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Photomicrography

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Introduction

Photomicrography is a specialized form of micro-projection where images of minute specimens, magnified by a microscope, are captured using some form of detector. For most of the twentieth century, the primary medium for photomicrography was the silver halide emulsion on glass or film. These materials served the scientific community well by faithfully reproducing countless images produced from the optical microscope beginning almost when photography started. Now, digital imaging has largely displaced film and is cheaper and easier to use than conventional photography. The term photomicrography should not be confused with microphotography, which describes the process by which miniature photographs are made of large objects, such as microfilms of books and documents.

The range of light detection methods and the wide variety of imaging devices currently available to the microscopist makes the selection of equipment difficult and often confusing. In particular, the characteristics of the imaging device have an important influence on the resolution achieved and on the dynamics of visualizing the specimen.

This section is intended to aid in understanding the basics of digital photography as applied to microscopy and as a guide to selecting a suitable imaging detector type appropriate for the variety of optical microscopy techniques.

Historical Perspective

Recording images with the microscope dates back to the earliest days of microscopy. During the late seventeenth century, Dutch microscopist Antoni van Leeuwenhoek and the famed English scientist, Robert Hooke, both produced exquisite and highly detailed drawings of miniature creatures and other materials observed in their single- and double-lens compound microscopes, respectively. Microscopes developed in this period where incapable of projecting images, so observation was limited to careful inspection of specimens through a single lens of very short focal length.

The first compound microscope capable of projecting images was developed by Henry Baker, who described a "solar microscope" based on the camera obscura in 1743. In 1771, George Adams, another English microscopist, produced one of the first projection microscopes employing artificial illumination from an oil lamp. However, there was no way to record the projecting images until 1802, when Thomas Wedgewood reported the use of a solar microscope to produce photomicrographs on paper treated with a sensitized coating of silver chloride. Unfortunately, these images were not permanent because no process of fixing had been discovered.

True photographic images were first obtained with the microscope in 1835 when William Henry Fox Talbot applied his then-experimental process to capture photomicrographs of natural subjects. These specimens were magnified less than 20 times and required exposures of about 15 minutes. The late 1830s and early 1840s witnessed an explosive growth in the application of photography to recording microscope images, using a variety of processes including the daguerreotype. In 1840 John Benjamin Dancer utilized a microscope with gas illumination to photograph a flea magnified enough to fill a 5×7 inch daguerreotype plate. Twelve years later, in 1852, the wet collodion process was first applied to photomicrography by J. Delves, and for the next 150 years, the capturing of images formed by a microscope evolved hand-in-hand with improvements in photographic technology. During the mid to late 1800s, many significant advances were made, especially in Germany, where Carl Zeiss and Ernst Abbe had perfected the manufacture of specialized optical glass and used them for many optical instruments including compound microscopes. Many describe this period as microscopy's golden age where many advances essential for photography through the microscope were developed.

Microscope Cameras

In principle, photographing with a microscope does not require a conventional camera, since the microscope produces its own image, which can be projected on to a suitably mounted detector. For film enthusiasts, the camera can be as simple as a light-tight box fitted with a focusing screen and a film holder, which is then supported over the microscope with a shutter and light-baffle near the eyepiece. This simple "purist" approach produces the best images in theory, since there are no superfluous optical components in the light path. However, almost any style of conventional camera can be used if the lens is set to infinity and the camera positioned very close to the eyepiece. Ideally the eyepiece's exit pupil should be located at the front nodal point of the taking lens. The resulting images

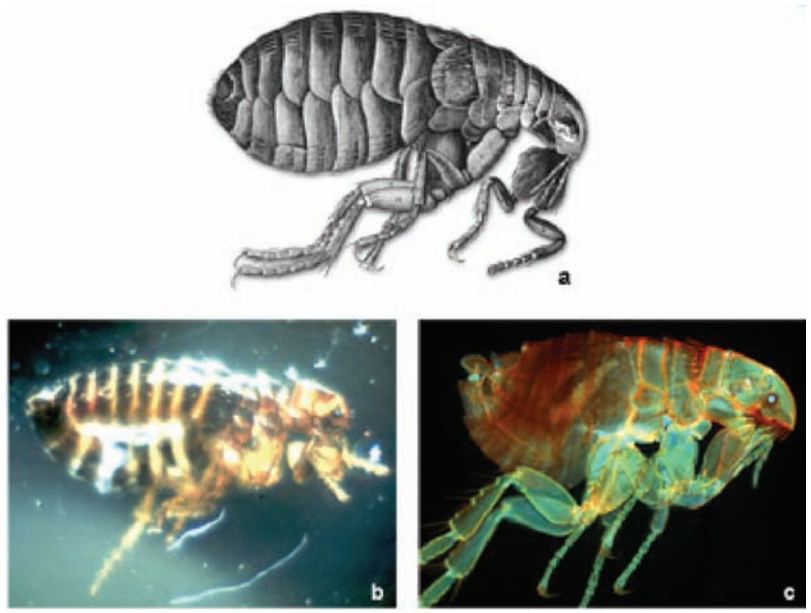

FIG. 82 Evolution of recording images with the microscope. (a) Drawing of a flea prepared by Robert Hooke from observations made in his compound microscope; (b) Photograph of a flea made with a modern camera using a Hooke microscope (note the poor contrast and specimen detail recorded by the camera compared to the articulate image prepared by Hooke) (c) autofluorescence in a flea recorded with a color charge-coupled device (CCD) camera system and a modern research-level microscope. (Photo courtesy of Professor Brian J. Ford, Beyond Distance, University of Leicester, Leicester, UK.)

from this system are likely to be vignetted since the eyepiece lens will make a small circle of good definition and not cover the imaging area. A wide range of adapters are offered by the microscope and aftermarket product manufacturers for attaching many of the popular "point-and-shoot" digital cameras to modern microscope eyepieces. This is one of the most economical solutions for amateur microscope enthusiasts.

Professional photographers who prefer to use film also have a number of choices when coupling an advanced camera system to the microscope. Many adapters for single-lens reflex cameras (minus the lens) are available for both generic and specific brand-name microscopes. These devices are divided into simple extension tubes with adapter couplings and more elaborate units that contain magnifying lens elements. Some adapters can be mounted directly on the central tube of a trinocular microscope, or coupled to the eyepiece tube with or without an imaging or photo eyepiece.

Until recently, microscope manufacturers marketed specialized eyepiece cameras in 35mm and 4×5 formats that coupled directly to the microscope without external support. These devices incorporated an eyepiece telescope, beam splitter, intermediate lenses, and film holding/transport mechanism. The beam splitter divided the light between the focusing telescope and film. This system enabled a sharp focus of the specimen to be achieved for both observation and at the film plane for recording. High-end models included programmable automatic exposure metering. These systems were costly, making them accessible only to researchers and serious professionals. Now digital cameras have taken over the consumer and scientific markets and these systems are no longer manufactured though they can still be found at used instrument dealers or on eBay.

In the late 1970s and early 1980s, video cameras were used with optical microscopes to produce time-lapse image sequences and real-time videos in much the same way as still cameras, with or without a lens. More recently, the CCD has heralded a new era in digital photomicrography and film is no longer used. These numerous and gradual changes in the way optical microscope images are recorded suggests that the term photomicrography may no longer be appropriate. It may be considered that the capturing of electronic images with the microscope may be best described as digital or electronic imaging.

Photographic Films

Although film is rapidly being supplanted by digital imaging, a number of microscopists still use it. Because the microscope is a relatively stable platform with good illumination properties, films in the 50 to 200 ISO range are commonly used. Modern film magazines have a so-called DX code that allows camera backs to automatically recognize the film speed and switch the control device to the proper setting. Microscopes can form photographically demanding images and films were designed or adapted to address the image contrast and color saturation

challenges of color photomicrography. One special emulsion was a Kodak aerial film that had additional contrast and enhanced blue and red color response and was repackaged as Kodak Photomicrography Color Film, a very popular emulsion in many microscopy labs for years. Because there were more daylight films produced than tungsten-balanced emulsions, microscope manufacturers often supplied a daylight conversion filter (e.g., Kodak Wratten 80A) as a standard accessory. Most research microscopes would have a daylight color temperature conversion filter built in. Creating color slides with a high degree of color saturation is difficult with film and a didymium glass filter was often used to bolster color reproduction of samples that had a pink or blue hue to them.

Film photomicrography in black and white was notably more difficult than color work, especially capturing small tonal differences in thin sectioned, lightly stained materials that are, for example, light pink and blue. Contrast filters such as Kodak Wratten 11 helped, but Kodak Technical Pan was a legendary emulsion for its application for microscopy. It could be rated as low as ISO 25 and up to ISO 400 depending on the developer used. This emulsion could be developed to a contrast index of 0.40 all the way to 3.0 and had the potential to resolve up to 200 lp/mm in the highest contrast situations. It was an ideal emulsion for chromosome mapping and the production of karyotypes as well as some many other challenging applications, and not only in microscopy. Other "home recipes" of emulsion and developer combinations were devised to photograph the near invisible in the optical microscope. One intriguing combination paired Kodak Kodalith film with 1 part of Kodalith A & B developer with an equal amount of paper developer to yield high contrast with midtones.

Digital Photomicrography

Since the mid-1990s, digital camera technology has dramatically improved and now over 60 manufacturers offer a wide variety of models and features to suit almost every application and budget. Similar advances have occurred with digital cameras designed for microscopes. These image directly onto an electronic image sensor without focusing eyepieces since the image may be displayed in real time at the imaging workstation.

The number of pixels in camera sensors directly influence the amount of information captured by digital cameras, and at this moment in time, it is impossible to differentiate images made using either technology.

One of the main advantages of digital imagery is that it offers many opportunities for digital image manipulation, perhaps more aptly described as clarification or visual improvement. This is a skilled process and care must be taken not to affect the scientific content of the image. Manipulation implies deceit while clarifying does not carry a negative connotation (see essay entitled *Image Manipulation: Science Fact or Fiction*).

Selection of an electronic camera requires careful thought about its proposed use. For example, this can include the consideration of whether fixed specimens or live specimens will be photographed, whether there is need for true color images or whether only grayscale images are required, whether the sensitivity to low light such as in fluorescence applications with associated long exposures will be encountered, whether sizable resolution is required for print application, whether speed of image acquisition is required, whether there will be qualitative or quantitative investigations, and whether there will be a video feed required into a computer (via frame grabber card) or VCR.

The two general types of electronic cameras available for microscopy incorporate tube types (Vidicon family) of cameras or CCD cameras, although the less rugged tube cameras are rapidly becoming obsolete. Either device can be intensified for increased sensitivity to low light, usually with increased image noise, which may affect image contrast and visibility. The silicon intensified tube (SIT), or ISIT, is useful for further intensification of tube cameras, while high-performance, research-level CCD cameras are often equipped with an electron multiplying gain register that enables them to work at low brightness levels. CCD chips can also be cooled to increase their sensitivity and improve signal-to-noise ratio for long exposures. These kinds of devices can be designed to respond to light levels undetectable by the human eye, and several companies have products for this type of imaging.

Selection criteria for a camera specifically for microscopy include consideration of the quantum efficiency of the chip, its signal-to-noise ratio, spectral response, dynamic range, and the linearity of response. Also important is shutter lag and the speed of image acquisition and display, as well as the geometric accuracy of images and the adaptability to the microscope. A very important criterion for the newer digital CCD cameras is digital resolution, not to be confused with optical resolution. Current chips range from as few as 64×64 pixels and more than 5000×5000 required for very specialized applications. Larger arrays are continuously introduced and, at some not too distant time, digital image resolution will be able to match the microscope optical resolution at all magnifications.

CCD cameras are usually of small size and generally feature no geometrical distortion, little or no shutter lag, and good linearity of response. Each pixel of the CCD camera is an individual sensor (or well) for storing the photoelectrons generated by the incoming photons for subsequent readout. The capacity of these wells determines dynamic range and thus influences the number of gray levels the camera can differentiate which is important in difficult to image situations. Binning of adjacent pixels by joining them together into super pixels can be employed to speed readouts in slow-scan CCD cameras.

An emerging technology that shows promise as the possible future of digital imaging is the active pixel sensor (APS) complementary metal oxide semiconductor (CMOS), also known as a camera on a chip. Mass production of CMOS devices is very economical and many facilities that are currently engaged in fabrication of microprocessors, memory, and support chips can be easily converted to produce CMOS optical sensors. In the center of the APS CMOS integrated circuit is a large array of optical sensors that are individual photodiode elements covered with dyed filters and arranged in a periodic matrix. Each pixel element is controlled by a set of

three transistors and an amplifier that operates simultaneously to collect and organize distribution of optical information. The array is interconnected much like memory addresses and data busses on a dynamic random access memory (DRAM) chip so that the charge generated by photons striking each individual pixel can be accessed randomly to provide selective sampling of the CMOS sensor.

The Microscope

Figure 80 is an illustration of a modern epifluorescence microscope equipped with an advanced megapixel digital imaging camera system with Peltier cooling designed to image specimens over a wide exposure range recorded in a 24-bit color using low light levels. This setup might be characterized as a high-end imaging microscope but in reality, any reasonably good compound or stereo microscope can be used for photomicrography and digital imaging. An instrument that is decades old may still be able to produce highly resolved images if it has good optical components. Probably the most important components of any microscope are the objectives and eyepieces, which should be of the highest quality affordable. Most important for digital or film imaging is the quality of the objectives and substage condensers, especially if they have high numerical aperture (NA). Equally important is the degree of correction of the eyepiece projection lens and the collecting lenses of the illumination system of the microscope.

Objectives

Microscope objectives are designed to form a diffraction-limited image at a fixed location (the intermediate image plane) defined by the microscope's tube length and located at a specific distance from the rear focal plane of the objective or from the front focal point of the eyepiece. Specimens are placed at a very short distance from the lens, which is defined as the front focal point of the objective, so the optical train is influenced by the medium that the sample is surrounded by or its refractive index. It is thus important to be aware of refractive aberrations. Samples are typically surrounded by air, water, glycerin, or specialized immersion oils. Microscope manufacturers offer a wide range of objective designs to meet the

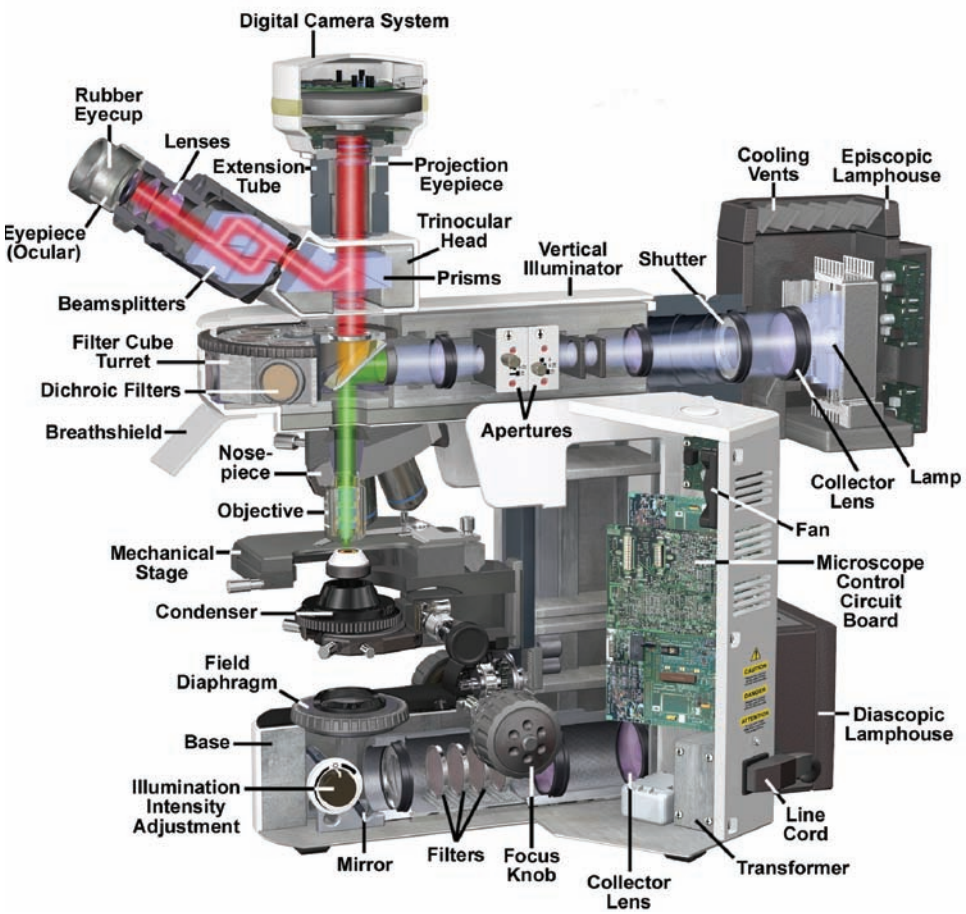

FIG. 83 Cut-away diagram of a research-level fluorescence microscope coupled to a modern color CCD digital imaging camera system.

performance needs of specialized imaging methods (discussed below) to compensate for cover glass thickness variations, and to increase the effective working distance of the objective.

The least expensive (and most common) objectives are the achromatic objectives, which are corrected for chromatic aberration in two colors (red and blue) and spherical aberration is corrected for one wavelength, green.

The limited correction of achromats leads to image artifacts that are especially obvious in color photomicrography. When the focus is optimized on a contrasty subject (or in green light), blue-red (magenta) haloes are evident. This is known as residual color. However acromats are satisfactory for black and white imagery if a green color filter is used.

A better level of correction is found in the more-expensive fluorite objectives. These have optical elements that contain low-dispersion materials such as fluorspar or newer synthetic substitutes to permit greatly improved aberration correction. Like achromats, fluorite objectives are also corrected chromatically for red and blue light, but their spherical aberration is corrected for two colors. This allows larger numerical apertures and consequently better resolution and contrast.

The highest level of correction (and expense) is found in apochromatic or APO objectives, which are corrected chromatically for three colors—red, green, and blue—effectively eliminating chromatic aberration. Spherical aberration is also corrected for two colors. Apochromats are an excellent choice for color photomicrography or digital imaging, but they are expensive. An achromat 10× PLAN lens might sell for $350 while its equivalent APO cousin could list for $3500. Many of the newer, high-end fluorite and apochromat objectives are both color and spherical aberrations corrected for four colors.

All these objective types suffer from field curvature to a greater or lesser extent, so their images are not in perfect focus across the field of view. However, lens designers have produced flat-field-corrected versions that have edge-to-edge sharpness. Such lenses are known as plan achromats, plan fluorites, or plan apochromats, and although this degree of correction adds to the cost, these objectives are best for photomicrography. Figure 84 reveals the important characteristics of a microscope objective, as well as an explanation of the nomenclature engraved or printed on a typical lens barrel.

Numerical Aperture

When characterizing a traditional photographic lens, a useful measure is to describe the maximum lens aperture as its f-stop and is used as an approximate indicator of light transmission. The aperture number is determined by dividing the focal length of the lens by the maximum clear diameter of that same lens when the lens is focused at infinity. Microscope lenses are also described by their light-transmitting potential; however, this information is provided as the objective's numerical aperture. There are several factors, which influence the objective's performance including the refractive index(es) of the material in which the lens is being used and the acceptance angle of the diffracted rays of light it can gather.

$$NA = n \text{ Sine } \mu$$

The NA of the objective is among other factors influenced by the focal length of the objective. Shorter focal length objectives will have a shorter working distance and consequently a greater angle of acceptance. As an objective's magnification goes up, so does its corresponding numerical aperture. The objective's NA will also influence the smallest of structural details that can be resolved when using the lens.

Eyepieces

Eyepieces (also known as oculars) work in combination with microscope objectives to further magnify the real intermediate image, allowing specimen details to be better observed. Better results in microscopy require that objectives be used in combination with eyepieces that are matched in correction with the type of objectives in the instrument. Inscriptions on the side of the eyepieces describe their particular characteristics and function. Often inferior and poorly corrected eyepieces are the cause of poor images.

Substage Condenser

The substage condenser is perhaps the least understood component in an optical microscope. This lens group gathers light from the lamp and produces a cone of light that illuminates the specimen with a uniform intensity spanning the entire view of field, while influencing the energy that will form optical resolution. It is critical that the condenser height be properly adjusted to optimize the intensity and angle of light entering the sample and subsequently the objective lens. Each time an objective is changed, a corresponding adjustment must be performed on the substage condenser aperture iris diaphragm

FIG. 84 Modern microscope objective specifications and nomenclature, including the definition for parfocal and working distances. A constant parfocal distance between similar objectives in a nosepiece ensures that each objective of increasing magnification will be in approximately the same focus as the others. Working distance is a measure of the free space between the objective front lens and the cover glass or specimen.

to provide the proper light cone for the numerical aperture of the new objective. Like objectives, condensers come in a variety of configurations and are classified as Abbe, flip or swing out, and/or Achro-Aplanic. There are numerous special types of substage condensers such as phase contrast and darkfield, just to name two.

There are two apertures on a compound microscope and aperture adjustment as well as proper focusing of the condenser is critical in realizing the full resolution potential of the objective. Specifically, appropriate use of the adjustable aperture diaphragm (incorporated into the condenser or just below it) is critical in producing uniform and even illumination, image contrast, image resolution, and image depth of field. The opening and closing of this aperture diaphragm controls the angle of illuminating rays that exit through the condenser, then enter the specimen, and then into the objective. Condenser height is controlled by a rack and pinion gear system that allows the condenser height to be adjusted for proper illumination. Correct positioning of the condenser with relation to the cone of illumination and focus on the specimen is critical to quantitative microscopy and optimum photomicrography. Care must be taken to ensure that the condenser aperture is opened to the correct position with respect to objective's NA. When the aperture is opened too much, stray light generated by refraction of oblique light rays from the specimen can cause flare and lower the overall image and system contrast. On the other hand, when the aperture is closed too far, the illumination cone is insufficient to provide adequate resolution, and the image looks as though it was made without critical focus and void of fine detail but full of image contrast.

The NA of the combined objective/condenser optical system determines the maximum useful magnification that an objective/eyepiece combination can achieve. As a rule of thumb, magnifications greater than about 1000× of the system numerical aperture enter the realm of 'empty magnification:' the image may appear bigger, but there is no new information revealed. However, this factor is somewhat dependent on both the wavelength of observation and on the viewing distance of the final image—images in blue or UV light usually have higher resolution than those made at longer wavelengths.

Resolution

The resolution in an optical microscope is defined as the shortest distance between two points that can be distinguished by the observer or camera system as separate locations. The resolution of a microscope's objective is described as its ability to distinguish between two closely spaced point sources of light (in practice Airy disks) found in the diffraction patterns produced by the illumination of the specimen. Three-dimensional representations of the diffraction pattern near the intermediate image plane reveal the point spread function.

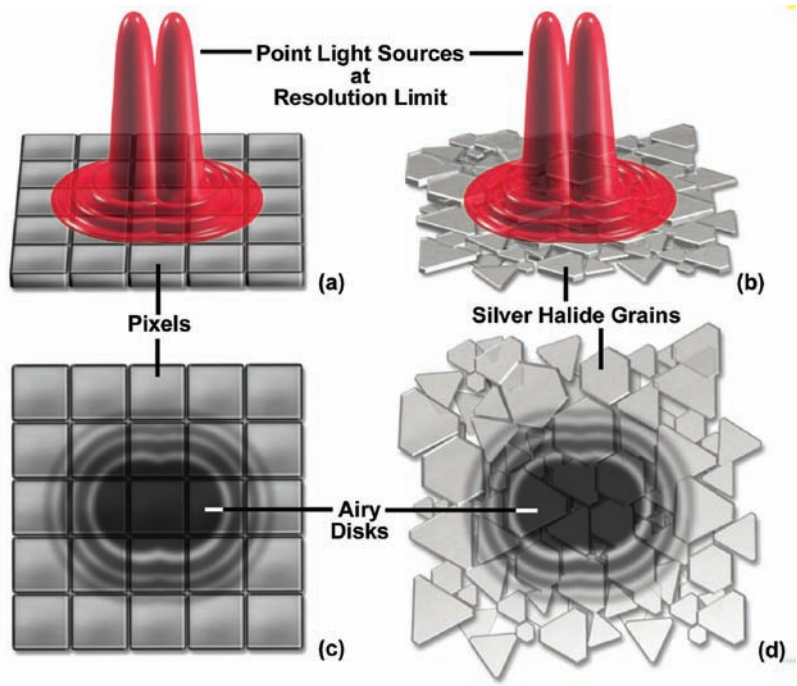

FIG. 85 Film grain size or CCD pixel size determines detector resolution. Superimposed on a CCD array (a) is the point spread function of two light points at the limit of optical resolution. The same situation for silver halide grains in film is illustrated in (b). The Airy disk patterns on the CCD (c) and film grains (d) indicate the affected sensor elements.

The final specimen image is represented by a series of closely spaced point light sources that form overlapping Airy patterns. The separation distance of these point sources on the detector surface in relation to the detection of spatial resolution (eye retinal cell, CCD pixel, or film grain size) determines the resolution of the detection system, as illustrated in Figure 82.

Correct alignment of the microscope optical system is also of importance to produce the system's maximum resolution. The substage condenser's NA must be matched to the objectives and the subsequent adjustment of the aperture diaphragm for accurate light cone formation and specimen illumination. The wavelength of light that is used to image a specimen is also a determining factor in the degree of resolution afforded by the microscope optics. Shorter wavelengths are more capable of resolving finer details than the longer wavelengths. The principal equation used to express the relationship between NA, wavelength, and resolution is

$$\text{Resolution } (d) = \lambda/\text{NA} + \text{NA}$$

In this expression, d is resolution (the smallest resolvable distance between two points), NA is the microscope objective and condenser numerical aperture, and λ is the wavelength of light. The resolution equation is based upon a number of factors (including a variety of theoretical calculations made by optical physicists) to account for the behavior of objectives and condensers and should not be considered an absolute value of any one general physical law. In some instances, such as confocal and fluorescence microscopy, the resolution may actually exceed the limits placed by any one of these calculations. Other factors, such as low specimen contrast and improper illumination may serve to lower resolution and, more often than not, the real-world maximum value of r (about 0.25 μm using a mid-spectrum wavelength of 550 nm) and an NA of 1.35 to 1.40 are not realized in practice.

The wavelength of light is important in determining the resolution of an optical microscope. At any given NA, shorter wavelengths provide higher resolution (smaller values for d). The highest practical resolving power is obtained in ultraviolet light, and it gradually diminishes towards the red part of the spectrum. Most microscopists use white light to illuminate the specimen, and the mean wavelength of the visible spectrum is at about 550 nm, which coincides with the maximum sensitivity of the human eye. With self-luminous and reflective sources (as in fluorescence and dark-field illumination), objects that are much smaller than the theoretical limit can be readily detected (but not resolved) against a dark background by their emitted, scattered or diffracted light.

Resolution is a somewhat subjective concept in optical microscopy because at high magnifications, an image may appear unsharp but still be resolved to its maximum. Numerical aperture determines the resolving power of an objective, but the total resolution of the entire microscope optical train is also dependent upon the NA of the substage condenser. In general, the higher the NA of the total system, including the objective and condenser, the better the system resolution.

As discussed above, the primary factor in determining resolution is the objective NA, but resolution is also dependent upon the type of specimen, coherence of illumination, degree of aberration correction, and other factors such as contrast-enhancing methodology either in the optical system of the microscope or in the specimen itself. In the final analysis, resolution is directly related to the useful magnification of the microscope and the perception limit of specimen detail.

Illumination

The microscope operation is built upon the concept of conjugate planes, which incorporate two interacting pathways that occur concurrently in the optical pathways of the instrument. One conjugate pathway consists of four field planes and is referred to as the image-forming path, while the other consists of four locations and is known as the illumination conjugate pathway. Proper alignment of the microscope illumination for photomicrography and digital imaging requires that these conjugate plane sets are focused (with the light beam centered) at the correct locations. Because various locations are in focus and others are not forms the basis of the Köhler illumination, named for its inventor August Köhler. Illumination is perhaps the most critical factor in determining the overall performance of the optical microscope. It is under the conditions of Köhler illumination that the requirements are met for having two separate sets of conjugate focal planes, field planes, and aperture planes in precise physical locations in the microscope. The details of adjusting a given microscope to satisfy the Köhler illumination conditions depend to some extent upon how the individual manufacturer engineers the microscope.

The basic requirements for setting up Köhler illumination are very simple (illustrated in Figure 83). A collector lens on the lamp housing must focus the lamp filament to the front aperture in the condenser while completely filling the aperture diaphragm. With the lamp's image relayed to the proper location, the sample should be focused to create the objective's proper working distance. With this condition met, the condenser must be adjusted to bring the image of the field diaphragm to the proper location found within the optical axis of the microscope (the sample is also in focus at this location). Meeting these conditions will result in a bright, evenly illuminated specimen plane, even with an inherently uneven light source such as a tungsten-halogen lamp filament (the filament will not be in focus in the specimen plane). With the specimen and field stop now in focus, the focal plane conjugates will be in the proper position so that aperture diaphragm can be set for optimized resolution and contrast.

The imaging and illumination ray paths through a microscope adjusted for Köhler illumination are presented in Figure 86, with the focal conjugates of each plane set indicated by crossover points of the ray traces. Illustrated diagrammatically in the figure is the reciprocal nature of the two sets of conjugate planes that occur in the microscope. The optical

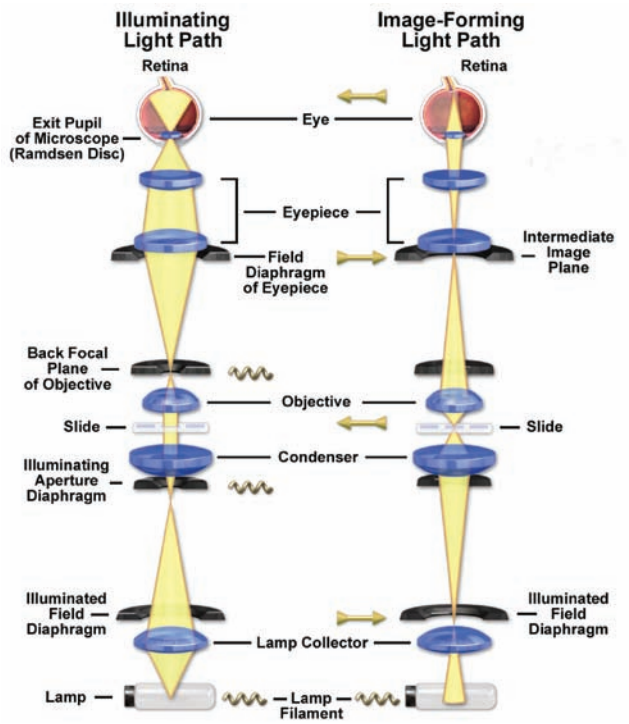

FIG. 86 Light paths in Köhler illumination. The illuminating ray paths are illustrated on the left side and the image-forming ray paths on the right. Light emitted from the lamp passes through a collector lens and then through the field diaphragm. The aperture diaphragm in the condenser determines the size and shape of the illumination cone on the specimen plane. After passing through the specimen, light is focused on the back focal plane of the objective and then enters and is magnified by the ocular before passing into the eye.

relationship between the conjugate planes is based upon the fact that, in the illuminating ray path, the spherical wave fronts converge and are brought into focus onto the aperture planes, while in the imaging ray path, the spherical waves converge into focused rays in the field planes. Light rays that are focused in one set of conjugate planes are nearly parallel when passing through the other set of conjugate planes. The reciprocal relationship between the two sets of conjugate planes determines how the two ray paths fundamentally interact in forming an image in the microscope, and it also has practical consequences for operation of the microscope.

Other Microscopy Techniques

There are many contrast-producing methods for both qualitative and quantitative microscopy, including darkfield, Rheinberg, phase contrast, polarized light, Hoffman Modulation Contrast, differential interference contrast (DIC) metallurgical and fluorescence. Many of the newer fluorescence microscopy techniques have also been further developed into high resolution imaging methods for biological applications.

The oldest and most common technique, brightfield illumination, is used with specimens with inherent contrast, which are often stained with naturally occurring or synthetic dyes. The resultant image will appear colored against a white field. Many times though the sample does not arrive in this condition. Sometimes creating contrast is required or the sample will remain invisible.

An older and more useful technique is the darkfield technique, which is used especially for transparent objects. It may also be referred to as darkground illumination. Darkfield illumination can make use of commercially available condensers or be achieved by removing the systems central order of light with opaque stops. They are usually placed near or in the condenser. The field and aperture diaphragm are completely opened and oblique angle light enters the sample. The object will be brightly illuminated against a black background. In the absence of an object the field is dark.

Rheinberg illumination, developed by British microscopist Julius Rheinberg, is a striking variation of low to medium power darkfield illumination. This technique uses colored gelatin or glass filters to provide rich and different colors to the specimen and background. The central opaque stop used in darkfield is replaced with a semi-transparent, colored, circular stop inserted into a transparent ring of a contrasting color. These stops are placed under the bottom lens of the condenser. The result is a specimen rendered in the color of the ring with a background having the color of the central spot.

Polarized light microscopy is an easy technique to configure and is readily available to both amateur and professional microscopists. A linear polarizer is placed beneath the condenser and a second polarizer—often referred to as an analyzer—is placed behind the objective (nearly anywhere in the light path) at a 90-degree angle to the first filter. This technique is excellent for examining and recording images composed of birefringent materials, such as recrystallized organic compounds, insect chitin, plastics, liquid crystals, and a wide variety of similar specimens. Materials with multiple refractive indices are described as anisotropic, while materials of a single refractive index are described as isotropic.

Phase contrast microscopy, developed by Frits Zernike in the early 1930s, was engineered to make unstained objects visible by producing contrast by changing the light phase relationships. Unstained specimens that do not absorb light are called phase objects. In the phase microscope, they alter the phase of the light diffracted by the specimen. In phase technique the zero order of illumination is retarded one-fourth of a wavelength relative to the sample beam of light passing through or around the zero order. Special objectives and condensers are needed for phase contrast. A substage phase contrast condenser equipped with phase annulus is matched to an objective with a complementary phase ring. Each objective condenser setting is matched. A universal condenser might be used in phase and usually has a brightfield position and a darkfield setting, as well as the various phase settings.

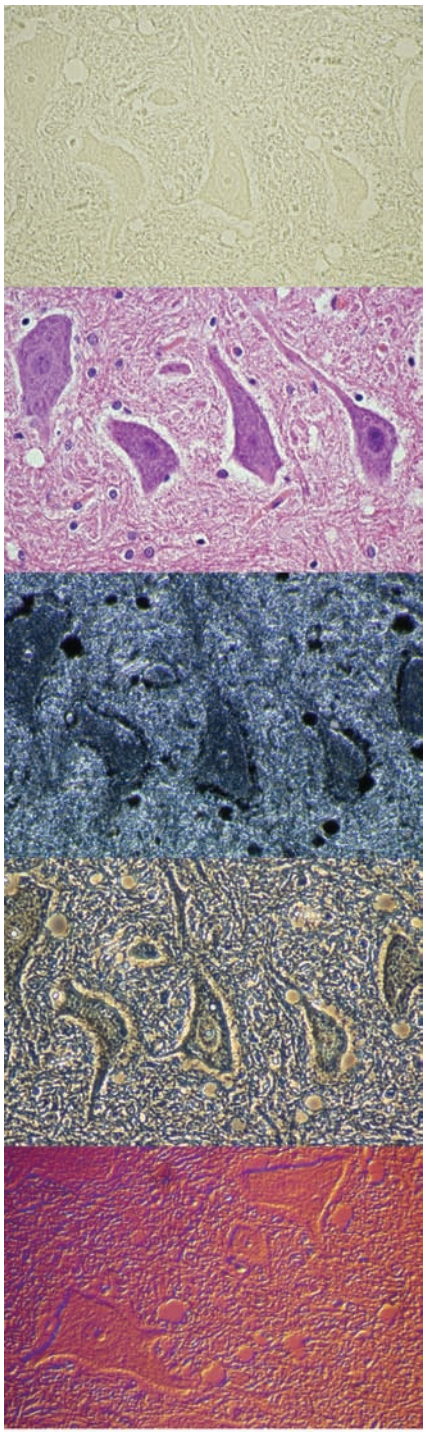

FIG. 87 This composite image reveals how a preparation of mammal nerve cells would appear when using a 10× objective. From the top: an unstained sample with brightfield illumination stained with hematoxylin and eosin; darkfield illumination phase contrast technique; and DIC technique. (Photographs by Michael Peres.)

The Hoffman Modulation Contrast (HMC) system, invented by Dr. Robert Hoffman in 1975, is used to increase visibility and contrast in unstained and living material by detecting optical gradients (or slopes) in the sample and converting them into variations of light intensities. An optical amplitude spatial filter, termed a modulator by Hoffman, is inserted at the rear focal plane of the objective. This filter is complemented by an off-center slit that is placed in the microscope condenser. Light intensity passing through this system varies in intensity above and below the median brightness, which by definition, is then said to be modulated. The system produces images that are very high in contrast with a high degree of coherence, capable of producing sharply focused sections. In the resulting images shapes and details are rendered with a shadowed, pseudo three-dimensional appearance. These appear brighter on one side, gray in the central portion, and darker on the other side, against a gray background. Some have called HMC poor man's Nomarski.

DIC (also known as Nomarski contract) is a specialized technique developed by the French optics theoretician Georges Nomarski in the 1950s for detecting optical gradients in specimens and converting them into intensity differences creating contrast. In DIC, light from the lamp is passed through a polarizing filter located beneath the substage condenser, in a location similar to that used in polarized light microscopy. Next in the light path (but still beneath the condenser) is a Wollaston prism, which splits the entering beam of polarized light into two beams traveling in slightly different directions. The two rays intersect at the front focal plane of the condenser where they travel in parallel and extremely close together with a slight path difference, but they are vibrating perpendicular to each other and are therefore unable to cause interference. The beams enter and pass through the specimen where their wave paths are altered in accordance with the specimen's varying thickness, structure, and refractive indices. When the parallel beams enter the objective, they are focused above the rear focal plane where they enter a second Wollaston prism that recombines them. For the beams to interfere, the vibrations of the beams of different path length must be brought into the same plane and axis. This is accomplished by placing a second polarizer (analyzer) after the second Wollaston prism. The image then proceeds toward the eyepiece where it can be observed and recorded as differences in intensity and color. Similar to HMC, the images appear obliquely illuminated and reveal an almost pseudo-relief.

Fluorescence microscopy is an excellent tool for studying material that can be made to fluoresce, either in its natural form (primary or autofluorescence) or when treated with chemicals capable of fluorescing (secondary fluorescence). This form of optical microscopy is now one of the fastest growing areas of investigation in microscopy.

The basic task of the fluorescence microscope is to expose a sample to excitation energy and then to separate the much weaker fluorescence signal from the brighter excitation light. In this manner only the fluorescence emission reaches the eye or other detector. The resulting fluorescence shines against a

dark background with sufficient contrast to permit detection. This separation of excitation from emission light requires the use of specialized filter sets. It should be noted that this is the only mode of microscopy in which the specimen, subsequent to excitation, gives off its own light.

Fluorescence microscopy has unique advantages not offered by other optical microscopy techniques. The use of fluorochromes, commonly called fluorophores, has made it possible to identify cells and sub-microscopic cellular components and entities with a high degree of specificity amidst non-fluorescing material. An extremely small number of fluorescing molecules (as few as 50 molecules per cubic micron) can be detected. Although the fluorescence microscope cannot provide spatial resolution below the diffraction limit of the objective, the presence of fluorescing molecules below such limits is made visible.

Fluorochromes are stains, somewhat similar to the better known tissue stains, which attach themselves to visible or sub-visible organic matter. These fluorochromes, capable of absorbing and then re-radiating light, are often highly specific in their attachment targeting and have significant yield in absorption-emission ratios. This makes them extremely valuable in biological application. The growth in the use of fluorescence microscopes is closely linked to the development of hundreds of fluorochromes with known intensity curves of excitation and emission and well-understood biological structure targets. When deciding which label to use for fluorescence microscopy, it should be kept in mind that the chosen fluorochrome should have a high likelihood of absorbing the exciting light and should remain attached to the target molecule. The fluorochrome should also be capable of providing a satisfactory yield of emitted fluorescence light. Fluorescence microscopy has given birth to a number of sophisticated techniques that rely on the fluorescent properties of biological molecules to produce images. Several of the more important techniques are now reviewed.

Confocal laser scanning microscopy (CLSM) is a popular mode of optical microscopy in which a focused laser beam is scanned laterally along the x and y axes of a specimen in a raster pattern (Figure 88). The emitted fluorescence (reflected light signal) is sensed by a photomultiplier tube and displayed in pixels on a computer monitor. The pixel display dimensions are determined by the sampling rate and the dimensions of the raster sensor. Signal photons that are emitted away from

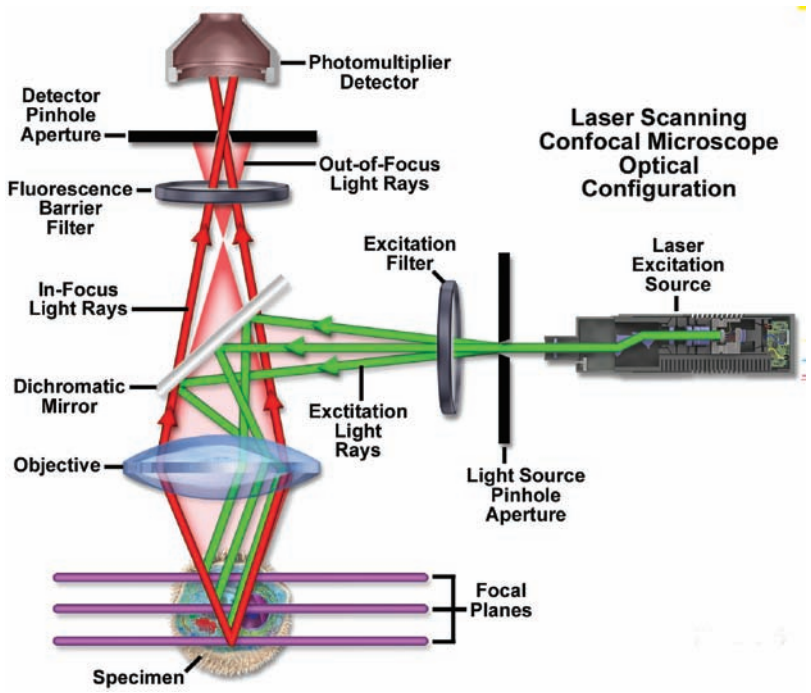

FIG. 88 The confocal principle in epifluorescence laser scanning microscopy. Coherent light emitted by the laser system (excitation source) passes through a pinhole aperture that is situated in a conjugate plane (confocal) with a scanning point on the specimen and a second pinhole aperture positioned in front of the detector (a photomultiplier tube). As the laser is reflected by a dichromatic mirror and scanned across the specimen in a defined focal plane, secondary fluorescence emitted from points on the specimen (in the same focal plane) pass back through the dichromatic mirror and are focused as a confocal point at the detector pinhole aperture.

the focal plane are blocked by a pinhole aperture located in a plane that is confocal to the specimen. This technique enables the specimen to be optically sectioned along the z-axis.

Deconvolution fluorescence microscopy is a technique that applies algorithms to a stack of images acquired which has been along the optical (z) axis to enhance photon signals specific for a given image plane or multiple focal planes in an image stack. The microscope must be equipped with a stepper motor attached to the focus gearset to guarantee image acquisition at precisely defined intervals between focal planes in the specimen. In a typical application, deconvolution analysis is utilized to deblur and remove out-of-focus light from a particular focal plane of interest using fluorescence excitation and emission (although the technique is useful for other illumination modes as well). The most sophisticated applications apply deconvolution analysis to an entire image stack to produce projection views or three-dimensional models.

Total internal reflection fluorescence microscopy (TIRFM) is designed to probe the surface of fluorescently labeled living cells with an evanescent wave generated by a light beam traveling between two media of differing refractive indices. In practice, an incident laser beam is reflected at a critical angle (total internal reflection) when it encounters the interface between a microscope glass slide and the aqueous medium containing the cells. Fluorophores within a few nanometers of the surface are excited by the evanescent wave, while those farther away are unaffected. This technique is commonly employed to investigate the interaction of molecules with surfaces, an area which is of fundamental importance to a wide spectrum of disciplines in cell and molecular biology.

Two photon (multiphoton) microscopy is a derivative technique of laser scanning confocal microscopy where fluorochrome excitation is based on an infrared or long wavelength visible light laser beam whose energy density is adjusted to allow frequency doubling or tripling at the point of beam focus in the specimen. Fluorophores in the specimen are simultaneously excited by two or three photons to produce excited state transitions that are equivalent to single-photon fluorescence. For example, two- and three-photon excitation at 900 nm is equivalent to excitation by higher energy photons of 450 and 300 nm, respectively. Multiphoton microscopy enables deep penetration into thick tissues and eliminates the need for a pinhole aperture because fluorescence emission is restricted to a single focal plane.

See also the following articles

Biological Photography; Fluorescence Imaging; Ophthalmic Photography

FURTHER READING

Hibbs, A. R. (2004). *Confocal Microscopy for Biologists*. New York: Kluwer/Plenum.

Murphy, D. B. (2001). *Fundamentals of Light Microscopy and Electronic Imaging*. New York: Wiley-Liss.

Rost, F. and Oldfield, R. (2000). *Photography with a Microscope*. Cambridge, U.K.: Cambridge University Press.

Police Photography

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The first known use of the camera for law enforcement purposes was in the mid 19th century, initially to record still images of arrested individuals and to document crime scenes. This is still important today, but police also now use camera and video to record interrogations, traffic stops, surveillance, public thoroughfares, and traffic accidents. They also frequently use cameras to document physical evidence at a crime scene before it is collected into evidence. Fingerprints developed with fluorescent powder or illuminated by an alternate light source (ALS, an intense light source with filters capable of illuminating a wide range of wavelengths from the short-wave ultraviolet (230 nm) through the near infrared (900 nm), commonly used to search for trace evidence at a crime scene) is an example of this. The police photographer must have an understanding of how the camera can record not only the visual and audio components of an interrogation or traffic stop but also how it can record images at invisible ultraviolet (UV) and infrared (IR) wavelengths. The police photographer must also have a good working knowledge of other specialized techniques such as close-up (macro) photographs, the effective use of fill-flash and bounce flash, and photography in less than desirable conditions such as night-time with limited ambient lighting.

Most police agencies use a hybrid system for their photographic needs. This hybrid system is composed of a digital camera, a 35 mm single lens reflex (SLR) camera, and a video camcorder. When the digital camera emerged in the late 20th century, it was touted as the replacement for traditional film cameras. This proved not to be true, at least into the early 21st century. Nonetheless, digital cameras are very useful in police photography. They are able to capture images in the invisible range, particularly in the IR. Other advantages of digital cameras include the ability of the photographer to see the immediate results of the photo, the ability to send digital photos over the internet and to computer-equipped police cars, the ability to record images over a wide range of the spectrum from the UV into the IR, and storage capability. However, digital cameras may not perform well in low light situations, when enlargements of greater than about 24 × 36 cm (11 × 14 inches) are required, or when a crime scene must be recorded at night using a painting-with-light technique. In these situations a film camera should be used. A 12 megabyte digital camera might be able to produce an acceptable 24 × 36 cm enlargement, but the cost of such a camera may be prohibitive for a police department. As a result, most law enforcement agencies use the same make of digital camera as their film camera in order to use the same accessories. For instance, if a police agency uses a Nikon 35 mm SLR camera then the digital camera is going to be a Nikon SLR so that the same lenses, filters, flashes, etc., can be used for both cameras. In this way, the department does not have to invest in different