and found its

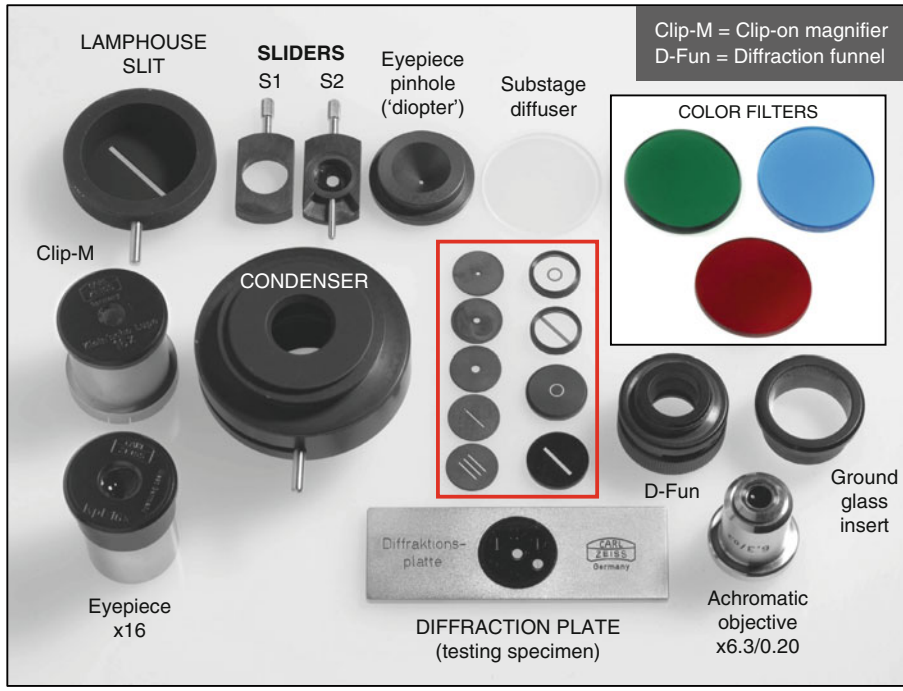


**Fig. 5** Abbe diffraction apparatus at rest. The "diffraction plate" hosts microscopic gratings as testing objects. The red box highlights (left) five aperture stops (masks), and (right) two phase ("retardation") plates and corresponding substage condenser diaphragms. Each of the masks and phase plates fits in the S1 slider inserted into the diffraction funnel (D-Fun) mounted above an objective. The S2 slider is fitted with a variable diaphragm, and enables experiments shown in Fig. 3 even if the objective is not fitted with the iris diaphragm. To directly examine the (intermediate) image of the specimen the eyepiece may be replaced with a ground-glass insert. The diffraction pattern generated by the specimen is inspected either with a clip-on magnifier (Clip-M) above the eyepiece (with an effect of either a centering ["phase"] telescope or Bertrand lens) or with an eyepiece pinhole ("diopter"). Details may be found in Ref. [21]. Image (unmarked) reprinted by permission of © The Trustees of the National Museums of Scotland (Edinburgh, UK) where this piece of equipment (made ca. 1970) is held

use, for example, in studying intracellular motility [29]. Eastman-Kodak Co. was supplying Wratten-Rheinberg filter sets for this microscope [8, 30]. Thread cells of hagfish slime gland [31, 32] could be conveniently visualized under Rheinberg illumination using custom-made color filters (Fig. 6D). It should be noted though that the Mikropolychromar is in fact a simple condenser, not a dedicated darkfield one, and as such only performs well at smaller magnifications. An excellent recent review about Rheinberg illumination is available [33].

A dedicated darkfield condenser capable of mixing brightfield and darkfield images was also made and referred to as the "Quick-Change-Over" condenser (Fig. 7A) [30], implying the ease of switching between brightfield and darkfield. The author has not encountered any images arising from its use. An interesting

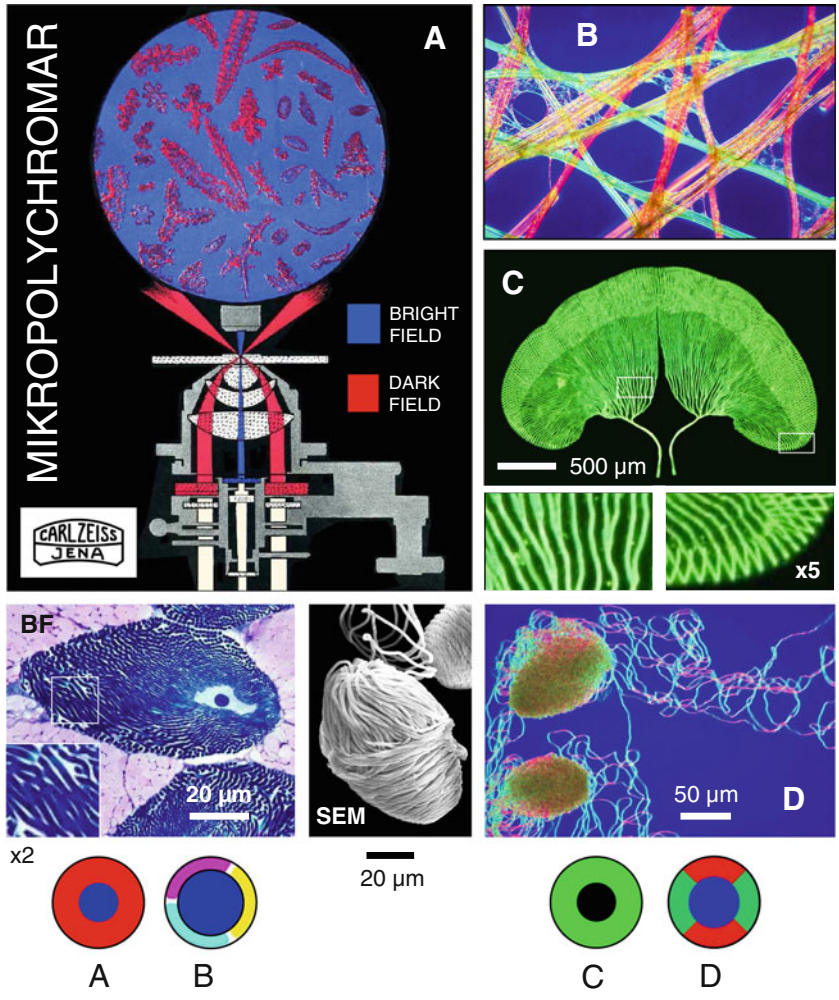


**Fig. 6** Rheinberg illumination. Color discs shown at the bottom (A-D) were used to obtain the images. (**A**) The Mikropolychromar condenser (aplanatic) capable of mixing brightfield and darkfield images. As it is not a dedicated darkfield condenser it is suitable for low-power objectives only (cf. Fig. 7a). Reproduced from Carl Zeiss catalogue, “Mikropolychromar” (Druckschrift “Mikro 493/II” from 1938). (**B**) Lens-cleaning paper. Original magnification ×50, image width ca. 2 mm. Courtesy of © Stephen W. Downing (University of Minnesota Medical School-Duluth Campus [Duluth, MN, USA]), originally presented in a 1980 photomicrography competition (https:/www.nikonsmallworld.com/people/steve-downing). (**C**) Proboscis of a house cricket (*Acheta domesticus*) in pure darkfield (the brightfield component is blocked). Courtesy of © Stefano Barone (Diatom Lab, Italy; www.diatomshop.com, www.testslides.com). Image originally presented in a 2014 photomicrography competition (https:/www.nikonsmallworld.com/people/stefano-barone), and a similar one elsewhere [28]. (**D**) Two thread cells of a hagfish slime gland, with the threads unwinding. Corresponding brightfield (*BF*) and SEM images are shown. Courtesy of © Stephen W. Downing (University of Minnesota Medical School-Duluth Campus [Duluth, MN, USA])

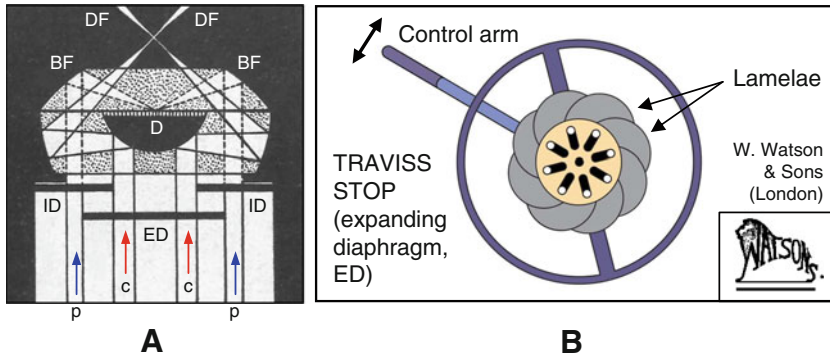


**Fig. 7** Darkfield microscopy aided by Traviss stop. (**A**) Dedicated darkfield condenser for mixing darkfield and brightfield illumination. It used to be manufactured by Leitz, and marketed as the "Quick-Change-Over" condenser. *BF* Brightfield. *C* Central rays for darkfield illumination. *D* Diffusely reflecting surface generating brightfield illumination. *DF* Darkfield. *ED* Expanding diaphragm (Traviss stop). *ID* Iris (aperture) diaphragm. *p* Peripheral rays for brightfield illumination. (**B**) The Traviss stop made the use of a darkfield condenser more straightforward, even though originally not part of the type shown here. Image A is adapted from Ref. [10]. Image B is based on Ref. [34] (copyright expired 70 years after author's death; Edmund J. Spitta, 1853–1921 [20]), as adapted in Ref. [44] (reprinted by permission of © Macmillan Magazines/Springer)

accessory of darkfield condensers is the so-called Traviss expanding stop, essentially an iris diaphragm working in reverse (Fig. 7B). Jointly with the standard iris diaphragm, light annulus of any diameter and thickness can be produced. This is helpful in correctly adjusting darkfield illumination [34]. The Traviss stop used to be manufactured by W. Watson & Sons (London) [8].

## 5 Heine Condenser and Incident Illumination

The examples shown in the previous section (Rheinberg illumination) illustrate the capabilities of darkfield microscopy. A more advanced option was available in the form of the Heine condenser (Fig. 8A, B). This was a variant of the cardioid condenser (the shape of its mirror is derived from the cardioid curve), that is, a dedicated darkfield condenser performing well not only at small magnifications. Additionally, it enabled easy switching not only to brightfield but also to phase-contrast imaging [35, 36]. The cardioid condenser alone (inset in Fig. 8B), that is, without the phase-contrast modality add-on, makes it possible to visualize, for example, single unstained microtubules (Fig. 8C), and to follow their dynamic instability (Fig. 8D). Darkfield imaging provides better contrast in images of single microtubules than other suitable label-free modalities such as interferometric scattering [37] or interference reflection [38] microscopy.

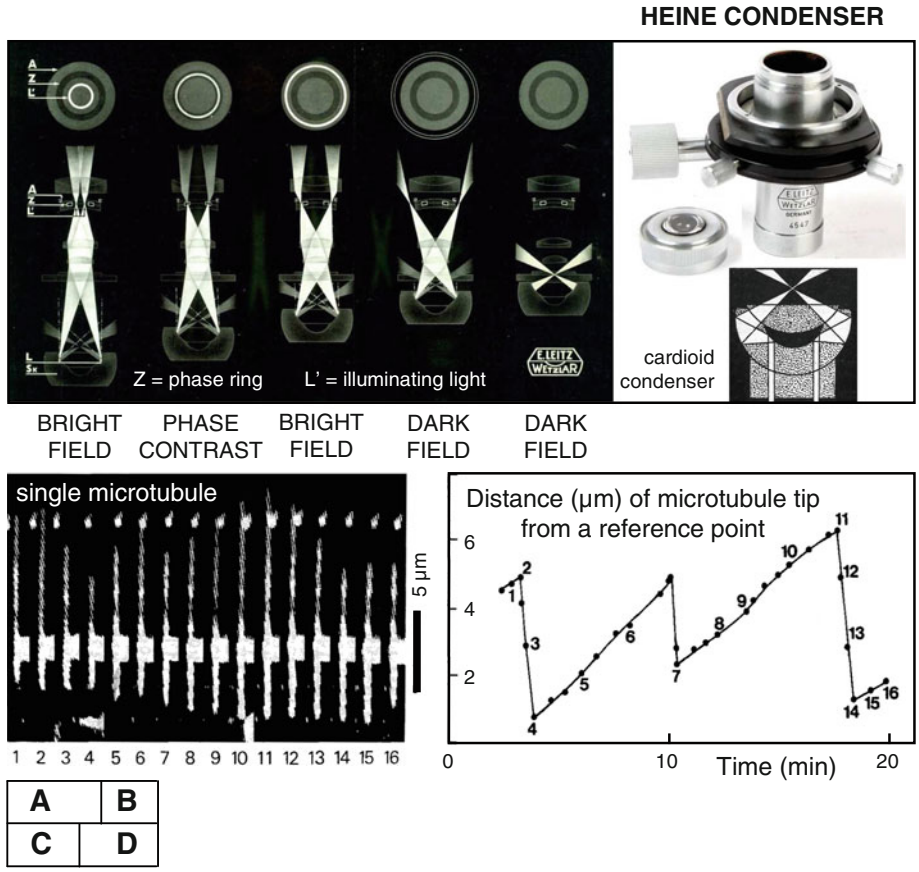


**Fig. 8** Heine condenser. (**A**, **B**) The Heine condenser is based on the cardioid (i.e., dedicated darkfield) condenser, and makes it possible to gradually change from one modality to another (brightfield, darkfield and phase contrast). The main image is reproduced from Leitz catalogue № 51.3-5a/Engl.–X/60/FY/L (early 1950s). The photograph of the condenser (complete with a screwable lens for use of immersion oil) was taken by Peter Höbel (Erlangen, Germany; http:/www.mikroskopie-ph.de/index-Heine.html). The ray diagram (bottom right) is reproduced from Ref. [10]. (**C**, **D**) Time-lapse imaging of a single unstained microtubule by darkfield microscopy, using the cardioid condenser (inset in B). Microtubule growth and shortening (dynamic instability) can be followed; microtubule tip is the plus end. Adapted from Ref. [45] by permission of © Nature Publishing Group (Springer)

More recently, a revival of the combined illumination scheme has been presented in an incident illumination setup (Fig. 9) better suited to inspect, for example, tissue surfaces. As it also provides darkfield and phase-contrast images, complementary image information can be conveniently obtained (Fig. 9B, C). It is inspired by the "Ultropak" device (Leitz) which did not offer the phase-contrast modality.

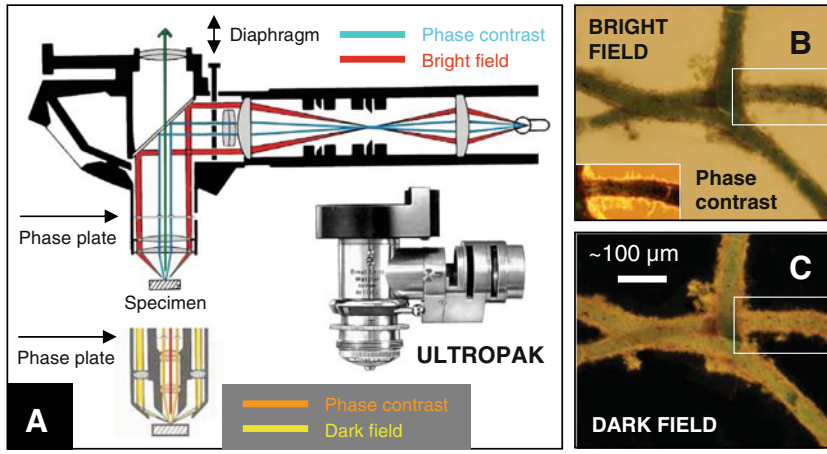


**Fig. 9** Incident illumination. (**A**) Vertical illuminators inspired by the "Ultropak" (Leitz) which was not fitted with a phase plate. Various modalities (color-coded in the image) and their combinations may be obtained, depending on the annular diaphragm(s) used (small, large or both). The drawings are adapted from Refs. [46, 47] by permission of © Cambridge University Press. The photograph is reproduced from Leitz catalogue № 513-36a/Engl (1965). (**B**, **C**) Filamentous algae in incident illumination; note complementary rendering in brightfield, darkfield, and phase-contrast. Reprinted from Ref. [46] by permission of © Cambridge University Press

## 6 Camera Lucida and Comparison Microscopy

The drawing attachment (tube) often referred to as *camera lucida*, literally "light chamber," used to be commonplace in microscopy laboratories even when photomicrography was already widely used. When using it, the microscopist is simultaneously viewing the cell under the microscope, and his/her drawing of that cell (Fig. 10A). Semi-transparent mirror (beam splitter) project the drawing into the eyepieces. Naturally, both images should be of comparable brightness, and built-in rotatable polarizing filters, for example, in camera lucida made by Carl Zeiss ("Zeichenapparat") facilitate that.

Nowadays, software is available to skeletonize cell images, that is, to convert their grayscale representations (typically 8-bit, 0 to 255) to line-drawing type images (one-bit models). Nevertheless, it is still often more convenient to draw the images manually with *camera lucida* (Fig. 10B). The drawings of neurons by Ramón y Cajal (1852–1934) of course represent a classical example.[3]

A need often arises to view two similar scenes (specimens) simultaneously. For this purpose, a so-called comparison microscope may be conveniently employed. It in fact consists of two separate microscopes connected with a special (dual) eyepiece

[3] The Beautiful Brain (Abrams Books 2017, ISBN: 9781419722271)

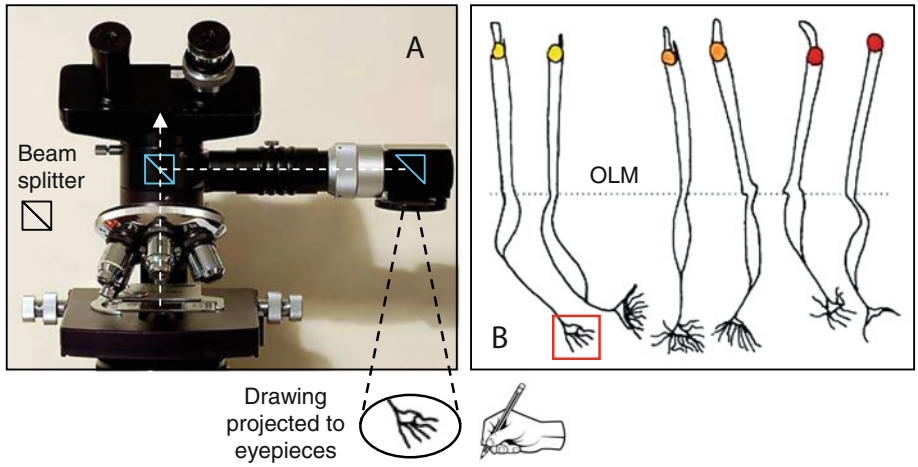


**Fig. 10** *Camera lucida* or drawing attachment. (**A**) Example of fitting to Wild M20-EB microscope. Adapted from Ref. [48] (© Ian Walker). (**B**) *Camera lucida* drawings of freshly isolated chick retina photoreceptor cells (cones) aligned by the outer limiting membrane (OLM). The color spots are oil droplets acting as color filters aiding color vision. Adapted from Ref. [49] (©López-López et al.)

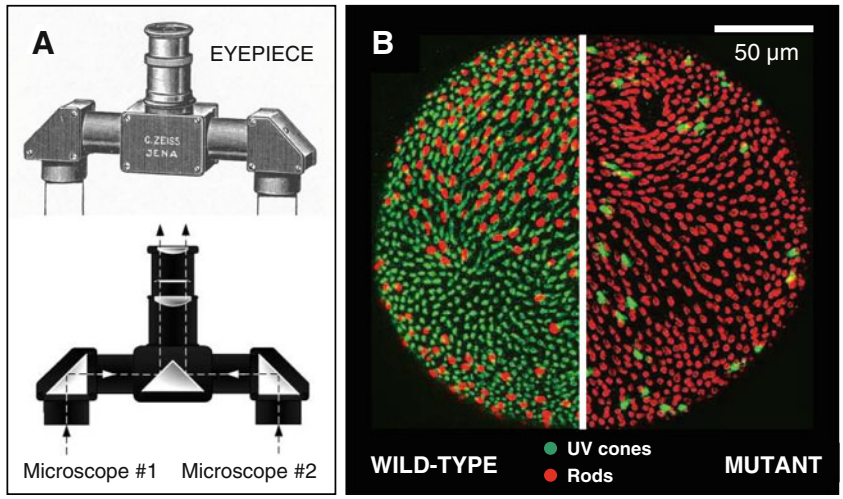


**Fig. 11** Comparison microscopy. (**A**) The comparison eyepiece fits two microscopes working in concert. The Zeiss drawing is reproduced from Ref. [10]. The optical diagram was drawn by Tamás Szőcs (https:/en.wikipedia.org/wiki/Comparison_microscope). (**B**) An example of use. As the author of the present chapter is not in possession of the comparison eyepiece separately obtained images are shown: Retinae of zebrafish larvae, wild-type versus lots-of-rods mutant (*lor*$^{p25bbt}$). Reproduced from Ref. [50] by permission of © Natl. Acad. Sci. USA

tube capable of merging the two images into one (Fig. 11A). An example of comparison microscopy is shown in Fig. 11B in which the retina of wild-type and mutant zebrafish larvae is presented.

As digital images can be easily acquired and displayed, the comparison eyepiece dating to at least 75 years ago [10] is

nowadays hardly encountered in biological laboratories. However, it is still useful in situations when only one digital camera is available, or when routine comparative observations are made. Indeed, dedicated comparison microscopes, inseparable from each other, have been commercially available at least since 1935. Examples of situations where comparative microscopy is useful include pathological, biological, forensic, and industrial laboratories specializing, for example, in the following [8]:

1. Examining tissue in health and disease
2. Identifying powdered adulterated drugs
3. Biological systematics (inspecting unknown vs. type specimen)
4. Identifying crystals, hair, or textile fibers from a crime scene
5. Comparing optical performance of two microscopes

## Acknowledgments

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## Appendix 1 (Hands-on Demonstration)

**Diffraction and Resolution**

Inspect a diatom specimen[5] using an objective fitted with a built-in iris diaphragm (e.g., Nikon ×100/0.50 − 1.30) at different settings (i.e., different *effective* numerical aperture, NA). In this way, it is possible to artificially reduce the objective's resolving power to the extent (NA = 0.40 in our example) that the finest details in the diatom image completely disappear (Fig. 3B).

Alternatively, use an ordinary objective (having no iris diaphragm) jointly with a diffraction funnel fitted under the ocular head of an upright microscope. Insert S2 slider (with a built-in iris diaphragm) into the funnel (Figs. 4 and 5).

Inspect the objective back focal plane using a centering ("phase") telescope or Bertrand lens[6] to monitor the diffraction maxima of different orders while closing and opening the diaphragm in (or above) the objective lens. Typically, 0th and 1st

[4] CZ.02.1.01/0.0/0.0/16_019/0000729

[5] Available, for example, from Diatom Lab (www.diatomshop.com, www.testslides.com)

[6] The Bertrand lens is an extra focusable lens that, when inserted into the optical path, works in conjunction with the eyepiece to form a small telescope to give a magnified view of the objective back focal plane.

order maxima will be visible (the former representing direct or undiffracted light).

Note that the diffraction patterns may be clearly observed only with the condenser aperture diaphragm closed as much as possible (down to a "pinhole").

Illuminate the diatom specimen with light of different wavelengths. Blue light (shorter wavelength, ca. 450 nm) is more likely to resolve fine details than red light, ca. 700 nm) as the resolving power is inversely proportional to NA. For this effect to be sufficiently prominent, the objective (or S2 slider) iris controlling the effective NA needs to be set to an appropriate value (0.80 in the example shown in Fig. 3C, D), as checked by viewing the diffraction patterns in the objective back focal plane.

Be aware that different diatom species feature structures of different periodicities.

## Appendix 2 (Exercise)

**Setting Köhler Illumination**

(minor adaptation of a text originally written by Dr. Lisa Cameron, Light Microscopy Core Facility, Duke University, Durham, NC, USA)

Following is a step-by-step protocol for Köhler illumination with transmitted light. An interactive version is also available at the online Microscopy U(niversity)[7] and elsewhere[8]. For information on focusing the light source, please see the "Focusing the light source" section further below.

First, open all diaphragms. Raise the condenser to its highest point (on an upright microscope). Put a well-stained specimen on the stage, and inspect it with a low power (10×) objective.

Focus the objective lens to obtain a sharp image of the specimen by using the coarse and fine focus controls. This first step sets the correct relationship between the specimen and objective lens.

On a binocular microscope, each user may need to adjust the eyepieces for their own eyes for optimal focus. At least one eyepiece will have an adjustment collar. Use one eye to look down the microscope and focus on some detail in the specimen while keeping the eye which uses the eyepiece with adjustable collar closed. Then switch and use the other eye. Turn the adjustable eyepiece collar to focus the same detail in the image as sharp as before. This procedure is referred to as diopter adjustment and is recommended every time a microscope is used, for the sake of microscopist's visual comfort.

[7] https://www.microscopyu.com/tutorials/kohler

[8] https://micro.magnet.fsu.edu/optics/timeline/people/kohler.html

Setting Köhler illumination is possible without it if using one eye only.

Focus the condenser by first closing the illuminated field diaphragm and then adjust the height of the condenser with the condenser focus knob until a sharp image of the field diaphragm is seen superimposed on the image of the specimen (Fig. 1C). Make sure that the condenser diaphragm is wide open. This adjustment sets the correct relationship between the condenser lens and the specimen. If the microscope has an Abbe condenser, this image will likely have a fringe of color around the field aperture.

Center the condenser lens. To do this, make the image of the field diaphragm concentric with the field of view (Fig. 1D) using the condenser centering screws. This adjustment makes the optical axis of the condenser lens coincide with that of the microscope as defined by the field diaphragm and the objective lens.

Adjust the area of the field that is illuminated. Open the field diaphragm until its image is just outside the field of view; readjust the condenser centering if necessary (as you open it). This ensures that illumination falls only on the area of specimen within the field of view, and that the diameter of the primary image is only a little larger than the field-limiting diaphragm as seen by the eyepiece. This prevents light from falling on the internal walls of the microscope to be scattered to produce hot spots and haze, reducing contrast in the final image.

Adjust the aperture diaphragm (illuminating aperture) in the condenser. To do this, remove the eyepiece, or turn the Bertrand lens into position if available—look down the microscope tube from ca. 100 mm above the tube, and observe the back focal plane of the objective, the disc of light at the base of the tube. More conveniently, use a centering ("phase") telescope in place of the eyepiece, in the same way as during adjustment of the annular diaphragm for phase-contrast imaging. Close the aperture diaphragm until the image of the iris is approximately 70–80% of the viewing field (the aperture of the objective). Replace the eyepiece (or remove the Bertrand lens). The working (effective) aperture of the condenser is now slightly smaller than the aperture of the objective lens. Do not close the diaphragm too far; this will cause a serious deterioration in the quality of the image.

Adjust the brightness of illumination using the control on the lamp power supply, or by inserting neutral density filter(s). These are usually found along the base of the microscope between the lamp and the field diaphragm. The microscope optical adjustments or diaphragms should not be used to control brightness. This will adversely affect the quality of the image. For instance, if the condenser diaphragm is closed too much, the image will appear too contrasty, as refractile structures will be highlighted too much due to diffraction effects; and with it wide open, there will be glare due to stray light (internal reflections). The resolution is poor in both.

In a microscope with absolutely no internal reflections the setting is optimal when the *effective* numerical aperture of the condenser (adjustable by its diaphragm) matches the NA of objective in use. As such microscopes do not exist in reality the abovementioned setting of ca. 70–80% is recommended. Image contrast is slightly improved yet the diffraction artifacts thus introduced are minimal.

**For a higher power objective:**
Rotate the nosepiece to the 40× dry objective. Owing to parfocality of objective design, the 40× objective should be almost in focus after aligning the microscope for 10×; it was not the case in very old microscopes.

As before, focus and center the image of the field diaphragm using the condenser focus knob and the condenser centering knobs. The aperture of the field diaphragm will need to be readjusted.

Remove an eyepiece (ideally replace it with the centering ["phase"] telescope), or use the Bertrand lens to observe the back focal plane of the objective. Notice that the area illuminated for the low power objective is much smaller than the diameter of the back aperture of the 40× objective.

Adjust the condenser diaphragm so that the effective NA of the condenser is about the same as the objective NA.

**For a high-power oil immersion objective:**
Rotate the nosepiece so that a high-power oil immersion objective is near-vertical. Just before it is clicked into place, stop and add oil to the coverslip, as close as possible to the optical axis (light beam coming from condenser prealigned at smaller magnifications, see above). Be sure the oil droplet does not have any bubbles. Use immersion oil provided by the microscope manufacturer, as there are some slight differences. Ideally, the refractive index of the oil, coverslip, and objective lenses should be the same.

Click the oil immersion objective into place. The space between the front lens of the objective and the coverslip should now be filled with oil.

Remove an eyepiece (ideally replace it with the centering ["phase"] telescope) or use the Bertrand lens to view the back aperture of the objective. Open the condenser diaphragm to almost fill the objective aperture.

Replace the eyepiece (or remove the Bertrand lens) and observe the specimen. Adjust the field diaphragm until the edge just matches the field of view. Strictly speaking, the condenser should again be readjusted as above (focus and centering). However, switching from ×40 to ×100 objective will rarely misalign the condenser beyond tolerable limit.

**Focusing the light source:**
Remove the diffuser from the lamp housing or along the base of the microscope stand, if possible, in order to see bulb and filament. Lamp illumination should fill most of the front aperture of the condenser. Put a sheet of lens paper on the specimen stage to help visualize the area of illumination. Focus light on the lens paper by moving the lamp-focusing knob. Then remove the eyepiece (ideally replace it with the centering ["phase"] telescope) or insert the Bertrand lens to view the back focal plane of the objective. Be sure the lamp filament is centered and focused in the plane of the condenser diaphragm. Adjust the collector lens on the lamp housing.

N.B.: Many student microscopes and more recently released modern research ones do not have illumination bulb adjustments, but are designed to deliver even illumination.

## References


1. Abbe E (1874/1875) A contribution to the theory of the microscope, and the nature of microscopic vision. Proceedings of the Bristol Naturalists' Society (new series) 1 (Pt 2):200–261 (translated and commented on by H. E. Fripp). https://biodiversitylibrary.org/page/7506100
2. Abbe E (1873) Beiträge zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung. (Schultze's) Archiv für Mikroskopische Anatomie (Bonn) 9:413–468. https://doi.org/10.1007/BF02956173. BHL:13290331. https://biodiversitylibrary.org/page/13290331
3. Sheppard CJR (2017) Resolution and super-resolution. Microsc Res Tech 80(6):590–598. https://doi.org/10.1002/jemt.22834
4. Köhler A (1893) Ein neues Beleuchtungsverfahren für mikrophotographische Zwecke. Zeitschrift für Wissenschaftliche Mikroskopie und für Mikroskopische Technik 10 (4):433–440. https://biodiversitylibrary.org/page/3013561
5. Köhler A (1894) New method of illumination for photomicrographical purposes. J Roy Microsc Soc (volume not numbered, April issue):261–262. https://biodiversitylibrary.org/page/49603250
6. Abbe E (1873) Ueber einen neuen Beleuchtungsapparat am Mikroskop. (Schultze's) Archiv für Mikroskopische Anatomie (Bonn) 9:469-480. https://doi.org/10.1007/BF02956177. BHL:13290387. https://biodiversitylibrary.org/page/13290387
7. Abbe E (1875) A new illuminating apparatus for the microscope. Monthly Microscopical J 13:77–82. https://biodiversitylibrary.org/page/14276730
8. Needham GH (1958) The practical use of the microscope. Charles C Thomas, Springfield, IL. https://lccn.loc.gov/57010440
9. Metzner P (1928) Das Mikroskop, 2nd edn. Franz Deuticke, Leipzig/Vienna
10. Wolf J (1944) Mikroskopická technika. Vesmír, Prague
11. Hostounský Z, Pelc R (2006) An efficient way of high-contrast, quasi-3D cellular imaging: off-axis illumination. J Biochem Biophys Meth 68(1):23–30. https://doi.org/10.1016/j.jbbm.2006.03.016
12. Hostounský Z, Pelc R (2007) Phase-contrast versus off-axis illumination: is a more complex microscope always more powerful? Adv Physiol Educ 31(2):232–235. https://doi.org/10.1152/advan.00028.2006
13. Axelrod D (1981) Zero-cost modification of bright field microscopes for imaging phase gradients on cells: Schlieren optics. Cell Biophys 3 (2):167–173. https://doi.org/10.1007/BF02788132
14. Töpler A (1866) Ueber die Methode der Schlierenbeobachtung als mikroskopisches Hülfsmittel, nebst Bemerkungen zur Theorie der schiefen Beleuchtung. (Poggendorf's) Ann Phys Chem 127(4):556–580. [Ann Phys 203 (4):556–580]. https://doi.org/10.1002/andp.18662030405
15. Hoffman R, Gross L (1975) The modulation contrast microscope. Nature 254:586–588. https://doi.org/10.1038/254586a0

16. Padawer J (1968) The Nomarski interference-contrast microscope. An experimental basis for image interpretation. J Microsc (Oxf) 88 (Pt3):305–349. https://doi.org/10.1111/j.1365-2818.1968.tb00616.x
17. Bretschneider F, Teunis PFM (1994) Reduced-carrier single-sideband microscopy: a powerful method for the observation of transparent microscopical objects. J Microsc (Oxf) 175 (Pt2):121–134. https://doi.org/10.1111/j.1365-2818.1994.tb03475.x
18. Inoué S, Spring K (1997) Video microscopy: the fundamentals. Plenum Press (Springer), New York. ISBN: 978-0306455315
19. Murphy DB, Davidson MW (2012) Fundamentals of light microscopy and electronic imaging, 2nd edn. Wiley-Blackwell, New York. https://doi.org/10.1002/9781118382905
20. Bradbury S (1996) The reception of Abbe's theory in England. Proc Roy Microsc Soc 31 (4):293–299. http://www.microscopist.co.uk/wp-content/uploads/2017/04/The-reception-of-Abbes-Theory-in-England.pdf
21. Nuttall B (2008) Image formation in the microscope: the Abbe diffraction apparatus. Bull Sci Instr Soc 96:27–29
22. Fripp HE (1876/1877) On professor Abbe's experimental demonstration of diffraction spectra in the microscope. Proc Bristol Naturalists' Soc (new series) 2(Pt 1):24–38. BHL:7545726. https://biodiversitylibrary.org/page/7545726
23. Stephenson JW (1877) Observations on professor Abbe's experiments illustrating his theory of microscopic vision. Monthly Microscopical J 17:82–88. BHL:14157879. https://biodiversitylibrary.org/page/14157879
24. Michel K (1964) Die Grundzüge der Theorie des Mikroskops in elementarer Darstellung (Physik und Technik), vol 1. Wissenschaftliche Verlagsgesellschaft, Stuttgart
25. Wetzel RA, Needham GH (1928) Some photographs of optical images after Abbe. Washington Meeting of the American Physical Society (Washington, D.C., 20–21 Apr 1928). Phys Rev 31(6):1108. https://doi.org/10.1103/PhysRev.31.1108
26. Zernike F (1955) How I discovered phase contrast. Science 121(3141):345–349. https://doi.org/10.1126/science.121.3141.345
27. Rheinberg J (1896) On an addition to the methods of microscopical research, by a new way of optically producing colour-contrast between an object and its background, or between definite parts of the object itself. J Roy Microsc Soc (volume not numbered, August issue):373–388. https://biodiversitylibrary.org/page/49597776
28. Gautier C (2012) Proboscis ("tongue") of a cricket (*Gryllus campestris*) under Rheinberg illumination. The Scientist (Image of the Day, 30 Oct 2012) https://www.the-scientist.com/image-of-the-day/image-of-the-day-40285 and Nikon Small World https://www.nikonsmallworld.com/people/christian-gautier
29. Bünning E (1935) Einige Beobachtungen über die Körnchenströmung in den Staubfadenhaaren von Tradescantia, nebst Bemerkungen über die Verwendbarkeit des Mikropolychromars bei zellphysiologischen Studien. Zeitschrift für wissenschaftliche Mikroskopie und für mikroskopische Technik 52(2):166–172
30. Shillaber CP (1959) Photomicrography in theory and practice, 5th edn. John Wiley & Sons, New York (Chapman & Hall, London)
31. Downing SW, Spitzer RH, Salo WL, Downing JS, Saidel LJ, Koch EA (1981) Threads in the hagfish slime gland thread cells: Organization, biochemical features, and length. Science 212 (4492):326–328. https://doi.org/10.1126/science.212.4492.326
32. Downing SW, Spitzer RH, Koch EA, Salo WL (1984) The hagfish slime gland thread cell. I. A unique cellular system for the study of intermediate filaments and intermediate filament-microtubule interactions. J Cell Biol 98 (2):653–669. https://doi.org/10.1083/jcb.98.2.653
33. Havics AA (2014) Contrast methods in microscopy: Rheinberg illumination. The Microscope 62(4):157–169
34. Spitta EJ (1907) Microscopy. The construction, theory and use of the microscope. E. P. Dutton & Co, New York
35. Gottschewski GHM (1953) Über die Anwendungsmöglichkeiten der neuen Leitz-Phasenkontrasteinrichtung nach Heine. Zeitschrift für Wissenschaftliche Mikroskopie und für Mikroskopische Technik 61(4):185–200
36. James P (2003) The Heine condenser (Part 1 and 2). Micscape, issue 95 http://www.microscopy-uk.org.uk/mag/sep03ind.html and 97 http://www.microscopy-uk.org.uk/mag/nov03ind.html
37. Andrecka J, Arroyo JO, Lewis K, Cross RA, Kukura P (2016) Label-free imaging of microtubules with sub-nm precision using interferometric scattering microscopy. Biophys J 110 (1):214–217. https://doi.org/10.1016/j.bpj.2015.10.055

38. Mahamdeh M, Simmert S, Luchniak A, Schäffer E, Howard J (2018) Label-free high-speed wide-field imaging of single microtubules using interference reflection microscopy. J Microsc 272(1):60–66. https://doi.org/10.1111/jmi.12744
39. Rottenfusser R (2013) Proper alignment of the microscope. Meth Cell Biol 114:43–67. https://doi.org/10.1016/B978-0-12-407761-4.00003-8
40. Bradbury S, Evennett PJ, Haselmann H, Piller H (1989) RMS dictionary of light microscopy (RMS Handbook №15). Oxford University Press and Royal Microscopical Society, Oxford. ISBN: 978-0198564218
41. Sanderson J (2019) Understanding light microscopy. Wiley (RMS series), Hoboken, NJ. ISBN: 978-0470973752
42. Wessenberg H, Reed MK (1971) The use of oblique illumination in microscopic observations of living protozoa. Trans Am Microsc Soc 90(4):449–457. https://jstor.org/stable/3225459
43. Michel K (1943) Grundzüge der Mikrophotographie. In: Zeiss Nachrichten, Sonderheft (special issue), vol 4, 2nd edn. Kommissionsverlag Gustav Fischer, Jena, pp 1–192
44. Amos WB (2000) Lessons from the history of microscopy. Nat Cell Biol 2(8):E151–E152. https://doi.org/10.1038/35019639
45. Horio T, Hotani H (1986) Visualization of the dynamic instability of individual microtubules by dark-field microscopy. Nature 321 (6070):605–607. https://doi.org/10.1038/321605a0
46. Piper T, Piper J (2012) Variable phase dark-field contrast – a variant illumination technique for improved visualizations of transparent specimens. Microsc Microanal 18(2):343–352. https://doi.org/10.1017/S1431927612000153
47. Piper T, Piper J (2013) Variable phase bright-field contrast – an alternative illumination technique for improved imaging in transparent specimens. Microsc Microanal 19(1):11–21. https://doi.org/10.1017/S1431927612013323
48. Walker I (2005) Wild M20-EB microscope. Micscape, issue 122 http://www.microscopy-uk.org.uk/mag/dec05ind.html
49. López-López R, López-Gallardo M, Pérez-Álvarez MJ, Prada C (2008) Isolation of chick retina cones and study of their diversity based on oil droplet colour and nucleus position. Cell Tiss Res 332(1):13–24. https://doi.org/10.1007/s00441-007-0572-6
50. Alvarez-Delphin K, Morris AC, Snelson CD, Gamse JT, Gupta T, Marlow FL, Mullins MC, Burgess HA, Granato M, Facool JM (2009) Tbx2b is required for ultraviolet photoreceptor cell specification during zebrafish retinal development. Proc Natnl Acad Sci U S A 106 (6):2023–2028. https://doi.org/10.1073/pnas.0809439106

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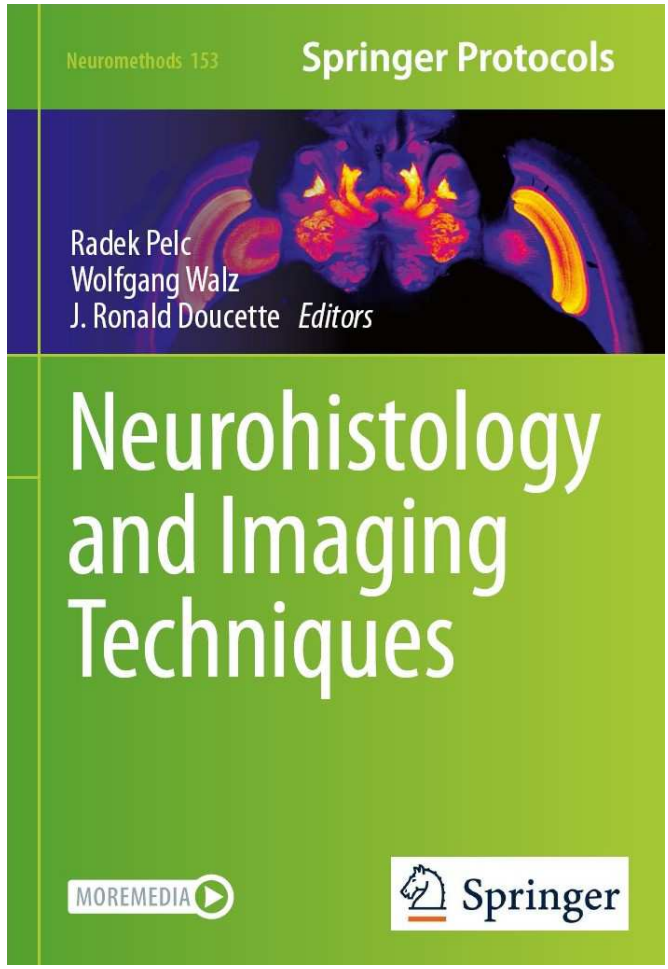


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