Light Microscopy – Brightfield and Darkfield Illumination

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The microscope image, especially its contrast, is profoundly influenced by the way that light is directed on to the specimen. The essential controls required are of intensity, evenness, area of illumination and angle of illumination.

Secondary article

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The condenser

The article 'Light Microscopy' describes the role of the condenser and gives definitions of terms used in this section. With his theories of microscope lens performance, Ernst Abbe determined the requirements of specimen illumination. Essentially, the light source should completely fill the back focal plane (bfp) of the objective. This is achieved by having a condenser for each objective, matching the objective numerical aperture (NA). The microscope illuminator will include a lens or lenses that may act as part of the condenser. This extends the complexity of the practical use of the condenser; manufacturers have devised many variations of condensers and illuminators, the choice of which depends on quality and price that can be afforded.

Source-focused illumination

Earlier instruments focused an image of the light source itself, such as the broad flame of an oil lamp, on to the specimen. Obviously, focusing the filament of a modern electric lamp on to the specimen will give very uneven lighting, and such a technique is not favoured today. In most microscopes of teaching laboratories, some modification of source-focused illumination is employed because of its cheapness. At its simplest, a ground glass filter is interposed between the light source and the condenser to present a more evenly illuminated specimen.

Köhler illumination

August Köhler (1866–1948) devised an illumination system specifically for photography through the microscope. It is the system now acknowledged by all manufacturers as being preferred for simple observation as well as for recording microscope images. It adds considerably to the cost of a microscope, but extends its performance and efficiency.

An illumination system for professional microscopy must satisfy the following:

- The specimen field should be fully and evenly illuminated.
- The intensity of the illuminated field should be variable within wide limits.
- The diameter of the illuminated field should be variable to match the fields of view of the more commonly used objectives, from about 0.2 mm to 4 mm.
- The angular aperture (NA) of the condenser should be variable to match the range of objective NAs, from about 0.1 to 1.3.
- Selection of wavelengths (colour) may be desirable for some aspects of specialized microscopy.

Köhler illumination employs a collector lens, close to the filament, to form an image of the light source in the condenser lens 'aperture', i.e. the plane of the condenser iris diaphragm (better called the aperture diaphragm). The filament image should completely fill the diameter of the aperture diaphragm. This enables an image of the light source to be formed in the bfp of the objective; the light source image will fill that plane, a requirement for the illumination of the full NA of the objective. The function of the aperture iris is to change the illuminating NA, and to match it to the objective in use.

Adjacent to the collector lens is the field (iris) diaphragm. The condenser lens focuses an image of the field diaphragm on to the plane of the specimen. As the field iris is opened and closed, the diameter of the illuminated specimen field is changed. To maximize contrast, no more of the specimen should be illuminated than is actually observed (or photographed).

All textbooks will have diagrams attempting to explain Köhler illumination (**Figure 1**) but they are quite intimidating until one understands that two different ray paths are being considered in the one figure. In addition, each of the

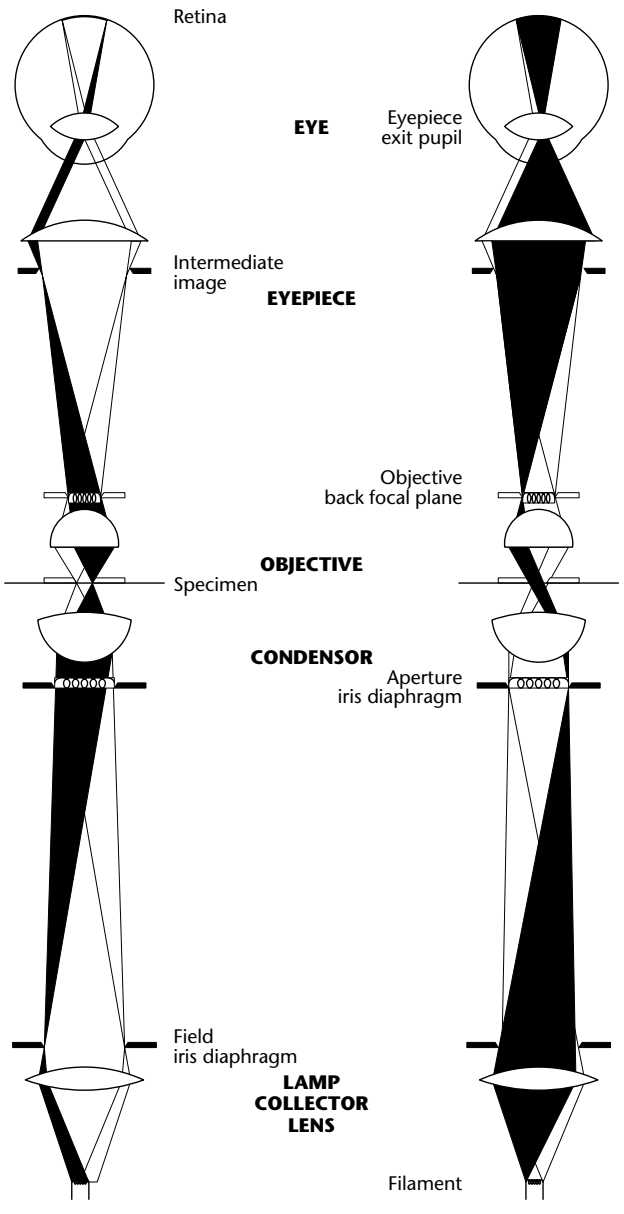

Figure 1 Köhler illumination. The diagram on the left highlights the image-forming ray path that incorporates the four conjugate planes associated with the specimen plane. On the right, the illuminating or aperture ray path is emphasized – four conjugate planes that incorporate the filament and the lens 'apertures'.

two ray paths has four conjugate planes, planes that are images of each other, images *on* each other of preceding planes. Familiarity with these two sets of four planes greatly facilitates understanding of the microscope and is assumed knowledge in the instructions for more elegant techniques such as differential interference contrast and fluorescence. Rather simple questions with quite difficult answers (such as Where should the eye be placed when looking down a microscope? How can I superimpose an image of a measuring device on the specimen?) may be answered with reference to Köhler illumination diagrams.

The separate functions of the two iris diaphragms, field and aperture, are not appreciated by many microscope users, and is a frequent source of serious error in microscope manipulation.

Brightfield (Köhler) Illumination – Practice

There is a complexity to the instructional diagrams and written instructions for Köhler illumination. In addition, each brand of microscope – each model – is likely to have a different set of instructions. But there are three sequential steps common to all systems:

1. An image of the filament is focused on to the (condenser) aperture diaphragm. In older microscopes, the lamp collector lens was focused to form a sharp image of the filament on the aperture diaphragm. Modern systems are said to have their light sources prefocused and precentred, and include groundglass filters to make it impossible to check this step.
2. An image of the field iris diaphragm is focused on the specimen. The condenser is raised or lowered for this step, and lamp-centring facilities will have to be engaged. The field iris is then opened or closed until its image matches the size of the observed specimen field.
3. The condenser NA is matched to the objective NA. The objective bfp is examined (after removing an eyepiece) to check the 'seven-eighths' position of the aperture iris (see the article 'Light Microscopy'). Imperfect specimens may demand further adjustment of the aperture iris to enhance contrast and/or depth of field.

With each change of objective, the NA and field diameter also change; strict Köhler illumination therefore requires re-adjustment of steps 2 and 3.

A condenser with NA greater than 1.0 is designed to be 'immersed' with a drop of immersion oil between the condenser top lens and the slide undersurface. Although, because it is messy, this is rarely done in routine microscopy, it is quite appropriate for those instances when the very highest level of microscope performance is sought.

Intensity control is by a variable transformer or rheostat; the aperture iris should not be used for this purpose.

Darkfield Illumination – Theory

It greatly limits the potential of the microscope to accept that the role of the instrument is merely to produce an image that is an exact, enlarged copy of the specimen. Especially with living material, contrast (i.e. variations in the image of colour or intensity) may be very poor, and the image practically invisible. It is a common experience that small dust particles in the air are easier to discern if light is directed not into the eye, but across the observer's line of vision. Although the dust is too small to be 'resolved' by the eye, particulate matter is seen, detected or made visible, even to the extent that measurement could be made of particle movement. The same principle is used in microscopy by preventing direct illuminating rays from entering the objective. Darkfield microscopy (many prefer the term 'darkground') renders the object as bright against a dark background, considerably enhancing the contrast and visibility of small objects (**Figure 2**).

Darkfield Illumination – Dry and Immersion Systems

The simplest darkfield attachment is a homemade patchstop (**Figure 3**), a clear disc with an opaque centre. The disc is inserted in the normal brightfield Abbe condenser, close to the position of the condenser aperture diaphragm; older microscopes had a filter holder convenient for this purpose. The aperture diaphragm itself must be left fully open. The opaque centre of the disc prevents 'direct' illumination from entering the objective; the diameter of the opaque region is not particularly critical, roughly one-half to two-thirds the diameter of the clear disc. The transparent outer margins of the patchstop transmit a hollow cone of light towards, but missing, the objective. Only if a specimen is present will diffracted or reflected rays be redirected and accepted by the objective. In spite of its rather amateur status, the patchstop is a very useful microscope accessory,

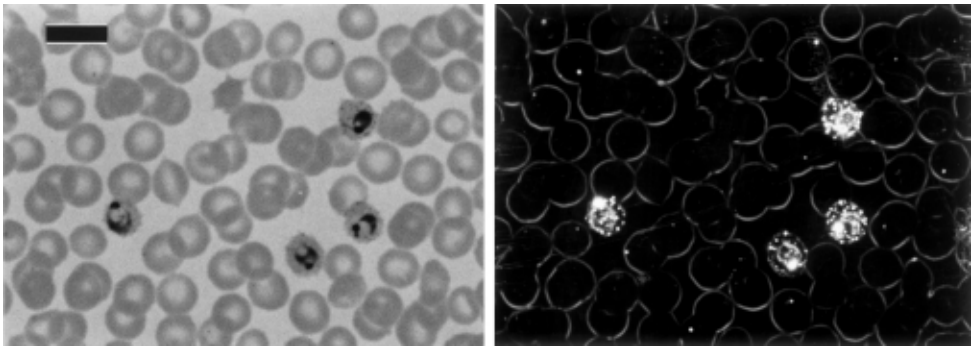

Figure 2 *Plasmodium vivax* in a human blood smear; brightfield (left), darkfield (right). The bar represents 10 μm.

Figure 3 Equipment for darkfield microscopy. From the left: a patchstop, a dry darkfield condenser, an immersion darkfield condenser, and an oil-immersion objective with iris diaphragm.

capable of serving objectives of NA less than about 0.7. If the microscope is equipped with a phase contrast condenser, the ×100 or ×40 annuli make excellent, centrable darkfield patchstops for the (phase or non-phase) ×4, ×10 and ×20 objectives. If the condenser NA is greater than 1.0, immersing the condenser to the slide (adding one or more drops of immersion oil between the condenser top lens and the bottom of the slide) may prove helpful by increasing the effective NA of the condenser.

Since the 1980s, dry darkfield condensers (**Figure 3**) have become available; they are called 'dry' to distinguish them from the immersion systems more fully discussed below. The immersion darkfield condensers were quite difficult to use, mainly because they were so messy; there is no such problem with dry systems. In addition, dry darkfield condensers do not have the centring problems sometimes associated with the patchstop. These darkfield condensers function by having reflective curved surfaces to give highly oblique lighting on the specimen (**Figure 2**).

Immersion darkfield condensers (Figure 3)

These are not so commonly used now that dry systems are commercially available. However, if an oil-immersion objective with an NA of 1.2 or more must be used for the observation, then the darkfield condenser must have an even higher NA, making oil-immersion condensers essential. These are made with mirrored surfaces so that the illuminating cone NAs are approximately 1.2 to 1.4, even more oblique than with the dry condensers. It is essential to immerse the condenser to the slide, otherwise total internal reflection occurs at the condenser glass/air boundary and no light gets to the slide. Immersed objectives and immersed condensers make for troublesome microscopy. Oil-immersion objectives intended for darkfield work should have an iris diaphragm in the objective bfp. For brightfield work the objective iris is left open; for darkfield work the objective iris is closed (just) sufficiently to exclude direct rays.

Rheinberg Illumination

Replacing the opaque area of the conventional patchstop by a deep blue transparent filter will change the black background of a darkfield image into a blue background. Similarly, if a red filter replaces the clear area of the patchstop, the specimen will appear red, rather than white. A colourless object now appears bright red on a deep blue background. Beautiful optical staining effects, loved by magazine editors, can be obtained by using combinations of coloured filters in this way – suggested first by Rheinberg in 1896.

Practical Applications of Darkfield Microscopy

At low magnifications, darkfield is extremely helpful to find and focus near-transparent preparations. The material does not even have to be in focus, a bright blur showing its presence.

An exciting prospect of darkfield microscopy is that particles below the resolving power of the objective might be revealed; it is suggested that the minimum visible diameter of a white point on a black background is limited by the intensity of illumination, not the NA of the optical system. More practically, the high contrasts generated by darkfield permit low magnifications, i.e. wider fields, for investigating the presence or absence of very small pathogens, protozoa, bacteria, etc. (**Figure 4**). Some caution may need to be exercised in interpreting the image. Although, in the recent past, darkfield has been of prime

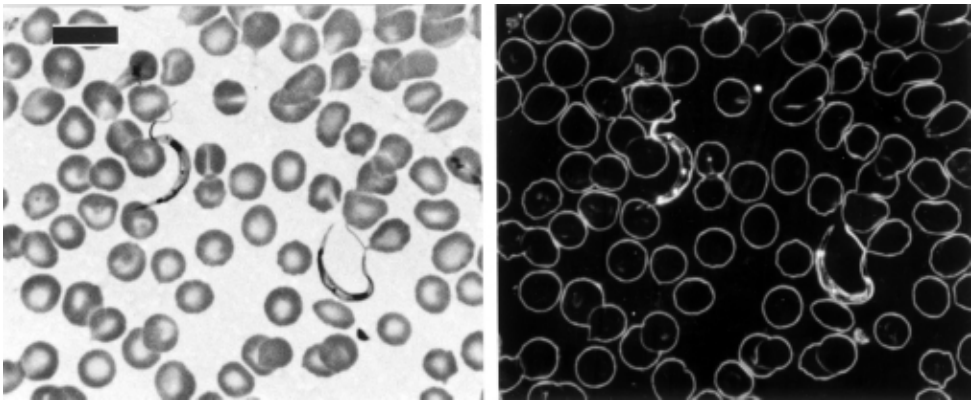

Figure 4 A blood smear with *Trypanosoma* sp.: brightfield (left), darkfield (right). The bar represents 10 μm.

importance in various areas of microbiology, the dairy industry and pathology, few darkfield systems are sold today. Biochemical and immunological tests have largely superseded the microscope for these purposes.

The system is extremely useful for low- and medium-power illustration of, for example, phytoplankton. The resolution of the microscope is maintained, the images are of high contrast and can be beautifully coloured. But extreme cleanliness of all glassware is essential, for there is no better way of demonstrating the presence of dust, detritus and objectionable smears on the slides and coverslips. To maximize contrast, the preparations should be thin, sparse, and heavily diluted if necessary. Limited depth of field may be a problem for it cannot be controlled.

Darkfield techniques are also available for the observation of opaque specimens in epi-illumination systems. They have some advantage for the detection of silver in autoradiographs.

Filters in Light Microscopy

There are several factors that will influence the selection of wavelengths for specimen illumination in microscopy. For all microscopy, especially with living cells, it is useful to have an infrared-absorbing heat filter in the light path. Optical systems generally, but more particularly the commonly used achromatic objectives, are designed to perform maximally in the green part of the spectrum. This is a good reason to employ green filters routinely in the microscope. The choice of short wavelengths, towards the blue end of the spectrum, is advantageous to promote minimum resolved distance (resolving power) as predicted by Abbe. This led to the investigation of ultraviolet light as an illumination source, later much extended into completely new principles of fluorescence microscopy. If the specimen selectively absorbs certain wavelengths, contrast can be optically enhanced with appropriate 'oppositely' coloured filters, both for observation and for black and white photography. The red/blue ratio of 'white' light defines its colour temperature, and this needs careful adjustment for colour photography. Other specialized microscopical techniques may employ polarizing filters, infrared-transmitting filters, diffusion filters or neutral grey filters.

Filters are commonly made of glass, gelatin or polycarbonate materials. Interference filters are particularly important for fluorescence microscopy, but in spite of their considerable expense they have spread into all aspects of microscope use.

Further Reading

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