Contrast Enhancement in Light Microscopy

Optical microscopes, which are among the oldest instruments of scientific discovery, continue to be key tools in both biomedical research and routine diagnosis. This remains true despite the development of a wide range of new imaging technologies, many with far greater resolution—ranging from electron microscopes to the multitude of scanning probe systems available today. The overall simplicity of optical microscopes, their minimally destructive impact, particularly on live specimens, and the many microscopy techniques available for enhancing or visualizing specific specimen features make this technique exceptionally useful in biomedical applications. In recent years, electronic imaging has both greatly enhanced the capabilities of light microscopes and placed ever-increasing demands on their optical performance.

A number of purely optical methods can be used to enhance feature extraction from biological material for both visual observation and subsequent electronic image processing. In the present discussion, a short primer on image formation in basic transmitted-light bright-field microscopes and their resolution limits will be followed by a brief outline of how to optimize the imaging conditions by using what is known as Köhler illumination, and finally by discussion of alternatives to bright-field microscopy.

KÖHLER ILLUMINATION

Köhler illumination is a method of focusing and centering a microscope’s light source and optics and setting its diaphragms to obtain the best image detail and contrast. It was pioneered in the late 1800s by August Köhler, a zoologist in Giessen, Germany, who in 1900 joined Carl Zeiss in Jena to head up the company’s microscopy group. Today, all manufacturers of high-quality microscopes provide Köhler illumination in their instruments.

Basic Concepts

Understanding the possible contrast enhancement techniques and appreciating their specific applications requires a clear grasp of the basic concepts of Köhler illumination. It is illustrative to follow the path taken by light through a microscope from source to final image (see Fig. 2.1.1). A single, off-axis source point is imaged by the collector into the front focal plane of the condenser (condenser aperture diaphragm), where an image of the light source is formed. Every point of this source image is projected by the condenser as a parallel pencil into infinity through the specimen. These pencils uniformly distribute the light intensity of each source point across their diameter, which in turn is set by the field diaphragm.

In the back focal plane of the objective, also called the objective exit pupil, the source is imaged again and relayed by the eyepiece to its exit pupil or eyepoint, which becomes the entrance pupil for the eye or imager. Four “source-conjugated” planes in which a source image appears can be identified, the latter two of which are modulated by diffraction in the specimen.

Superimposed over this illumination beam path is the image-forming beam path. The specimen is conjugated to the field diaphragm and imaged by the objective into the real intermediate image plane, then projected by the eyepiece into infinity and reimaged by the detector’s objective (eye lens or camera lens) onto the final sensor (retina, film, or camera).

There are several advantages to this dual optical train. The field stop permits control over internal stray light and internal reflections by limiting the diameter of the illumination and imaging bundles to that needed for a given specimen field diameter. Contrast control can be achieved by controlling the condenser aperture, which directly influences the coherence and participation of diffracted wavefronts in the formation of the final image: closing the condenser diaphragm increases contrast and decreases resolution. An experienced microscopist will attempt to find the best compromise between resolution, contrast, and, to a lesser extent, depth of field.

Most microscopes have built-in illuminators, so the steps for obtaining good Köhler illumination are simple:

1. Achieving sharp specimen focus;
2. Achieving substage or condenser focus and centering via the field diaphragm, and setting that diaphragm to cover a given objective’s and eyepiece’s field of view; and
3. Setting the condenser aperture diaphragm for best contrast, resolution, and depth of field, based on the nature of the specimen itself.

Resolution and Contrast

The light path for Köhler illumination can be explained by geometric optics, a way of thinking about how light travels in a straight
line as a ray. To understand the limits of resolution and the contrast transfer from object to image, it is necessary to consider the electromagnetic wave nature of light, its wavelength ($\lambda$), and the interaction of wavefronts with the specimen’s structures. The theory of image formation through a microscope was developed by Ernst Abbe, who distinguished between two types of specimens: self-luminous objects (light sources, luminescence, or fluorescence), which follow the Rayleigh criterion, and illuminated objects, which follow the Abbe criterion. According to the Rayleigh criterion, which is based on diffraction in the objective, the point-to-point resolution, $\delta$, (in $\mu$m) is given by:

$$\delta = \frac{0.61\lambda}{NA_{objective}}$$

In the case of an illuminated object, the Abbe criterion defines the resolution, $\delta$, as

$$\delta = \frac{\lambda}{2NA} \quad \text{or} \quad \frac{\lambda}{NA_{objective} + NA_{condenser}}$$

where $\lambda$ is the wavelength of the emitted or illuminating light and NA is the numerical aperture of objective or condenser, given by the
sine of half its collection angle times the refractive index of the medium between specimen and objective.

With a self-luminous object, diffraction in the objective itself causes the smallest image point to expand into what is known as an Airy disk (Fig. 2.1.2), whose diameter, $D$ (in $\mu$m), will be:

$$D = \frac{1.22\lambda}{NA}$$

With an illuminated object, diffraction is caused by the structural features of the specimen and their spacing. The diffraction angle ($\alpha$) is determined by the wavelength and the spacing or distance ($d$) between features.

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

The diffraction angle that an objective is capable of collecting is directly related to its numerical aperture. Abbe proved that for a given structural spacing to be resolved, at least two orders of diffracted light produced by this spacing need to participate in the image formation. Interference between diffracted and nondiffracted wavefronts in the intermediate image plane resolves structural detail and determines the contrast at which the image is rendered.

**Bright-Field Microscopy**

Bright-field microscopy is the optical technique most commonly used. With a microscope set up and adjusted for Köhler illumination, bright-field microscopy is ideally suited to the study of specimens whose features are clearly differentiated by differences in absorption. Such specimens are also called “amplitude specimens,” because they primarily change the intensity or amplitude of the illuminating light. Either inherent absorption or absorption induced by staining will change the gray level or color contrast if spectral differences in absorption exist. The contrast appears against a white, bright background.

Histochemically stained tissue sections of all sorts, cytology and bacterial stains, and naturally absorbing specimens are best studied under a bright-field microscope.

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**Figure 2.1.2** (A) A typical Airy disk. (B) Computer graph of the intensity distribution of the Airy disk.
Oblique and Anaxial Illumination

Just as the rising or setting sun will better reveal the topography and mountain ridges of a landscape than the noonday sun, obliquely illuminating a specimen with limited internal contrast can greatly enhance structural differences in optical density or refractive index and turn an otherwise flat or almost invisible object into an image of striking relief and apparent three-dimensionality with clearly enhanced contrast (Fig. 2.1.3).

To obtain oblique illumination with some degree of reproducibility, a “turret condenser,” which allows the condenser aperture diaphragm to be shifted laterally, is helpful. This lateral displacement combined with the best setting for the aperture’s diameter varies with the objective’s numerical aperture and, in wave-optical terms, changes the mix of direct, non-diffracted wavefronts and diffracted light to produce pseudorelief and enhanced contrast. For more subtle enhancement, a similar effect can be achieved by decentering the light source. Care must be taken to retain even, uniform illumination over the field.

Oblique illumination is a simple, inexpensive means to enhance contrast in unstained, transparent sections, sediments, or casts and can be a useful tool for finding focus in highly transparent specimens.

Dark-Field Illumination

Dark-field illumination greatly enhances a microscope’s ability to detect minute structures or particles, often far below the theoretical limits of resolution—that is, even though the size and spacing of the structures cannot be resolved, their presence is obvious: they appear bright on a dark (black) background. This dark

![Figure 2.1.3 Axial versus oblique illumination and the effects of aperture on resolution. (A) Low-aperture axial illumination will not resolve structures that generate diffraction angles $0/+1$ or $0/-1$. (B) Shifting the same aperture to the side permits the objective to collect diffraction order $0$ and $-1$, resolves the structure, and, with only one side band of diffracted light participating, generates a relief effect.](image)
background is achieved by excluding all direct, nondiffracted light from the objective. Specifically, the dark-field condenser produces a hollow cone of illumination with an aperture higher than that of the objective. This can be accomplished by an annular diaphragm in the condenser aperture or by specific dark-field condensers, such as “paraboloid” (dry) or “cardioid” (oil) condensers (Fig 2.1.4). Objectives of high numerical aperture require a built-in iris or a funnel stop to reduce their numerical aperture below that of the condenser. Because the image is formed by diffracted light only, a contrast reversal takes place. As a result, dark-field microscopy is exceptionally sensitive to contamination; it is therefore imperative that condenser, slide, and objective front lens be perfectly clean. Contaminants, bacteria, cell and urine casts, and blood, among others, lend themselves well to dark-field studies.

**Hoffman Modulation Contrast and Varel Contrast**

Hoffman modulation and Varel contrast techniques are sophisticated methods for oblique illumination. Images generated by these methods exhibit a striking three-dimensional effect produced by converting directionally opposing specimen gradients in refractive index or thickness into opposing gray-level differences. The two techniques differ mainly in the geometry of a special attenuator for the

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**Figure 2.1.4** The dark-field condenser’s hollow cone of illumination passes by the objective. Only light diffracted or refracted by the specimen is collected.
nondiffracted, zero-order direct light in the back focal plane of the objective and the corresponding illumination aperture in the condenser; the position of the attenuator determines the direction in which the gradients are best contrasted.

In Hoffman modulation contrast a straight, bar-shaped attenuator or modulator is placed on the periphery of the objective’s aperture and absorbs ~85% of the direct light coming from a slit in the condenser aperture properly aligned to superimposition over the modulator (Fig. 2.1.5).

In Varel contrast, an annular attenuator in the very outer back aperture of the objective absorbs ~85% of the direct light coming from a corresponding segment of an annulus in the condenser. Only direct light is attenuated, while diffracted light passes fully for a strikingly improved contrast generation.

In both techniques, oblique brightfield can be added when specimens of relatively high inherent contrast are studied. Unstained live tissue and cell cultures in either glass or plastic vessels make ideal specimens for these techniques; the improved depth perception of the resulting images also facilitates micromanipulation or microinjection.

**Differential Interference Contrast**

Differential interference contrast is the most sophisticated, most flexible, and potentially most highly resolving technique available; it converts specimen gradients into gray-level differences and produces a striking pseudo-three-dimensional effect. The system employs polarized light and special prisms called Wollaston prisms to produce two slightly sheared or separated wavefronts, which traverse the specimen (Fig. 2.1.6). The amount of shear is usually below the resolution of a given objective and is a function of the Wollaston prisms in the condenser and objective. Specimen gradients in refractive index or thickness result in an optical path difference between the two sheared wavefronts. When the wavefronts are recombined and made to oscillate in a common plane by an analyzer, different amounts of constructive or destructive interference produce distinct gray-level differences for opposing gradients, with the greatest contrast along the direction of shear. The system can be set to maximum contrast for any specific specimen gradient by adjusting one of the prisms or using a special compensator. It is often desirable to use the full objective and condenser apertures, particularly when using video-enhanced imaging to extract the very smallest contrast differences so as to detect and visualize intracellular organelles such as microtubules.

Differential interference contrast (largely the version proposed by Nomarski) has contributed greatly to the study of live cells and tissues and is now an indispensable tool in develop-

**Figure 2.1.5** Basic principle of modulation contrast.
mental biology, physiology, neuroscience, and many other disciplines. Because it employs polarized light, plastic specimen vessels should not be used for this method, as they tend to show birefringence and depolarize the sheared wavefronts. To avoid strain or stress in the condenser and objective, it is important to use components recommended by the manufacturer.

**Phase Contrast**

Phase contrast microscopy is designed for the study of thin, unstained sections or live cultures—i.e., transparent specimens with minimal inherent contrast. Unlike amplitude or absorbing specimens, for which the diffracted wavefronts are phase-shifted by one-half of a wavelength, such so-called phase specimens generate shifts of only one-quarter of a wavelength. The interference conditions between diffracted and direct wavefronts are neither constructive nor destructive, and the image contrast is poor. The Dutch physicist Frits Zernicke won the Nobel prize for his proposal to add to condenser and objective elements that

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**Figure 2.1.6** Principles of differential interference contrast. The separation between the two sheared beams is greatly exaggerated.
shift the phase of nondiffracted, direct light by one-quarter of a wavelength, and at the same time attenuate the intensity so as to greatly enhance the interference conditions for the image rendition. The result is an image wherein “positive-phase” contrast areas of higher refractive index appear darker. Specific gray levels optically “stain” areas of specific refractive index and thickness. This is accomplished by an illumination annulus in the aperture plane of the condenser along with a conjugate phase ring in the back focal plane of the objective that acts as both attenuator and phase shifter. A green filter further enhances the contrast (Fig. 2.1.7).

In contrast to differential interference or oblique illumination techniques, which optically “stain” specimen gradients and generate

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**Figure 2.1.7** Phase contrast. The annulus, R, in the condenser aperture is superimposed on the phase plate behind the objective, which is both an attenuator and a phase shifter.
a pseudo-three-dimensional effect, phase contrast produces a two-dimensional image of index- and thickness-specific gray levels.

The limitations of phase contrast are determined by the illumination aperture, a function of the condenser’s phase ring, and by the “halo” effect along steep specimen gradients, which limits the section thickness for phase contrast to ~5 µm.

**Reflection Interference**

Reflection interference microscopy looks at the interference pattern that is naturally present between a cell or tissue and its substrate (cover glass). Wavefronts reflected at the cell surface interfere with those reflected at the substrate and are one-half wavelength out of phase for those areas where the cell adheres to the cover glass, resulting in destructive interference and darkness (adhesion plaques).

Using reasonably monochromatic incident light obtained by filtering light from a tungsten halogen or, better, a mercury lamp through a green filter, along with good Köhler illumination, produces a striking contrast that allows direct analysis of a cell’s proximity to the substrate on which it grows. Limiting the illumination aperture further contrasts intracellular features based on the varying path differences they generate. Video enhancement of the contrast can also considerably improve the results (see Key References for suggestions for further reading on this topic).

**Polarized Light Microscopy**

For a wide range of biological materials, visualization can be considerably improved by using either simple polarized light illumination or polarization contrast, whereby a polarizer below the condenser (usually oriented “east-west”) linearly polarizes incoming wavefronts. An analyzer behind the objective is oriented at 90° to the polarizer; without a specimen the field of view is dark. Specimens with distinct structural orientation, such as muscle, nerve, or bone tissue, are birefringent—i.e., they display different refractive indices in different directions. Depending on their thickness and orientation to the polarizer and analyzer, such specimens will alter the plane of vibration of incoming polarized light and change it to some form of elliptically polarized light, part of which will then be able to pass the analyzer. This results in bright specimen images on a dark back-

**Figure 2.1.8** Epifluorescence is made possible by a dichromatic filter that reflects the exciting radiation down on the specimen and allows the emitted radiation to pass upward to the eye.
When the full visible spectrum of light is used, specific wavelengths will be suppressed and others enhanced as a function of the path difference the specimen has generated between its two orthogonal vibration directions. This can result in vibrant interference colors, which in turn provide information about the specimen’s birefringence and thickness.

A detailed discussion of polarized light microscopy—especially the quantitative aspects of the analysis of specimen birefringence and directional and structural orientation—would require far more in-depth treatment than can be contained in this brief overview; the reader is encouraged to consult the relevant literature (see Key References) for further information.

**Fluorescence Microscopy**

One of the fastest-growing tools in biomedical microscopy is fluorescence. The exceptional sensitivity of this technique, combined with the ever-growing list of very specific protein markers and fluorophores covering a wide range of different colors that are available, have made it indispensable for qualitative and quantitative diagnosis. Autofluorescent or fluorophore-labeled specimens are excited with short-wavelength radiation and almost instantly convert some of the absorbed exciting radiation to emitted longer-wavelength fluorescence.

In present-day microscopes, excitation is provided almost exclusively by either incident light or epillumination. The light source is usually a mercury or xenon gas discharge lamp. Special filter/reflector combinations isolate the fluorophore’s specific emission wavelength from the exciting radiation to maximize the efficiency of both and thereby produce a bright, sharp fluorescent signal on a black background (Fig. 2.1.8). Ideally the microscope should be equipped with objectives of high numerical aperture, magnifications just high enough to see the areas of interest, and good chromatic correction. Much of the image capture is done with sensitive cameras either in real time or, for low light levels, with long-term signal integration.

A multitude of publications, textbooks, and reprints detailing fluorescence microscopy are available in bookstores and from most of the major microscope manufacturers. For a more in-depth look into these interesting and useful microscope methods the reader is encouraged to contact these sources (see Key References).
Reﬂection Interference Microscopy

Polarized Light Microscopy

Fluorescence Microscopy


Photomicrography

Video Microscopy

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