Introduction to MICROSCOPY BY MEANS OF LIGHT, ELECTRONS, X RAYS, OR ACOUSTICS

SECOND EDITION

Theodore George Rochow
and
Paul Arthur Tucker

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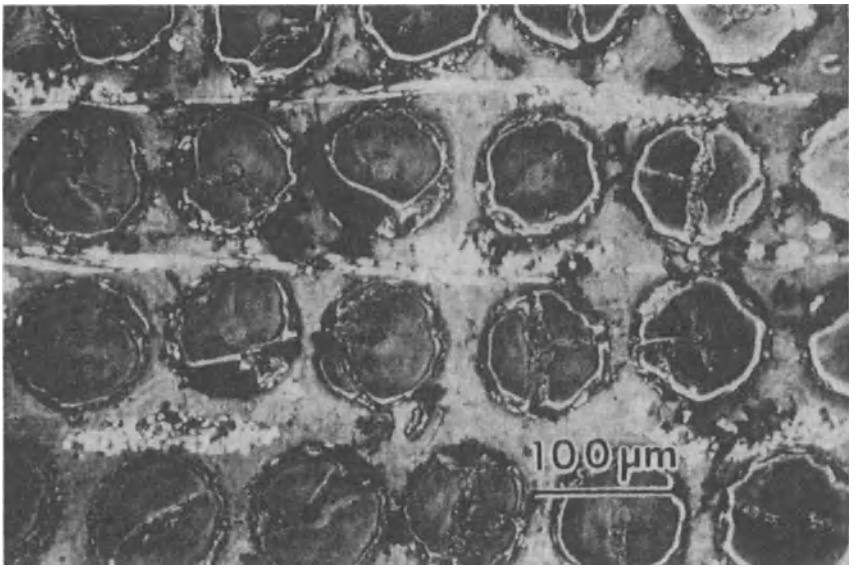

Acoustic micrograph of a composite of boron and glass fibers in an epoxy matrix. Boron fibers are seen in cross-sectional view and the smaller glass fibers are in both the longitudinal and cross-sectional view. The photomicrograph was taken with 400-megahertz acoustic radiation. (Courtesy of D. A. Downs, A. El-Shiekh, M. H. Mohamed, P. A. Tucker, and J. C. Russ of North Carolina State University.) (See Chapter 18.)

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To the North Carolina
State University at Raleigh

Preface

Following three printings of the First Edition (1978), the publisher has asked for a Second Edition to bring the contents up to date. In doing so the authors aim to show how the newer microscopies are related to the older types with respect to theoretical resolving power (what you pay for) and resolution (what you get).

The book is an introduction to students, technicians, technologists, and scientists in biology, medicine, science, and engineering. It should be useful in academic and industrial research, consulting, and forensics; however, the book is not intended to be encyclopedic.

The authors are greatly indebted to the College of Textiles of North Carolina State University at Raleigh for support from the administration there for typing, word processing, stationery, mailing, drafting diagrams, and general assistance. We personally thank Joann Fish for word processing, Teresa M. Langley and Grace Parnell for typing services, Mark Bowen for drawing graphs and diagrams, Chuck Gardner for photographic services, Deepak Bhattavahalli for his work with the proofs, and all the other people who have given us their assistance.

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Raleigh, North Carolina

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A Brief History of Microscopy

1.1. INTRODUCTION

All kinds of microscopy have a common beginning in mankind's intellectual goal to see better. Visible light was the first medium, and visibility was limited to the unaided eye until the first century A.D., when Seneca discovered[1] that by looking through a clear spherical flask filled with clear water "letters however small and dim are comparatively large and distinct."[2] This idea led to simple magnifiers, whether a single large lens to accommodate both eyes together or two lenses of a smaller diameter to accommodate each eye separately.

During the next dozen centuries, spherical segments of clear minerals were set in frames as eyeglasses to help older people see better. In about the year 1300, clear silicate glass was made in Italy, and superstition prohibiting its use in eyeglasses for far-sightedness was overcome.[3–5] By the sixteenth century concave lenses were also for sale to help near-sighted people. The availability of both convex and concave lenses led the Dutch to the linear combination of the two as a crude compound microscope. Just who invented the Dutch microscope is still not known; credit generally is given to three Dutch spectacle makers around the turn of the seventeenth century.[6,6a] The principle of a concave lens serving to amplify the objective is still used today to provide a flat field in some instruments for projection and photomicrography.

In 1611 the Dutch microscope came to the attention of the German mathematician and astronomer Johann Kepler (1571–1630). Kepler was quick to realize that enlargement could also be obtained by combining a convex (instead of concave) ocular with the convex objective but that the image would appear inverted rather than upright. The Kepler ocular serves to enlarge the real image of the objective, which is the principle of the modern compound light microscope.[2] Kepler also explained the diop-

trics of the eye and showed how convex spectacle lenses correct for hyperopia, while concave lenses correct for myopia. By 1619 Cornelius Drebbel in London had made a compound microscope with a biconvex eye lens and a plano-convex objective the size of a small cherry, stopped down by a diaphragm opening the size of a thick needle. The microscope produced an inverted image.[7] In May 1624, Galileo received a Drebbel microscope; he demonstrated how it worked and indicated that he had made similar but better "occhiali" (eyeglasses). In the same year Galileo ground lenses, mounted them into microscopes, and sent them to friends.[7,7a,8] Galileo exhibited his cleverness when demonstrating his telescope, and some observers doubted that what they saw was real. He simply turned the telescope toward something very familiar nearby and invited the doubters to take a look. Thus he demonstrated that telescopy, like microscopy, is the *interpretive* use of the instrument by the *observer.*

The word microscope was coined by Giovanni Faber on April 13, 1625.[6] It implies subjectively seeing (resolving) small things or their parts. However public dictionaries to this day define microscope objectively as a powerful magnifier. This definitely was the reason for using a microscope in the days of Robert Hooke (1635–1703). Hooke owned a microscope (made by his friend Christopher Cock) with an objective lens and an ocular lens. Hooke also possessed a third (field) lens, which he called his middle glass. He experimented with the relative placement of these lenses for more magnification while viewing such materials as cork. His skill as an artist is illustrated in Figure 1.1. Indeed one of Hooke's drawings, *Eyes and Head of a Grey Drone Fly,* is included in the 1991 exhibition of sixteenth- and seventeenth-century art *The Age of the Marvelous.*[8]

Robert Boyle was particularly impressed with Hooke's ability and recommended him, at the age of 27, to be life-time curator of experiments for the Royal Society. Consequently King Charles II requested a "handsome book" of Hooke's reports. In his resulting book *Micrographia,*[9] Hooke also described how to make a simple (single-lens) microscope by means of a very small spherical bead:

> Draw a thread of glass, run it into a bead in the flame, and then snap off the apex. Grind that region flat with jeweler's abrasives. Fit the tiny lens in a hole made in a flat piece of metal. Impale the specimen or hold it with wax (or place a drop of a liquid specimen) on a spike hand-made into a screw for focusing the lens.[9,9a]

As with all simple microscopes, the object is placed very close to the lens. Likewise the eye must be placed close to the lens to see the whole field.

Antony van Leeuwenhoek (1632–1723), the Dutch draper, was 40 years old before he began making and using simple microscopes (for 50

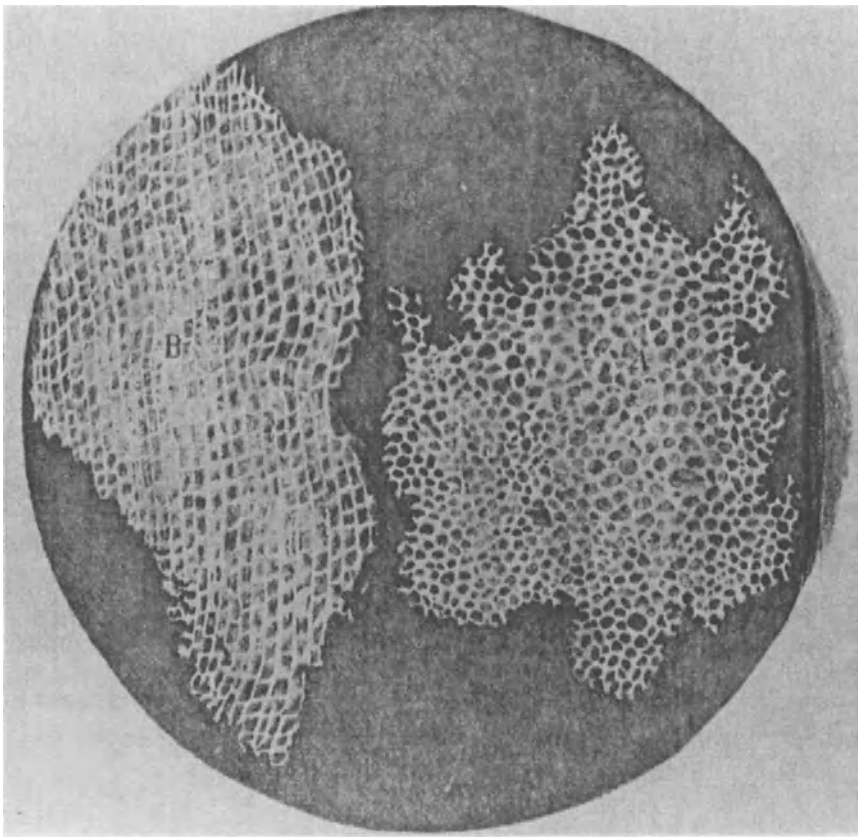


FIGURE 1.1. Hooke's drawings of cork showing the cells, a word he used because the tiny spaces reminded him of monks' cells.[8]

more years). He followed Hooke's directions[9] and made more than 400 microscopes, of which only nine remain. These range in numerical apertures from 0.11–0.37 (69×–266×). The one at the Museum of Science at the Utrecht University is the best, and it has been evaluated recently.[10]

These two microscopical giants of their time, Hooke and Leeuwenhoek, differed personally in attitude. The naive Dutchman, with his simple microscope, persisted in searching for microscopic objects, such as "animalcules." For two-and-a-half centuries however, there have been those who doubted that Leeuwenhoek could have discovered bacteria and other microorganisms—and with only a single-lens microscope held in his hand. However recent work, especially that of Brian J. Ford and his colleagues,

using Leeuwenhoek's surviving microscopes and modern versions of simple microscopes, has completely substantiated Leeuwenhoek's work and results.[11–13] Why did Leeuwenhoek succeed in resolving such small dimensions? The main reason seems to be that he held specimen, lens, and his eye all very close together. This rule remains today for all simple microscopes (and magnifying glasses).

Hooke preferred to use a *compound* microscope exclusively, which resembled Galileo's telescope, with objective and eyepiece considerably further apart[8] than today's standard tube length. Moreover Hooke was convinced that more and more *magnification* was required to see more detail than was already known. For example he tried to examine green "tarnished" water with his long compound microscope to see "whether this was like moss—yet so ill and imperfect are our microscopes, that I could not certainly discriminate any."[9] The main reason for the difficulty was that his compound microscope magnified aberrations along with the images.

Throughout the seventeenth century experiments in empirically arranging two, three, or more lenses in a tube were continued, but the salient advance for us today was the advent of the Huygens eyepiece, invented by the Dutch physicist and astronomer Christiaan Huygens (1629–1695) and his brother. The Huygens eyepiece has two planoconvex lenses, each with the convex side facing the objective. The lower (field) lens modifies the real image from the objective to give a brighter but smaller image, which is then examined by the upper lens. The Huygens design is still the principal one for microscopical eyepieces of magnifications of 10× and less. Reticles of linear, areal, or angular scales are used now in such eyepieces for micrometry, as they were in Huygens' day.

The hand-held crude wooden or iron microscopes of the seventeenth century evolved into elegant eighteenth-century table microscopes, made chiefly of brass. The importance of the evolution of microscopical stands centers on the illumination of the object: Mirrors were developed to transmit light through transparent objects, while others were made to reflect light from opaque objects. Some stands have mirrors to direct a beam of light through transparent objects; other stands have mirrors that direct a beam of light on opaque objects so that it may be reflected to the objective.

The problems of viewing through compound microscopes persisted into the nineteenth century. Some microscopists and manufacturers preferred the simple microscope with only a one-lens system that was achromatically corrected. Robert Brown, the great botanist, not only preferred the microscope without an eyepiece but also without a condenser. Instead he used a double-sided mirror mounted on a rotating collar to increase the

variation of illumination in both kind and extent, as illustrated in Figure 1.2. He traveled widely, exploring Australia and Tasmania, and returned with over 4000 species to identify. In his reports on this work in 1827 and 1829, he mentioned the "active molecules," the phenomenon now known as *Brownian* movement (motion.)[14]

Brown's conclusion about active molecules, like Leeuwenhoek's about animalcules, has been sharply criticized, but *both* have been substantiated and publicized by Brian J. Ford.[11–14] Besides, Brown himself asked George Dolland (1774–1853) to use his uncle's fine triple achromatic objective lens (1764) to confirm the Brownian phenomenon. He did. Ford writes, "There is a belief that nowadays we can do things which of course could not have been possible in those unrefined, far off, and primitive times. Yet as I have shown elsewhere, the images that can be obtained even with a Leeuwenhoek lens are not so different from those you would obtain with the best optical microscope available today."[14] Microscopy is the interpretive use of the microscope.

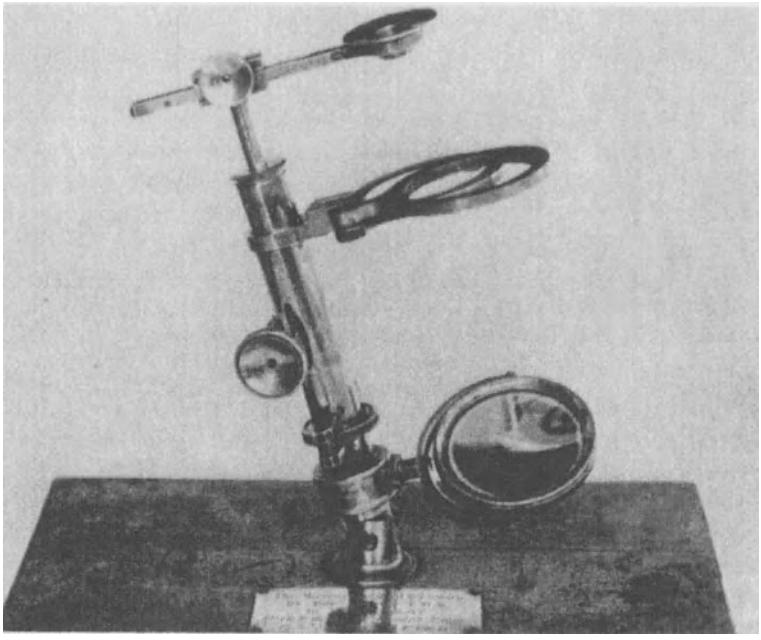


FIGURE 1.2. The microscope with which Robert Brown observed active molecules, now called Brownian movement (or motion).[14] Courtesy of McCrone Research Institute.

1.2. CORRECTING FOR ABERRATIONS

During the seventeenth century there was little optical improvement because the causes of aberrations and artifacts were not understood. However, at the turn of the eighteenth century, David Gregory (1661–1708), a friend of Sir Isaac Newton (1642–1727), noted something that had escaped the master: Different kinds of glass spread out the colors of the spectrum to different extents.[4] Gregory suggested that a proper combination of two kinds of glass might produce no spectrum at all. The first commercial achromatic objectives appeared in 1824 and were of French manufacture. The early ones had a smaller numerical aperture than the corresponding uncorrected objectives,[2] so these produced less brilliant images and were at first unpopular. However achromatic objectives of higher aperture were perfected rapidly during the nineteenth century.[15–21] A vivid example of this progress is evident from comparing Figures 1.3a and 1.3b.[7]

In Figure 1.3b, use of the achromatic objective of 1850 has increased the resolution in Figure 1.3a, but contrast has decreased and glare has increased. During the next hundred years, resolution was further improved by means of the apochromatic objective. With improvements in illuminating systems and other parts of the light microscope, contrast was increased, glare decreased, and resolution brought to very nearly theoretical limits.

Meanwhile there was corresponding improvement in oculars. In 1782, Jesse Ramsden (1735–1800), an English telescope maker, produced an ocular of new design. Like the Huygens' eyepiece, Ramsden's contained the two planoconvex lenses, but the convex sides were turned toward each other, and they were close together for better correction of spherical aberrations. Both lenses were mounted so that they were above the focal plane of the objective's real image, where the cross hair or micrometer scale normally is placed; thus the ocular could magnify both. The Ramsden type of eyepiece is used today for most work requiring eyepiece magnification of about 12× or greater.[18]

By the beginning of the twentieth century, microscopists were setting up standards for measuring resolving power,[22] employing such natural structures as the striated scales of butterflies, followed by diatoms and then by artificial rulings.[21] Thus it was discovered that resolution increased with angular aperture in the objective and condenser.

One of the chief investigators, Giovanni Battista Amici, combined the qualities of theorist, practical optician, and microscopist. Among his important innovations was the use of a single hemispherical front lens in the microscopical objective (in 1844), a principle that still prevails. In 1830 Joseph J. Lister (1786–1869), father of the famous surgeon, discovered the

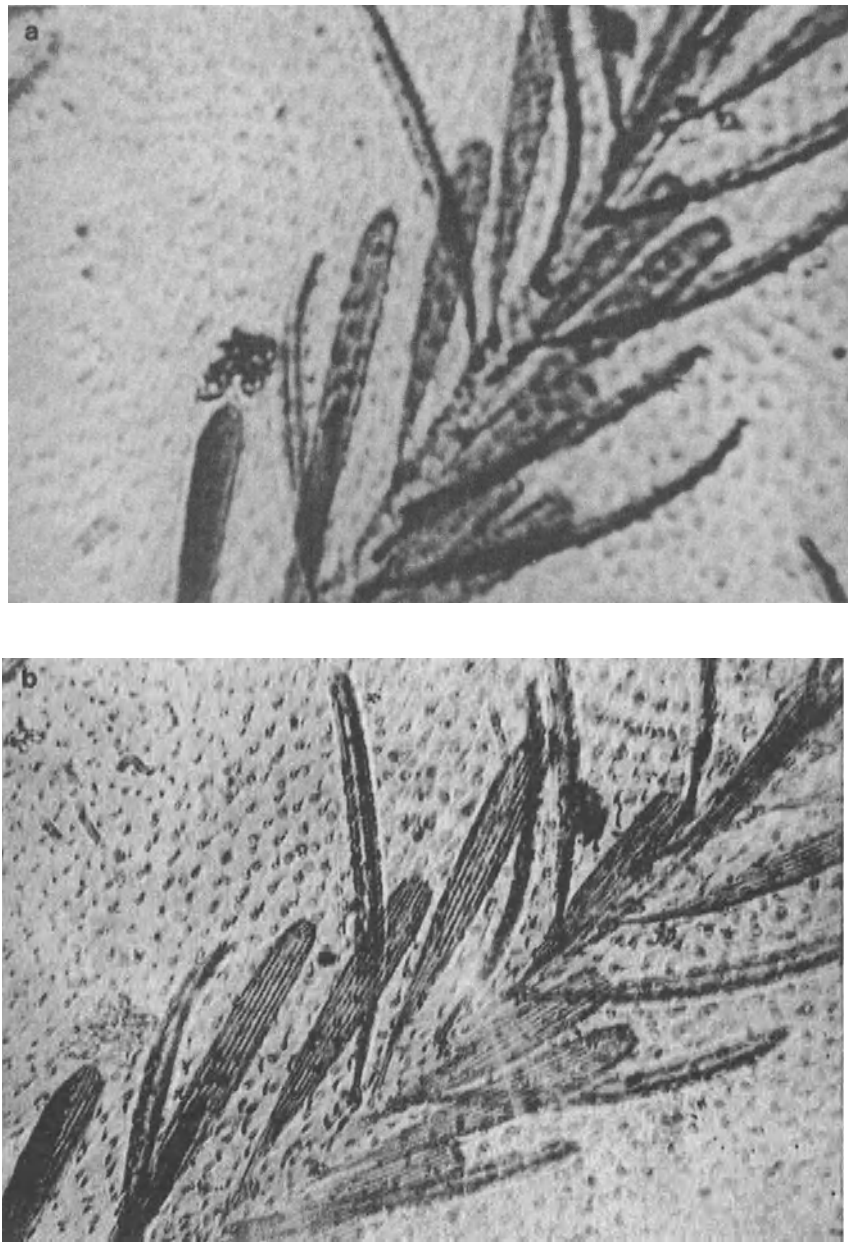


FIGURE 1.3. (a) Gnat's wing as seen with an uncorrected Cuff objective, ca. 1750. Photomicrograph courtesy of Dr. Maria Rooseboom, director of the National Museum of the History of Science, Leiden, the Netherlands. (b) Gnat's wing as seen with an achromatic Oberhauser objective, ca. 1850. Photomicrograph courtesy of Dr. Maria Rooseboom, director of the National Museum for the History of Science, Leiden, the Netherlands.

two aplanatic foci of achromatic doublets. He worked on the problem of the increasing influence of cover-glass thickness when using objectives of increasing aperture. Different solutions to the problem were found. In 1837, Andrew Ross made objectives with movable lens separations to correct for varying thicknesses of cover glasses. A decade later Amici showed that immersing the objective in a liquid of appropriate refractive index could eliminate problems in the cover glass.[7]

Until the early nineteenth century, it was thought that perfecting lenses alone would lead to the resolution of infinitely small structures. Then, in 1834, the astronomer George B. Airy (1801–1892) showed that light from a star would never be focused at a single point but only in a disk. Therefore in microscopy there must also be a limiting angular distance between self-luminous objects to make them appear separate. This angle would depend on the radius of the lens and the wavelength of light. In 1873, Ernest Abbe (1840–1905) came to essentially the same conclusion for periodic structures, thereby giving practical meaning to the concept of numerical aperture. Abbe worked with the Carl Zeiss Company in Jena in 1846; his many inventions include apochromatic objectives (1885) and their compensating eyepieces. Along with Otto Schott, he succeeded in greatly improving the quality of optical glass. In 1857 the British Royal Microscopical Society introduced a standard size and pitch of screw thread for objectives but met with difficulty in perfecting taps to be supplied to manufacturers. In an effort to perfect the standard, the American Microscopical Society worked with the Royal Microscopical Society and eventually arrived at a joint standard. Today machining practice is also standardized. Virtually every manufacturer in the world now produces objectives and microscopes fitted with "Society" threads, and to this extent interchangeability is ensured.

Standards for eyepieces and substages have not been universally accepted; however there is a set of standards for these and other parts under the supervision of the British Standards Institution.[23] In the United States interchangeability of microscopical parts is within the scope of the American Society for Testing and Materials.[24]

1.3. CONDENSERS

Until about 1880 practically all microscopes using visible light transmitted through the specimen were without condensers, which were later introduced for two reasons: the growing importance of bacteriology and the availability of the Abbe condenser produced by Zeiss.[21] As bacteriologists demanded more and more resolving power, they were demanding

more and more numerical aperture in the illuminating cone of light, even at the expense of some contrast.

E. M. Nelson was among those who advocated using the condenser to focus light onto the specimen itself—a process known as Nelsonian illumination. While this use of the condenser produces the brightest field, it is only as uniform as the source. Thus an incandescent filament as the light source focuses unevenly (the image of a coil filament). Such illumination can also be glary. In 1893, August Köhler published a paper advocating focusing the light source, such as an incandescent filament, in the entrance-plane of the condenser (on the iris diaphragm if it has one). In such a case manipulating the diaphragm also controls the size of the illuminated field, according to the numerical aperture of the objective in use, thus excluding glare.[21]

1.4. DARK-FIELD MICROSCOPY

It is reported[12] that Leeuwenhoek sometimes used dark-field illumination sometimes, but this is not certain. Two centuries after Leeuwenhoek, Lister (in 1830) used dark-field microscopy to take advantage of light scattered by surfaces and other discontinuities.[1] Special dark-field condensers for higher powered objectives were made available by Francis H. Wenham (1823–1908),[2] who used a paraboloid condenser and central stop to produce a hollow cone of light directed outside the angular cone of the objective.

1.5. POLARIZED LIGHT MICROSCOPY

The double refraction of calcite was described by Erasmus Bartholin (1626–1697) in 1669, and Etienne L. Malus (1725–1812) coined the term polarized light. In 1828, William Nicol (1768–1851) used calcite to make his famous polarizing and analyzing prisms. Other designs followed, but all plane-polarizing calcite prisms became known as nicols. Polarizer plus analyzer are used as a pair in a polarimeter to study the optical activity of liquids, or in a polariscope to study the optical properties of solids. Louis Pasteur (1822–1895) used the pair with his microscope on tartaric acids and their salts to discover a whole new class of chemical isomers.[25,26]

The Englishman Henry Clifton Sorby (1826–1908) employed polarized light microscopically to study thinned sections of limestones and other transparent rocks and identify their mineral constituents.[27,27a] Sorby and Pasteur represent a group of scientists in the golden nineteenth cen-

tury whose microscopical research went far beyond detailed description of specimens. They postulated mechanisms, drew broad scientific theories, and pointed the way to a variety of technologies.[26,26a]

By the twentieth century the light microscope had reached the limit of highly developed theory for resolving power and contrast not only for visible light but also for the ultraviolet and infrared regions. By the 1930s light microscopy was playing a part in every science and technology. Great professors, such as Ernest Abbe in Germany, had worked with outstanding producers of optical glass, namely, Schott, and of microscopes, namely, Zeiss.[21] Abbe's experiments with phase differences and amplitude contrasts in microscopical images were followed by Frits Zernike's phase-amplitude microscope, for which he won the Nobel prize in 1953.[28]

The development, production, and use of interference microscopes followed a similar pattern. From Newton's general observations of interference phenomena in the seventeenth century, Thomas Young developed the fundamental theory of interference in 1801. During the first quarter of the twentieth century, Albert Michelson (1852–1931) developed an interferometer for reflecting objects, and Ernest Mach and Zender developed an interferometer for examining transparent objects. By the middle of the century, several companies offered interference microscopes.[29–32]

In 1932, Edwin H. Land (1909–1991)[33] invented Polaroid® polarizing film, which has supplanted calcite as polarizer and analyzer in modern polarized-light microscopes, used in petrography, chemistry, resinography, metallography, and nowadays in biology.

At the turn of the century and well into the twentieth century, petrographers and chemists were also changing their microscopes to examine specimens.[18] The condenser occasionally needed a central, eccentric, or colored stop. This requirement called for a substage rack. As the Nicol polarizer became widely used, so did the condenser need to accept it mechanically. Later, the advent of the Land Polaroid® polar simplified the mechanics of the substage, and by the same token, there was more room for filters.

Among Land's many inventions[33] are Polaroid® self-processing photosensitive film and the Polaroid® camera to use it. These have influenced not only light microscopy but all other microscopies and indeed all of photography. For example Land and his associates published a paper in 1949[34] on combining the Polaroid® photographic system with a Zeiss reflective objective. Another reflective objective was used as condenser, and the entire microscopical system functioned in the strictly ultraviolet region. Moreover these particular objectives are apochromatic for three different wavelengths strictly in the ultraviolet region. These investigators made three separate Polaroid® transparencies, employing the three sep-

arate (invisible) wavelengths in the ultraviolet region, then they substituted three beams of different wavelengths in the visible range. Thus they obtained color-contrast and higher resolution by employing apochromatic, strictly reflective lenses.[34]

These results gave Robert C. Gore the idea of employing the Zeiss reflective microscopical lenses in the infrared region (1949) to perform infrared spectrometry on microscopic samples.[35] In the 1950s, the Perkin-Elmer Company, pioneer builder of infrared spectrometers, added a microscope to their single-beam infrared (IR) spectrometer. This in turn led to Fourier transform-infrared (FT-IR) microscopy as described by John A. Reffner.[36,36a]

1.6. NEAR-FIELD SCANNING LIGHT MICROSCOPES

These light microscopes without lenses were invented at Cornell University by Professor Michael Isaacson and his associates, who realized that the resolving power of lenses is not only limited by the wavelength(s) of the emanation, but also by the aberrations imposed on an image formed relatively far from the object—that is, in the far field. Therefore, instead of focusing light with a lens, Isaacson passed light through a very small hole and through a sample so close to the hole that the light beam had no chance of spreading out. Using yellow green light (γ = 550 nm), Isaacson's team obtained a resolution of about 40 nm with this microscope, so the team is not resolving atoms (as with a scanning tunneling microscope) but using light. The limit to resolution is not so much the wavelength of the light but the fact that the amount of sample is so small as to be undetectable. Aaron Lewis at Hebrew University in Jerusalem and Raoul Koppelman at the University of Michigan plug the 50-nm hole with a tiny crystal of anthracene because it fluoresces in ultraviolet light, yielding excitons that pass easily through the tip and turn into visible light (fluoresce). The result is a much brighter image, say, of a biological cell, from a near-field light microscope.[37]

1.7. LIGHT MICROSCOPE MANUFACTURERS

Figure 1.4 shows the development of the light microscope industry from 1970–1992. In particular the Karl Zeiss Company of Jena, East Germany, and the Carl Zeiss Company of West Germany were recently reunited.[38] The Leitz Company of West Germany and the Wild Company of Switzerland became Leica,[39] then Leica was joined by the microscopical

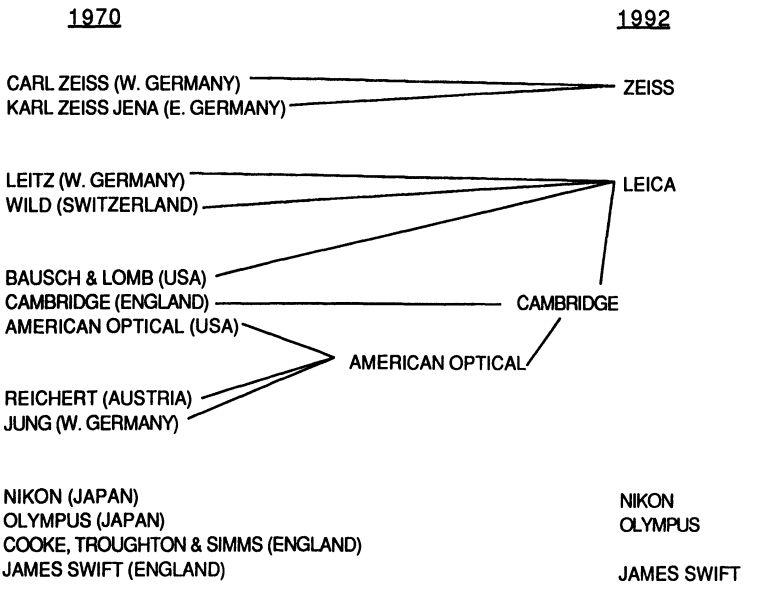


FIGURE 1.4. Manufacturers of Light Microscopes. Courtesy of Don Felty and Robert Martin.

part of Bausch and Lomb in the United States. The American Optical Company merged with Reichert of Austria and Jung of West Germany. That conglomerate joined the Cambridge Company, which merged with Leica.[39] Cooke, Troughton, and Simms of England joined with James Swift of England. Nikon[40] and Olympus,[41] both of Japan, have expanded into the United States.

1.8. TRANSMISSION ELECTRON MICROSCOPES

Two events in the 1920s brought about the development of the electron microscope. One was the realization from the de Broglie theory (1924) that particles have wave properties and very short wavelengths (e.g., 0.05 Å) are associated with an electron beam of high energy. The other event was the demonstration by Busch in 1926–1927 that a suitably shaped magnetic field could be used as a lens to create electron microscopes.[42,43] Busch and Ernst Ruska initiated studies of electromagnetic lenses in 1928–1929 and published a description of an electron microscope in 1932. In 1934, Ruska described the construction of his type of electron microscope, which surpassed for the first time the resolution of light microscopes. In

1938, Ruska and von Borries designed and built a practical microscope for the Siemens and Halske Company, but World War II prevented its sale and use outside of Germany. In 1986, the Nobel prize in physics was awarded jointly to Ruska for his pioneering work and to Heinrich Rorer and Gerd Binnig for the subsequent development of the scanning tunneling electron microscope (see Section 1.12).

Independently at the University of Toronto in Canada, under the supervision of E. F. Burton,[44] A. Prebus and James Hillier built an electromagnetic electron microscope, which they described in 1939. A similar instrument built in the United States was described by Cecil E. Hall.[42] In 1934, Ladislaus L. Marton built an electron microscope in Brussels with which he took the first electron micrographs of biological objects, such as bacteria. With James Hillier and Vance at the Radio Corporation of America, Marton helped build an electron microscope (1940) under the direction of Vladimir K. Zworykin, the inventor of the television picture tube. The first RCA commercial model of this electron microscope went to the American Cyanamid Research Laboratories in Stamford, Connecticut, on December 9, 1940.[45]

In 1940, the Columbian Carbon Company built an electron microscope at the University of Toronto under the terms of a Research Fellowship for William A. Ladd, another of Burton's students. The microscope was moved to the Columbian Carbon Research Laboratories in 1941, where pioneering industrial research was performed.[46,47]

In England, Metropolitan Vickers produced a prototype electron microscope in 1939, which was developed into a series of improved models.[48] The Phillips commercial electron microscope was being developed in the Netherlands at the same time. In the 1940s, companies in France, Germany, Japan, and other countries developed and produced this type of microscope, now known as the scanning transmission electron microscope (STEM).

The word transmission suggests one of the most important practical problems: obtaining specimens thin enough to allow sufficient electrons' transmission to affect the photographic material satisfactorily without affecting the specimen detrimentally (by heat absorption of electrons).[48] In the vast science of biology, the development of practical electron microscopy depended primarily on a corresponding improvement in microtomy, so that sections of tissue could be sliced much more thinly than those required in light microscopy. Differential stains also had to be developed on the basis of differential electron absorption by elements of relatively high atomic number rather than by differential light absorption (color). Films, whether in the form of specimens, substrates, or replicas, must be sufficiently thin. In the 1940s, in both biological and nonbiological sciences,

there were also problems in obtaining contrast; shadows were made by preferentially evaporating a metal onto the specimen in a high vacuum. Such problems were complicated by obtaining and maintaining a high vacuum in the electron microscope itself. There were other important mutual problems, such as maintenance, repair, resolution, magnification,[49] and interpretation.[50] Whereas light microscopists had struggled for centuries over interpreting images, including macroscopic ones, this problem was newly introduced to electron microscopists in 1945 (see Figure 1.5).[50] See Chapter 20 on specimen preparation.

In 1938 M. von Ardenne added scan coils to the STEM. Applications were limited to specimens thin enough to transmit electrons to activate the photographic medium.[51] Historically the chief importance of these scan coils may lie in the experience that led to the scanning (reflection) electron microscope (SEM), whose great depth of focus allows quantitative evaluation of specimens topography.

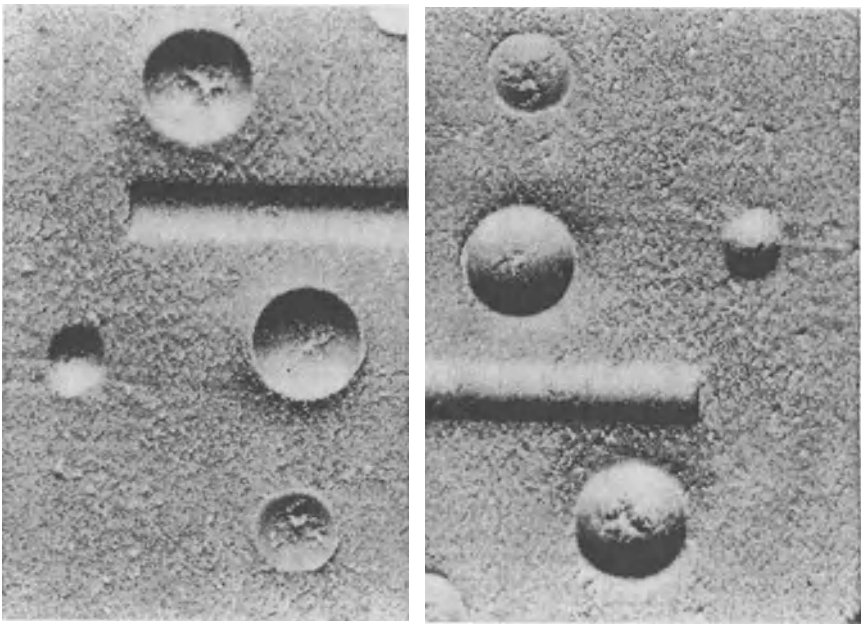


FIGURE 1.5. Two different orientations (180° apart) of the same photomacrograph of depressions in wet sand; (left) apparent depressions; (right) apparent elevations.[50] Taken to demonstrate the importance of orientation in interpreting images.

1.9. SCANNING ELECTRON MICROSCOPES

Instead of transmitting the primary electrons to form an image, in 1942 Zworykin, Hillier, and R. L. Snyder of the Radio Corporation of American used the *secondary* electrons reflected from the surface of a specimen to produce an image of the topography. The limiting resolution however was only 1 μm, less than that of the light microscope (0.1 μm). By reducing the size of the scanning spot and making other improvements, they increased the resolution to 0.05 μm. Further development of the SEM was suspended during World War II.

In 1948, C. W. Oatley at the University of Cambridge became interested in the SEM. He and D. McMullen built one with a resolving power of 0.05 μm. K. C. A. Smith (1956) made several technological improvements, and T. E. Everhart and R. F. M. Thornley (1960) made use of a light pipe to reduce noise. R. F. W. Pease (1963) and W. C. Nixon (1965) produced a prototype of Cambridge Scientific Instruments' Mark I.[51,52]

In 1942, Zworykin, Hillier, and Snyder tried a cold field-emission sharp cathode as the source of electrons in their experimental SEM to reduce the size and improve the intensity of the source. Instability however forced these experimenters to return to the thermionic electron gun. The cold-emission point source was finally improved by A. V. Crewe in 1969 to the point of successful application in the SEM. Another type of electron gun, developed by A. N. Broers, employs a heated, pointed rod of lanthanum hexaboride (LaB_6) because it is brighter and lasts longer than a tungsten filament. The requirement of a higher vacuum however prohibits incorporating the LaB_6 gun in some types of SEM.[42]

Advances in the sixties and seventies have involved contrast mechanisms not available in other types of instruments. Better crystallographic contrast was produced by crystal orientation, lattice orientation, and lattice interactions with primary beams by D. G. Coates in 1967.[51] Since 1969 the TEM has been modified with features of the SEM, resulting in the scanning transmission electron microscope (STEM).[51]

In a reflection microscope the contrast between features is often too low, but it can be enhanced by processing the digital signal. Early processing was done by nonlinear or differential amplification. Derivative signal processing (differentiation) was introduced in 1970 and 1974. Image storage circuits have been developed so that one can observe the image and/or operate on it off-line. Grain sizes, physical–chemical phases, and other analytical features are emphasized by computer evaluation and scanning electron microscopical images (CESEMI). In fact computer interaction with the SEM has yielded many benefits.

1.10. ELECTRON PROBE MICROANALYZERS

A close relative of the SEM is the scanning electron probe microanalyzer (EPMA). Instead of recording the scattered electrons, the microanalyzer records the *emitted X rays* and sorts them according to wavelength with a Bragg spectrophotometer. A quantitative analysis is made of a chemical element as the scanner picks up the distribution of the element. At the same time, an enlarged image may be displayed if the X-ray microspectrometer is part of a scanning electron microscopical system.[51]

1.11. FIELD-EMISSION MICROSCOPES

In 1897, R. W. Wood described the phenomenon of the field emission of electrons, the process of emitting electrons from an extremely small area of a cathodic surface in the presence of a strong electric field. In 1936 E. W. Müller (1911–1977) applied this principle to a negatively charged very fine tip (< 1-μm radius) of tungsten wire in the high vacuum of a cathode-ray tube. In this *field*-electron microscope, Müller obtained a pattern on the fluorescent screen that represented the array of atoms (see Figure 1.6).

In 1950, Müller charged the acicular tip positively, introduced helium into an extremely high vacuum, and formed He^+ ions at the tip. Some of the atoms hopped off the tip and activated the fluorescent screen to form a pattern typical of the *field*-ion microscope.[53,54]

1.12. SCANNING TUNNELING MICROSCOPES

The history of microscopy has already taught that the limit of resolution of any microscope depends on the limiting *wavelength* of the particular radiation used. Thus electron microscopes manifest their high resolving power because the wavelengths of electron beams are so short, for example, 0.5 nm (0.05 Å) for an electron beam accelerated by 60,000 V.

However electron beams, as well as light, also manifest the *particulate* nature of electrons. In 1973 Brian Josephson won a share of the Nobel Prize in physics for explaining the phenomenon called tunneling: If two electrically conducting surfaces are brought close enough together, the electron wave forms (clouds) surrounding their atoms overlap. If a small voltage is applied between the conductors, electrons *tunnel* from one cloud to another. This is true even though the voltage is much lower than classical physics would require.

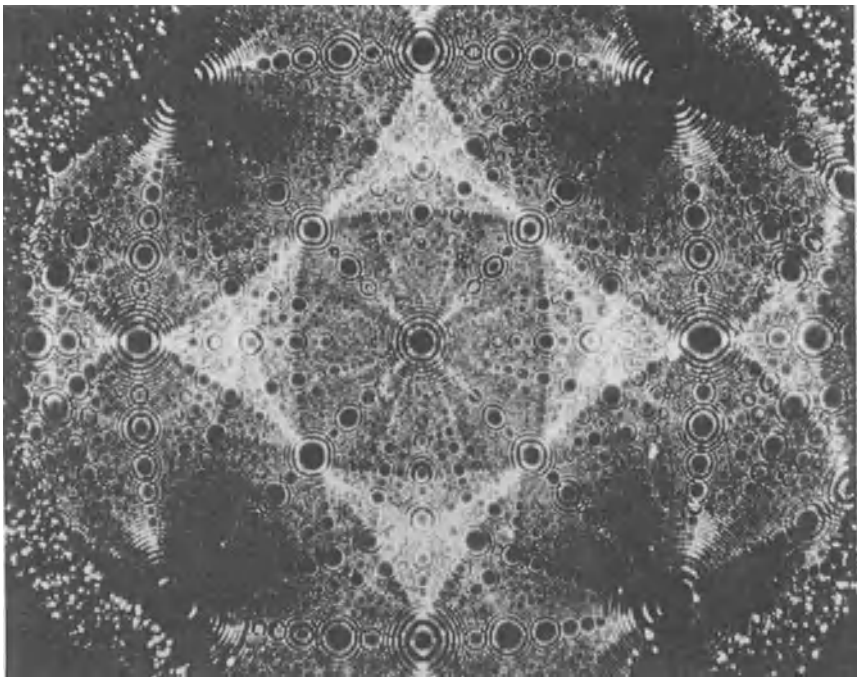


FIGURE 1.6. Field-ion micrograph of a platinum crystal by the late Professor Erwin W. Müller[54] of the Pennsylvania State University.[2] Courtesy of Professor T. T. Tsong,[53] Department of Physics, the Pennsylvania State University.

In the mid-1970s Heinrich Rohrer at IBM Research Laboratories in Zurich turned his attention from phase transitions, critical phenomena, and magnetic fields to electrical and mechanical layers of very thin oxides. He and his new research assistant, Gerd Binnig, probably needed to visualize inhomogeneities on a much smaller scale than microns (micrometers) or even a hundred angstroms (10 nanometers). It seemed natural in late 1978 to use the tunneling effect by making a sharp point and trying to tunnel through a vacuum. If the tip's position could be controlled accurately and scanned accurately over a surface, one would have an imager (microscope) delivering a detailed atomic map. In mid-1979 Rohrer and Binnig submitted their first patent disclosure on a scanning tunneling microscope (STM). In 1986, Rohrer and Binnig received half of the Nobel Prize in physics.[55–57] The STM however does not work well with nonconducting surfaces.

1.13. SCANNING ACOUSTIC MICROSCOPY

There are two types of acoustical microscopes, both based on the idea of the Russian scientist S. Sokolov, who in 1936 proposed using short wavelengths of *ultrasonic* energy instead of light to look directly inside an opaque specimen. This idea was not put into actual practice until the 1970s, when the manufacture of working models was begun by L. W. Kessler and associates. They produced the scanning laser acoustic microscope (SLAM).[55] Meanwhile C. F. Quate and associates[55,56] developed a mechanical scanning acoustic microscope (SAM); see Chapter 18.

1.14. ATOMIC FORCE MICROSCOPES

The atomic force microscope (AFM) was invented in about 1986 by IBM Zurich's Binnig and Berger in collaboration with Calvin Quate at Stanford University. The AFM (like the STM) scans a surface by moving a tip along one straight line at a time, but the tip is in actual contact with the material and senses the minute forces between atoms. The original AFM used a diamond tip mounted on a tiny gold foil cantilever spring. (This arrangement is like an old-fashioned phonograph arm fitted with a diamond needle—but with only one millionth of the weight.) When the tiny diamond tip goes over an electron "bump," the tip goes up, and the tip goes down over a void. To measure these tiny deflections, inventors sandwiched the diamond tip and its gold spring between the sample and an STM tip! As the diamond of the AFM rises, there is more tunneling current; while there is less tunneling current when the diamond goes down. The diamond tip, only one atom or so wide, is obtained by smashing an inexpensive diamond and selecting the right fragment by means of a light microscope.[57]

However the tunneling AFM has already been replaced by the optical-lever AFM—at IBM Zurich Research Laboratory and independently at IBM Research in Yorktown Heights, New York. The optical lever is a laser beam bounced from a small mirror mounted on the diamond tip. "Light reflected from the moving mirror is detected by a sensor; minute deflections are amplified, or 'levered,' by the geometry of the system so that the unit can detect a vertical change in a surface of only 0.1 angstrom" (0.01 nm).[6a]

1.15. X-RAY MICROSCOPY

X-ray microscopes were probably imagined soon after Wilhelm Röntgen discovered X rays in 1895. But the great penetrating power of X rays

was offset by the inability to refract or reflect X rays. Therefore images were obtained, and still are obtained today, particularly by the medical profession, by contacting photosensitive film with the object. The result is simply a macroradiograph. However electron microscopes in the 1940s could be adapted to provide a point source for X rays. In 1958, Martin C. Botty and Fred G. Rowe patented an adapter so that the objective lens of an electron microscope could focus its electrons onto a target to produce X rays instead of its original function.[58,59] Subsequently electromagnetic lenses were employed to market point-projection microscopes.[60]

Another important advance has been to focus X rays by means of a Fresnel zone plate with alternating transparent and opaque rings whose spacing diminishes outward from the center. Fresnel zone plates had been used to focus light, radio waves, sound, and even neutrons, and in 1960 Albert V. Baez at the Harvard–Smithsonian Astrophysical Observatory proposed using Fresnel zone plates to focus X rays. Achieving resolution of detail greater than that of light microscopy requires spacing the zone plate for X rays about 1/20 the wavelength of light. This is being done by procedures that bring us to the present day; see Chapter 17.

1.16. X-RAY LASER MICROSCOPES

Descended from the electron microscopes of the 1940s, X-ray laser microscopes use a special mirrored surface to focus X rays beamed through a specimen. The result is a sort of relief map based on the differential absorption of X rays. Although the X-ray laser microscope does not have the resolving power of electron microscopes, it has the advantage of not killing live specimens, such as living cells. Thus the X-ray laser microscope greatly facilitates the study of the structure and function of biological cells.[61]

1.17. MICROSCOPY SOCIETY OF AMERICA

It began in 1942 as the Electron Microscope Society of America (EMSA) "to increase and to diffuse the knowledge of the science and practice of electron microscopy."[49] In 1992, while EMSA celebrated its fiftieth anniversary, the name was changed to the Microscopy Society of American (MSA) so as to express the importance of "confocal, infrared, fluorescence, x-ray, scanning, tunneling, atomic force, ion, and acoustic microscopies, as well as highly sophisticated methods for chemical and

structural analysis that utilize the interactions of photon, electron, or ion beams with matter."*

1.18. SUMMARY

The earliest microscopists studied mostly biological objects. Hooke (1635–1703) studied cork, for example, and coined the word cell (see Figure 1.1). Leeuwenhoek (1632–1723) studied many natural objects, including stagnant water. He discovered microorganisms, which he termed beasties. Robert Brown (1827–1829) reported "active molecules," which we now term Brownian movement or motion. Around this time, Sorby (1826–1908) used reflected light to study cast iron and its ores. He used polarized transmitted light to study thinned sections of limestone, etc. Pasteur (1822–1895), originally a chemist, used polarized light to discover a whole new class of chemical isomers.

At the turn of the century, more and more mineralogists and petrographers were using polarized light with the petrographical microscope. Meanwhile more and more chemists were using the polarized light (chemical) microscope in research and development of chemicals, drugs, polymers, plastics, textiles, paper, etc. Today the chemical microscope and petrographic microscope have become about the same. Today's consolidated list of manufacturers is given in Figure 1.4.

Meanwhile, in the 1940s, the TEM was developed and commercially produced. Its optics have been improved with regard to aberrations, illumination, practical resolution, and contrast. Scanning electron microscopy followed closely and pushed the resolving power further (see Figure 2.2).

The resolving power and contrast of the TEM is limited by the lenses; therefore the SEM was developed, and the progress in resolving power has been steadily increased from the 1940s to the present (see Figure 2.2).

Since the 1970s, STMs have pushed the resolving power toward the level of atomic spacing (see Figure 2.2). The STMs include AFM, which employs an extremely sharp diamond mounted on a gold foil spring that goes up and down over atoms in the object. The tunneling AFM has been succeeded by the optical-lever AFM, which uses a laser beam bounced from a small mirror on the diamond tip, thereby reducing the resolution to 0.1 Å (0.01 nm).

While X rays cannot be refracted, they can be reflected and focused by means of a Fresnel zone plate, thus achieving resolution of detail greater

*Statement (1992) of the Microscopy Society of America, P.O. Box EM, Woods Hole, MA 02543.

than with the light microscope. The X-ray laser microscope employs a special mirrored surfaced to focus X rays beamed through, say, a live specimen without killing it.

Within its broad range of capabilities, light microscopy continues to serve both biological and nonbiological sciences and technologies. With the advent of *reflective* optics, both infrared and ultraviolet techniques are included in the visible range. The fundamental value of light microscopies, used alone or together with any of the high-tech microscopies, lies in the use of *polarized* light to show structure (however complex) in either biological or nonbiological material.[62]

2

Definitions, Attributes of Visibility, and General Principles

2.1. DEFINITIONS

Microscopy is the interpretive use of microscopes.[1,1a,2] No matter what kind of microscope, employing whatever medium, in whatever manner, with whatever kind of specimen, microscopy also requires a *primary* observer to interpret the image. Accordingly, microscopy can be interpreted as science,[2a] art,[3,3a] or a game—what is it?

Interpretation requires both a sharp eye and an active brain.[1a] Together they form the subjective, *microscopical* part of microscopy. The specimen on or in a microscope is the objective, *microscopic* part of microscopy.[1]

A *microscope* is an instrument that increases resolution over that of the human eye alone (about 150 μm between two points or lines). *Resolution* is the distance between two specific points or parts of the object as viewed by the eye, microscope, camera, or video. Actually resolution is the revelation of the two points or lines on two individual receptors (rods and cones),[4,5] separated by at least one other of these receptors situated on the retina of the eye (see Figure 2.1). The rods are very sensitive at low levels but respond only to white light. In the center of the retina is a small spot only about 1.5 mm wide, called the fovea. Its center contains densely packed cones that are sensitive to colors.[5a] Variation in the kind and extent of the cones' color sensitivity among microscopical observers probably accounts for variations in personal conclusions about color.[6]

Resolving power is the ability to distinguish two points of an object as separate in an image (with their diffraction discs not overlapping more

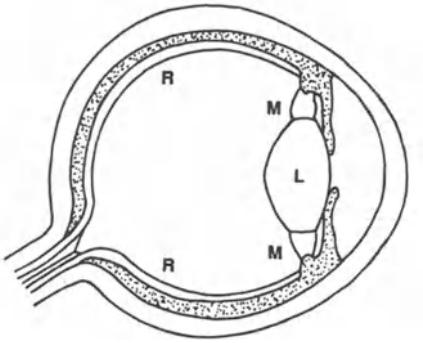


FIGURE 2.1. Diagrammatic section through eyeball; *M*: ciliary muscles; *L*: lens, elastic; *R*: retina.

than half their diameters).[1,2] Figure 2.2[6a] indicates the *potential* resolving powers of various kinds of microscopes since 1850 and projected to the year 2000. Data are based on Abbe's diffraction theory of resolution. Abbe and the Zeiss Company were the first to design and make apochromatic objectives (corrected for spherical aberration at two wavelengths of light and for chromatic aberration at three wavelengths).[1]

Practical *resolution* needs adequate *contrast;* the Zernike phase-contrast method led the way in light microscopy at the turn of the twentieth

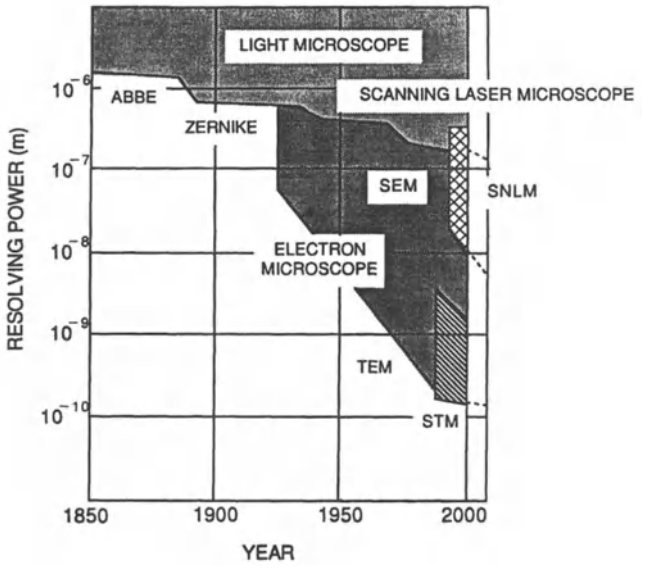


FIGURE 2.2. Potential resolving powers of various kinds of microscopes.[6]

century, as shown in Figure 2.2. In the middle of the twentieth century, the TEM displayed practical resolving powers from 10^{-6}–10^{-10} m. The SEM and the STEM offer advantages in both resolving power and contrast, as do the scanning laser microscope (SLM) and the scanning near-field light microscope (SNLM).[6]

Figure 2.2 shows where *resolvabilities* of various microscopes overlap or join; actually light microscopy begins where the resolvability of the unaided eye ends (ca. 150 μm). The degree of resolution may lie within:

- A biological *organism* or nonbiological *material*; electron microscopies begin somewhere around the limit (see Figure 2.2) of light microscopy (ca. 10^{-6} m)
- A biological organ or material *part* of the whole
- A biological *skin* or *membrane* or material *surface* or interface; several kinds of microscopies can be involved
- A biological cell or polymer; a material polymer, monomer, or atom itself.[7,8]

2.2. ATTRIBUTES OF VISIBILITY

2.2.1. Correcting for Aberrations

An aberration in any kind of microscope is the failure of an object's point to be imaged as a point.[1] With a lens transmitting light, visible or invisible, there are five kinds of aberrations: spherical, coma, astigmatism, curvature of field, and distortion. The most important aberration is spherical aberration; these are all discussed in Chapter 3. Electron microscopical lenses possess aberrations, too, and these are discussed in Chapter 14.

2.2.2. Sample Quantity and Quality

When selecting the kind and extent of microscopy, the size of the object and nature of the problem can be paramount. As Chapter 13 explains infrared spectrometry and infrared microscopy were combined in the late 1940s when all-reflective objectives and condensers became available for samples as small as the milligram range and smaller.[9] Micromanipulators have been developed for use with many kinds of microscopies (see Chapter 12).

2.2.3. Focus Depth

With far-field microscopes (light/electron/X ray) *focus depth* in the image space[1,2] refers to the other side of a particular lens with respect to the field depth in the object, as shown in Figure 2.3. Although the terms focus depth and field depth may be considered synonymous,[10] they are discussed separately here, since field depth is objective while focus depth is subjective with respect to the microscopist. When working visually it may be an advantage to have an image equal to the great focus depth of the human eye with its low numerical aperture (NA) of 0.002. With a deeper image (see Figure 2.3), the observer does not have to adjust the focusing controls as often; in such cases choose an objective of low NA.

On the other hand, great focus depth is a disadvantage in optical sectioning when studying spatial arrangements in structures. In such studies a very thin field depth is required to observe sharp changes in the object's contours with very small changes in focus. Hence select an objective with a high NA.[10]

2.2.4. Focus

In visual microscopy the eye (see Figure 2.1) should be focused so that the ciliary muscles (*M*) controlling the thickness of the eye's lens (*L*) are relaxed and the individual feels at ease when the object is in focus.[11] If the microscopical image goes out of focus, refocus the objective rather than accommodate it with the ciliary muscles.

Objectively focusing means turning mechanical wheels or electronic knobs to move the object or objective or change the optics. Subjectively focusing is a personal choice of the image by the direct observer, photo-

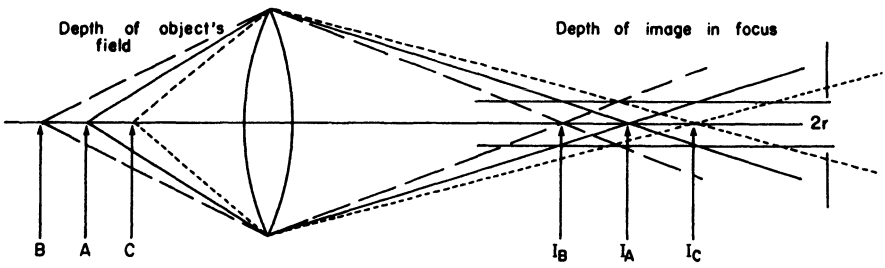


FIGURE 2.3. Field depth and focus depth employing light as an example. Courtesy of Microscope Publications.

micrographer, or armchair microscopist. Focusing does not always mean choosing the sharpest image; sometimes it is the process of over- or undershooting to gain contrast, resolution, or some other attribute to visibility. Whatever else it may be, focusing boils down to each individual's personal choice in the interpretation of images.

2.2.5. Illumination

Illumination is very important, and in light microscopy there are several types, as illustrated in Figure 2.4.[12] There are two principal methods of illumination by transmitted light: Nelson's method and Köhler's meth-

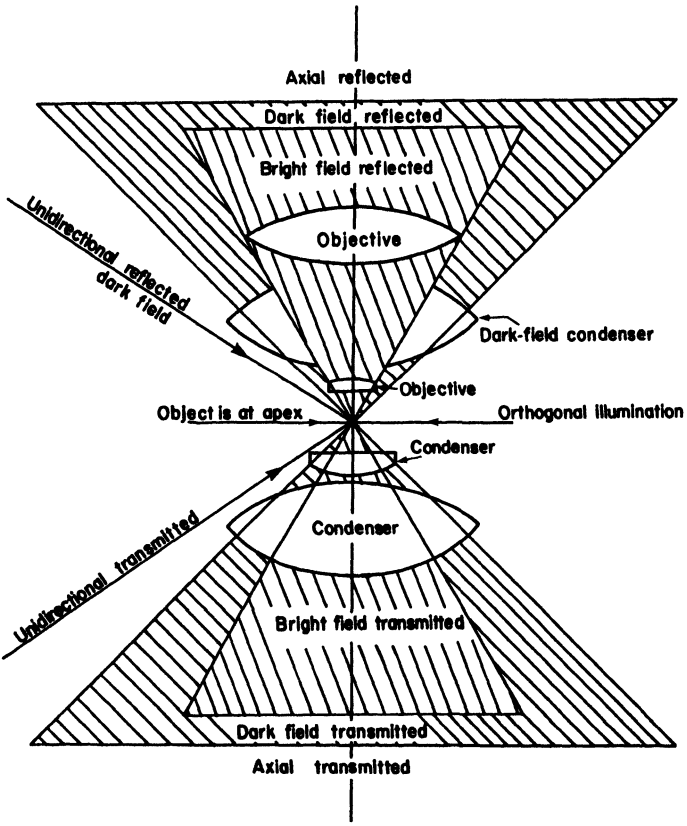


FIGURE 2.4. Principal types of illumination. Directional beams lie on or within a main cone.[12]

od. Nelson's "critical illumination" was devised in the days before electricity to focus the side of a burning oil wick in the *plane* of the specimen by means of a substage condenser.[13] Nelson's method remains satisfactory in these days of electricity *and ribbon filaments* for either light or electron microscopies. However there is the problem of unevenness if a *coil filament* is sharply focused in the plane of the specimen, as shown in Figure 2.5.[10] Throwing the coil filament out of focus and/or inserting a ground-glass diffuser in the lamp's housing compromises the purposes of Nelsonian illumination, which is to illuminate evenly the whole area of the specimen in focus; yet Nelsonian illumination remains common in light microscopy.

In Köhler's illumination (see Figure 2.6), the source F is equipped with its own *auxilliary condenser* lens and field *diaphragm* A_2–A_2. These may be housed as a unit illuminator or as part of the microscope itself. The important point is to focus the field diaphragm's opening at A_1–A_1, the *iris diaphragm* of the *substage condenser* (not on the specimen).[14]

Referring to Figure 2.6, the substage condenser is moved to bring the image of the lamp's iris diaphragm A_2–A_2 into the plane of the object F_1. Then the lamp's diaphragm A_2–A_2 is opened just enough to fill the objective's field of the object at F_1. Another advantage is that the lamp's filament is out of focus, so that the field is evenly illuminated. The field stop A_2–A_2 also enables the observer to center the optical axis to coincide with that of the objective. The substages of microscopes are generally equipped with centering devices for the condenser system. When both F and the object are in focus, the centering screws should be used to center the field-stop image with the field of view at F_1.[15]

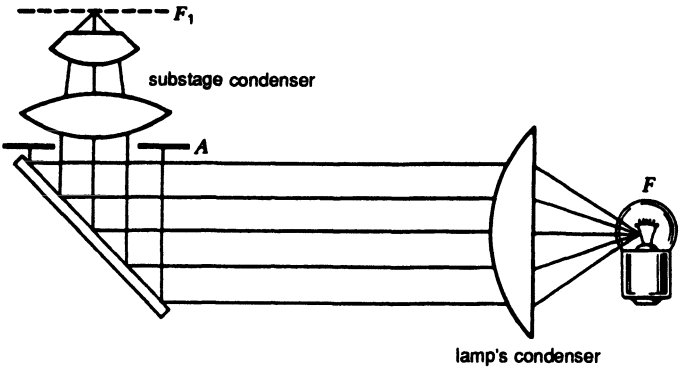


FIGURE 2.5. Modern version of Nelson's method of critical illumination whereby the source of illumination (F) is focused in the plane of the object as F_1 by means of the lamp's condenser and the substage condenser, whose aperture A is controlled by its iris diaphragm to equal the field of view.[10]

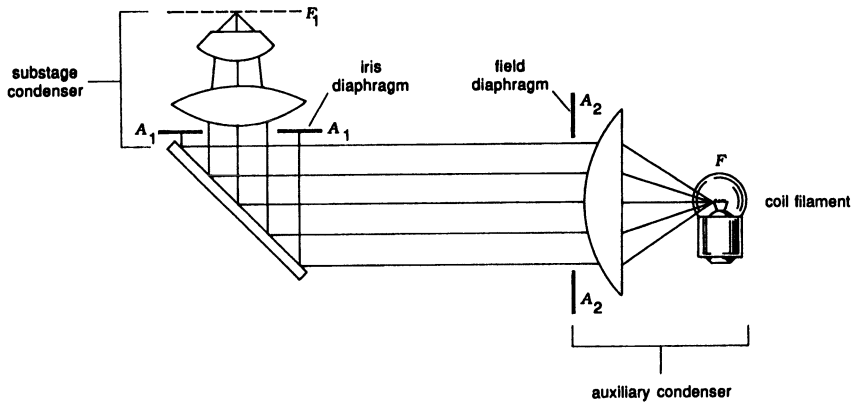


FIGURE 2.6. Illumination system for transmitted light (Köhler illumination).[15]

Routine procedure for Köhler's illumination involves the following steps:

1. Focus on the specimen (see Figure 2.6).
2. Open the iris diaphragm A_1–A_1 of the substage condenser.
3. Almost close the field diaphragm A_2–A_2.
4. Focus the substage condenser until the field diaphragm A_2–A_2 is simultaneously in focus with the object at F_1.
5. Open the field diaphragm A_2–A_2 so that all of the field is in view but no more.
6. Recenter the substage condenser if necessary.

Caution: Do not open the field diaphragm A_2–A_2 any farther to avoid burning the retina or paralyzing the tiny muscles controlling the size of the iris diaphragm. This precaution is especially important in photomicrography when light sources of high intensity are likely to be used.

Köhler's method is available in the electron microscope by means of automated controls that select the appropriate aperture and lens conditions to illuminate homogeneously only the imaged area. An advantage in electron microscopy is that Köhler's illumination protects the specimen from heat damage[14] (see Chapter 14).

2.2.6. Radiation

Microscopy employs light, electrons, X rays, or acoustic energy in an expanding number of analytical or synthetical fields. The present changes

in the kinds and varieties of radiation have resulted from the interaction of equipment, research, and application. During the first half of this century, ultraviolet invisible light was used to gain fluorescence, resolution, or contrast.[12] However electron microscopy surpassed the advantages of ultraviolet (UV) light microscopy. Not all the effort expended on UV microscopy was lost, since it was possible to take three UV photographs and convert them to a color photograph of excellent contrast.[16] The same reflective optics also led to advances in infrared microscopy[17] and thence to FT-IR microscopy[9] (see Chapter 13).

With UV and infrared regions of light, there are of course fewer options with illumination, and these are discussed in succeeding chapters. With other types of radiation there are not many variations in kinds of illumination. These are discussed in the chapters that follow.

2.2.7. Anisotropy

Anisotropy (re light) may originate in a structure in one or more manifestations of influence by polarized light:

Molecular anisotropy resulting from the orientation of dipoles, usually in long or flat molecules[18]

Birefringence[1] in units of all anisotropic crystals

Polarization patterns, such as the permanent cross seen in spherulites[1,2] like starch grains

Flowed, streamed, or *grown* masses of long or flat units (not necessarily crystalline) in a medium of different refractive index[18,19]

Regions of local *strain anisotropy* photoelastic effect in a normally isotropic substance, such as inorganic or organic glass under stress.

Many materials owe their anisotropy to two or more of the preceding origins. Cotton fibers are a good example, as shown in Figure 2.7. All fibers except inorganic glass manifest eight independently determinative properties based on their anisotropy. Therefore microscopical examination reveals both structure and morphology (see Chapter 6).

Crystals reveal even more optical properties than fibers, due to the variations in crystallographic symmetry, as discussed in Chapter 7. Liquid crystals are in a class by themselves with regard to their many optical properties based on anisotropy.[18] These are also discussed in Chapter 7.

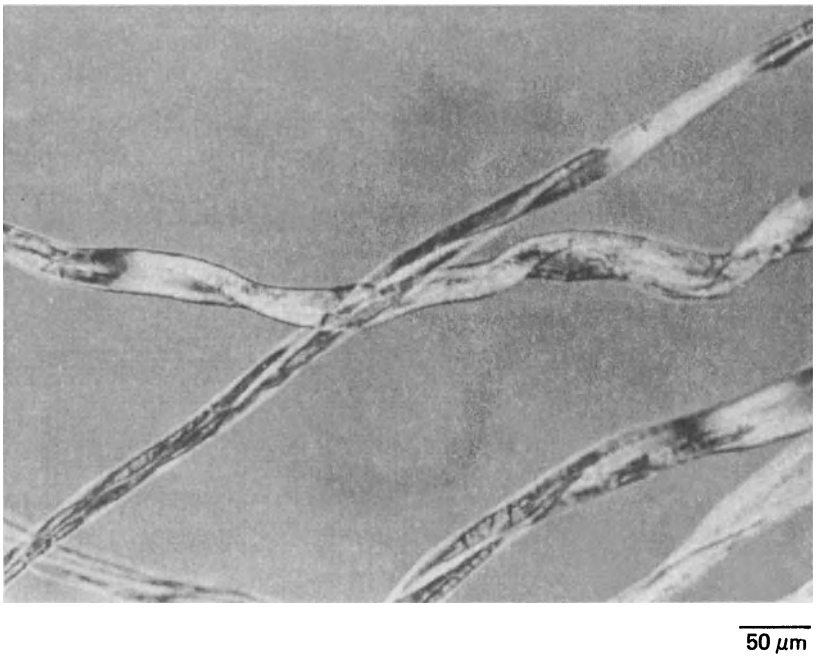


FIGURE 2.7. Cotton fibers, mounted in mineral oil and shown between partially crossed polars, revealing the convolution of fibers resulting from the complex structure. Taken in class by Mrs. G. Berry with modest equipment, ca. 1975.

2.2.8. Magnification

Useful *magnification* is incidental to the resolving power (NA) of the objective and the eye; the approximate relationship is

$$\frac{\text{maximum NA (microscope)}}{\text{minimum NA (eye)}} = \frac{1.40}{0.002} = 700\times$$

which can also be stated as

$$\frac{\text{limit of resolution (eye)}}{\text{limit of resolution (microscope)}} = \frac{0.15 \text{ mm}}{0.0002 \text{ mm}} = 750\times$$

The theoretical optimum magnification for light microscopy is therefore about 750×. Useful magnification in practice may be somewhat higher to assure that adjacent points in the object are imaged on separated receptors

(rods and cones) on the retina. Separated receptors mean that the two nerve endings receiving images from different points of the object are not adjacent; instead there must be at least one other nerve ending between the two. The necessary magnification may be as high as 1000 times the NA of the objective.[10]

The chief function of an eyepiece is to increase the incidental magnification of the objective to a total magnification that is useful to the eye:

$$\text{magnification (objective)} \times \text{magnification (eyepiece)} = 1000 \times \text{NA (objective)}^{[10]}$$

2.2.9. Stereoscopy

Stereoscopy involves simultaneously seeing from slightly different angles a slightly different image with one eye than with the other eye. The brain combines the two images to give the impression of depth and solidity. Achieving this effect with any kind of microscopy involves taking two micrographs at slightly different angles and then viewing them with a stereoscope. This simply holds the two illuminated micrographs, which are then viewed through a pair of spectacles at the personal interpupillary width. Of course stereoscopic microscopes are independent of dual micrographs and holders.

With the electron microscope, two slightly different views are routinely taken by tilting or turning the specimen after taking the first micrograph. While the procedure for some of the newer kinds of microscopy may not be well established, microscopical stereoscopy has been very convincing, especially in consulting work.

In light microscopy, stereomicrographs are usually taken with a stereoscopic microscope, sometimes called a binocular (like a field glass). However, not all microscopes fitted with binocular eyepieces are stereoscopic. There must be a corresponding pair of objectives (at the angle of close vision) or else a common objective broad enough to include two separate effective views of the object.[20,21]

2.3. GENERAL PRINCIPLES

2.3.1. Specimen Structure

To the biologist *structure* is practically synonymous with morphology; to the crystallographer[22,23] structure describes the kind of periodic ar-

rangement of the units of organization, whereas morphology pertains to the size and shape of the resultant architecture. Structure and morphology are the two coordinates in Figure 7.1.[22]

Abbe's fundamental theory of limiting resolving power assumes that the specimen has microscopic periodic structure. Diatoms are such structures, and these have been involved in the history of microscopical development. For example the test diatom *Pleurosigma angulatom* (Quekett) is composed of hexagonally close-packed cells. If the resolving power of the light microscope is not quite adequate, the pores appear to be hexagonally shaped, as in Figure 2.8.[24] Other investigators[25,26] demonstrated that the structure of the unit cell itself is too complicated to test the resolving power of a light microscope. While the TEM has helped reveal the complicated structure, scanning electron microscopy has also helped[27] (see Figure 15.20).

2.3.2. Specimen Morphology

The *morphology* (size and shape) of a specimen[1,2] affects its visibility: The smaller the size and the more complex the shape of an object, the greater the resolving power, contrast, correction of aberrations (and most of the other attributes contributing to visibility) required to obtain a clear,

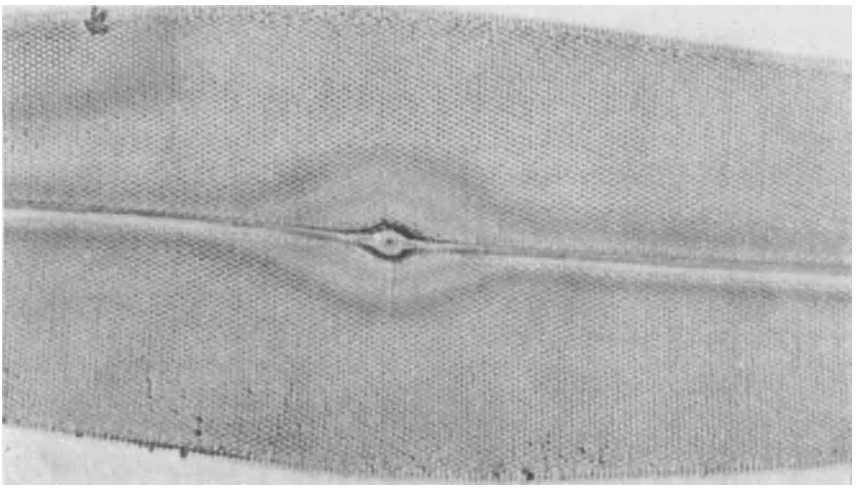


FIGURE 2.8. *Pleurosigma angulatum,* test diatom.

useful image. A regular polygon with n number of sides is resolved only if its diameter is n times the limit of resolution.[10]

2.3.3. Specimen Information

Information about the specimen is often of utmost importance. What are we looking for? Why? Is the sample representative or nonrepresentative (exaggerated)? Is it of a type already on the market? Then information on possible solutions to the problem can be gathered from technical literature.

2.3.4. Experimentation

Experimentation with equipment and varied conditions surrounding a specimen may be necessary not only to improve visibility but also to justify image interpretation. To obtain optimum resolution, contrast, cues to depth, depth of focus, etc., one must experiment with objectives, oculars, condensers, polarized light, different illumination, and all the rest. If all the equipment is not available, one can improvise.

Of course keeping good, orderly records is also important for arriving at satisfactory conclusions. Good records and easy retrieval save a lot of future experimentation.

2.3.5. Specimen Preparation

Every sample should be examined immediately *as received.* If precautions to preserve it are specified, these should be taken. If the sample is cleaned or fractionated in any way, all fractions, including "dirt," should be saved and labeled.

Some preparations are routine and need no deliberation, such as mounting in an inert liquid of chosen refractive index to improve transparency and visibility, and cutting or otherwise preparing a surface quickly. More drastic and time-consuming preparations should be considered carefully and used on only part of the sample (see Chapter 20).

2.3.6. Specimen Behavior

Behavior means change in the sample with time, temperature, weather (natural or simulated), humidity or dryness, atmosphere (or vacuum), etc.

Even during storage or shelf life, samples should be examined often for change and to interpret these in terms of the problem.

2.3.7. Photomicrography

Photography at best is an illustration of the appearance of the specimen under very specific conditions. However photography has its own advantages and limitations of resolution, contrast, color, and emanation sensitivity introduced by grain size or color sensitivity of the photographic film or paper and by its development conditions. Hence *photomicrography* is a separate discipline that combines the art of the photographer with the science of microscopy. There are frequent references to photomicrography[28] in later chapters.

2.3.8. Video

In *television,* too, the picture has its own resolution, contrast, and color characteristics. These and other attributes are variable, and they contribute to microscopical conclusions.

2.4. SUMMARY

Microscopy is the interpretive use of microscopes. Interpretation can be science, art, or a guessing game. In any case interpretation requires a sharp eye and an active brain, which together form the subjective *microscopical* part of microscopy. The specimen is the objective *microscopic* part of microscopy.

A *microscope* is any instrument that increases the resolution of the human eye. *Resolving power* (what you pay for) is expressed *theoretically* as the *minimum* distance between two adjacent, separate points in the object. *Resolution* (what you get) is the *actual perception* of the two separate points because their separate images fall on two receptors separated by at least one other receptor on the viewer's retina. The potential resolving power of today's light microscope is close to the theoretical value (0.2 μm). The potential resolving powers of the scanning laser microscope (SLM) and the scanning near-field microscope (SNFM) lie between the highest for light microscopes and the lower ranges of the TEM; the STM has the highest potential resolving power of all. While resolution is the most important attribute to visibility, *contrast* is almost as important: If two points are

separated by a particular microscope, but their images are no brighter (or darker) than the space in between, they will not be visible. Therefore most of Chapter 2 is devoted to various ways of obtaining resolution with adequate contrast.

The greatest problem in the development of light microscopy was *correcting for aberrations* (the failure of an object's point to be imaged as a point); at least some of these problems remain in electron microscopy. Problems of *sample quantity* and *quality* are addressed by combining infrared light microscopy with infrared spectrometry to create FT-IR microscopy. With far-field microscopes (light/electron/X ray), *depth of field* and *depth of focus* are considered separately, since the former is *objective* and the latter is *subjective.* The more depth of field, the less resolving power, or vice versa. In visual light microscopy, the eye should be relaxed (focused on infinity). Focusing on the object should be done by the microscope, *not* the eye.

There are many types of illumination in light microscopy, including Nelson's critical illumination, where the source, an incandescent filament, for example, is focused on the specimen by the substage condenser. In Köhler's method the filament or other source is focused in the plane of the iris of the substage condenser, and the image of the lamp's field diaphragm is focused in the plane of the specimen, thus controlling centering and the size of the *evenly illuminated field.* Up to 1990, electron microscopes were illuminated by Nelson's method, but since then Köhler's method has been introduced.

3

Simple and Compound Light Microscopes

3.1. LIMITS OF RESOLUTION BY THE EYE

The limiting resolution d, the distance between two points barely resolved by the human eye, is called *visual acuity.* It varies directly with the distance D between the object and the eye. In Figure 3.1 the eye's viewing angle V is purposely exaggerated, and the distance D from object to eye is not drawn to scale with respect to d, which separates the barely resolved points P and P. Figure 3.1 demonstrates that when distance D' to the nearer points P' and P' is exactly half the distance D, then the barely resolved distance d' between P' and P' is exactly half of d. This direct relationship persists as the object is brought closer and closer to the unaided eye, until the object is brought to a certain minimal distance, approximately 250 mm from the normal eye. At this distance of closest vision, the eye can resolve two points that are about 0.15 mm apart. These limits are set by the NA = 0.002 for the eye with its iris diaphragm wide open. At this setting the eye's lens is as thick as physiologically possible.[1]

A far-sighted person, whose eyes cannot accommodate objects much closer than arm's length, requires either eyeglasses prescribed for hyperopia or a magnifying glass to achieve normal visual acuity. Following the same principle, a person with normal eyesight can increase his/her visual acuity by using a magnifying glass. The simple microscope, illustrated in Figure 3.2, follows the same principle. The auxiliary lens bends the rays from the object so that they fall within the eye's viewing angle and are focused on the retina rather than beyond it.

Combining the focal length f in Figure 3.2[2] with distances D and D' in Figure 3.1 permits calculation of the expression[3]

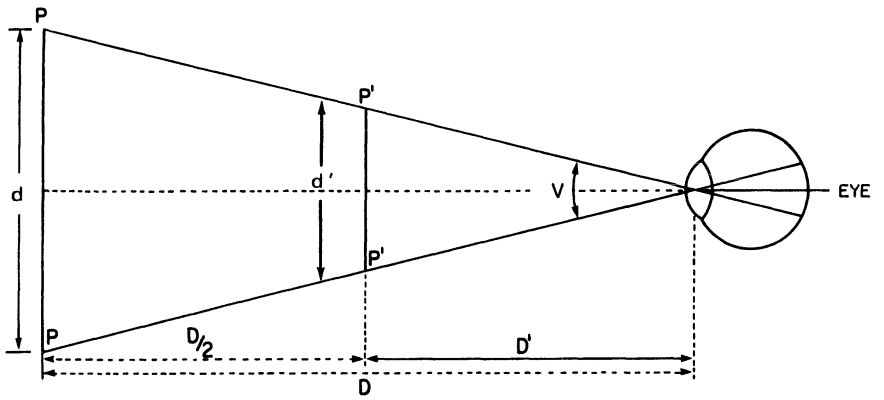


FIGURE 3.1. The viewing angle. The relationship between the distance d between barely resolved points P and their distance D from the eye. Courtesy of Microscope Publications.[2]

$$\frac{1}{f} = \frac{1}{D} + \frac{1}{D'}$$

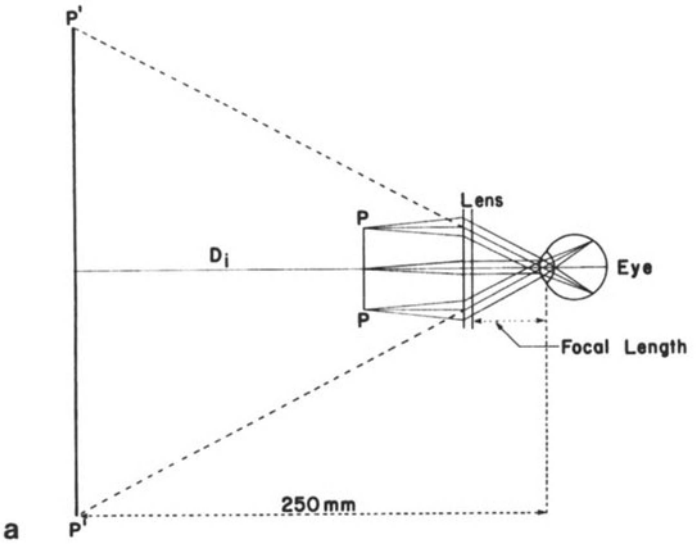
where D equals object distance from the lens and D' equals image distance from the lens if the lens is assumed to be thin. Magnification M equals $PP/P'P' = D/D'$ By eliminating D or D', the magnification equation is[3]

$$M = \frac{f}{(D - f)} = \frac{(D' - f)}{f} = \frac{D'}{f} - 1 = -\left(\frac{D'}{f + 1}\right)$$

Real distances are positive, and virtual distances are negative. With this equation we can estimate magnification from knowledge of only the object distance from the lens and the magnification without actually measuring the size of the formed image. If we examine this equation with the value of $D' = 25$ cm as the distance of distinct vision for most people and assumes $f =$ 5 cm, as in many simple lenses, magnification equals six times. A Leeuwenhoek lens of $f = 1$ mm gives a magnification of 251 times (approximately 250, as often reported). Of course this lens does not have corrections for the lens defects or aberrations, but this high magnification helps explain why it was initially superior to early compound microscopes.

3.2. SIMPLE MICROSCOPES: ONE-LENS SYSTEMS

Simple microscopes have only one lens unit, as indicated in Figure 3.2a. These are inexpensive, have relatively large fields, and give upright



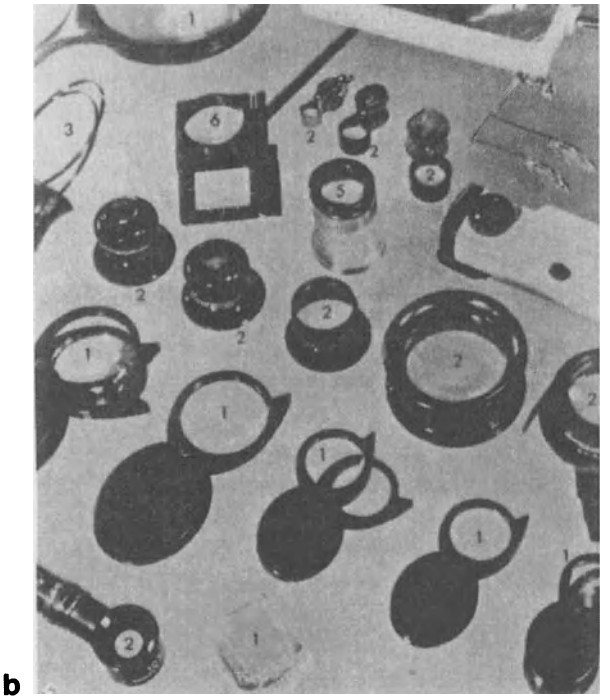


FIGURE 3.2. (a) A simple microscope.[1] Courtesy of Microscope Publications. (b) Some magnifiers and simple microscopes for use in macroscopy: 1-magnifying glasses; 2-simple microscopes: pocket and desk sizes; 3-auxiliary lenses; 4-double loupe for eyeglasses; 5-transparent base magnifier, with or without reticle; 6-vertical (table model) magnifier with reticle. Courtesy of Bausch and Lomb.[2a,4]

images. There are many uses for the simple microscope, as many as there are kinds: magnifying glasses, magnifiers with reticules (many kinds), pocket magnifiers with carrying cases, simple binoculars (nonprescription eyeglasses), simple- and double-eyeglass loupes, for example[2a] (see Figure 3.2b). With all simple microscopes (as with compound microscopes), keep the eye(s) close to the eyelens(es)! The tendency to violate this rule becomes greater the larger the lens is. Follow Leeuwenhoek!

3.3. COMPOUND MICROSCOPES: TWO OR MORE LENS SYSTEMS

Compound microscopes are composed of two or more lens systems. Some compound microscopes contain only the minimum: an objective plus an eyepiece (see Figure 3.3). The objective forms a real image $P' - P'$ in the eyepiece plane.[1] The eyepiece is a magnifier that causes the images of two separate points P and P to fall on separate receptors P'' and P'' on the retina.

Figure 3.4 illustrates an uncomplicated compound microscope being used by hand in micrometry. Even smaller models, scarcely larger than a fountain pen, are available.[4] Portability is their main advantage, but low

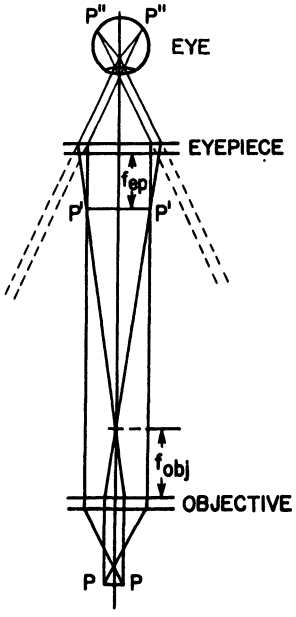


FIGURE 3.3. Light path of two characteristic rays through a compound microscope comprised of only objective and eyepiece. Courtesy of Microscope Publications.[2]

FIGURE 3.4. Hand compound microscope being used in micrometry by reflected light for opaque specimens. Courtesy of Helen Clapp.

NA = 0.06–0.10 is their main limitation (see Table 3.1). Moreover hand microscopes cannot substitute for those with sturdy stands, so most microscopical work is done with compound microscopes mounted on convenient stands and equipped with accessories necessary for proper illumination and precise focusing.[5]

Field microscopes are also held in the hand, but they have a stage and other facilities for transparent specimens, as indicated in Figure 3.5. The model shown comes with three objectives: 4×, 10×, and 40×. It is intended

TABLE 3.1
Theoretical Resolving Powers of Light Microscopes

Focal Length (mm)	Numerical Aperture (NA)	Maximum Useful Magnification	Approximate Theoretical Limit of Resolution (d)[a]		Depth of Field (μm)
			Axial (μm)	Oblique (μm)	
250	0.002	Eye alone, 1×	150	—	—
25	0.10	Hand lens, 10×	10	—	42
32	0.10	Compound microscope, 100×	5	2.5	25
16	0.25	Compound microscope, 250×	2	1	3.8
8	0.50	Compound microscope, 500×	1	0.5	0.86
4	0.95	Limit, air-immersion objective, 1000×	0.52	0.26	0.083
3	1.38	Oil immersion (visible, 500 nm), 1500×	0.36	0.18	—
3	1.38	Oil immersion (UV, 270 nm), 2000×	0.20	0.09	0.04

[a]Based on Abbe's theory $d = \lambda/(1\text{–}2\ \text{NA})$ disregards aberrations). This is about *maximum* obtainable resolution and assumes adequate contrast. Any reduction of aperture of objective or condenser increases d. Photography has its own limitations.

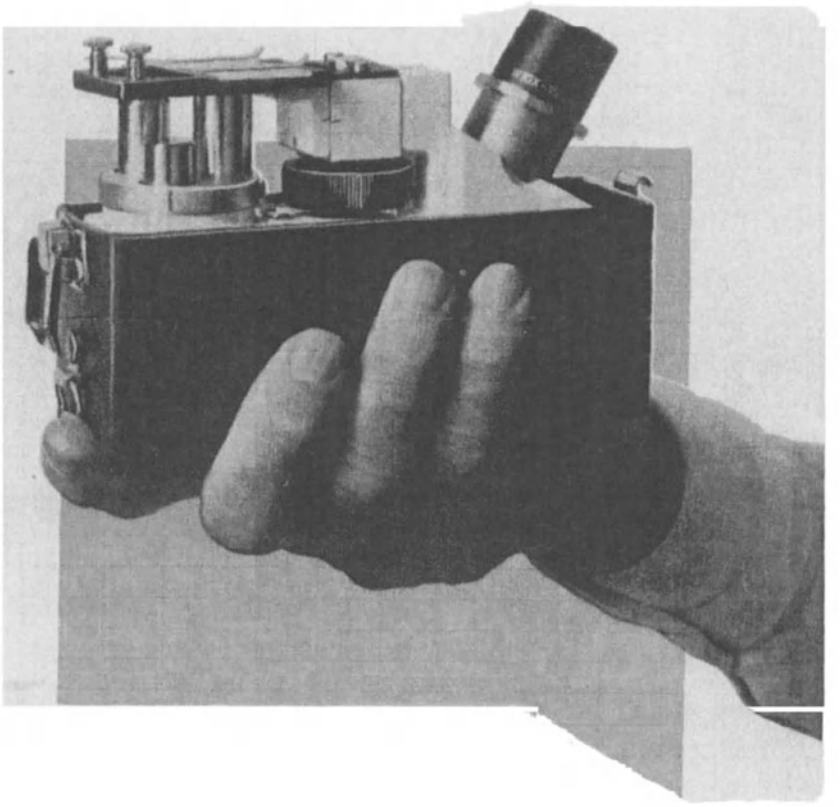


FIGURE 3.5. Swift field microscope supplied with carrying case, three objectives (4×, 10×, and 40×), and a battery-powered illumination; available with phase optics, objectives with longer working distances, adaptors for photography.[6] Courtesy of Swift Instruments.[6]

for use in the home, field, factory, pilot plant, or at the scene of an accident or crime.[2a] The main advantages are portability and handiness. The various limitations involve stability, versatility, kind and extent of illumination, lack of polarized light, etc., as discussed later in this chapter and in Chapters 4 and 5.

Most light microscopical work is done with compound microscopes mounted on convenient stands and equipped with accessories necessary for proper illumination and precise focusing. Figure 3.6 illustrates a compound microscope that may be suitable for students in introductory biology or for anyone satisfied with its limitations and comparatively low price. The microscope's sturdy stand has an up-to-date appearance and built-in light source. It is binocular but *not* stereoscopic because both eyes

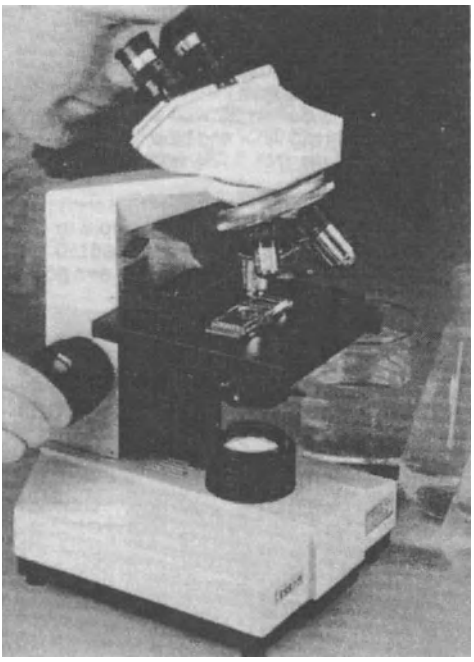


FIGURE 3.6. Compound microscope at a relatively modest price. Courtesy of Edmund Scientific Co.[4]

receive the same view from the single objective in use. The several objectives are parfocal and satisfy the standards of the Deutsche Industrie Normung (German Standardization Institute), as do all the other optical components. The binocular eyepiece is at 45° to the vertical axis for personal comfort and satisfaction, and it is 360° rotatable for viewer convenience. The eyepieces are also adjustable for interpupillary distance.[4]

3.4. STEREOCOMPOUND MICROSCOPES

Microscopical stereoscopic vision is obtained by means of two compound microscopes, one for each eye, at about the ordinary interpupillary angle for reading or writing. This aspect alone makes for comfort and ease. The stereoscopic effect results from a slightly different angular view for each of the eyes. Moreover the image is erect, and the depth of field in focus is relatively long to make it easier to manipulate tiny tools when operating on small objects.

Stereomicroscopes are essentially composed of two plain compound microscopes, such as those shown in Figures 3.7a and 3.7b. Hence there are two kinds of compound stereomicroscopes: the binobjective-binocular

(Greenough) type and the monobjective-binocular (common objective [CMO]) type.[7] The binobjective-binocular type is really two compound microscopes mounted at a slight angle to each other on a single stand. As shown in Figure 3.7a, this binocular microscope has an objective and an eyepiece for each eye, mounted at the interpupillary angle of close vision. The natural stereo effect of the binocular system is enhanced by a pair of erecting prisms, adjustable to individual interpupillary distance between eyepieces. The paired objectives are slender, compact, and close together, providing easy access to the specimen. Furthermore, aberrations are relatively easy and inexpensive to correct because each of the two beams enters its respective lens *axially*.[7]

Disadvantages of the binobjective-binocular system are

Intermediate images are inclined from the plane of the specimen stage and tilted toward each other, so that only the central portions of the two images are in simultaneous focus.

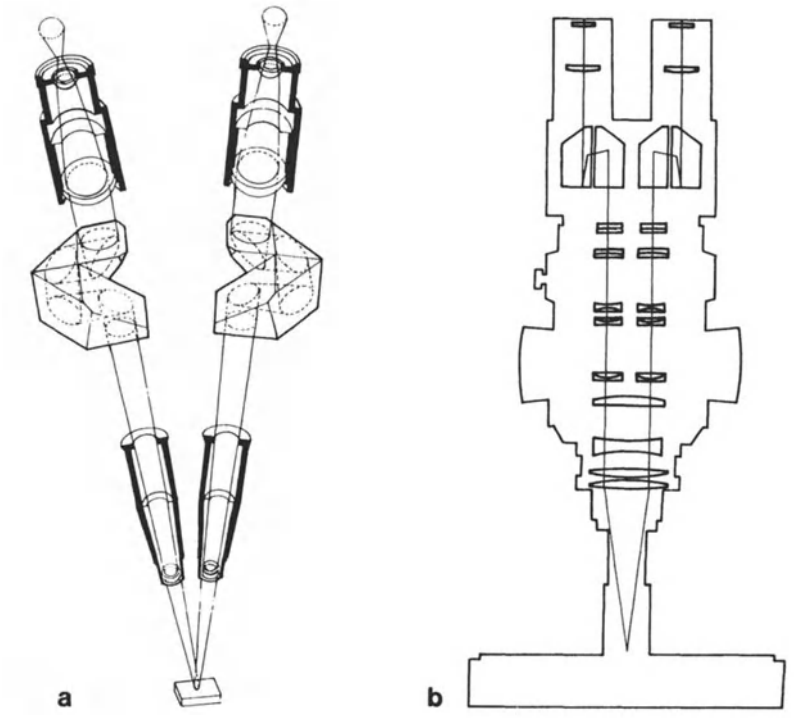


FIGURE 3.7. (a) Greenough stereomicroscope system: A diagram of the beam path.[7] (b) The common main objective stereomicroscope system: A schematic diagram of the beam path.[3]

Both images are tilted unless the specimen is tilted for only one image.[7]

The other type of stereomicroscope has only one objective, but it is large enough to yield different views to the two eyepieces, as shown in Figure 3.7b. This type may be equipped with erecting prisms like the Greenough type. The advantage of the CMO is that the intermediate image planes are parallel to the plane of the object without tilting toward each other. Therefore both images are in simultaneous and complete focus, and consequently accessories are easily adapted. However since the two imaging beams traverse the large common objective obliquely, corrections for aberrations are relatively difficult and expensive to make.[7]

Either the monobjective or the binobjective type of stereomicroscope can be equipped with old-style stepwise changes in objectives or with the newer style zoom objectives with a lens system of continuously variable magnification.[7] One built-in advantage to the stepwise system of objectives of fixed magnification is in micrometry. Once each objective has been calibrated with a given micrometer eyepiece by means of a standard-stage micrometer, the calibrations are fixed.[7]

The corresponding disadvantage of variable magnification in the zoom objective is obvious, but it is partially overcome in a newer design by click-stops on the rotating collar. The manufacturer of the design has published information about its zoom stereomicroscopes relating NA, resolution, depth of field, and definite magnifications. Data are reproduced as Figure 3.8 and Table 3.2[7] With reference to Chapter 2, NA is independent of the eyepiece's magnification (10× in Table 3.1).

The relatively great depth of field of either type of stereobinocular microscope is a distinct advantage in examining surfaces that have suffered fracture, cracking, corrosion, erosion, weathering, etching, sawing, or abrading. It is also an advantage in examining, sampling, separating, dissecting, selecting, and orienting biological materials, crystals, soils, sands, powders, flours, spots, and specks, particularly on uneven surfaces.

Such an instrument may also be used to examine, at least in a preliminary way, natural, synthetic, or artificial composites and their constituents. The utmost advantage of the stereomicroscope lies in the realistic three-dimensional image of any specimen whose third dimension is within the relatively great depth of focus. A further advantage is the characteristically large field. Ordinary kinds of illumination may be employed, and since their effects are known, the image is easy to interpret. Also the image is erect, and therefore manipulating the specimen is easy. All these advantages come with a compound microscope, which is comparatively inexpensive.

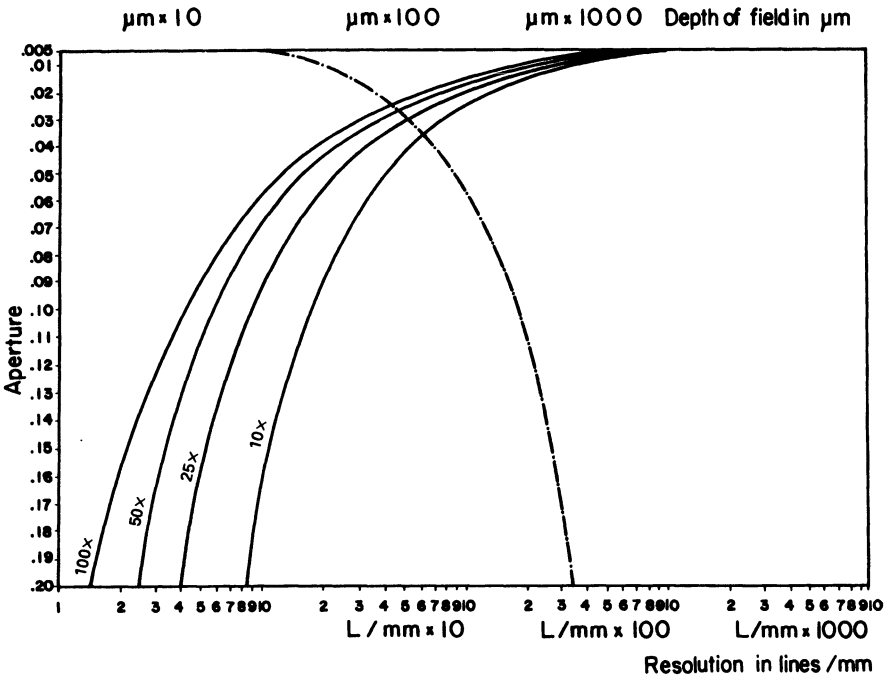


FIGURE 3.8. Resolving power and field depth in stereomicroscopes. The solid curves drawn specifically for total magnifications (as shown) obtained with the M5 stereomicroscope, are applicable in principle for other stereomicroscopes made by Wild Heerbrugg. The dash-dotted curve, representing the correlation between NA and resolving power expressed in lines per millimeter, has general validity for reflected light observation. Courtesy of Wild Heerbrugg Instruments.[7]

TABLE 3.2
Numerical Apertures of Various Optical Combinations for the M5 Stereomicroscope[a]

Optical combination	Magnification-changer position[b]			
	50×	25×	12×	6×
Main objective alone	0.0800	0.0750	0.0445	0.0223
Main objective with 2.0× attachment objective	0.1650	0.1500	0.890	0.0446
Main objective with 1.5× attachment objective	0.1200	0.1125	0.0667	0.0334
Main objective with 0.5× attachment objective	0.0400	0.0375	0.0223	0.0112
Main objective with 0.3× attachment objective	0.0240	0.0225	0.0134	0.0067

[a]Table courtesy of Wild Heerbrugg Instruments, Inc.[7]

[b]The magnification-changer position indicates total power when 10× eyepieces are used. It should be noted that numerical apertures for each position are independent of the eyepiece power.

The chief limitation of the stereomicroscope is its relatively low resolving power. There is no substage or condenser, and therefore the kinds, and intensities of transmitted illumination are limited. For reflected light, vertical bright-field illumination is very limited in its ability to gain contrast.

The most convenient laboratory stand for the stereomicroscope is separable at the joint between the specimen stage and the base. After separation the whole microscope may be placed directly on a large flat object, thus avoiding destructive sampling. In this position ordinary incident oblique illumination is usually employed. For transmitted light the base carrying the mirror and the glass window is put into place. Double hand rests are usually supplied for convenience in manipulating the specimen and/or instruments.[7] The complete microscopical head should be removable to go onto other stands, one of which is a heavy stand with a long crossarm, so that the microscope can be swung over an irregular object or part of a large machine or other assembly (see Figure 3.9).

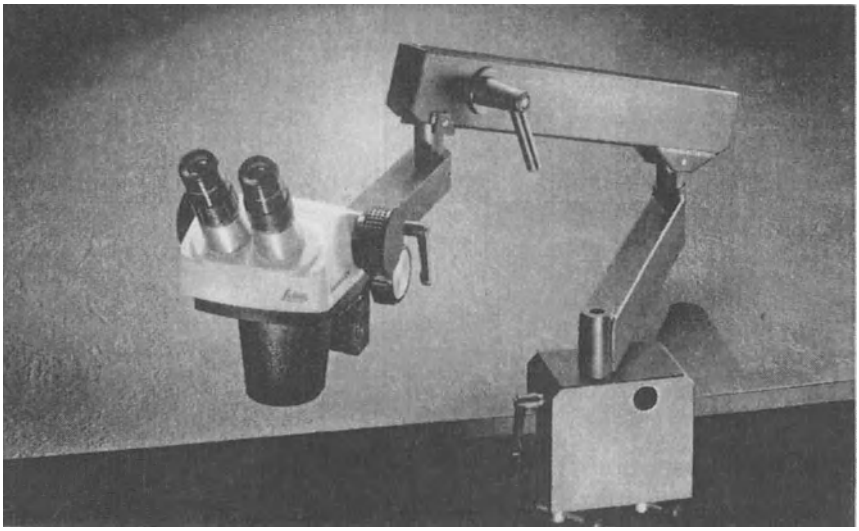


FIGURE 3.9. Leica SZ4 stereomicroscope on a Flex-Arm stand featuring two different configurations: one, a rigid 12-in. (31-cm) arm section for applications that require only horizontal movement; and two, a 13.33-in. (34-cm) counterbalanced section with an adjustable weight range of 4–21 lb (2–9 kg) is used for situations demanding both horizontal and vertical movement. By permission of Leica.[8]

3.4.1. Illuminating with Stereomicroscopes

Lighting a specimen by oblique illumination to use a stereomicroscope can be performed with an ordinary desk-type microscopical lamp fitted with a field condenser and diaphragm. Lately a fiber-optic cable has been offered instead;[8,9] the resulting beam is only about 5° from vertical illumination.[9] Such illumination is useful, even necessary, for examining metals, ores, rock, concrete, bones, etc. (see Figure 3.10).[8]

A great deal can be done under the stereomicroscope with unsophisticated tools. For example R. B. Scott[10] simply taped an acrylic fiber onto a microscopical slide under a stereomicroscope at about 60×. Then with a hand-held razor blade, he made a cut, as illustrated in Figure 3.11, and peeled off the skin, which could be examined separately. By making a second cut (see Figure 3.11b), he peeled off a longitudinal section only 5–10 μm thick and examined it between crossed polars (see Chapter 5). The success in making these longitudinal sections may be attributed chiefly to the layered structure of the fiber that allows it to be peeled, thin layer after thin layer. The phenomenon is all the more remarkable considering that a filament of only about 20 μm in diameter was operated on under a limiting resolving power of about 5 μm (see Table 3.1). The attributes contributing to visibility are often surprising.

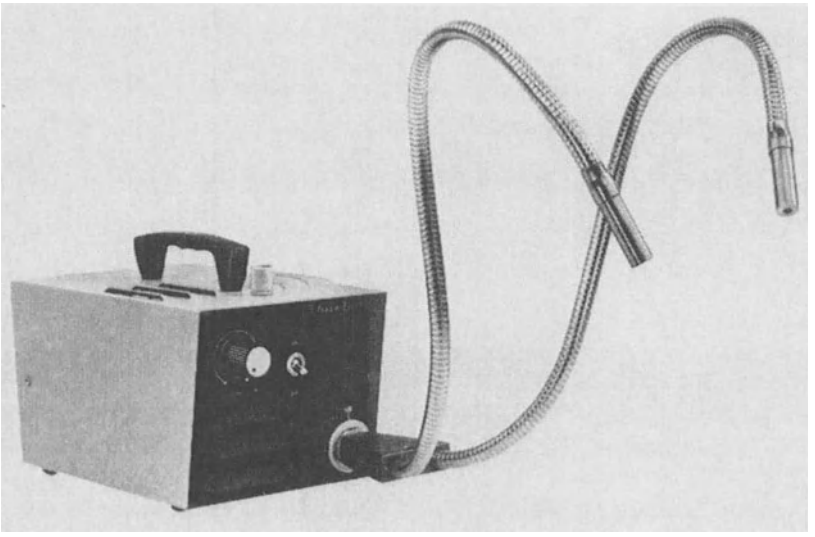


FIGURE 3.10. Leica Fiber-Optic Illuminator, which has many potential uses, such as nearly vertical illumination with a stereomicroscope.[9] Courtesy of Leica.

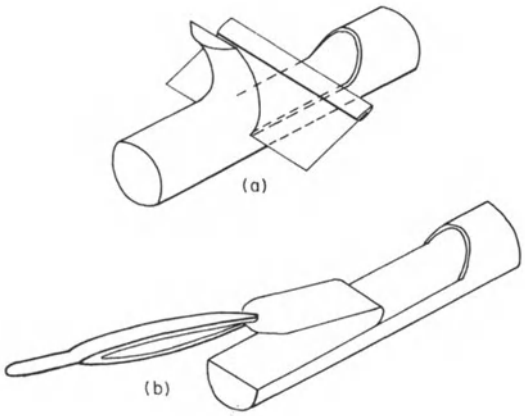


FIGURE 3.11. Peeling a fiber to yield (a) skin and (b) longitudinal section.[10]

3.4.2. Preparing the Specimen

For preliminary examination with a stereomicroscope, no preparation is desired, much less required. The idea is to look and see exactly what has been received. Anything done to the specimen will change it. Besides any action will take time, which alone may cause the specimen to change.

If the specimen will not stand by itself, stick it in modeling clay or flatten a bit of it to make a base. If the specimen is smooth enough for its surface to be within the depth of focus of a low-power objective but not all in focus with a higher power objective, rather than smoothing it (and changing the surface), revert to a lower power objective and choose a higher power eyepiece.

For the reasons just pointed out, no loss of numerical aperture or resolution results from this practice. To expose the interior of the sample or to prepare a fresh surface, try a simple appropriate method: If the sample is soft enough, cut it with a knife or razor blade, if rubbery, use a pair of scissors or shears. If hard, try breaking, sawing, or abrading it. If the resulting surface is too rough or glary and refining the surface is inappropriate, wet it with an inert liquid and cover with a cover glass.

3.5. BIOLOGICAL MICROSCOPES

In Chapter 1 we saw that historically biology was the first science to grow up with microscopy, particularly through the nineteenth century.

This dual growth was quite separate from developments in petrography and chemistry, where different requirements for the microscope were being met. Thus the distinctive instrument called the biological microscope appeared. It is a compound microscope, highly developed in its best models in accordance with most of the theoretical principles expressed in Chapter 2. The objectives are manufactured and described primarily in terms of NA. (Magnification in objectives is of secondary importance; it can be bolstered if desired by means of the eyepiece.) Next to NA in importance is the kind and degree of correction in the objective (achromatic versus apochromatic). Competent biological microscopists also understand the use and care of oil-immersion objectives as well as air-immersion objectives.

Relatively recently professional light microscopical stands were enlarged. Thus, the lamp, its field condenser, and its field diaphragm are aligned and enclosed, as illustrated in Figure 3.12a.[11] Figure 3.12b is a diagram of the corresponding optical system[12] of the corresponding kind of microscope. Figure 3.12a shows that one end of the tube of the microscope carries the objectives on a rotatable nosepiece. The other end of the tube is to contain the eyepiece. If instead there is a binocular (but not stereoscopic) arrangement, it is, by means of prisms, to divide the rays equivalently to form a whole image for each of the two eyes. If there is a third tube, it is primarily for a photomicrographical attachment. The microscope is attached to a heavy housing to minimize vibrations.[12] As a biological microscope, the specimen rests on a fixed stage, which usually carries a (graduated) mechanical device for moving the specimen in x–y directions. There is a hole in the center of the stage to pass the beam of light from the source, through the condenser and specimen, into the objective. Usually a rotatable nose piece carries three objectives of choice.[12]

3.5.1. Objectives

These are the most important optical part of a professional light microscope. The principal attributes of an objective are (NA) and degree of *correction* (achromatic, semiapochromatic [fluorite], and apochromatic). Having chosen an objective of a given NA and degree of correction for aberrations, any convenient increase in magnification, as in micrometry, should be made by choice of eyepiece.

Chromatic aberration is due to the greater refraction of shorter wavelengths than longer ones; hence the focal length of a simple lens is shorter for blue rays than for red ones. This dispersion causes color fringes in the

image field of a lens with chromatic aberration. The phenomenon is diagrammed in Figure 3.13a, and the approximate compensation is suggested in Figure 3.13b to form an *achromatic* objective. As indicated achromats are corrected chromatically for red and blue light and also for spherical aberration for green light. Thus achromats are best used visually with a green light filter and photomicrographically with the green filter and black and white film.[12]

By using mineral fluorite to some degree among lens elements, spherical aberration can be corrected for two colors, so that fluorite objectives (semiapochromats) are better than achromats for photomicrography with color film. The best (and most expensive) objectives are the apochromats, which are composed of many elements, as indicated in Figure 3.13a.[12]

3.5.2. Eyepieces

There are two principal kinds of eyepieces: Huygenian (negative) and Ramsden (positive). As shown in Figure 3.14a, the Huygenian eyepiece has only the upper (eye) lens above the diaphragm, whereas the Ramsden type (see Figure 3.14b) has both lenses above the diaphragm; incidentally this is an easy way of distinguishing between the two different types, especially if there is no labeling. The simple Huygenian eyepiece is suited for use with ordinary achromatic objectives (with focal length of 32, 16, 8, or 4 mm). The Ramsden eyepiece has both the eye lens and the field lens above the diaphragm; the two lenses may be cemented together. Ramsden eyepieces are generally corrected better than the Huygenian type. Some Ramsden eyepieces are designed to compensate for residual aberrations in fluorite and apochromatic objectives. Compensating eyepieces are also used to advantage with fluorite objectives of focal length 4 mm or shorter. Because Ramsden eyepieces have all their lenses above the diaphragm and can be sharply focused on its plane, they can all be used with any kind of pattern placed on the diaphragm: a calibrated linear scale, a grid of cross lines, a pattern of angles, etc.; thus the Ramsden eyepiece is used in quantitative linear or areal analysis and when comparing an object with some other pattern or type.[12]

3.5.3. Condensers

Figure 3.15 shows three kinds of condensers, distinguished by the degree of correction: Abbe, aplanatic, and aplanatic/achromatic.[12] Used

properly, the Abbe condenser is generally satisfactory with achromatic objectives. With all oil-immersion objectives, all condensers require immersion oil between the upper lens of the condenser and the *underside* of the microscopical slide.[12]

Figure 3.16a shows two adjacent points that diffract a ray of a certain wavelength in the dry object at point O thus subtending an angle a', which happens to be less than half the angular aperture AA of the objective. Therefore the ray a' can enter the objective and do its part toward resolving the two particular points in the object.

In Figure 3.16b it is assumed that the object has been mounted in a

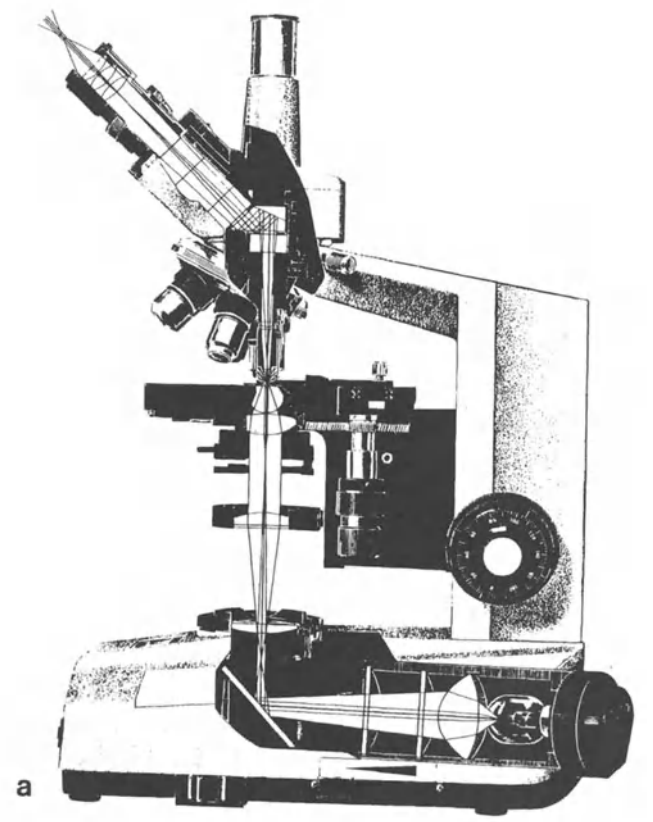


FIGURE 3.12. (a) "Biological" type of compound microscope.[12] Courtesy of Olympus Corporation.[11] (b) Diagram pertaining to Olympus microscope shown in Figure 3.12a.[12] Courtesy of Olympus Corporation.[12]

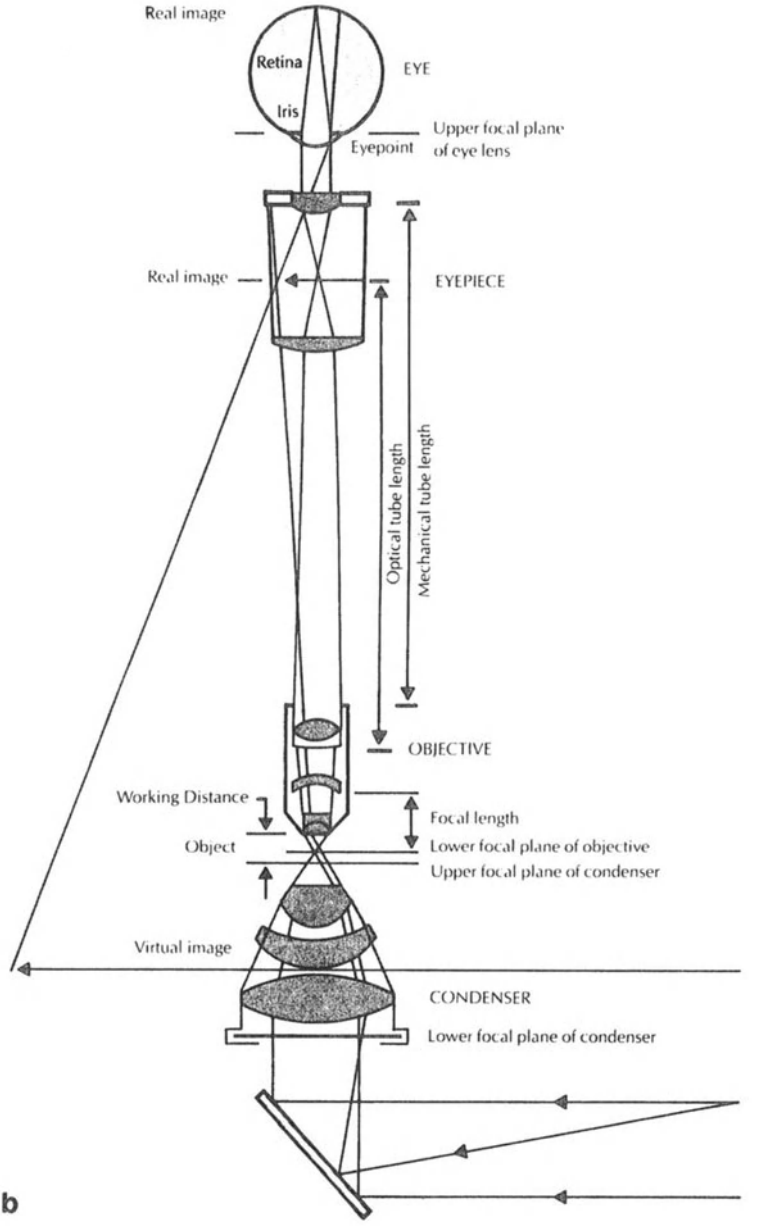


FIGURE 3.12. (*Continued*)

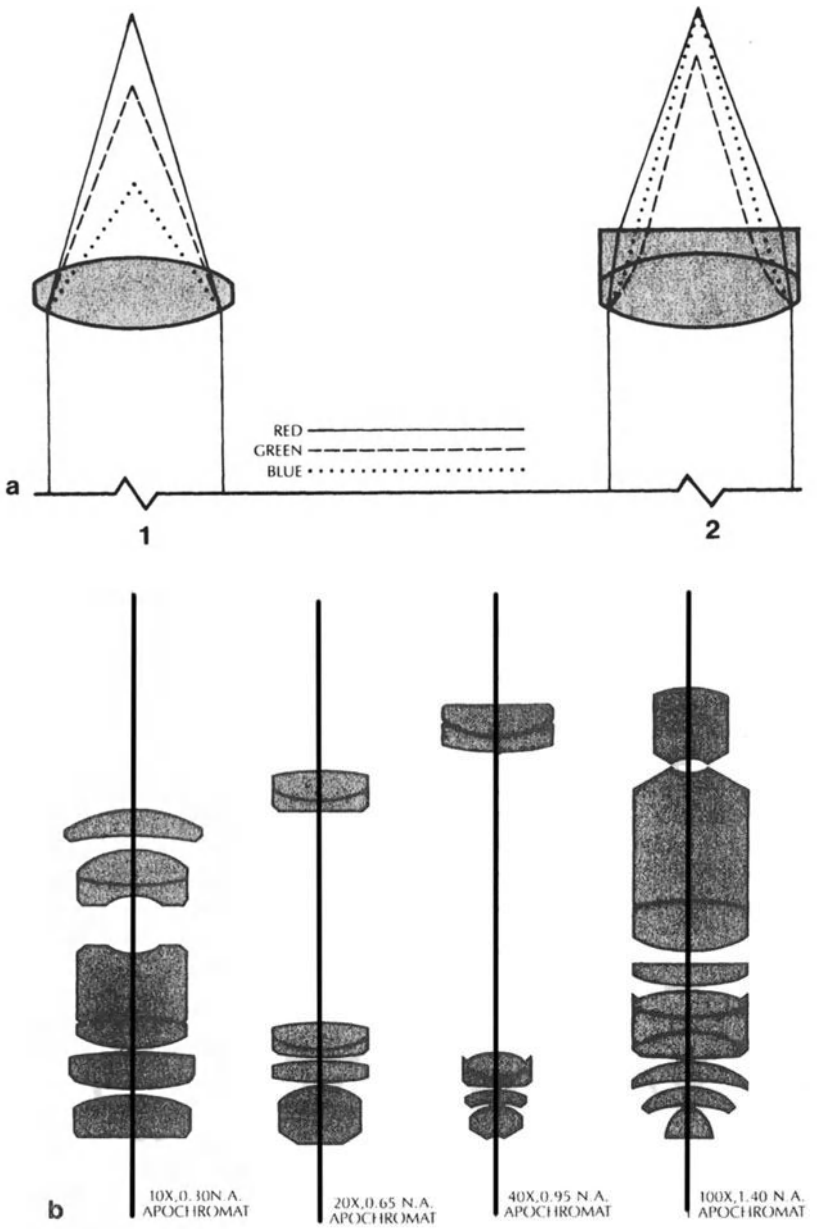


FIGURE 3.13. (a) Chromatic aberration: Failure of a simple lens to bring light of different wavelengths to a common focus (1) can be compensated for in part by using an achromatic lens (2).[12] Courtesy of Mortimer Abramowitz and the Olympus Corporation.[12] (b) A series of apochromatic objectives showing the number, shapes, and kinds of segments.[12] Courtesy of Mortimer Abramowitz and the Olympus Corporation.[12]

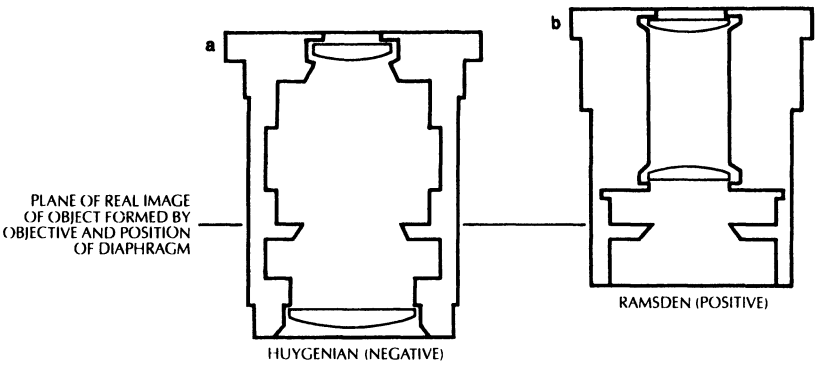


FIGURE 3.14. (a) Huygenian eyepiece in cross section. (b) Ramsden eyepiece in cross section.[12] Courtesy of Mortimer Abramowitz and the Olympus Corporation.[12]

liquid of the same refractive index as that of the cover glass (ca. 1.55). Therefore angle *a″* is less than *a′* (see Figure 3.16c), but on reentering air angle *a″* becomes *a′* again. However if the space between the cover glass and an *oil-immersion objective* in Figure 3.16c is filled with immersion oil (n = 1.55), the narrower angle *a″* is maintained. Therefore if the *AA* of the particular objective is totally filled, smaller units can be resolved with a properly employed oil-immersion objective than those resolved with the best air objective. However it is assumed that optical homogeneity is maintained *from object to the front lens of the oil-immersion objective and from the top lens of the condenser to the underside of the object slide.**

*See the advantages of a water-immersion objective: M. Brenner, *American Laboratory*, pp. 14, 16–19, April, 1994.

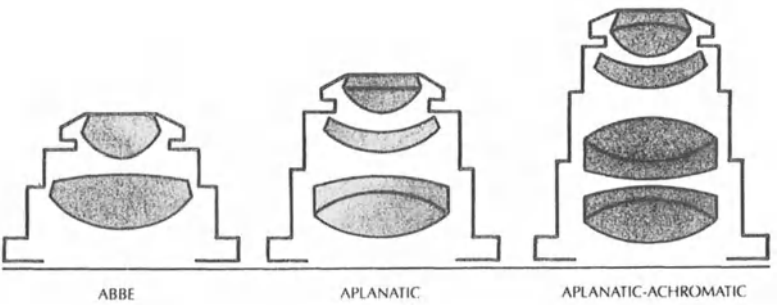


FIGURE 3.15. Three types of light-microscopical, substage condensers, arranged in order of degree of correction for aberration(s).[12] Courtesy of Mortimer Abramowitz and the Olympus Corporation.[12]

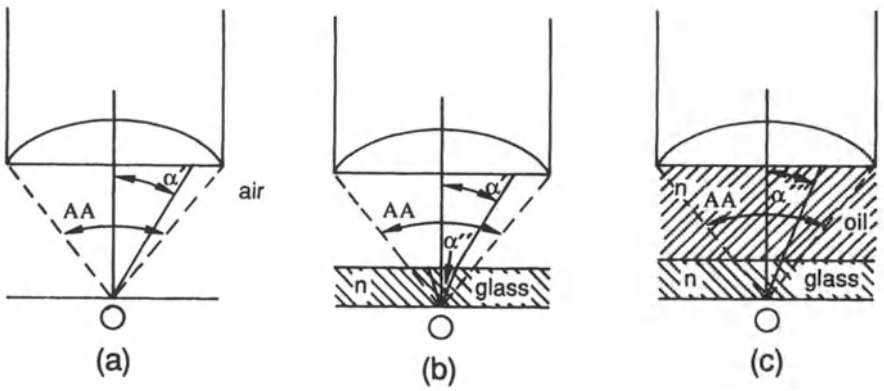


FIGURE 3.16. Effective angular aperture of (a) dry, (b) covered, and (c) homogeneously immersed specimens.[1]

3.5.4. Illumination

In Chapter 2 illumination is discussed in terms of Nelson's method versus Köhler's. Nelson's method is satisfactory with a ribbon filament as the electrical source. However most if not all light-microscopical lamps employ electric light bulbs with *coiled* filaments, which by Nelson's method produce an image of the coil! That is, the microscopical field is unevenly illuminated. Throwing the image out of focus defeats the purpose of critical (focused) illumination. With Köhler's method the coil filament is imaged by means of the *lamp's lens* (see Figure 3.17) onto the back aperture

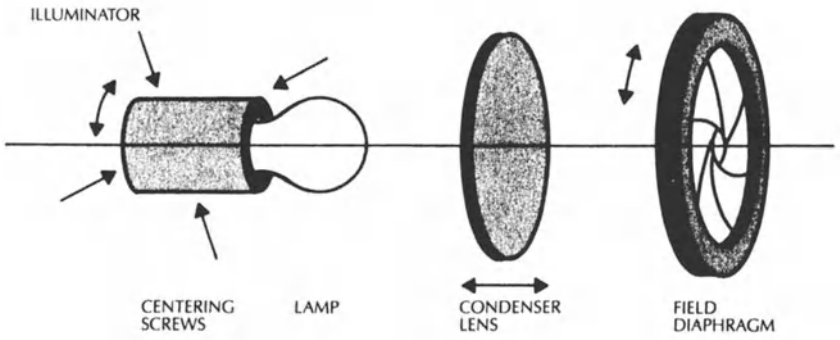


FIGURE 3.17. Microscopical illuminator. As a separate lamp the housing contains its condensor lens and field iris diaphragm.[12] Courtesy of Mortimer Abramowitz and the Olympus Corporation.[12]

(iris diaphragm) of the microscope's condenser; thus the condenser's lens provides uniform illumination to the object. Moreover the condenser's diaphragm limits the size of the illuminated area to exactly that of the particular objective in use.[12]

Figure 3.18 summarizes the optical requirements of Köhler's illumination,[12] particularly regarding a housed lamp separate from the microscope's housing, which is the case in classrooms and laboratories where the set-up is repeatedly taken down and set up again. The relevant advan-

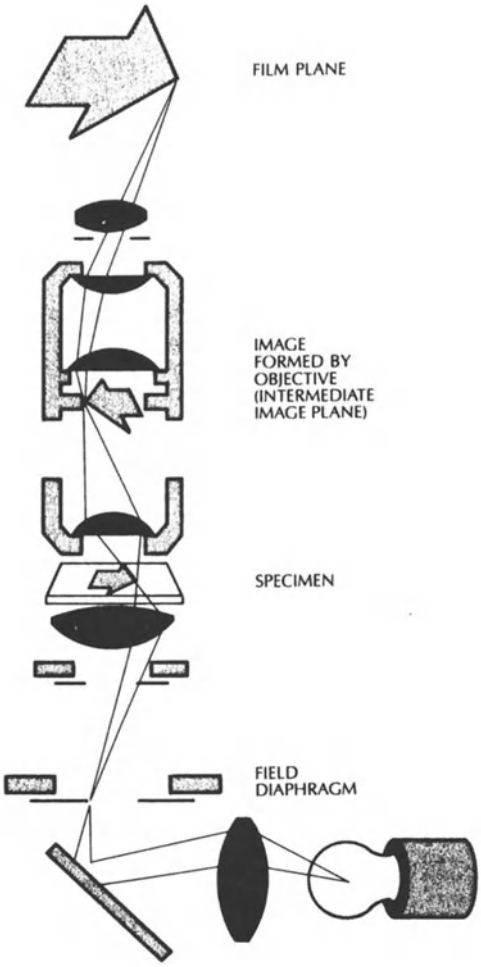


FIGURE 3.18. Setup for Köhler's illumination. Two ray paths are traced from the filament of the light bulb to a point on a photomicrographical film.[12] Courtesy of Mortimer Abramowitz and the Olympus Corporation.[12]

tages of housing the lamp and microscope together are seen in Figure 3.18.[12,13]

3.5.5. Ultraviolet and Infrared Light

Ultraviolet light is used in biology to excite fluorescence in the visible range.[19,20] For such radiation there are special objectives through which the rays are guided by *reflection.* Reflecting objectives (see Figure 3.19) are without chromatic aberrations throughout the entire spectrum of light, including the UV region for fluorescence microscopy and the infrared region for FT-IR microscropy[23] (see Chapter 13).

3.5.6. Anisotropy

Anisotropy may exist in biological specimens in any or all of the manifestations previously described in Section 2.13. However equipment for viewing these phenomena is not usually included in a strictly biological microscope. The usual instrument has no polars, no rotatable stage, nor any slot for retardation plates, and adding these to a biological microscope is usually unsatisfactory even if possible. Besides the objectives and other optical parts provided on biological microscopes are not necessarily strain free, and they tend to interfere with polarization phenomena. Therefore further discussion of anisotropy is reserved for Chapter 5, devoted to polarizing microscopes.

3.5.7. Magnification

Magnification is still emphasized by biologists rather than resolving power and other attributes contributing to visibility. This is evident whenever biologists persist in using magnification as the first criterion in de-

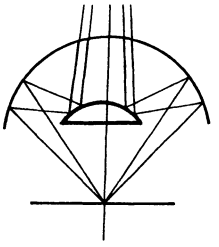


FIGURE 3.19. Reflecting objective.[1] Courtesy of Microscope Publications.

scribing any objective, e.g., 40× regardless of whether the objective has an NA of 0.65, 0.75, or 0.95 or it is achromatic, apochromatic, or in between.[1] Magnification has its place, especially in micrometry, but a distinction must be made between useful and empty magnification.

3.5.8. Photomicrography

Nowadays *photomicrography* is so involved with microscopies of all kinds that cameras either are built into or easily attached to the microscope. The question is what type of film is used: 32-mm roll film, Polaroid® type, or both? Manufacturers anticipate that the answer is both because the professional microscopist has to be prepared to present positive micrographs on occasion and also to take micrographs to be presented either as slides for viewing on a screen or as enlargements for reports and publication. Thus it is wise for the microscopist to be on the mailing list of photomicrographical research and development companies.[14,15,16]

3.6. SUMMARY

Visual acuity, which is the inherent resolving power of the human eye, increases (up to a point) as the eye is brought closer to the object. The limiting point is reached at a distance of about 25 cm, where the eye's lens is contracted as much as possible without strain. Since the limit of resolution of the eye is inversely proportional to its distance from the object, the only way to increase resolution is to supplement the lens of the eye with an external lens or lenses, thereby bringing the eye closer to the object than the natural limit of 25 cm.

The simplest device for accomplishing this is the common magnifying glass, which has its limitations: It cannot be held steady, the focus point keeps changing and is seldom optimum, and the image at the circumference of the lens is greatly distorted. When the lens is corrected and supported and brought into proper alignment and focus, it is called a *simple microscope*, and it is a very useful instrument. With it the limit of resolution is improved from 150 μm (for the unaided eye) to 10 μm (for the simple microscope of highest practicable NA).

The *compound microscope* allows increased NA and great improvement in resolution by using two or more lens systems. An objective (usually a multiple-lens system of short focal length) forms a tiny real (but inverted) image in the focal plane of a second lens system called the eyepiece, which magnifies that image to a point where it can be resolved on the retina of

the eye. For optimum utility the lenses are corrected according to the optical principles of Chapter 2 and mounted in a sturdy stand equipped with a light source, a condenser, a suitable stage for the specimen, a system of focusing, and a variety of accessories for micrometry, special illumination, micrography, and so on.

Compound microscopes vary in kind and extent of their contributions toward optimum visibility. The two general types considered in this chapter are the stereomicroscope and the biological microscope. Stereomicroscopes are very popular because they allow both eyes to be used to perceive depth, and they are usually equipped to give an upright and hence easily interpreted image. Two designs are available, the first with two objectives, two eyepieces, and two body tubes inclined at a slight angle to each other, so that the lines of sight converge on the object. This is the classical Greenough type, constructed with dry objectives of long working distance, so that almost any object can be handled, turned, treated, and even dissected during observation. Since the left and right images are formed separately in axial lens systems, the systems can be fully corrected. One disadvantage is that both axes are inclined to the plane of the specimen, so that at maximum resolution, only a portion of either field can be in focus at one time. A greater disadvantage is that there is no substage condenser, for the simple reason that it is impossible to shape a biaxial lens. Hence there can be no critical illumination, and resolution is severely limited. The other design of stereomicroscope can indeed have a substage condenser because it uses only one large objective and splits the light from that objective into two parts that (after passing through image-erecting prisms) are observed through separate eyepieces in separate parallel body tubes. Both images are in simultaneous focus, and various accessories for different kinds of illumination are easily fitted.

The biological microscope is traditionally a nonpolarizing compound microscope designed for examining transparent or translucent specimens and demonstrating their parts and structures. The modern biological compound microscope is housed in a single stable unit containing the illuminating system designed for the Köhler type of precise illumination. The more elaborate models are equipped for two eyepieces by means of erecting prisms for one person using two eyes (or two persons using one eye each). Since only one objective can be employed at a time, there can be no stereoscopic effect. A third tube may be provided for photomicrography at additional cost.

Chapter 3 explains the design and proper use of various objectives, condensers, eyepieces, and accessories. Biologists now recognize the advantages of polarized light, and its use is becoming more common in microscopes. This subject is included in Chapters 5–7.

4

Compound Microscopes Using Reflected Light

4.1. STUDYING SURFACES BY REFLECTED LIGHT

Microscopy by reflected light may be used for a number of reasons: to look at a natural surface such as a leaf, feather, skin, shell, or fossil; to compare surfaces after aging, usage, weathering, or other exposure; or to prepare an inside surface for studying an opaque substance, such as bone, metal, coal, ore mineral, ceramics material, or pigmented plastic. Microscopy can answer such questions as how many layers are there in a seashell, tree's growth, laminated paper, or board? What is the structure of a sponge, tree cone, botanical cane, zoological organ, fossil, rock, ore (Figure 4.1), brick, cement, or plastic filled with biological material (Figures 4.2–4.5)?[1–5] Indeed the specimen may be a particulate material, such as seeds, tiny insects, sand, rock dust (Figure 4.6[1]), or small crystals. Such specimens may be better embedded in a dark, pigmented resin for reflected light than in a clear, colorless resin for transmitted light.

Experience, habit, or equipment may determine whether the light should be reflected from the prepared surface of a thick specimen or transmitted through a prepared thin section. Metallographers,[2,2a,3] ore-dressing engineers,[4,5] and resinographers[6–8] are experienced in using reflected light, while biologists are more experienced in using transmitted light.[9] If the biologist has to use reflected light, he/she should gain sufficient experience in solving microscopical problems.

Reflected illumination may reveal more or different information than transmitted light. For example, anthracite coal may be petrographically prepared thin enough to be examined classically by transmitted light. However a thick section may be examined by reflected light as polished or

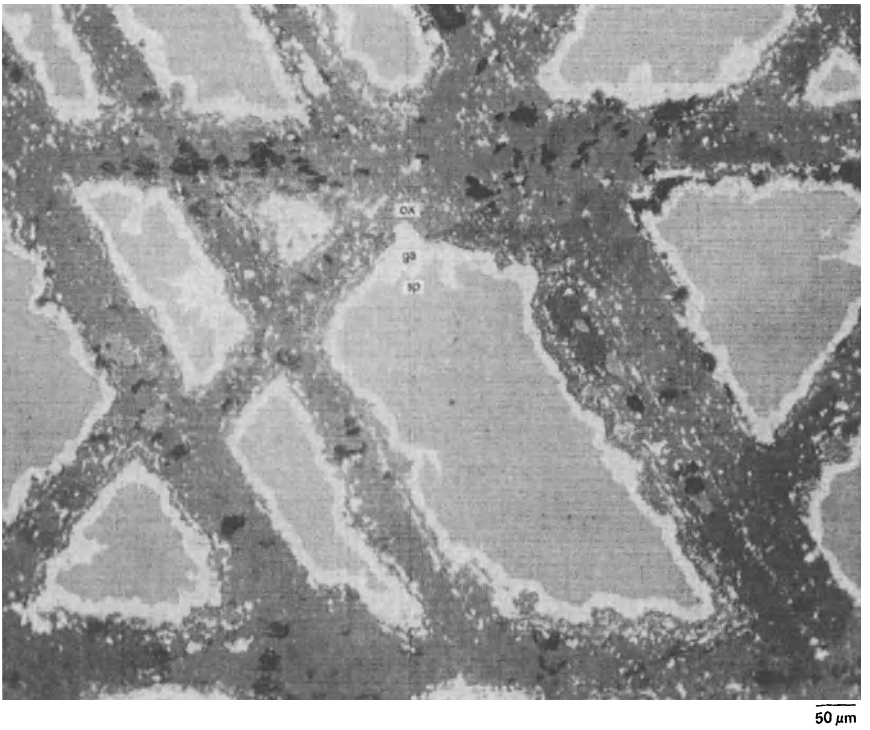


FIGURE 4.1. Lead–zinc deposit, South Africa. Sphalerite (sp) replaced along its cleavage planes by lead–zinc oxidation products (ox) and by galena (ga). Polished thick section, reflected light. Courtesy of American Cyanamid Co.

as etched, with or without polarizing the light. Figure 4.7 shows the differences under reflected light by varying the methods of examination.

With the increasing development of man-made composites of opaque and transparent, hard and soft, large and small components, organic and inorganic plastics, came greater use of reflected light on strictly transparent specimens[10,11] (Figure 4.8)[12].

4.2. RESOLVING POWER

The *resolving power* of microscopical objectives for use by reflected light can be slightly higher than those intended for use with transmitted light and a cover glass. No cover is necessary on a prepared surface

FIGURE 4.2. Wood flour mounted in black phenolic resin. Polished cross section, reflected light. The fibers are straight in lengthwise section, and some cross sections occur in groups.[1]

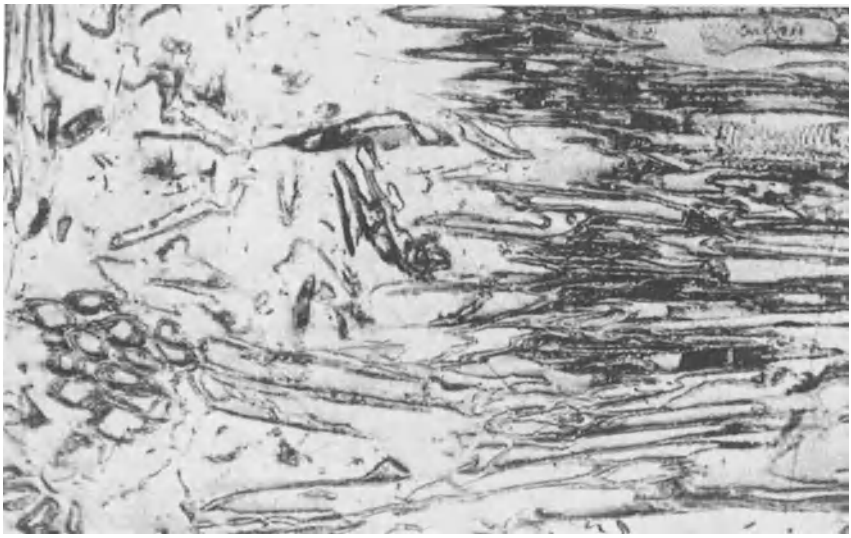


FIGURE 4.3. Sugar cane (bagasse) mounted in black phenolic resin. Polished cross section, reflected light.[1]

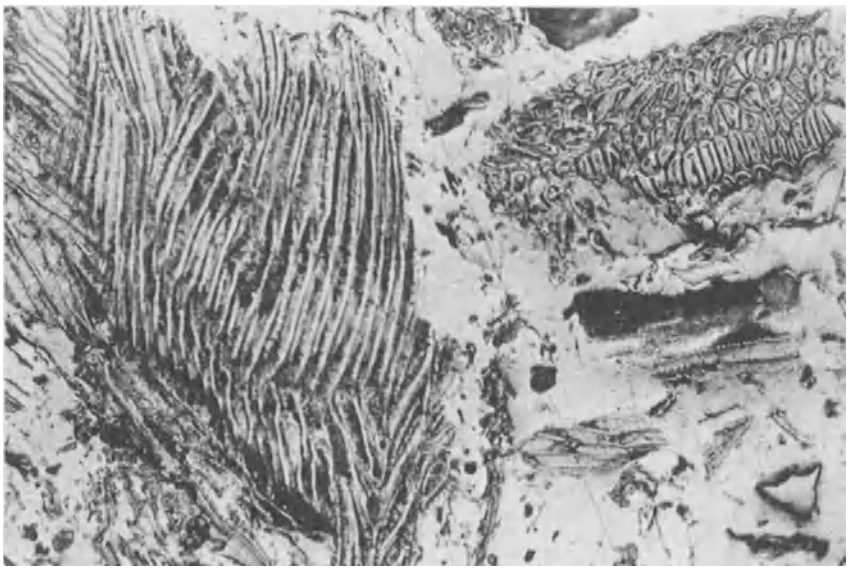


FIGURE 4.4. Peanut shells mounted in black phenolic resin. Polished cross section, reflected light, showing plates of short, interlocked fibers in longitudinal and transverse sections.[1]

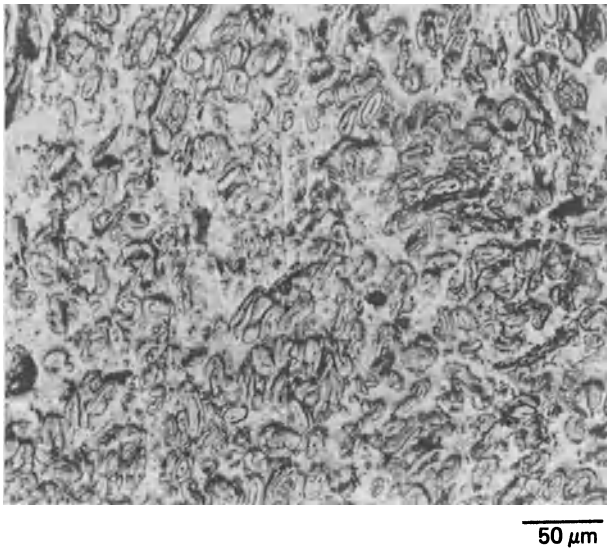


FIGURE 4.5. Cotton mounted in black phenolic resin. Polished cross section, illumination by reflected light. Most of the fibers are shown in cross section. Oval cross sections are shown with a slit in the center. Actually they are cross sections of *collapsed* cylindrical tubes.[1]

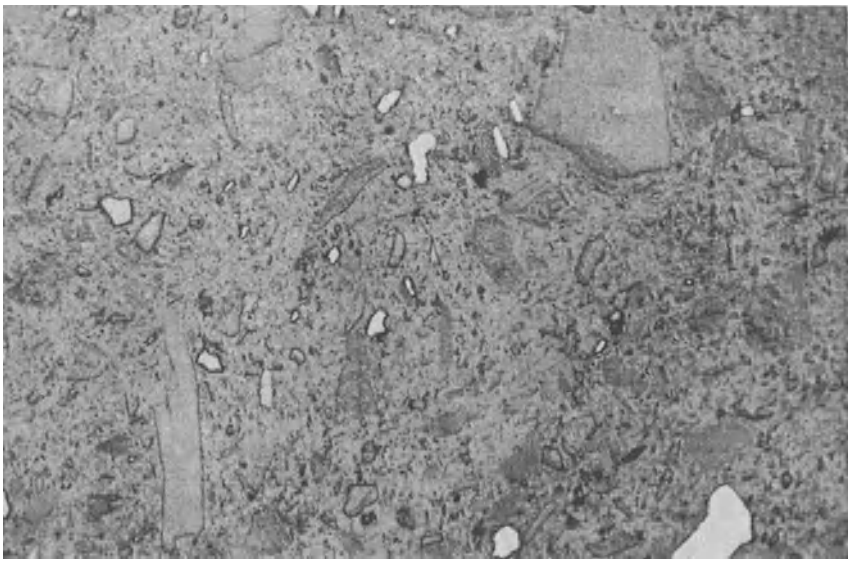


FIGURE 4.6. Dust from Vermont chrysotile asbestos fiber. Illumination by vertical reflected light. Gray particles are chrysotile asbestos or massive serpentine. White particles are associated magnetite. Matrix is Melmac® melamine–formaldehyde resin.[1]

examined by reflected light, so the refractive index n in the formula for numerical aperture

$$\text{NA} = n \sin \alpha$$

is not restricted to 1.52 for glass or any other cover. Accordingly an immersion objective of NA = 1.60 was made for use with monobromonaphthalene (n_D = 1.66)[11] as the immersion liquid, with corresponding high resolution. Unfortunately this kind of objective is no longer manufactured; evidently there was insufficient demand for it, presumably because prospective users did not understand its optical advantages.

The resolution of fine structures by reflected light begins with proper objectives and their proper use.[13] If the objectives are corrected for use without a cover glass, no cover should be used. Reflected-light objectives are usually in short mechanical mounts for optical reasons and bear the manufacturer's information code. It is essential to know the code and use the information. If the objective is corrected for infinity, it is probably not interchangeable with those of a microscope of any other make (or perhaps other models of the same make). If an objective is corrected for use with a

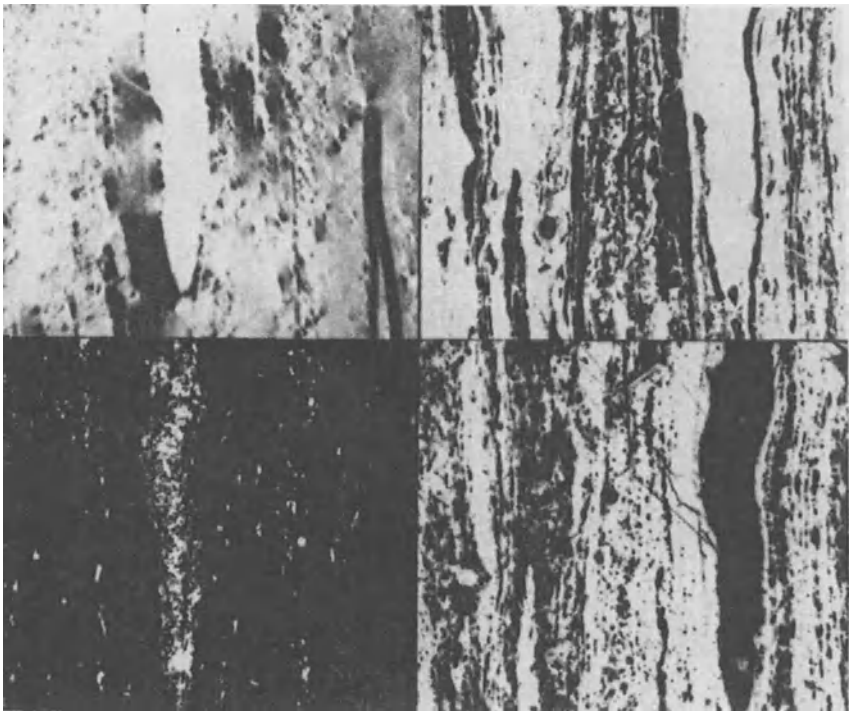


FIGURE 4.7. Four ways to view a field of polished anthracite coal by reflected light: (upper left) between crossed polars; (upper right) unpolarized bright field; (lower left) unpolarized dark field; (lower right) bright field after etching with chromic acid (for microscopy with polarized light, see Chapter 5).

certain eyepiece or projection piece, use it. If an objective is to be immersed, make sure that the immersion fluid has the proper refractive index.

4.3. CONTRAST

Contrast in images by reflected light is low because of many partial reflections from various surfaces as the beam goes down through the objective to the specimen and back through the objective, illuminating reflector, and eyepiece. With highly reflecting specimen surfaces like those of polished metals and ores, there is still plenty of contrast. However poorly reflecting, highly scattering surfaces like those of paper, textiles, and wood cause much glare.[11] Even so as shown in Figures 4.1–4.8,

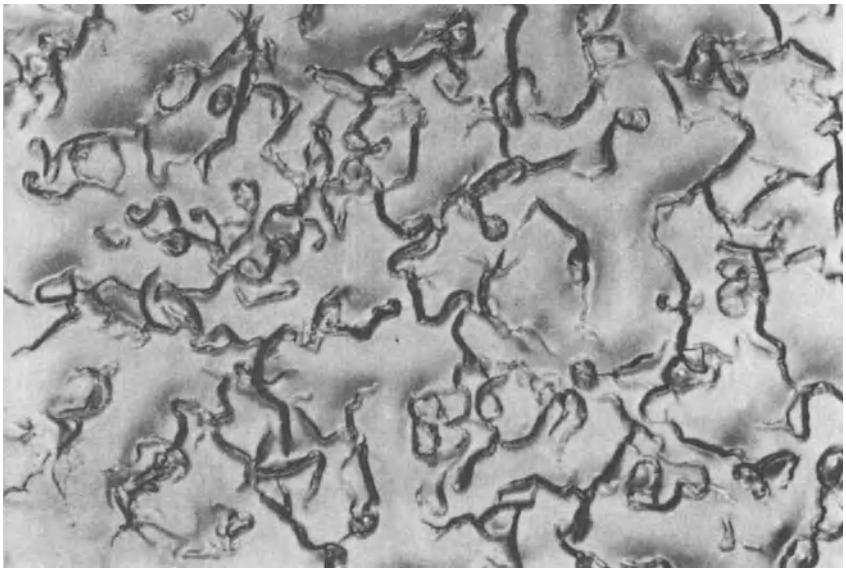


FIGURE 4.8. Wax on glass, cooled in about 20 seconds. Micrograph by reflected light. Courtesy of Continental Oil Co.[12]

enough contrast and sharp resolution can be obtained from transparent material by reflected light to provide valuable information to those who will work for it.[12]

4.4. CORRECTING FOR ABERRATIONS

Corrections for aberrations in dry objectives of NA = 0.5 or more for use by reflected light are designed with the assumption that no cover glass will be used.[13] On the other hand, corrections for immersion objectives assume that any cover should have the same refractive index as the immersion fluid. Ordinary oil-immersion objectives assume that the oil will have n = 1.52, so ordinary glass covers can be used with oil-immersion objectives when covering will help the visibility. For example, the surfaces of paper, textiles, and wood scatter much less light if covered with the proper immersion fluid in conjunction with an immersion objective. If the specimen is fibrous, particulate, or dusty, the particles may be held in place by a proper cover slip and immersion liquid.

4.5. SPECIMEN CLEANLINESS

Cleanliness of the specimen is a special attribute contributing to microscopical visibility of a surface by reflected light.[5] Dust, abrasives, films, and even fingerprints will scatter light enough to add glare to the image and confusion to the interpretation. Precautions should also be taken with regard to the illuminating reflector inside the microscope, which is usually directly under the eyepiece or projecting unit. It is especially important that the tube *always* be covered to protect the illuminating surfaces from dust and dirt, and they must be inspected and cleaned periodically.

Cleanliness in a broader sense includes protection of the specimen from contamination and damage, care of the equipment, knowledge of optical principles, and order in general. All aspects of cleanliness are especially important in working with reflected light because so many optical surfaces are involved twice, first in illumination and again in image formation. Proper alignment of the parts and adjustment of diaphragm apertures are important for the same reason.

4.6. FOCUS

Focus itself is perhaps not so important in working by reflected light as by transmitted light, provided the specimen's surface is flat and oriented exactly 90° to the axis of the microscope. Yet there are times when a deep etch or relief polish and unidirectional oblique illumination are desirable to produce shadows. In such cases selecting the plane of focus is arbitrary and therefore critical.

Depth of focus is characteristically important in images formed by reflected light because the tilt of the illuminator is critical. If the tilt is not exactly 45° to the axis of the microscope, all of the image will not be aligned 90° to the axis. The eye has great depth of focus, so if the depth of focus in the objective is great enough, all of the image may be in acceptable visible focus. Yet in photomicrography the eye is not directly involved, making the depth of focus in the objective critical for obtaining a good photomicrograph.

4.7. ILLUMINATION

Illumination is the most determinative attribute contributing to microscopical visibility by reflected light. As Figure 2.4 shows, reflected illumination can be either dark field or bright field; it can be unidirectional,

symmetrical, or in between. Unidirectional dark-field illumination can be obtained from an ordinary reading lamp with a stereomicroscope (see Chapter 3) or from a smaller lamp and an ordinary compound microscope. Symmetrical dark fields with such microscopes can be obtained with the Lieberkühn type of concentric, concave mirror (Figure 4.9)[14]; from a ring-shaped lamp fitting around the objective, such as the old-fashioned wreath of small incandescent lamps[13]; from the newer circular fluorescent lamp, small enough to fit close to the objective; or from a circle of fibrous light wires[14,14a] surrounding the objective. In any case with dark-field illumination, a mirrorlike surface appears dark because light is reflected away from the objective, but a light-scattering surface like paper appears bright.

With stereoscopic or monobjectives of far enough working distance, bright-field (vertical) illumination can be obtained simply by placing a partially reflecting, clearly transmitting sheet (such as a thin, flat cover glass) in front of the objective at 45° to its axis and to the flat surface of the specimen, as shown in Figure 4.10[13]; the photograph in Figure 4.11 was taken this way. It shows a ball bearing of high-carbon steel retained in both halves of its case-carburized steel race.

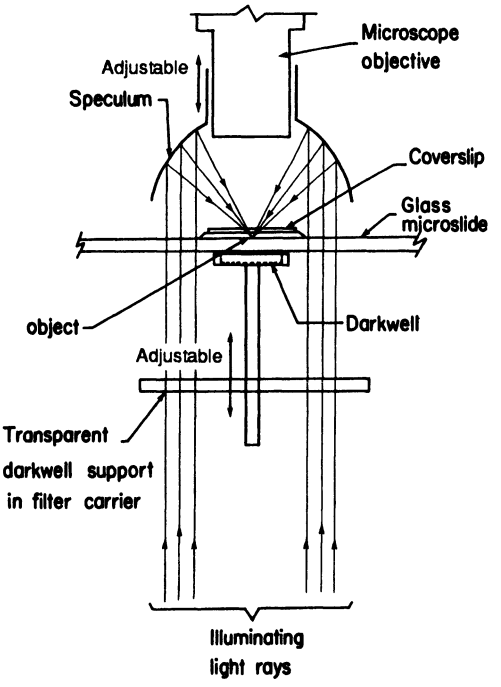


FIGURE 4.9. The Lieberkühn type of illuminator.[14] Courtesy of Microscope Publications.

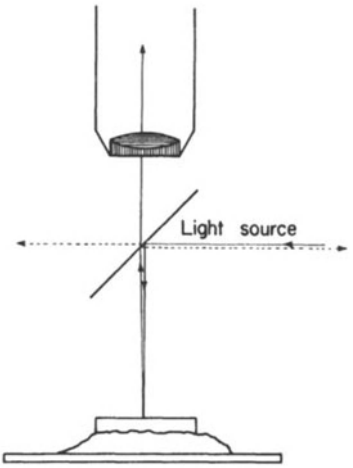


FIGURE 4.10. Vertical illumination by a transparent reflector between objective and specimen.[13]

For higher resolution the transparent reflector must be placed beyond the objective. As shown diagrammatically in Figure 4.12, the central mirror *MM* is for bright-field illumination of the specimen SP, which returns some light to the transparent reflector *MM*, allowing light rays to pass through the system to form an image. Light from the source goes through a series of lenses and apertures then enters the side of the microscope. There the light strikes the transparent mirror *MM*, and is reflected into the objective, which acts as a condenser. In the manner of Köhler's illumination, the field iris diaphragm *FF* is focused in the plane of the object. The iris *FF* is opened just enough to fit the field of the particular objective. Light is reflected from the object through the objective, this time through the mirror *MM* to form

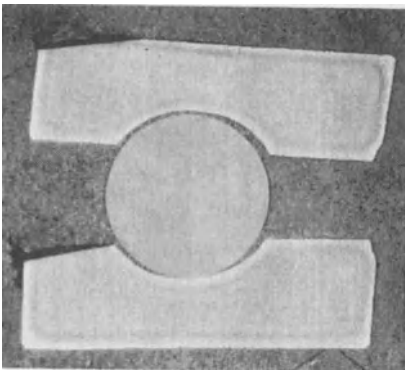


FIGURE 4.11. High-carbon-steel ball bearing in its low-carbon, case-carburized race, mounted in bakelite, sectioned, polished, and etched. Illuminated as shown in Figure 4.10.

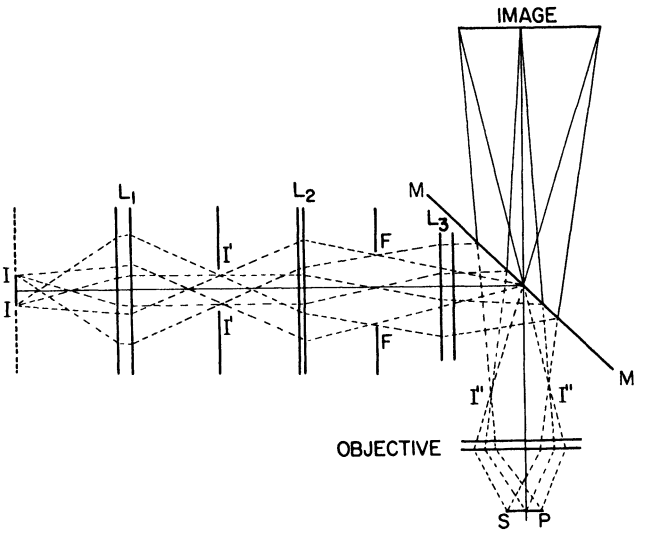


FIGURE 4.12. Vertical bright-field Köhler illumination by reflected light.[15] Courtesy of Microscope Publications.

a real bright-field image.[15] This image is enlarged by the eyepiece or projection piece (neither is shown in Figure 4.12). By shifting *FF* and/or tilting *MM*, oblique bright-field illumination forms shadows on ups and downs in the object.[1,13] Figure 4.2 illustrates the use of slightly oblique reflected illumination in observing a transparent object.

Figure 4.13 shows a current model[15] of a microscopical stand fitted to illuminate a specimen by reflection, thereby producing either bright-field or dark-field illumination with its vertical illuminator (see Figure 4.14) and dark-field (DF) and bright-field (BF) sliding adapters (see Figure 4.15).[15] The sliding adapters DF and BF allow for quick and easy interchange. Moreover by sliding out both of them and replacing the high dry objectives with cover glass or oil-immersion objectives, the microscope is ready for transmitted light.[15]

4.8. RADIATION

The *radiation* most used by reflecting microscopes continues to be produced by an incandescent tungsten filament. Olympus microscopes usually employ a 12-volt tungsten halogen lamp of 50 or 100 watts[15]; other photomicroscopes employing incident light are shown as Figures 4.16–

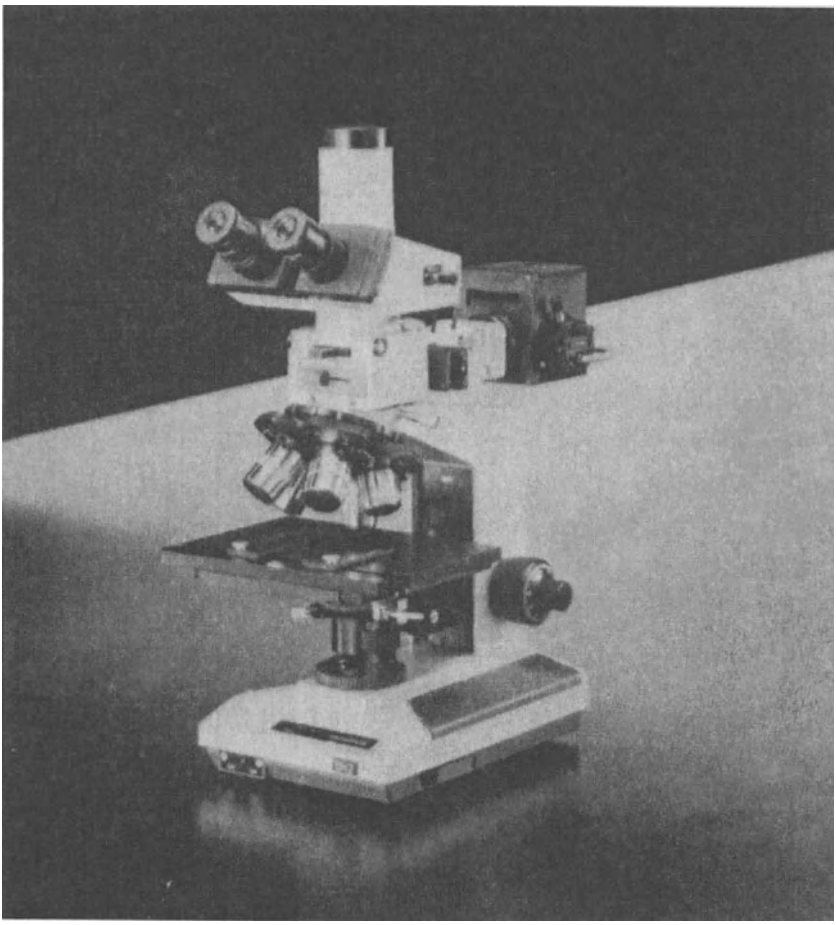


FIGURE 4.13. An incident-light microscope with vertical illuminator in place. Courtesy of Olympus Corporation.[15]

4.18.[16] Figure 4.19 shows an inverted microscope, designed to hold a polished, briquetted specimen[17] with its plane perpendicular to the axis of the microscope. The inversion of the plane-polished specimen is one of the advantages of metallographs, which are nevertheless outmoded because they are large, heavy, and limited in use.

Many minerals and other opaque crystalline materials are anisotropic, which means that both reflectance and color vary with the vibrational direction of polarized light with respect to the orientation of each crystal

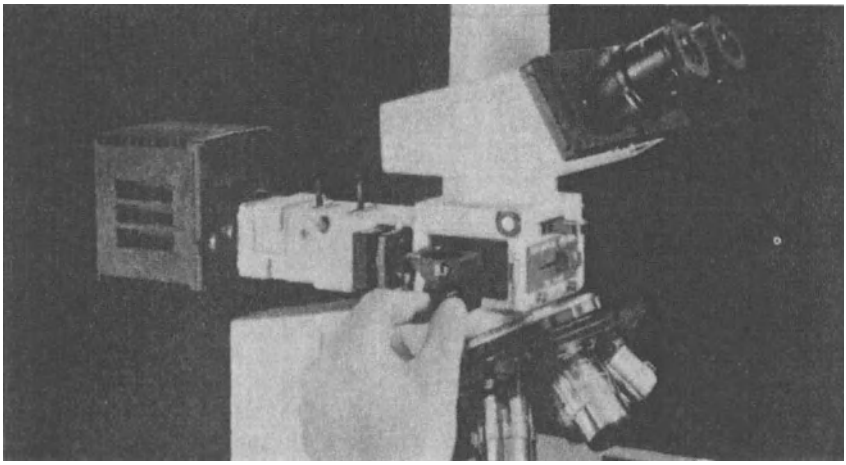


FIGURE 4.14. Vertical illuminator on Olympus microscope employing incident illumination.[15] Courtesy of Olympus Corporation.

grain. Anisotropic crystals also have positions of extinction between crossed polars (see Chapter 5). These phenomena mean that polished anisotropic crystalline grains present various kinds and degrees of visibility as they are rotated between crossed polars. At the back aperture of an objective with a high NA, an interference figure may be seen[18] (see Figure 4.20). To observe such phenomena, a microscope must be fitted with a polarizer, an analyzer, and an adequate source of illumination[14] (see Chapter 5).

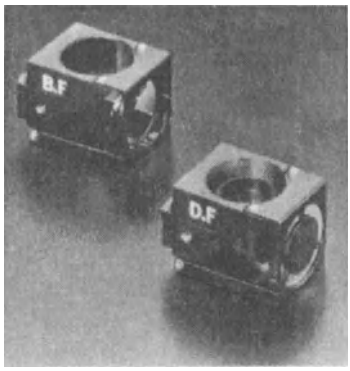


FIGURE 4.15. Bright-field and dark-field semitransparent mirrors in mounts for insertion in Olympus microscope.[15] Courtesy of Olympus Corporation.

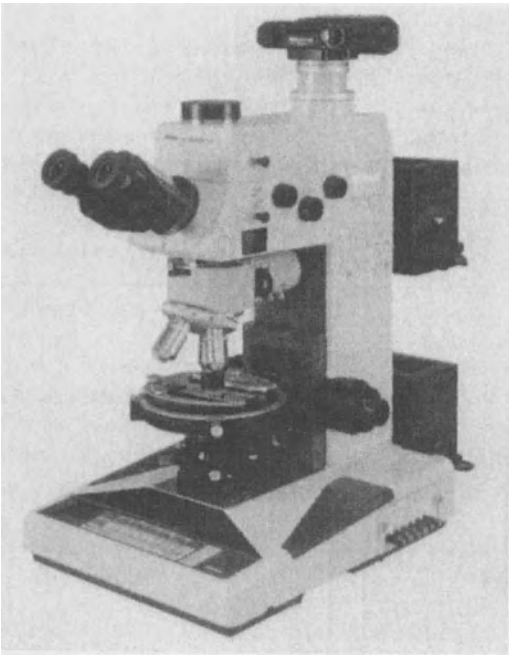


FIGURE 4.16. Microscope equipped for photography by incident and reflected light. Courtesy of Nikon.[16]

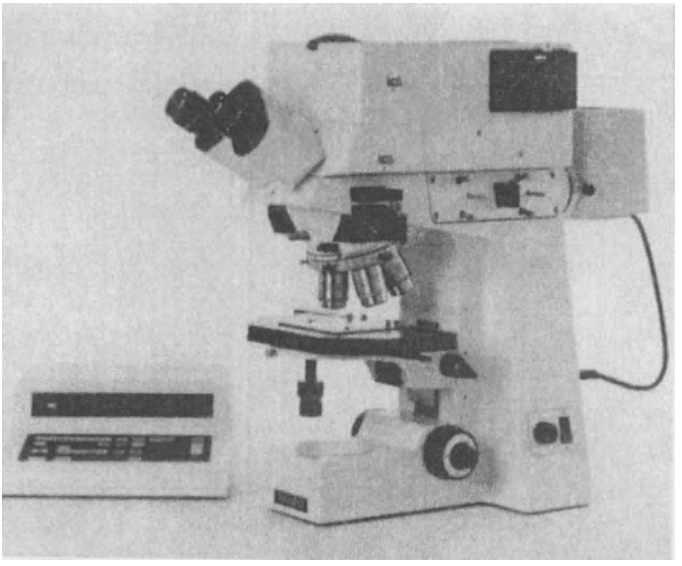


FIGURE 4.17. Microscope equipped for photography by reflected light. Courtesy of Carl Zeiss.[16]

FIGURE 4.18. A metallographic microscope with reflected and transmitted illumination with Nomarski interference contrast optics plus automatic camera. Courtesy of Olympus Corp.[15]

4.9. MAGNIFICATION

Exact *magnification* is determined by reflected light in the same way as by transmitted light[14]: by comparing the real or virtual image with the graduations of a standard scale placed as the object on the microscopical stage. However, when using reflected light, spaces between graduations on the standard scale must be specularly reflecting (silvered) instead of transparent (clear). Real images of the standard scale, such as on a ground-glass projection screen or photomicrograph, are measured in enlargement by a commensurately larger scale ("ruler"). Virtual images (those observed directly in the eyepiece) are measured by a secondary micrometer (reticle) in the eyepiece. Such a micrometer must be calibrated against the standard scale placed as the object on the stage for the particular objective, tube length, and eyepiece. During calibration make sure that unit graduations at both ends of the scale have the same value (within your tolerance) as

FIGURE 4.19. An inverted microscope arranged so that the briquetted specimen lies flat on its plane, polished face. Also fitted with large and small cameras.[16] Courtesy of Nikon.

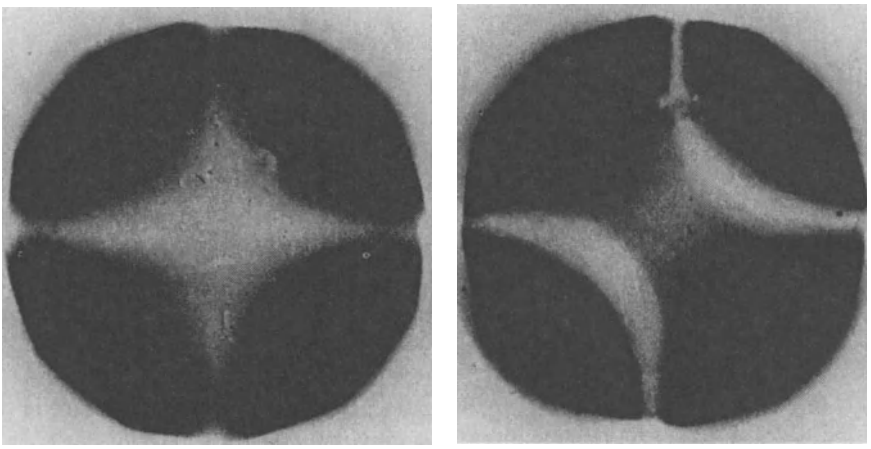


FIGURE 4.20. Figures obtained by reflected light with pyrite (isotropic, left) versus arsenopyrite (anisotropic, right), with an objective of high NA. Courtesy of American Cyanamid Co.

those graduations in the middle of the scale. If not, return the scale to the manufacturer for one that does. For very accurate work an institution should have at least one stage micrometer that has been checked and corrected by the National Bureau of Standards in Washington, D.C.

Designation of magnification is essential in all kinds of micrometry. Figure 4.21 is typical of the measurement of layers on case-hardened low-carbon steel. The scale at the measured magnification is on a negative image of the specimen during printing.

Knowledge of the magnification is essential when estimating grain size in metals and alloys.[19] Likewise magnification must be known when measuring the microhardness of materials[20] (see Figure 4.22).

4.10. FIELD OF VIEW

The *field of view* should be large enough to tell the story, as in Figures 4.21 and 4.22. On the other hand a picture should be cropped of extraneous

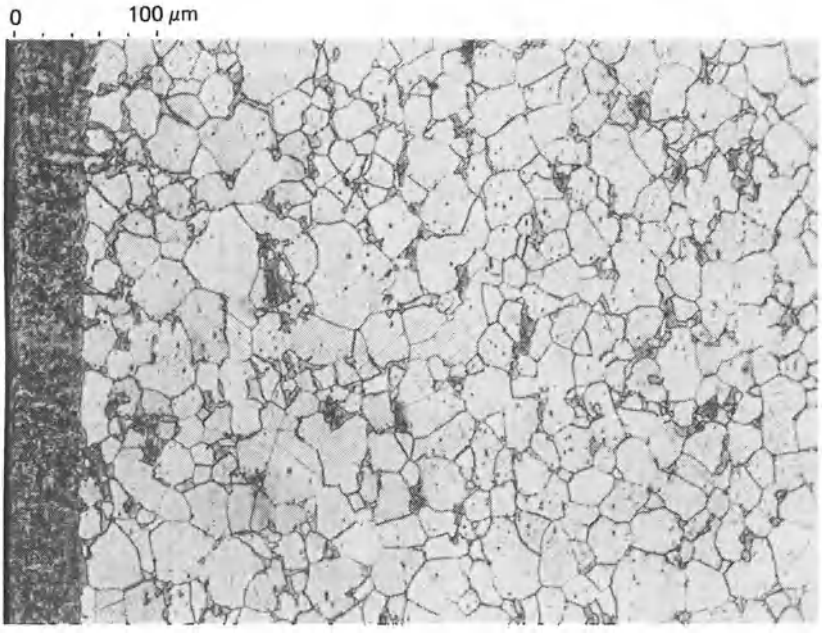


FIGURE 4.21. Bright-field, vertical illumination of a case-hardened specimen of very low-carbon iron.

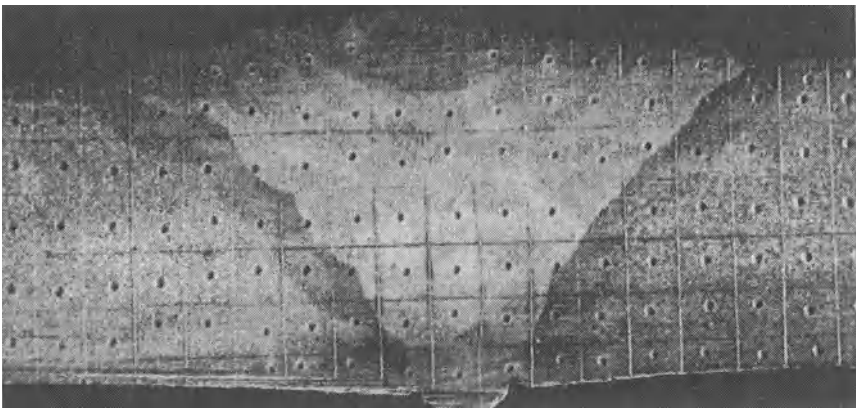


FIGURE 4.22. Hardness differences in a weld by means of indentation. Courtesy of A. White.

or distracting portions if they are on the periphery. By the same token the most important part of a picture should be placed in the center of the field of view if that is feasible.

4.11. GLARE

Glare from reflected illumination is much greater than from transmitted illumination because there is much more attenuation of the light as it goes through the objective (or dark-field condenser) to the object and back through the objective. Even in the least complex system, there is some scattered light. The problem becomes worse as we add lenses, thick glass prisms for illumination, polars, interference filters, etc. To see how much glare there is, focus on the lighted specimen, then remove it, and with the light still on, look again. You may see as much as one-third of the original intensity due to scattered light. Removing the eyepiece and looking down the tube may reveal specific reflections from metallic holders or frames for the reflector, objective, polars, etc., suggesting that some jet black paint in the right places may help remove glare.[13] Make sure the illumination is centered and the field diaphragm has not been closed to frame the field of view and exclude stray light (Köhler illumination).[13,15]

The intensity of partially reflected light, such as from the glass-to-air, interfaces of objectives, field lenses, and filters, can be reduced by coating such interfaces with a thin film of a substance (such as magnesium fluor-

ide) that is less refractive than the glass. The intensity of partially reflected light at the illuminator may be increased by coating it with a substance more highly refractive or more highly reflective (e.g., silver).[11] Reducing the aperture behind the objective to cut out the most oblique, glare-producing rays often restores acceptable contrast. Of course another objective of lower NA will do the same thing but at lower objective magnification.[15] Scattering at the surface of the object can also be reduced by covering it with immersion fluid and using the proper immersion objective. On the other hand, visibility may be better with dark-field illumination, since scattered light rays are then purposely collected by the objective and turned into the image, while specularly reflected rays are rejected.[11]

Some of the reflected light is polarized and can therefore be eliminated by orienting a polar so that its direction of vibration is crossed with the direction of vibration of most if not all of the polarized glare (see Chapter 5). The optimum effect is obtained by rotating the polar or specimen empirically until the glare is minimized.

4.12. DEPTH

The best *cue to depth* with reflected light is attained by casting shadows inside depressions and outside elevations, shallow as they may be. This can be done with bright-field illumination by tilting the mirror. With dark-field illumination shadows are created either by shutting off half of the dark-field beam or by decentering the hollow mirror. Either way relief polishing or deeply etching the specimen will lengthen the shadows.

Of course etching or relief polishing must not be so severe as to exceed their *field of depth* of the particular objective. This requirement is usually achieved when the specimen is first prepared as a smooth plane and then etched or relief polished lightly and progressively until the desired effect is obtained. Usually a flat surface attained by commercial planing or rolling yields an image within the field depth limitations of the optical system without further treatment. This is true of most samples of sheet metal, wooden boards, laminated paper, and cardboard, with or without paint, lacquer, varnish, or other finish. Of course there will always be some specimens that are too rough for a particular objective's depth of focus. The task then is to prepare a smoother surface that will remain significant and representative.

Lack of field depth with high-power objectives on upright microscopes using reflected light can also be a problem if the prepared surface is not sufficiently flat, a partial shim under the low side or corner may be all that is needed to bring all of the field into focus. Mechanical clamps are

available, or can be made, for holding the surface against a hole whose frame is parallel to the base. For low powers at least, an irregularly shaped specimen may be propped up with a little modeling clay and a modicum of patience. With *inverted* microscopes and metallographs (see Figure 4.23), the specimen rests on its plane-polished surface against the stage, which is perpendicular to the axis of the microscope.[11]

4.13. WORKING DISTANCE

Working distance between the specimen and objective for reflected light is usually not a severe problem, and as a rule there is no cover glass (≈0.18 mm thick or thicker), since the specimen is usually smooth and flat without peaks to take up working distance. Considerable working distance is needed in performing many kinds of operations and tests by reflected light, so provision has to be made. For example, in Figure 4.24 space is provided for a dental drill to remove, for a microchemical test, a certain particle in a complex mixture of ore minerals and gangue in a polished section.[1] Incidentally, the micromanipulator is used to control other tools, such as a tiny magnet, dissecting needles, or syringes. Another kind of test that requires considerable working distance is one for microhardness,[20]

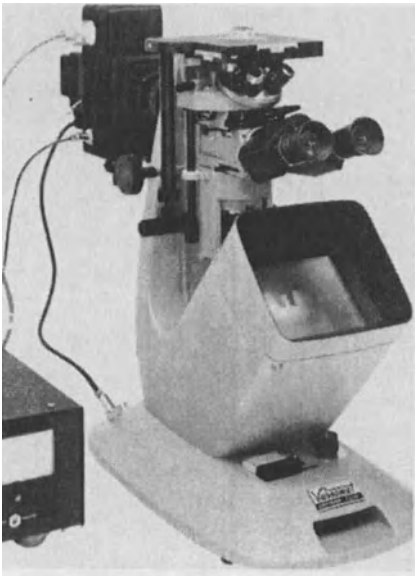


FIGURE 4.23. The microscope is inverted and illuminated with a tungsten lamp or a xenon arc. Courtesy of Unitron Instruments.

FIGURE 4.24. Dental drill and micromanipulator. The chuck of the dental drill is held in a special clamp attached to a micromanipulator.[1]

either by scratching or indenting with a diamond. Measuring the width of a scratch or indentation also requires considerable field depth.

4.14. STRUCTURE

Studying *structure* by reflected light is the very basis of metallography,[2,21,21a] ore mineralogy,[4,5] resinography,[7] wood technology,[10] and a host of other technologies. In metals, alloys, ores, and some plastics, we often have granular structures and sometimes fibers. In natural tissues, such as woods, reeds, bark, and shells, we have characteristic cells and fibers. The visible *kinds* of structure and their distribution are of fundamental importance. We must also consider the *orientation* of these structures, as shown in Figure 4.25.

Crystal structure is fundamental for visibility. Practically all metals, alloys, minerals, and starches are crystalline; consequently there are certain properties and phenomena derived specifically from the crystalline structure of such materials. For example, in Figure 4.25 the granular structure of a metal is brought out by etching, principally because of the differential solubility of crystals in different orientations within the mass. Such characteristic orientation of crystal grains is inherent in the crystalline structure. Likewise, the slippage direction of the planes of atoms in a

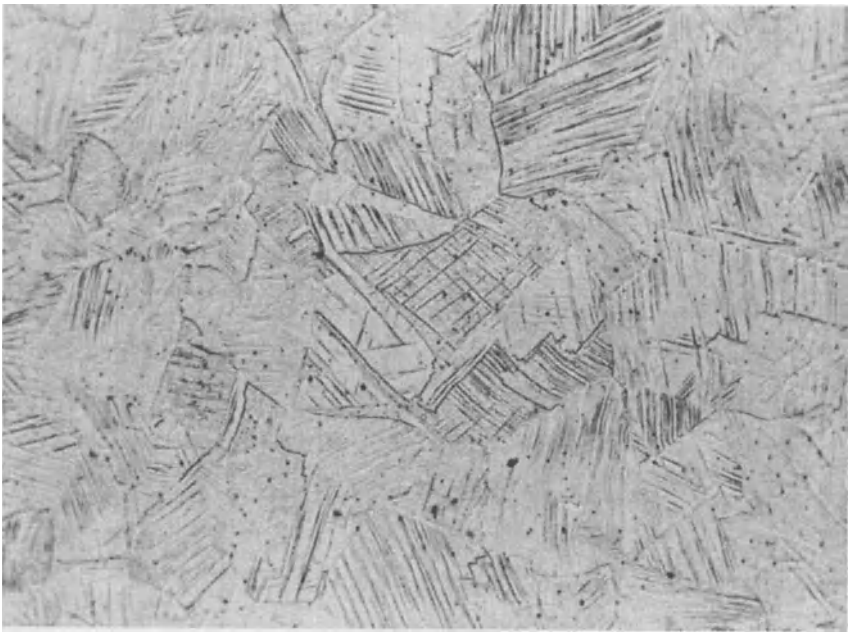


FIGURE 4.25. Stainless steel 18Ni + 8Cr, cold-rolled, polished, and etched, showing one, two, or three slip directions, depending on the specific orientation of each crystalline grain with respect to the direction of cold working.

crystal of stressed metal varies with the crystallographic orientation of each grain with respect to the direction of stress.

Although the potential visibility of structure lies within the specimen, other attributes depend on the microscopy and microscopist. In Figure 4.25, structure visibility is aided by adequate resolution, contrast, shadows, field of view, preparation, and photography.

4.15. MORPHOLOGY

Visibility of the internal features of the metal in Figure 4.25 is aided by appropriate *morphology:* size and shape of the grains, distance between slip lines (representing the slip plane in section), and thickness of intergranular material. In a similar way morphology of various phases in heat-treated, plain-carbon steels is made visible by reflected-light microscopy. This has enabled metallographers to understand the principles of precipitation hardening, summarized in Figure 4.26, which illustrates the metallog-

raphy of heat treating 0.89% carbon steel at various rates of increasing and decreasing temperatures. On the other hand, the precipitation hardness of aluminum alloys was not understood by light microscopy because the morphology of the precipitates was too fine. Nevertheless, experience with steels and brasses led metallographers to imagine what to look for using electron microscopy, and then their efforts were successful.

4.16. INFORMATION ABOUT THE SPECIMEN

Information about any specimen contributes to its visibility; in microscopy by reflected light, there is hardly an exception. Usually sufficient information can be obtained firsthand to enable the microscopist to decide how to go about solving the present problem. If there is insufficient information, it is best to go to the primary literature about the material before starting the investigation.

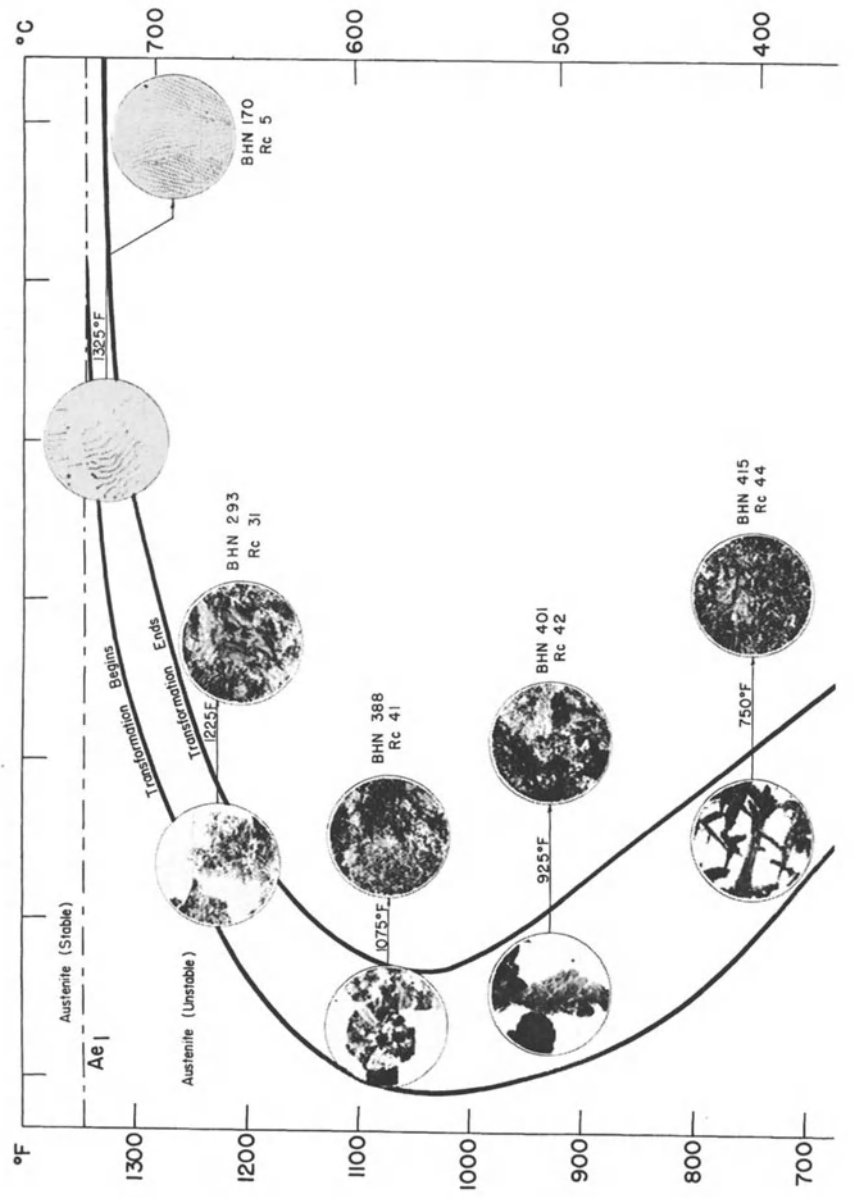
Figure 4.23 shows a microscope (one of several varieties) that allows quick choice between reflected and transmitted illumination. Figure 4.27 shows a table model of a polarizing microscope that also offers this choice. Whether reflected or transmitted light (or both) are used depends on the descriptive information accompanying the sample, what microscopical experiments are to be performed, the behavior of the specimen on the microscope and in the room during the scheduled time, and how much preparation is necessary.

4.17. EXPERIMENTATION

Microscopy involves *experimentation* as well as observation, and some possible experiments are suggested in Figures 4.7, 4.21, 4.22, and 4.24. Almost any experiment feasible by transmitted light can be performed by reflected light, with the added advantage of having the specimen uncovered so that the microscopist can pick, poke, scratch, cut, turn, or roll it. A small magnet, a tiny "soldering iron," a thermocouple, or any other active point can be applied. The specimen can be placed on a hot or cold stage,[13] and it can be stretched, pushed, or twisted.

4.18. SPECIMEN BEHAVIOR

Specimen behavior during observation may be fortuitous, since the specimen is uncovered and often exposed to a hot beam of intense light.


°C
700
600
500
400
°F
1300
1200
1100
1000
900
800
700
Austenite (Stable)
Ae 1
Austenite (Unstable)
Transformation Begins
Transformation Ends
1325°F
1225F
1075°F
925°F
750°F
BHN 170
Rc 5
BHN 293
Rc 31
BHN 388
Rc 41
BHN 401
Rc 42
BHN 415
Rc 44

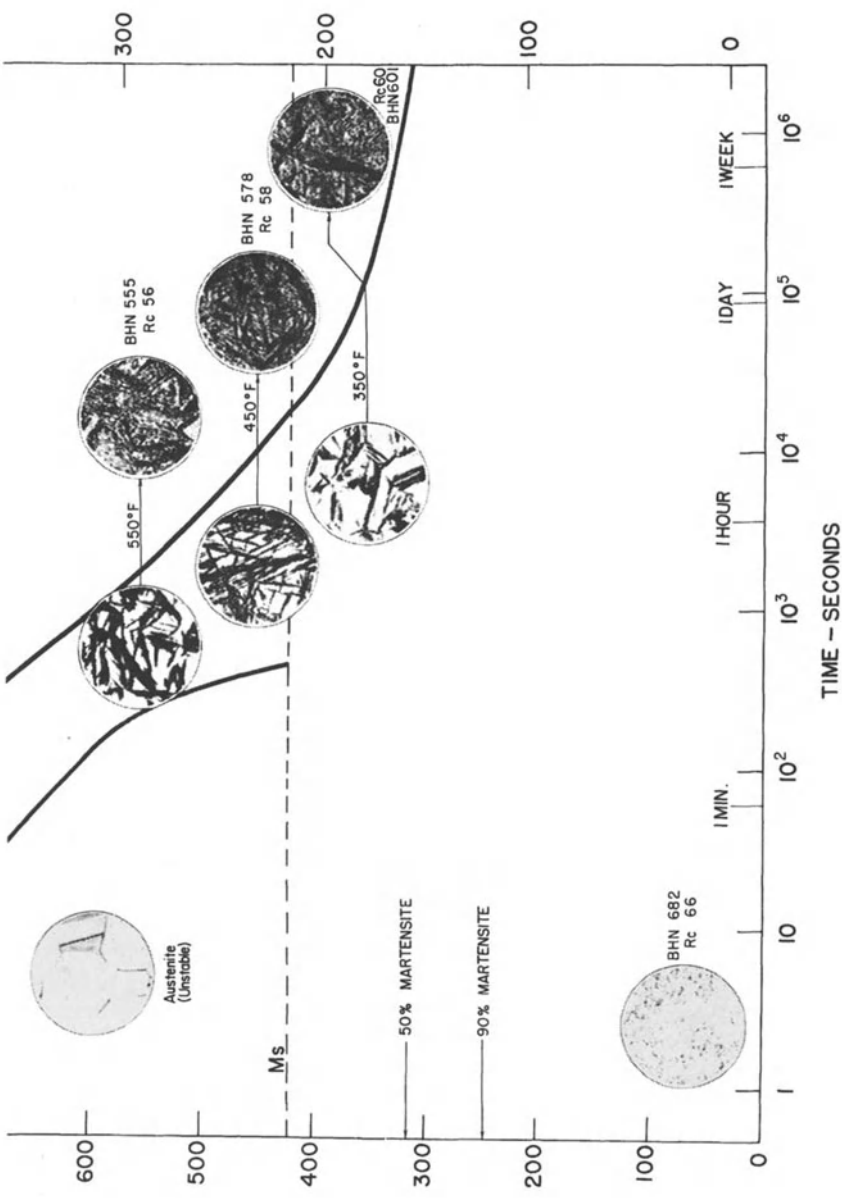


FIGURE 4.26. Isothermal transformation diagram of eutectoid carbon steel; C, 0.89% Mn, 0.29%. Austenitized at 1625° F. Grain size: 4–5. Photomicrographs originally 2500x. Courtesy of U.S. Steel Research Laboratory.

FIGURE 4.27. D. W. Davis using an all-purpose polarizing compound microscope, used in industrial microscopy with either reflected or transmitted illumination, bright field or dark field. Courtesy of American Cyanamid Co.

Nevertheless, any change in the appearance should be noticed, recorded, and explained. The uncovered, freshly prepared specimen may have been stored a long time in an ordinary or extraordinary atmosphere, dry or humid, before or after etching, and it may have undergone change. Behavior differences should be noted just as though the exposure were planned, as well it might have been.[10] Some reactions to look for are efflorescence, deliquescence, oxidation, sulfiding, and polymerization.

The chief limitation of microscopical examination by reflected light is the condition of the surface, so any change in that surface is significant. The surface must be flat enough and smooth enough for whatever objective is required to do the job, which is why a preliminary look at low power is so important. Even if the problem is not solved then and there, we can get an idea of how well the surface, as received, is suited to reflected illumination; how much preparation will be needed to see more; and how the surface has changed during storage and observation.

4.19. SPECIMEN PREPARATION

If the surface is dusty or dirty, clean it with a stream of air or some inert liquid. The safest liquid is water and a little wetting agent, perhaps using a brush, soft at first, then stiff if needed. If the surface has too many

scratches or pits and it is hard enough, a fine abrasive (1 μm or less) may improve the image, but any substantial abrasion will reveal the interior rather than the exterior.

To expose the interior of a sufficiently solid, cohesive material, we can cut, smooth, and polish it. This is the way most metals, many rocks, ores, ceramics, cements, glasses, and plastics are prepared. If a specimen is powdery, particulate, fibrous, thin, porous, or irregularly shaped, it should be mounted by molding it in a block of resin. A molding press and mold are available for compression-molding bakelite and similar resins and provide a convenient method of mounting such a sample.[1] If compression molding is not feasible, casting in a polymerizable liquid is recommended.[22,23]

Etching is the key to the light-microscopical examination of all metals. Its primary purpose is to remove metal that has been modified by preparatory procedures.[13,14,24]

4.20. PHOTOMICROGRAPHIC TECHNIQUES

Photomicrographic techniques by reflected light include those of general photomicrography and metallography. A special problem may be poor contrast between phases that do not differ much in reflectivity. Improved contrast can be obtained by using high-contrast photosensitive materials and under extreme conditions, a high-contrast developer.[16]

4.21. SUMMARY

Since many materials are opaque, their microscopical examination must be accomplished by using reflected rather than transmitted light. For example a metal cannot be examined by transmitted light no matter how thin a section is prepared or how thin a foil is chosen. Reflected light offers the only chance of learning something about the structure and morphology of the metal. In other situations only the *surface* of a transparent or translucent material may be important, and surface examination by reflected light may be the method. For such cases equipment and techniques for microscopical observation by reflected light have been developed to just as high degree as those for transmitted light.

The simplest optical arrangement for studying a specimen by reflected light is a polished glass plate mounted between the objective and the specimen at an angle of 45° to the axis of the microscope. Light from a source off to the side, at right angles to that axis, is reflected onto the

sample. Different components of the specimen's surface then reflect the light in a different and characteristic manner through the glass plate and into the optical system of the microscope, forming an image of that surface. Obviously, this arrangement works only for objectives of low power and long working distance. For more detailed study the 45° reflector must be positioned behind the objective, within the microscope body. The reflector may then be a mirror, a prism, or even a polarizing crystal positioned to reflect light downward on the sample. One advantage of this arrangement is that the objective automatically becomes condenser as well as objective (and a better corrected condenser of higher NA than most external ones), so that excellent illumination is assured. Furthermore, in most instances a cover glass is not necessary, so that an immersion liquid of suitable refractive index can be used between the objective and the sample, thereby improving resolution.

Light sources for reflective microscopy must be more intense than those used in transmissive work, due to greater losses in the optical system. Tungsten–halogen lamps, mercury or argon arcs, and even old-fashioned carbon arcs are preferred. Illumination may be bright field or dark field, according to various optical arrangements described in the text and shown in diagrams. With sufficiently intense sources and appropriate filters, advantage may be taken of some special spectral portion of the illuminating radiation to produce differential fluorescence (or other exaggerated response) within the sample components.

One drawback of reflective microscopes is that part of the light is reflected or scattered by the many surfaces it meets on the way to and from the sample, so there is likely to be extraneous light (called glare) that reduces contrast and impairs visibility. The amount of glare can be reduced by scrupulously cleaning optical components, hunting down and blackening any reflective metal parts in the microscope, using coated lenses, and (if absolutely necessary) constricting apertures or installing diaphragms to eliminate wide-angle reflected light. Since such light is partially polarized, it can be reduced in intensity by installing an eyepiece polar and rotating it for maximum effect.

Not only existing surfaces but also the *interior* structure and morphology of such opaque materials as bones and teeth can be studied by reflective microscopy. Suitable sections are sawed or cut, and the fresh surface is smoothed and then polished until all scratches are invisible and the surface features can be seen. Etching the polished surface of a metal is essential for distinguishing the structural components and studying the morphology. Etching also helps study plastics, polymers, minerals, and ceramics. Reference to a complete list of etchants for metals is given, and precautions for their use are included in ASTM Standard E407, Microetchants for Metals and Alloys.[24]

5

Microscopy with Polarized Light

5.1. OVERHEAD PROJECTORS

The historic observation of double imaging by a highly birefringent crystal, such as that of calcite (see Chapter 1), can easily be repeated on an overhead projector. A small dot of black paper is pasted on the projector's window near its center, then a cleavage rhombohedron of clear calcite is placed over the dot. Two images of the dot appear on the projection screen, which means that the incident beam is being divided into two beams that do not interfere with one another because they are vibrating in different (perpendicular) planes. When the calcite rhombohedron is rotated on whatever face it happens to rest, one image of the dot is stationary while the other image curiously traces a circle around the first image (see Figure 5.1). The fact that the so-called extraordinary image is displaced from the ordinary image means that the extraordinary ray travels at a different velocity than the ordinary ray. The fact that the extraordinary ray traces a circle rather than a spot confirms that the *difference* in velocities varies with the orientation of this crystalline species. The ordinary image is stationary because its velocity is constant with all orientations of the crystal, and accordingly the ordinary refractive index ω is constant. The *various* values for the refractive index in the path of the extraordinary ray can be given a general symbol, such as ε', or specific symbols, such as ε_1, ε_2, ε_3, etc.

The overhead projector may be converted into a polariscope[1–3] by placing two polarizing sheets (polars),[1] such as Polaroid®, over the illuminated window. The two polars are separated vertically by about 10 cm by means of two blocks, so that the specimen can be placed and turned by hand between the polars. If the polars are crossed with respect to their directions for vibration of light, the field will be black because none of the

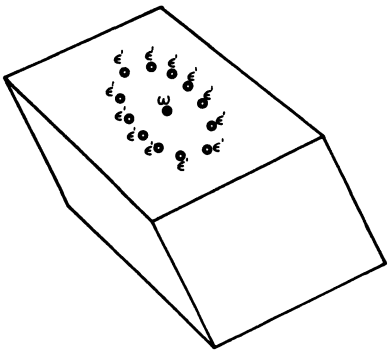


FIGURE 5.1. Single crystal placed over a single dot, showing double imagery: (1) The central imaged dot is stationary, since it is represented by the ordinary ray (n_1 = ω) inside calcite. (2) The circular ring of imaged dots indicates that there is a circular path as the calcite rhombohedron is rotated over the single object dot ($n_{||}$ = symbol, such as ε').

polarized light from the first polar (polarizer)[1] is vibrating in the direction for the light to vibrate through the crossed polar (analyzer).[1] An anisotropic crystal, such as a calcite rhombohedron, will appear bright in all positions of rotation except the four times in a revolution when the direction for ω or ε′ corresponds to the polarization direction of the polarizer or analyzer, as indicated in Figure 5.2. In all other positions of rotation, the crystal appears bright because there is a vector corresponding to the direction of vibration in the analyzer, so that some light appears.

The extinction displayed by calcite, shown in Figure 5.2, is called

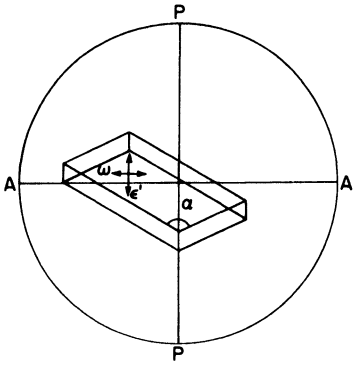


FIGURE 5.2. Calcite rhombohedron in one of four analogous positions of extinction (darkness) when vibration direction ω or ε′ in calcite corresponds to vibration direction *PP* in polarizer or *AA* in analyzer.[4]

symmetrical extinction because at extinction the obtuse angle α is bisected by *PP* or *AA*, the direction of vibration in the polarizer or analyzer. Some other species of crystal display parallel extinction, which occurs when *PP* or *AA* corresponds to a prominent edge of the crystal; otherwise an anisotropic crystal manifests oblique extinction. This and other optical properties in microscopic crystals call for microscopical examination by means of a polarizing microscope. Such a microscope is employed in optical crystallography,[4–7] mineralogy, petrography, chemistry, physics, and biology.[8] Anisotropy is also observable in liquid crystals[9]; strained glasses[10]; stressed plastic materials and models; partially crystallized resins and polymers[10]; reflecting surfaces; flowing colloids; man-made filaments; and biological fibers, cells, and tissues.[11,12]

5.2. ANISOTROPY

There are five kinds of *anisotropy:*

The optical anisotropy[1] of each *single anisometric crystal* indicates that the bonding of the unit molecules, ions, radicals, or elements is different in at least two of the crystallographic directions. Such crystals as those of calcite, quartz, melamine, and sucrose display 10 or more individual optical properties that can be detected and measured on these microscopic crystals. Such properties are very useful in characterizing materials in synthetical and analytical science and technology, as discussed in Chapter 7.

Multiples of anisotropic crystals have optical characteristics above and beyond those of the individual crystals. The simplest multiples are *twins* (see Figure 5.3)—two crystals sharing a single "composition" plane[4] but also manifesting individual properties. Figure 5.4 shows twins and higher multiples of subcrystals within crystals. Spherulites[1] have still higher multiples of crystals, with each one contributing to a unit effect on polarized light. Such spherulites are manifested for example by a single grain of raw starch or of polyoxyethylene.

Molecular birefringence is manifested by long or flat molecules, especially by macromolecules (polymers). In *long* molecules the atomic dipoles are primarily arranged in chains. By induction the dipoles are stronger than if the atoms were widely separated. When polarized light is vibrating lengthwise to the chain, the average strength of the dipoles is greater than when light is vibrating crosswise. The optical properties of a system of parallel long molecules are derived from those of the individual long molecules.[9]

Light vibrating parallel to a molecular chain or the axis of a (fibrous)

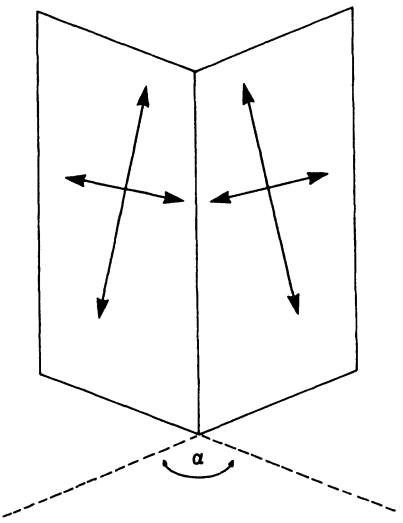


FIGURE 5.3. Twinned crystals (with separate extinction directions).[4]

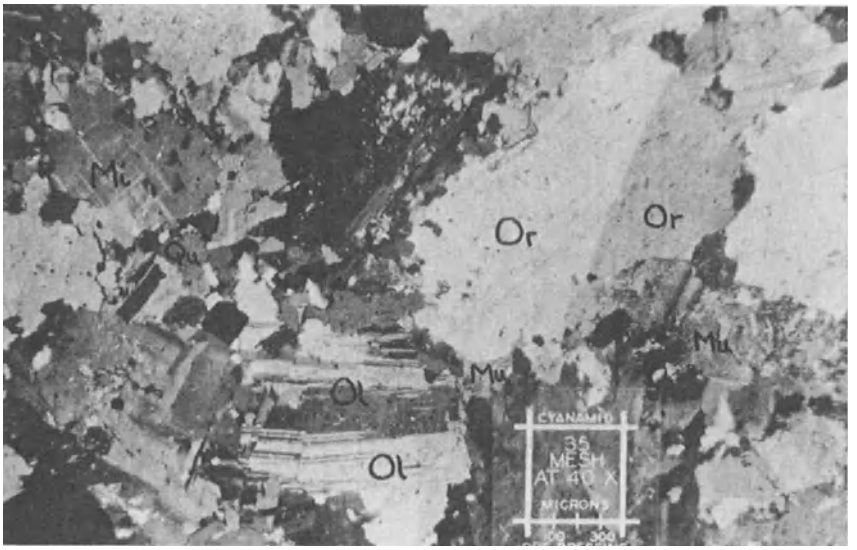


FIGURE 5.4. Thin section of pegmatitic rock (granite) between crossed polars, showing crystalline grains. The large grain Or–Or is a twin. The grains Ol–Ol and Mi are multiple crystals. Ol, oligoclase; Or, orthoclase; Mi, microcline; Mu, muscovite; Qu, quartz. Courtesy of American Cyanamid Co.

system of aligned long molecules travels more slowly than light vibrating crosswise, that is, the refractive index for light vibrating lengthwise ($n_{||}$) is higher than for light vibrating crosswise ($n_{\perp}$). This is conventionally called positive birefringence in fibers (see Chapter 6), and it is strong in natural cellulose fibers and many man-made fibers. Of course *side chains* on the molecule tend to reduce the strength of birefringence in the main chain of the molecule and therefore that of a fiber made up of such branched molecules[9] (see Chapter 6).

Flat molecules tend to slow down light that vibrates in their planes. If these molecules are arranged with their planes parallel, as in a film or foil, light vibrating in the plane of the arrangement has a refractive index higher than the refractive index of light vibrating perpendicularly to the planar arrangement. Symmetrically planar molecules (such as ring configurations) are isotropic in their planes, so that films or foils made of symmetrically planar molecules are isotropic when viewed perpendicularly to the sheet. Although long *flat* molecules tend to be anisotropic in their planes if they are oriented at random, the resultant sheet is still isotropic in its plane. However, if the long flat molecules are oriented with their lengths parallel and their planes parallel, their sheets tend to be birefringent in their plane as well as perpendicular to their plane.[9]

If the long flat molecule also preferentially *absorbs* polarized light *strongly* in one direction (generally lengthwise), the film made with such molecules tends to be polarizing like Polaroid® polarizing film.

Form birefringence,[9] also called *rod* or *plate* birefringence,[4] is manifested by a *two-phase* system whose mass of long or flat particles, *not necessarily anisotropic,* has been *oriented* in a medium with a *different* refractive index by a flowing, streaming, or growing process. The slower vibration direction (higher refractive index) of the system is parallel to the length of the rods or in the plane of the plates. This kind of anisotropy is found in some fibers, films, and other colloidal systems. It is detected by a *change* in degree of birefringence *while* the specimen is mounted in a liquid (such as a standard of refractive index). Such a liquid changes the refractive index of the continuous phase, thereby changing the difference in indices between phases. Soaking in such a liquid may be sufficient to eliminate all of the form birefringence, leaving only the true double refraction. If soaking produces isotropy, all of the original anisotropy was due to form birefringence.[4]

The *photoelastic effect* is the local anisotropy manifested as strain resulting from stress on normally isotropic materials, such as an inorganic or organic glass[10] (see Figure 5.5).

Anisotropy observed in a material can provide more visibility in microscopical *images* than ordinary unpolarized light as well as *patterns* of

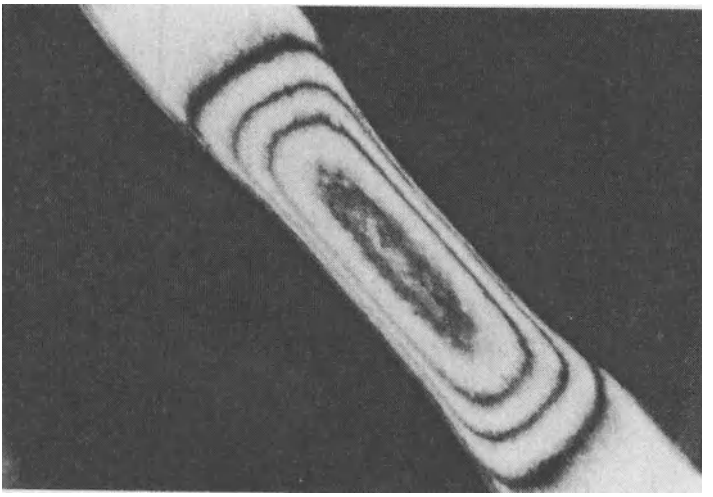


FIGURE 5.5 Interference pattern in the neck produced by pulling a fiber of nylon by hand. Taken between crossed polars at 45° from position of extinction by a student.

light and dark, usually in colors. The need for both kinds of information led to the development of the *petrographical* microscope, which has a variety of accessories[11–16] added to the ordinary compound microscope. Chemists later found they needed the same information, so the chemical microscope followed. In addition to petrographical and chemical microscopes, the newer research instruments and universal microscopes for use in all sciences also carry polarizing equipment.

5.3. NUMERICAL APERTURE AND INTERFERENCE FIGURES

The NA takes on special significance, particularly in the search for a principal kind of pattern, the *interference figure* (see Figure 5.6), sometimes called a directions image[4] because it is the pattern formed by summing the *directional effects* of polarized light rays on the specimen within the limitations of the *angular aperture* of the objective or its condenser, whichever is the smaller. Thus when producing interference patterns, the value of NA transcends that of the resolution of fine detail in a pictorial image. The purpose is to obtain as large a solid cone of rays as is commensurate with other attributes contributing to visibility. To obtain data from interference figures that are compatible with conventional data conversion charts, some authors[6] standardize on an NA of 0.85.

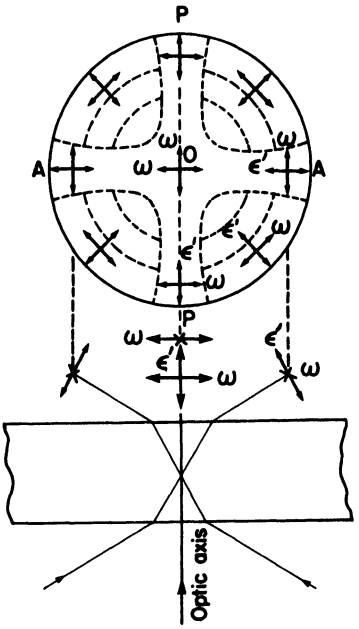


FIGURE 5.6. Diagram of an interference figure; the pattern is formed by the summation of effects by the specimen on the polarized light rays coming from all the directions included in the angular apertures of objective and condenser.[4]

5.4. RESOLUTION: SPECIMEN INTERACTION AND POLARIZED LIGHT

Resolution of the specimen's effects on polarized light depends on producing plane-polarized light with the *polarizer* and analyzing effects with the *analyzer*.[12] At present both polars[1] are usually made of polarizing film, e.g., Polaroid®, sandwiched between two protective pieces of thin glass. For transmitted light the polarizer *P* is generally placed in front of the iris diaphragm of the substage condenser, as shown in Figure 5.7.[9] The analyzer *A* is usually placed in a slide fitting in or out of the tube of the microscope above the objective *O*.

Resolution of the specimen's effects on polarized light means that everything else in the usual optical path must have no effect: Objectives, eyepieces, condensers, microscopical slides, and cover glasses must be *strain free*. The test is to remove the specimen and with the light source turned on and the polars crossed, look into the microscope. There should be no light. If there is a spot of light that does not fill the field, turning one optical element at a time may indicate which element is strained. If the spot does not move, remove one element at a time to find which ones are strained.[4] The manufacturer tries to supply all polarizing microscopes

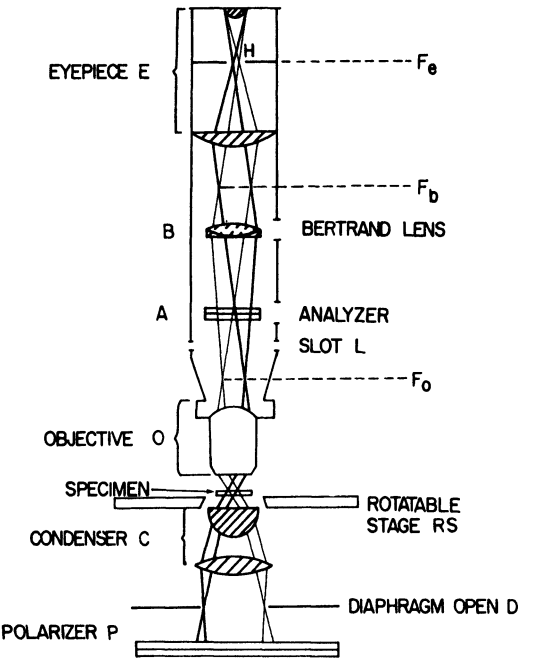


FIGURE 5.7. The polarizing microscope as used for conoscopic observations.[9] Courtesy of Microscope Publications.

with strain-free components, but sometimes objectives, eyepieces, and condensers are switched in a laboratory. The *objectives* carry some symbol, such as *P*, to indicate that they are for polarized light.

The *eyepieces* can all be of the Huygenian type because their magnifications and corrections are usually high enough for a polarizing microscope. There should be at least three eyepieces: one eyepiece equipped with cross lines and a lug on the eyepiece to engage in a notch in the tube of the microscope, so that cross lines are oriented W N E S to the observer; another eyepiece fitted with a linear micrometer (to be calibrated as explained in Chapter 4) for distances on interference figures, etc., as discussed later. A third eyepiece should be clear of reticules for photomicrography or whenever no reticule is desired. However other reticules, such as a protractor for estimating angles or cross-hatching for estimating areas, may be kept on hand, ready to drop into the eyepiece *E* at the focal plane F_e in Figure 5.7.

The *condenser C* should have an easily removed upper lens, such as one that flips out of the way and back into position. The iris diaphragm *D* is essential. The polarizer *P* does not have to be removable; it should be

rotatable but also have a catch of some sort to indicate when the vibration direction of light through it is parallel to one of the cross lines.

Slot *L* in Figure 5.7 is designed to accommodate retardation plates such as a first-order red plate, or a compensator, such as the quartz wedge used to make Figure 5.8. The *B* in Figure 5.7 indicates a Bertrand lens, a simple lens system that, with eyepiece *E*, constitutes a compound microscope for looking at an enlarged interference figure at the back aperture of objective *O*. When not in use the Bertrand lens can be slid or flipped out of the way.

5.5. CONTRAST: MICHEL–LÉVY INTERFERENCE CHART

Contrast, color or neutral gray on a dark background, is an outstanding attribute of viewing an anisotropic object between crossed or partially crossed polars, as illustrated in Figure 2.7. The light (first-order gray or high-order white) or colors (see Figure 5.8) is an interference phenomenon. Figure 5.9 diagrams how interference comes from polarizer *P* and analyzer *A*. The polarizer feeds plane-polarized light to the birefringent specimen *S*, which splits the beam into two components.[19] The specimen is in a position of brightness; that is, it is oriented on the rotatable stage (*RS* in Figure 5.7). Thus its two kinds of rays are vibrating in different azimuths from those of *P* and *A*, yet both bundles have to go through analyzer *A*. For some wavelengths (colors) two wave trains will be in phase and reinforce each other. For other wavelengths the two trains will be out of phase and interfere with each other. The potential result is Newton's series of interference colors, depending on how much the slower component falls behind the faster component. It is as though we have two runners, one faster

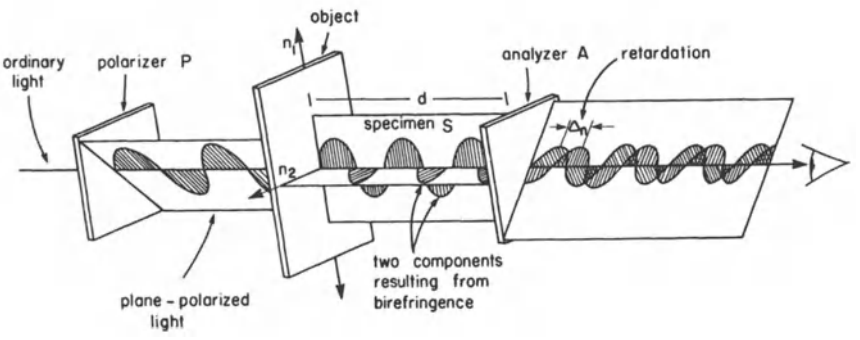


FIGURE 5.9. Diagram used to explain the functions of the polarizer and the analyzer.[4]

than the other. Assuming that their speeds are constant, how much the slower runner falls behind the faster one depends on their difference in speed and how far they run. If we know two of the three variables (difference in speed, distance traveled, and distance between the two), we can determine the third variable.

The difference in light ray velocity is expressed by the difference in refractive index n_1–n_2. The distance the rays travel is the thickness of the specimen, expressed in micrometers (formerly microns). The distance the slower light ray falls behind the faster one (retardation) is expressed in nanometers (formerly millimicrons). These three variables can be charted as a Newtonian series of interference colors, such as displayed by a quartz wedge showing several orders of retardation colors.

The classical chart is the Michel–Lévy scale of birefringence[4] (see Figure 5.8). This chart was originally designed for petrographers' thin sections of rock, with the standard thickness of professional sections, 30 μm, in the middle of the ordinate. But the chart is also appropriate for fibers, since they run commercially from 10–40 μm and even greater for thicker fibers used in carpets. The general equation for Figure 5.8 is

$$\text{retardation } (r) = \text{thickness } (t) \times \text{birefringence } (\Delta_n).^{[7]}$$

The abscissa on the bottom is graduated in nanometers of retardation. The scale is organized into orders; each order ends with red, about every 565 nm of retardation. The first order (up to $\Delta_r \approx 565$ nm) of course starts with black ($\Delta_r = 0$) and goes from dark gray to light gray to white of the first order (1°). Some people see dark gray as dark blue, which is just as diagnostic as dark neutral gray. It is important to recognize grays and white of the first order. Around $\Delta_r = 275$ nm the specimen in positions between crossed polars appears to be yellow, then at about 450 nm, it appears orange, then red, and finally magenta. The second order contains the most brilliant colors, from blues to greens, to yellows, to oranges, and finally to red. The third-order colors are less brilliant (less saturated). In the fourth order, colors are still less brilliant, i.e., nearly pastel. To many people fourth-order blue seems faded. By the fifth order (see Figure 5.8) blues are invisible to most people, and by the sixth order only pink (faint red) can be seen. After the eighth order retardation is visible only as high-order white, so the sequence of orders of retardation can no longer be detected by color. We then need special accessories, some of which measure as many as 30 orders of retardation; more is said about them later in this chapter.

The upper abscissa of the Michel–Lévy chart is expressed in terms of birefringence, n_1–n_2. The scale moves horizontally from 0.001–0.040 and

then vertically down to 0.20. Usually the problem is to obtain the birefringence from the thickness and retardation. Low orders of retardation are determined ordinarily with the aid of retardation plates.

5.5.1. Personal Interpretation of Interference Colors

Why does interpretation of interference colors differ? Subjectively people differ physiologically in their sensitivity to various colors, as explained by Land's retinex theory.[16a]

In the Michel–Lévy chart of birefringence (see Figure 5.8), colors represent the complex admixture of rays varying in wavelength and intensity. The total interference color observed for any value of retardation is the *sum* of the intensities at *various* wavelengths along the ordinate axis. In other words there are no pure colors. For example a straight line through 260 nm of retardation from violet to red reveals enough of each color to render first-order white. On the other hand a vertical line through the intensities shown in Figure 5.10 at 1300 nm indicates third-order green because there is so little of the other wavelengths (colors). Thus the more objective color differences there are, the more they affect color differences among observers.

As the caption to Figure 5.8 explains, there is a slight discrepancy between the subjective appearance of interference colors of the quartz wedge to the human eye and the objective rendition of the colors. The version of the Michel–Lévy chart in Figure 5.8 was obtained by photographing the microscopical image of a standard quartz wedge, so sensitivity to individual colors was based on the particular color photosensitive film employed. Further discrepancies appeared during printing, and as a result an artist's skill was added. Based on successful use of the first edition of this book in the classroom, the chart is reproduced in this edition.

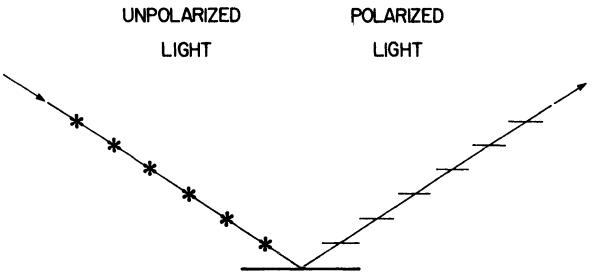


FIGURE 5.10. Light partially polarized by reflection from a surface.

5.5.2. Retardation Plates

Most polarizing microscopes are sold with two *retardation* plates as standard equipment: a first-order (1°) red plate and a quarter-wave (0.25λ) plate. The 1° red plate is also called the sensitive-tint plate because just a very little retardation added to, or subtracted from, that plate turns the retardation color a blue or yellow, respectively. Adding retardation means that the slower component of the specimen is parallel to that of the 1° red plate. Subtraction means that the two slower components are crossed. The vibration direction of the slower component is marked on the 1° red plate; this provides a means of detecting the vibration direction of the slower component as it emerges from the specimen.

Similarly the vibration direction of the slower component is marked on the 0.25λ (1° white) plate, and hence that plate can also be used to detect the vibration direction of the slower component from a specimen with a first-order polarization color. A specimen manifesting first-order white for example would be dark gray or black (compensated) when its slower component is crossed with that of the 0.25λ plate and bright white or yellow when the slower components are parallel. But if a white polarization color *remains white* while the specimen is rotated between crossed polars and the 0.25λ plate is in place (usually NW ↔ SE), the white is of high order.

Compensation is exactly matching retardation in the specimen to a standard retardation device. Criterion for matching involves observing darkness when the specimen is in a position of brightness until the retardation device renders the specimen black. Compensation would occur fortuitously with a 0.25λ plate if the specimen had a retardation of exactly 0.25λ and the two slower components were crossed. Compensation also occurs fortuitously with a 1° red plate if the specimen has first-order retardation and the two slower components are crossed. In either fortuitous situation, *and only then,* the retardation plate can be termed a *compensator.*

A true compensator is a device of variable retardation, such as a quartz wedge, say, of three orders. If the specimen, such as a fiber of nylon, is rotated into the NW or SE quadrant, and the quartz wedge is slid into its slot until the specimen becomes black, the compensating order and hue may be determined. From these data the approximate retardation may be estimated by using the Michel–Lévy chart. The Senarmont compensator[17] is more accurate because a *graduated* quartz wedge is slid over the first one until compensation is reached.

Another type of compensator uses the principle of varying the *direction* of the incident light by tilting an anisotropic material of constant thickness. One variety is called the Berek compensator.[17]

5.5.3. Specimen Thickness

If the exact path length is not known, it must be measured. Many old-fashioned and some new microscopes are equipped with fine-focusing wheels graduated in units, such as microns. With such a microscope, measure specimen thickness by using an objective of high NA (shallow depth of focus) and focusing upward from the *bottom* of the object. After reading the micrometer's scale with the bottom in focus, focus upward to the *top* of the object, then read the scale again. Focusing is always done in the same direction of knob-turning to take up any slack or backlash in the focusing mechanism. The difference in the two readings must be multiplied by the refractive index of the specimen because top and bottom images are displaced by that much. Some specimens can be measured for thickness before mounting by means of a thickness micrometer.[18,19]

If vertical distance cannot be measured, distance must be measured *horizontally.* If the specimen happens to be a cube, a cylinder, or a sphere, diameter is measured horizontally. Otherwise the specimen has to be turned on its side by micromanipulation[18] a spindle stage, or a universal stage.[20] Horizontal measurements are made with an eyepiece micrometer, calibrated for tube length and the particular objective in use by a certified stage micrometer.[18]

5.6. CORRECTING FOR ABERRATIONS DUE TO STRAIN

It is apparent by now that the most important *correction for aberrations* in the polarizing microscope is that of color. For visual work achromatic objectives are adequate, but apochromats are preferred for photography. The ordinary uncorrected condenser has such severe chromatic aberrations that its colors may be misinterpreted as retardation colors. Centering the illuminating beam and the condenser helps reduce aberrational color in an ordinary condenser. At the same time the color temperature of the illuminating beam should be controlled by using a daylight color filter.

All optical glass in the polarizing microscope system must be *strain free.* Objectives certified as strain free by the manufacturer are marked with a symbol, such as *P.* All other objectives should be tested between crossed polars. Eyepieces, condensers, slides and cover glasses, auxiliary lenses, retardation plates, and compensators should also be tested for strain; likewise windows, such as those in hot stages, should be tested for strain. (If strained, try loosening the retaining screw ring.)

Reflected light and all glare are partially polarized. One probable source of these is from overhead light entering in, or reflected from, the eyepiece. Once investigated, such aberrations are usually easy to correct.

5.7. CLEANLINESS: FREEDOM FROM INTERFERENCE FILMS

Cleanliness is especially important in working with the polarizing microscope. Thin films of oil, air, or other matter may result in interference colors that can be misinterpreted as polarization colors because they belong to Newton's series of interference colors (see Figure 5.8). Care must be taken to leave no air spaces or bubbles between the slide and cover glass because the appearance of the specimen in air may be misinterpreted for that in a liquid, which has a much higher refractive index than air.

5.8. FOCUS

We have seen that the optical properties of an anisotropic material vary with the direction of the incident light. Theoretically in determining these properties only axial rays should illuminate the specimen. With objectives of low NA and the top lens of the condenser flipped off, illumination is close enough to ideal, and focus depth is adequate. With high-aperture objectives however, the iris diaphragm of the condenser should be closed as much as feasible, and again focus depth is a bonus. Of course resolution is decreased, and if resolution is inadequate, fine detail should be studied separately with the iris diaphragm open.[5]

When anisotropic material is studied between crossed polars in a position of brightness (by rotating the stage), the material is bright against a black background, as in dark-field illumination (but for a different reason). In both instances the specimen appears to be self-luminous, like the moon at night. Such images are seldom in sharp focus. The sharpest image is obtained by first focusing with parallel polars and then crossing them to obtain the polarization image. Since interference figures are not images, the specimen need not be in sharp focus, and a thick specimen can usually be in focus.

5.9. ILLUMINATION

Illumination needed to obtain the optical properties mentioned in Figure 5.10 is called orthoscopic.[5] Except that the light is polarized, orthoscopic illumination is the axial, bright-field transmitted illumination dis-

cussed in Chapters 2 and 3. The image is the same with unpolarized or polarized light. By crossing or partially crossing the polars, we obtain polarization images as in Figure 5.6.

When an objective of moderate or high NA is filled with light by an adequate condenser, as in Figure 5.7, illumination is appropriately called *conoscopic*.[5] Such illumination is primarily used for observing polarization figures at the back aperture of the objective. Small but bright polarization figures are viewed with the eyepiece *out*. Larger but less brilliant figures are best seen with both the eyepiece *E* and Bertrand lens *B in*, as shown in Figure 5.7.[5]

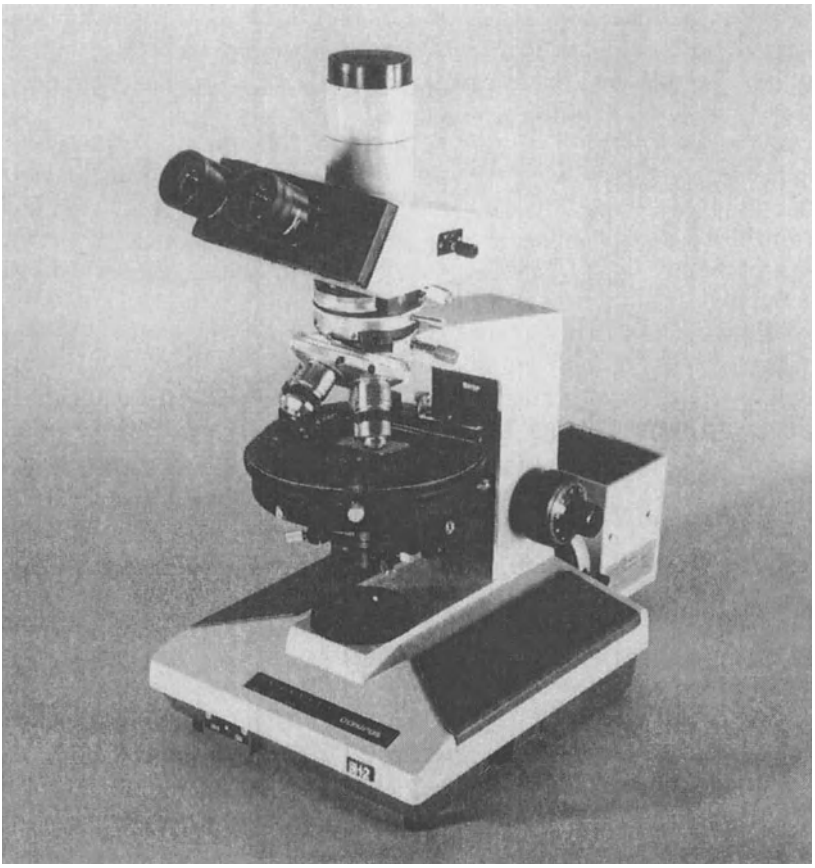


FIGURE 5.11. Olympus polarizing microscope.[15] Courtesy of M. J. Abramowitz and the Olympus Corporation.[16]

With either orthoscopic or conoscopic illumination, and with the polars crossed, incident light must be intense enough to see the effect that the specimen is producing. Even if 100% efficient, a Nicol polarizer absorbs half the light in order to polarize it. Common polarizing films absorb 65% of the light reaching them. The analyzer absorbs at least another 50–65% of the light that comes through the specimen, however transparent. So with only 17–25% of the original light available, even if the specimen fills the whole field (which it usually does not), the initial intensity of illumination has to be high.

5.10. RADIATION

Usually, a special 6-V incandescent lamp of the type already described is amply intense. Intensity is regulated by reducing the voltage as necessary by means of a variable transformer, but the radiation so obtained will be redder. A daylight filter stabilizes the color.

Something should be said here about determining the direction of light vibration from the polarizer, which must be known to detect and measure optical properties of anisotropic materials discussed in Chapters 6 and 7. To date manufacturers of polarizing microscopes have differed about whether the N–S or the W ↔ E cross line represents vibration direction in the polarizer when it clicks into place. To determine which is actually the direction, use light reflected from a flat black surface, such as the black enameled foot of a microscope stand. As shown in Figure 5.10, *part* of the light reflected from a plane surface is polarized, and the vibration direction lies in that surface at right angles to the reflected beam. So if the polarizer (or analyzer, whichever is easily removable) is held in the hand, looked through, and turned to bring the reflected beam to maximum darkness, the vibration direction of the light from the polar is at right angles to the plane of reflection.

Figure 5.12 also explains why in reflected-light microscopy (see Chapter 4) turning an analyzer eliminates some of the glare coming from the specimen and adds contrast to the image.

5.11. MAGNIFICATION

The total *magnification* of a polarization image or pattern should be no greater than is useful (≈1000 × NA).[4] Empty magnification[4] only decreases intensity of the image, which may already be low.

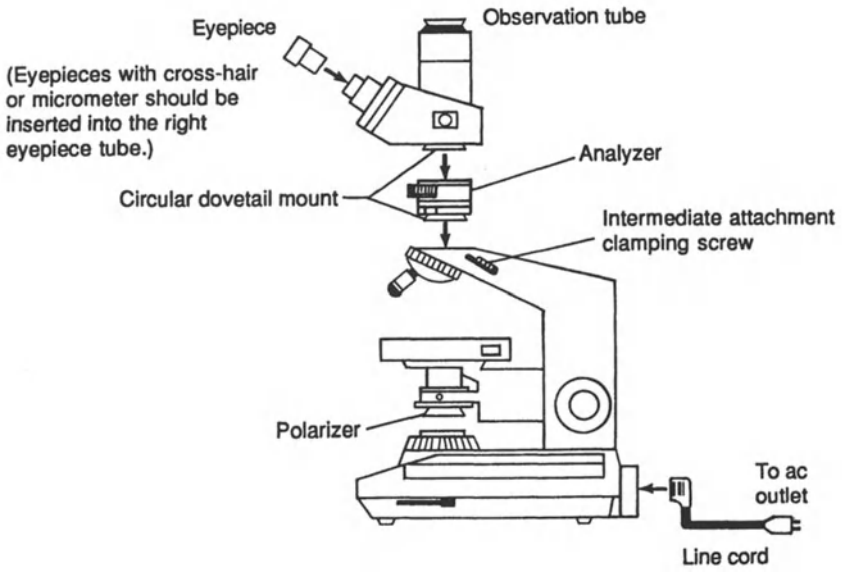


FIGURE 5.12. Diagram of the Olympus polarizing microscope.[15] Eyepieces with a crosshair or micrometer should be inserted into the right-hand eyepiece tube. Courtesy of the Olympus Corporation.[16]

5.12. FIELD OF VIEW OF AN INTERFERENCE FIGURE

The *field of view* of an interference figure should include only the one crystal involved, lest the figure be confused by effects of surrounding crystals. With small crystals, grains, or particles, a pinhole cap can be inserted in place of the eyepiece (with the Bertrand lens also removed). The smaller the hole in the cap (2 mm or less), the smaller the field of view. The Wright cross-slit diaphragm is more convenient because the size and position of its opening can be adjusted to contain only the one selected crystal observed while looking through the hinged eyepiece (replacing the regular eyepiece). When the single crystal has been framed, the Wright eyepiece can either be swung away or kept in place to work with the Bertrand lens.[4]

5.13. GLARE

With reflected illumination (see Chapter 4), there is some *glare* from the specimen itself. Since a portion of any reflected light is polarized (see

Figure 5.12), this much of it can be removed by rotating a properly placed polar until glare is at a minimum. As indicated before the polar is also effective on light scattered from other surfaces, including lenses in the objective. In any case the lower the objective NA, the less glare. Achromatic objectives present less glare than apochromats of the same NA because they have fewer air–glass interfaces.

5.14. DEPTH

As already illustrated in Figure 2.15, images of anisotropic specimens obtained between crossed or partially crossed polars can be a depth cue. The three-dimensional twists and spirals of cotton fibers present various orientations to axial polarized light to vary the degree of birefringence. Varying fiber depth changes the path length contributing to the fluctuating retardation and consequently introduces a variety of polarization grays and colors. Figures 5.4 and 5.5 (among others) also illustrate the fact that polarization effects are cues to depth.

In anisotropic crystals, since retardation of one of the double rays behind the other depends on the thickness of the specimen as well as the extent of birefringence, it is important for the thickness to be within the acceptable focusing power of the objective. As discussed in Section 5.8, with a self-luminous object the focus cannot be so sharp as with a dark object against a bright background. Therefore the *field depth* obtained with crossed polars is not so important as that obtained with bright-field illumination. It is important for the thickness of the specimen to be known or constant. In petrography thin sections are standardized to a thickness of 30 μm (the middle of the range in Figure 5.8). Fortunately most fibers used for paper, textiles, and cordage are also well within the range of Michel–Lévy chart.

Specimens for obtaining polarization figures may be even thicker than those for imaging specimens between crossed polars because polarization figures are images of the back aperture of the objective, not of the specimen. In fact the depth of the interference figure need be no shallower than the (long) depth of focus of the Bertrand lens (see Figure 5.7).

5.15. WORKING DISTANCE

The *working distance* of an objective used on a polarizing microscope should be within the range of specimen thickness in Figure 5.8 plus the cover glass thickness.[21] These are ordinary considerations for designers of microscopical lenses. The practicing microscopist however should always

be ready to use a hotstage[22] on a polarizing microscope, and he/she must have adequate working distance above it. In general working distance is precious, and the more of it the better.

Some extra-long working objectives are designed and built primarily for universal stages.[20] These objectives are also very handy for use with hot and cold stages, microdynamometers,[23] and other setups for dynamic experiments[24] requiring objectives with extra-long working distances.

5.16. SPECIMEN STRUCTURE

Specimen structure is the most fundamental concern when employing polarized light to study a material. An anisotropic material is sure to reveal different information when it is restricted to unpolarized light. This attribute was discussed in Section 5.2, and some of its practical aspects are continued in Chapters 6 and 7.

5.17. SPECIMEN MORPHOLOGY

Specimen morphology is also important. If there is a choice of thickness, choose one well within the limitations in Figure 5.8. If only thicker specimens are available, think about preparation methods that will reduce thickness but not change structure. If the sample is a powdery mixture of different-sized particles, optimum-sized particles can probably be screened out or otherwise separated.[24]

Crystals should always be examined as received under a microscope even though they are likely to be aggregated, broken, or rounded. Recrystallization should always be contemplated but employed *after* the preliminary examination.

5.18. INFORMATION ABOUT THE SPECIMEN

Information about the specimen should include history that may have brought on more or less anisotropy: stress, annealing, weathering, congealing, or precipitation due to short shelf-life, etc.

5.19. EXPERIMENTATION

Experimentation has already been discussed in Sections 5.15, 5.17, and 5.18. For example, if information indicates a working hypothesis that will help solve the problem microscopically, experiment!

5.20. SPECIMEN BEHAVIOR

Specimen behavior may be planned purposely for experimentation, but it may also be observed incidentally or fortuitously. For example both phases in a commercial sample of trisodium phosphate (TSP) were clear when received. Between crossed polars one phase was isotropic, and the other phase was anisotropic. While being examined under a reading lamp, the anisotropic phase turned white (dehydrated into light-scattering pseudomorphs). This behavior called for experimentation and preparation to confirm the identity of the two phases.

5.21. SPECIMEN PREPARATION

One of the most useful methods of preparing a sample for observation with a polarizing microscope is recrystallization. Every microscopist should know how to do it well and quickly,[4] using only a few tiny crystals and a single drop of water or other solvent. For recrystallization from water, the impure or imperfect material is added to a drop of water on a corner of a microscopical slide and dissolved by warming the slide over a microheater[2] while stirring with a drawn-down tip of a slender glass rod. When all is dissolved, the slide is allowed to cool until crystals begin to form. If necessary, the slide can be warmed again to evaporate some of the water until well-formed crystals appear. Placing a cover glass on the drop will then retard further evaporation.

5.22. PHOTOGRAPHY

Photomicrography employing polarized light is somewhat demanding with respect to the kind and extent of equipment. Resultant images are usually colored by various and variable wavelengths of light. Accordingly the condenser, objectives, eyepieces, and all other optical equipment should be chromatically correct. These and later remarks pertain to Figures 5.11, 5.12, 5.13, and 5.14. Figure 5.13 depicts a microscope employing transmitted polarized light, like Figure 5.11, but it is equipped with a sliding Bertrand lens, which together with the eyepiece, permits the observation and photomicrography of interference figures. These and other polarization colors require careful attention to the color sensitivity of photographic film.[25–29]

Figure 5.14 depicts a microscope employing reflected polarized light by means of a vertical illuminator.[14] Such a microscope can be employed

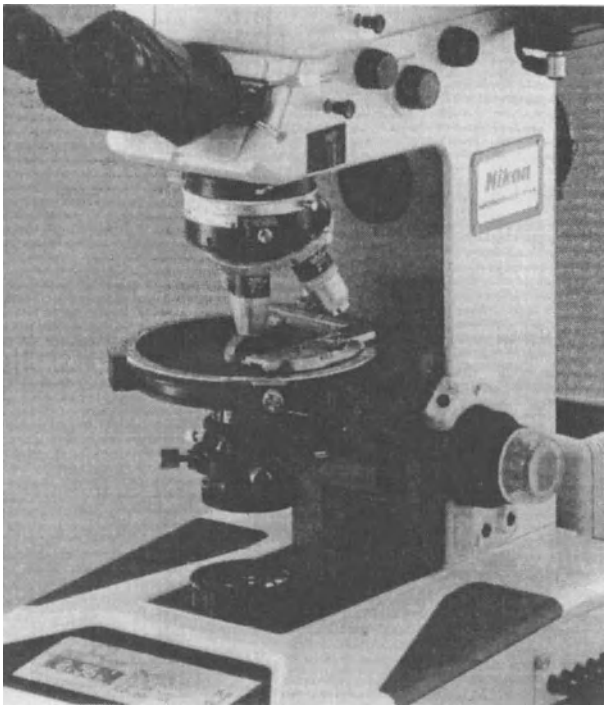


FIGURE 5.13. Nikon microscope precisely designed for microscopy by transmitted, polarized light, including photomicrography and television.[14] Courtesy of Nikon Instrument Group.[14]

to differentiate between anisotropic and isotropic opaque, polished surfaces, such as arsenopyrite versus pyrite (see Figure 4.16).

5.23. SUMMARY

Light is unique among all other kinds of emanation: It can be *polarized* to vibrate in only one direction. Consequently light microscopy is unique among all other kinds of microscopies. By polarizing the incident light, kind and extent of birefringence (double refraction) can be detected and analyzed according to the three principal perpendicular directions in the specimen. This *structural* information is then compared with the morphological information in biology, crystallography, description or analysis of natural versus man-made fibers, foils and films, stress and strain during test and use of materials, etc.

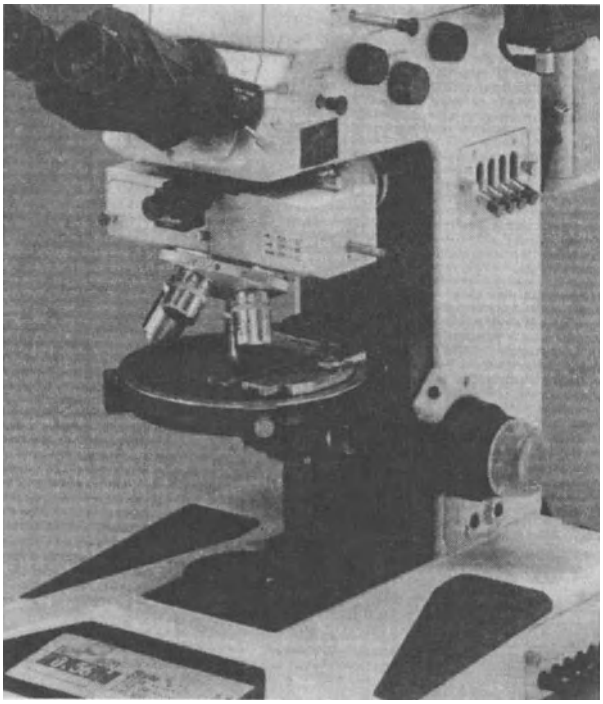


FIGURE 5.14. Polarizing microscope equipped for use with reflected light. Courtesy of Nikon.[14]

Illumination with polarized light reveals five kinds of *anisotropy:*

1. Optical anisotropy of a single anisotropic crystal in at least two of the three crystallographic directions. Such crystals as calcite, quartz, melamine, and sucrose display 10 or more separate properties that can be measured on microscopic crystals.

2. Multiples of anisotropic crystals, such as *twins, grains* of metal, rock, ore, and spherulites (such as starch grains, characteristic of the plant species).

3. Molecular birefringence as manifested by long or flat molecules, especially as arranged in a film or foil.

4. Form birefringence, also called rod or plate birefringence, as manifested in a two-phase system where long or flat particles are *oriented* in a medium of different refractive index by a flowing, streaming, or growing process.

5. The photoelastic *effect* is the local anisotropy manifested as strain resulting from stress on normal *isotropic* materials, such as inorganic or organic glass fibers, films, or solids. Small units can represent big units; indeed fibers can represent rods or girders. Films can represent sheets, and particles can represent rooms or even buildings, with respect to *both* structure (architecture) and morphology (size and shape), as different as these are.

6

Microscopical Properties of Fibers

6.1. INTRODUCTION

Fibers are unique units of biological tissues, mineral habits, or spinning processes; examples include muscle and nerve fibers, wool, fur, hair, cotton, linen, natural silk, natural and regenerated cellulose, asbestos, spun silicate glass, and man-made polymeric fibers. Although fibers vary widely in chemical nature, they are physically alike, being much longer than wide, very strong for their small cross sections, and anisotropic. The microscopically determinative properties of fibers are both morphological and structural. The distinctive morphology of a fiber type includes sizes and shapes in both longitudinal and cross-sectional views.

6.2. FIBER MORPHOLOGY

The determinative morphology of fibers was developed in the latter nineteenth century and the first-half of the twentieth century by means of light microscopy. Methods have been somewhat standardized, and determinative light micrographs have been published.[1–3] Transmission electron microscopy brought more detail of the morphology of fibers[3]; however, scanning electron microscopy has brought even more contrast and depth to the determinative micrographs of fibers.[4,5] Some examples are shown in Figures 6.1–6.13.[5]

In both longitudinal and cross sections, Figures 6.1–3 show similarities and slight differences in wool from merino sheep, blackface sheep, and cashmere goats. Together however the three species show the very char-

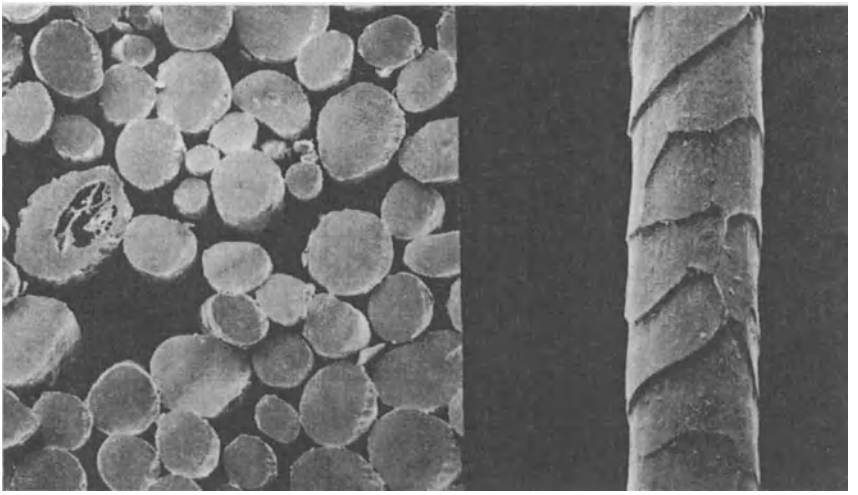


FIGURE 6.1. Scanning electron micrographs of wool (merino). Courtesy of Shirley Institute.[5]

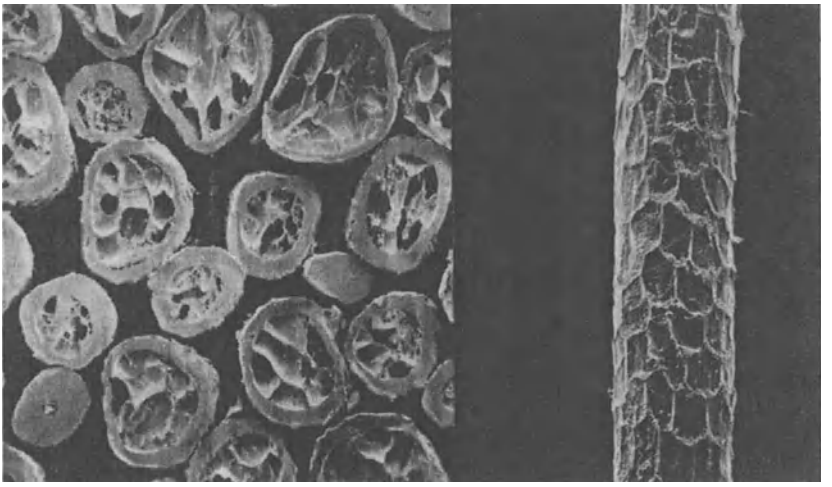


FIGURE 6.2. Scanning electron micrographs of wool (blackface). Courtesy of Shirley Institute.[5]

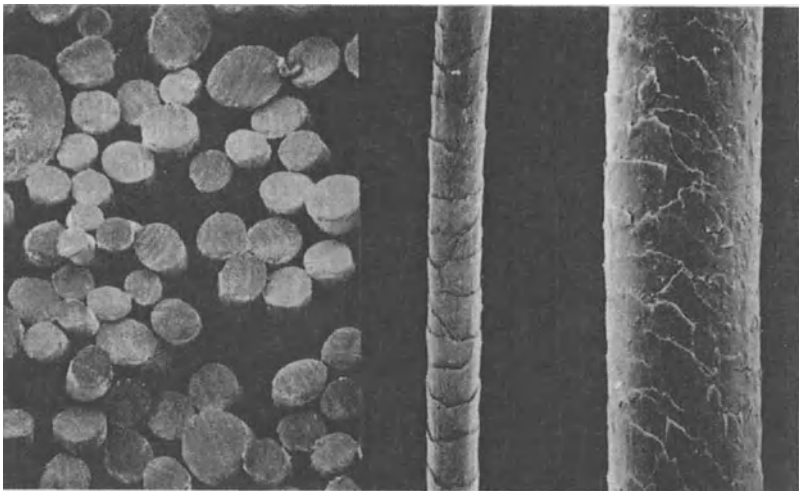


FIGURE 6.3. Scanning electron micrographs of a cashmere goat. Courtesy of Shirley Institute.[5]

acteristic exterior scales that hook on to one another during spinning to make yarn.

The next three scanning electron micrographs have a botanical origin: flax, cotton, and mercerized cotton (Figures 6.4–6).[5] Figure 6.4 shows cross and longitudinal sections of flax fibers for making linen. The cross sections are very irregular in size, depending on how many fibrils these contain. The longitudinal views of flax include nodes, which are dislocations of the fibrils. Figure 6.5 shows cotton fibers; their cross sections show tiny, collapsed tubes, curled to look like *C*s, *U*s, and *O*s. Longitudinal views of cotton look like tiny twisted ribbons. Mercerized cotton fibers (see Figure 6.6b) are swollen to the size of cylinders or thick-walled tubes.[5]

Figure 6.7 shows silk in cross sections, which are irregularly shaped three- four-sided polygons. Longitudinal views of silk fibers are striated (see Figure 6.7).[5]

Figures 6.8–6.13 show man-made fibers. Their morphologies reflect not only chemical composition, but also the manufacturers' design of the spinnerette and treatment of the filament(s). Viscose fibers are characteristically serrated in the cross section and grooved longitudinally (see Figure 6.8).[5] Terylene® polyester fibers (see Figure 6.9) vary in cross section from approximately polygonal to circular. Nylon cross sections are more

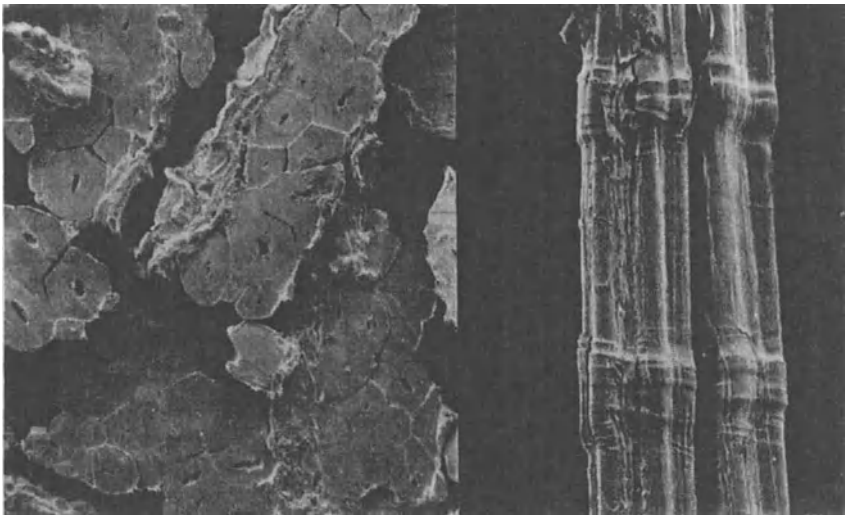


FIGURE 6.4. Scanning electron micrographs of flax. Courtesy of Shirley Institute.[5]

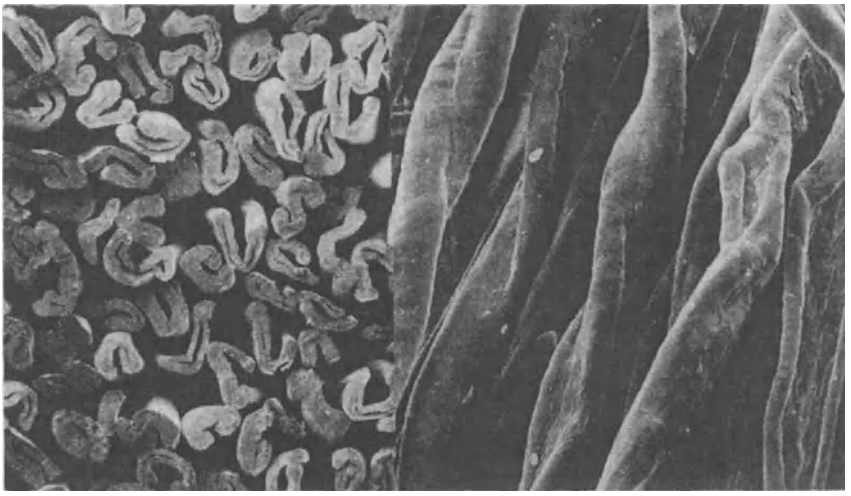


FIGURE 6.5. Scanning electron micrographs of cotton. Courtesy of Shirley Institute.[5]

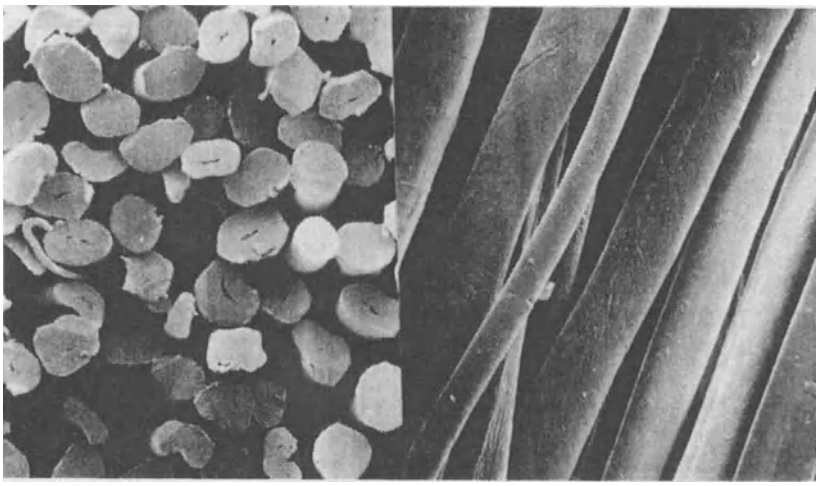


FIGURE 6.6. Scanning electron micrographs of mercerized cotton. Courtesy of Shirley Institute.[5]

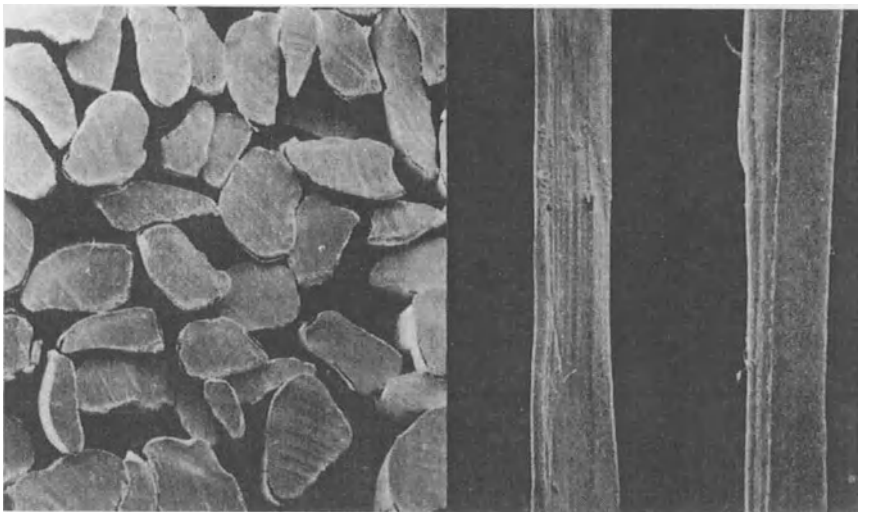


FIGURE 6.7. Scanning electron micrographs of silk. Courtesy of Shirley Institute.[5]

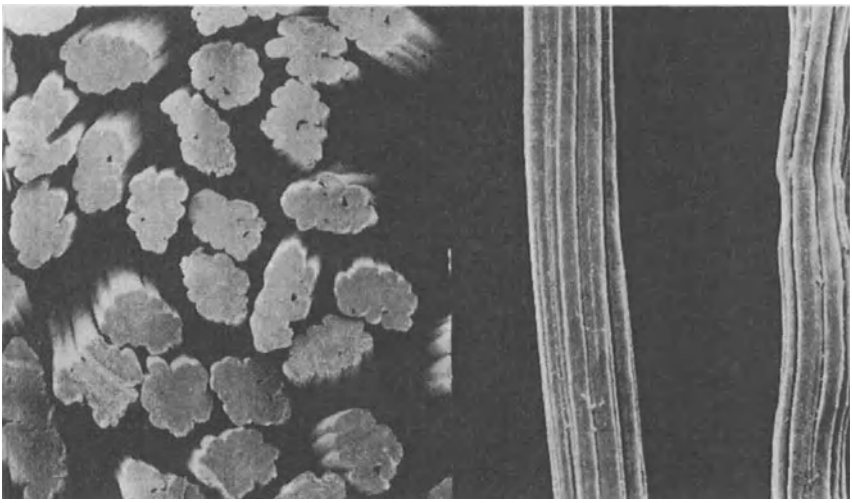


FIGURE 6.8. Scanning electron micrographs of viscose rayon fibers. Courtesy of Shirley Institute.[5]

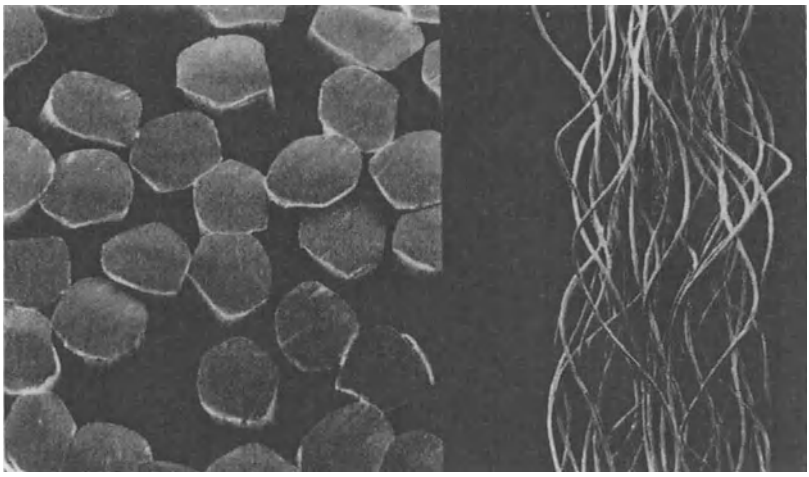


FIGURE 6.9. Scanning electron micrographs of Terylene® polyester fibers. Courtesy of Shirley Institute.[5]

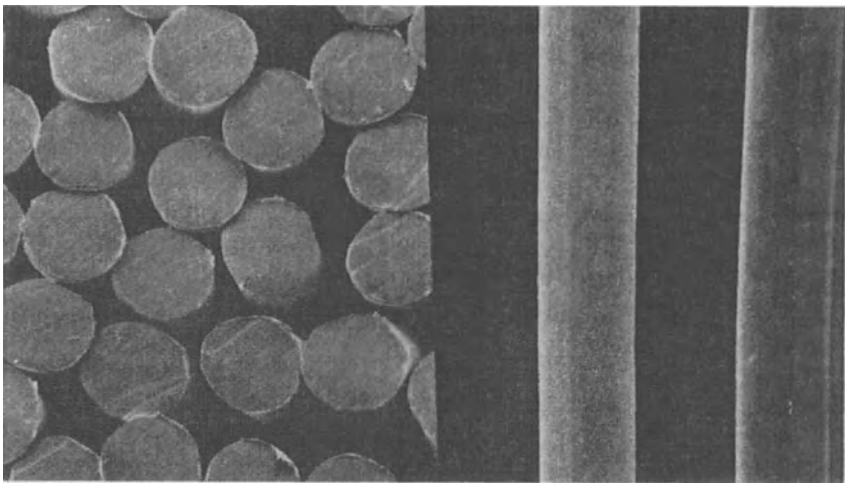


FIGURE 6.10. Scanning electron micrographs of nylon polyamide fibers. Courtesy of Shirley Institute.[5]

nearly round (see Figure 6.10).[5] Orlon® 42 acrylic fibers (see Figure 6.11) resemble the figure eight in cross section but are slightly rough longitudinally.[5] Acrilan® acrylic fibers are somewhat kidney-shaped in cross section and outwardly quite smooth longitudinally (see Figure 6.12).[5] Dicel® acetate fibers* (see Figure 6.13) are quite nondescript [whether viewed by SEM, TEM, or light microscope (the old standby)]. Thus we see that the man-made fibers are not especially distinguished by morphology alone. However, many of them are very distinguishable by means of their optical properties.

For a light-microscopical examination of longitudinal views of fibers, the general procedure is to mount the fibers on a microscopical glass slide in mineral oil of refractive index 1.48 and cover with a standard glass

*Terylene®, Orlon®, Acrilan®, and Dicel® are examples of *trademarks* registered with the U.S. patent office. Each trademark belongs *exclusively* to a single owner (usually a corporation), for use with a specific product or products. Trademarks are not nouns, so they should be followed by descriptive terms; for example there are Polaroid® cameras, films, or polars. *Trade name* (two words) is used for a *type* of product, such as nylon for a polyamide fiber, yarn, or fabric, without regard to the producer, see *A Guide to the Care of Trademarks*, U.S. Trademark Association, 6 East 45th St., New York, NY 10017.[6]

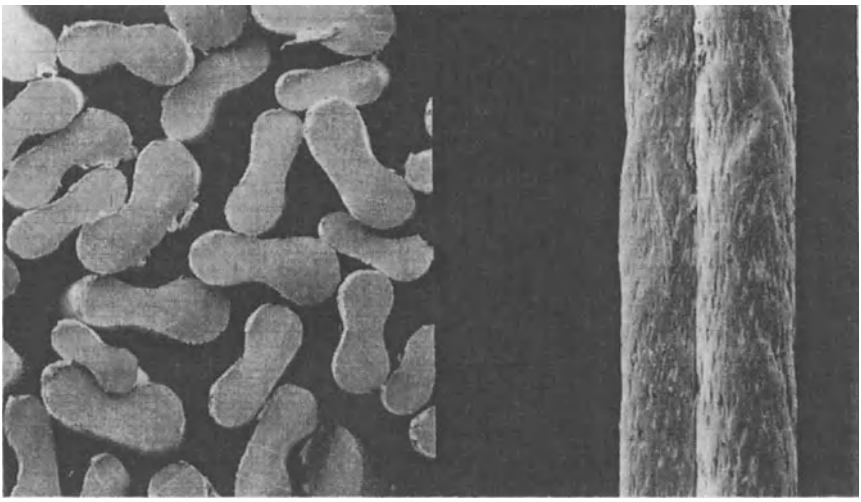


FIGURE 6.11. Scanning electron micrographs of Orlon® acrylic fibers. Courtesy of Shirley Institute.[5]

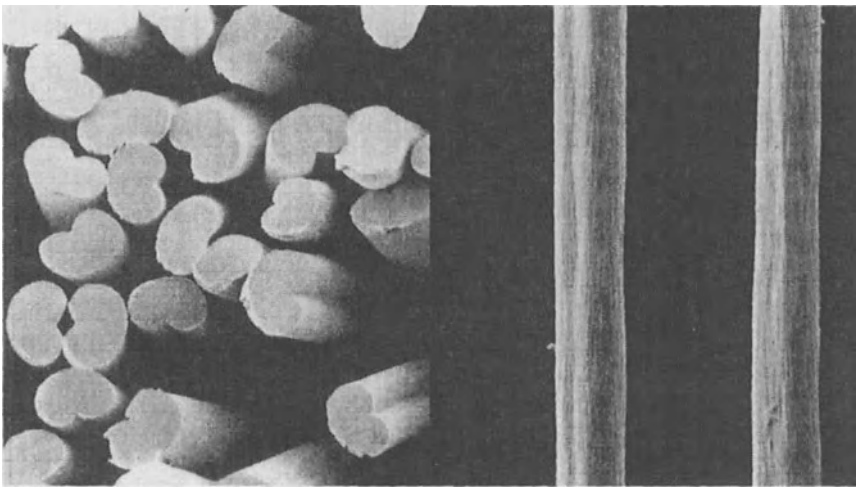


FIGURE 6.12. Scanning electron micrographs of Acrilan® acrylic fibers. Courtesy of Shirley Institute.[5]

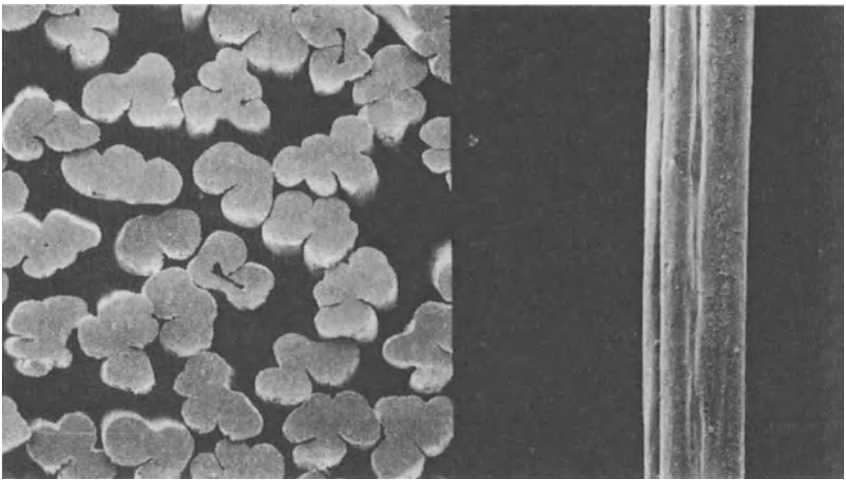


FIGURE 6.13. Scanning electron micrographs of Dicel® cellulose acetate fibers. Courtesy of Shirley Institute.[5]

cover. Procedures for making fiber cross sections are covered in Chapter 20.

Morphology is usually sufficient for either describing or identifying *natural* fibers; for *man-made* fibers however, the morphology may depend more on spinning and drawing processes and on how the spun fiber was physically treated than on composition of the material being spun. Nevertheless, every kind and variety of fiber has as many as eight different optical properties and these properties can be described and measured by means of *polarized light* and a *polarizer* and an *analyzer.* However the kind and degree of each property is not so constant as with crystals. With man-made fibers optical properties vary in kind and degree with variation in treatment during and after spinning; but there is sufficient constancy to be useful in both describing and identifying fiber species.

Figure 6.14 shows a simple fiber whose light waves vibrate in two privileged directions: one vibration direction parallel (ll) to the fiber's axis and the other vibration direction perpendicular ($\perp$) to the axis. To consider the two directions simultaneously, a few fibers are cut to lengths of 1 or 2 cm and mounted in a liquid to reduce light scattering by the fiber in air (refractive index n = 1.00). Water (n = 1.33) is handy for mounting such hydrophilic fibers as cotton, and mineral oil (n = 1.48) is commonly used for such hydrophobic fibers as nylon. Fibers should be examined im-

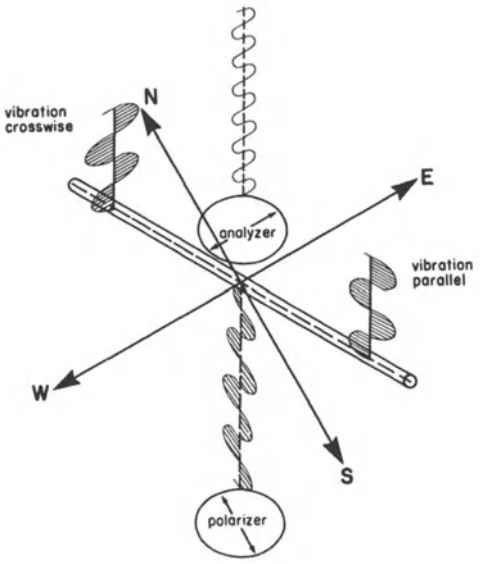


FIGURE 6.14. Fiber in position of brightness between crossed polars. Drawn by C. Miller.

mediately on being mounted in liquid, because they may change. Cotton for example swells in water with corresponding changes in optical properties, and nylon swells in some organic liquids. Such changes should be noted as behavioral information, but initial characteristics are to be compared with determinative literature.[2] At the same time subsequent behavior in liquids may indicate that more experiments are worthwhile.

With fibers properly mounted, the eight determinative optical properties can be observed in succession with the aid of a polarizing microscope. The fibers are first brought into focus with the analyzer out, and then observed between crossed polars while the stage is slowly rotated. The eight properties to be noted follow.

1. *Brightness* or grayness (instead of darkness) in some or all positions of rotation *between crossed polars.* Most fiber types show brightness in only certain rotation positions (see Figure 6.15), but cotton is bright in all positions because its structure is spiral instead of axial. Inorganic glass fibers show no change when rotated between crossed polars. Glass fibers are too thin to show detectable retardation ordinarily, although thicker glass rods and bottles do. The fact that glass fibers appear to be isotropic, even though they are not strictly so, should not detract from the analytical importance of their being the only kind of fiber that comes so close to showing no retardation under the microscopical conditions just set forth. That alone is determinative.

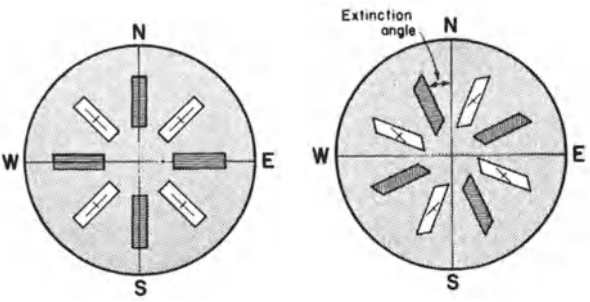


FIGURE 6.15. Positions between crossed polars of brightness and extinction (darkness); (left) parallel extinction; (right) oblique extinction.

2. *Extinction* (darkness): complete or incomplete, every 90° of rotation between crossed polars (see Figure 6.15). Cotton, with its spiral structure, shows no substantial extinction. Linen (flax), hemp, certain other bast fibers, and some mechanically treated man-made fibers show extinction in certain segments at a time.

3. The extinction may be *parallel or oblique* to the fiber axis, and the angle is determinative. Some fibers display segmented extinction, as in bast fibers, silk filaments, and some crimped man-made fibers (see Figure 6.15).

4. *Retardation*: the distance in nanometers that one wave train falls behind another (see Figure 6.14) is manifested as grays, whites, and colors (see Figure 5.8), some of which (reds and pinks) are visible between crossed polars to the eight order of classification of the interference between slower and faster ray bundles. Some polyester fibers show pinks of such high order.

5. *Sign of the birefringence* depends on which of the two ray bundles is the slower. Most fibers are positive, signifying that the rays vibrating lengthwise are slower (i.e., have the higher refractive index, n), but an important minority are negative. The sign is determined between crossed polars using a *retardation plate.*

6. *Quantitative birefringence,* the numerical difference between the high and low refractive indices, is independent of path length (thickness, denier). It can be determined from the retardation per unit path length by means of *compensators*[6a] (see Chapter 5). Birefringence can also be determined at the fiber *surface* by measuring the two separate refractive indices using immersion techniques.

7. $n_{\parallel}$, the refractive index for light (from the polarizer) *vibrating parallel* to the length of the fiber, can be measured by comparing $n_{\parallel}$ with the refractive index of a liquid standard. The resulting datum is determinative.

8. $n_{\perp}$, the refractive index for light *vibrating perpendicularly* to the length of the fiber, is measured in the same way as $n_{\parallel}$ but with the fiber oriented at right angles to the position used for measuring $n_{\perp}$. This datum is determinative and can also be used to determine the quantitative birefringence as in Property 6.

Brightness results when an anisotropic fiber on the rotatable stage is oriented between crossed polars so that there is a vector of each vibration direction parallel to the direction of vibrating light rays in the analyzer (see Figure 6.14). The vector varies from nothing (darkness) at positions of extinction to maximum brightness at a position of 45° between extinctions. When there is no extinction, as in the case of a spiral structure such as that of cotton, there is always brightness between crossed polars. In most kinds of fibers, the brightness is strong enough for the microscopist to recognize with confidence that the fiber is anisotropic. In a few cases, such as with some cellulose acetates and some acrylics, the brightness may be so weak as to leave doubt of anisotropy. In fact some cases of *iso*tropy have been reported in the literature for some trademarked products of these generic types. When in doubt, bring the sensitive tint of a first-order red plate into play. Also put a daylight blue filter into the path of yellowish artificial light, so that the field will be bright red of the first order. If the fiber *is at all anisotropic* in one diagonal set of quadrants, the fiber will be blue or at least magenta. In the opposite set, the fiber will be yellow or at least orange (see Figure 6.16). No further proof of anisotropy is needed.

Complete extinction (darkness) means that the whole fiber extinguishes between crossed polars every 90° of rotation. Occasionally natural silk fibers show some short portions that do not extinguish in the same positions of rotation as the major portions. Characteristically these positions occur where the still-soft (recently spun) filament was crossed by another part of the same filament as the silkworm was crisscrossing its double filament to prepare its cocoon. Flax and other bast fibers are really *bundles* of fibrils. Like a bundle of straws when bent, some of the fibrils will be dislocated. Each dislocation results in a local relocation of extinction, which shows up as a short, cross-hatched section. The number of dislocations indicates the number of bendings. Thus old linen can be distinguished from new linen, and linen-rag paper can also be recognized.

Parallel extinction means that the axis of the fiber is parallel (or very closely parallel) to one of the two cross lines in the eyepiece when the fiber becomes dark during rotation of the circular stage between crossed polars. It is assumed that the cross lines have been tested with a standard specimen of parallel extinction so that one cross line in the eyepiece represents the vibration direction of light from the polarizer (*e.g.*, $\overset{N}{\underset{S}{|}}$) and the other

cross line represents the direction in the analyzer (e.g., W↔E) (see Figure 6.15).

Parallel extinction is shown by practically all man-made fibers, *as spun*, because the structural units are arranged in the direction of flow through the spinnerette. Treatments, such as crimping or texturizing, locally alter parallel extinction if the parallel arrangement is disturbed.

Retardation of one of two wave trains behind the other is the total effect on the particular fiber by polarized light. In Figure 6.14, the polarizer is set so that the vibration direction of emerging light waves is north–south, applying terms of a magnetic compass to the cross lines of the eyepiece. The fiber is either oriented NW\SE or SW/NE because (having parallel extinction) it has maximum brightness in these two positions when the analyzer is crossed (W↔E) with the polarizer $\overset{N}{\underset{S}{\updownarrow}}$. Referring to Figure 6.14 the birefringent fiber splits the polarized beam into two polarized rays, one vibrating lengthwise (||) to the fiber and the other crosswise (⊥). They travel through the fiber at different velocities without interfering with each other because (as shown in Figure 6.14) they are in different planes. Yet they must squeeze through the analyzer, which is set for vibration in a single direction (W↔E). Two rays, one from each wave train, each with the same wavelength, may travel through a fiber of just the right thickness (denier) to enter the analyzer in such a phase that they completely cancel each other; that is, the color corresponding to that wavelength is lost in the analyzer. But at some other wavelength, the rays will be in such a phase that in going through the analyzer they reinforce each other. If, for example, the velocity difference between the rays vibrating lengthwise and those vibrating crosswise is such and the thickness of the fiber is such that the slower ray is in phase but about 525 nm behind the faster ray, the color of the light emerging from the analyzer will be red of the first order, as shown in the Michel-Lévy chart (see Figure 5.8). If retardation is about 1050 nm, the color is red of the second order; at about 1600 nm, third-order red; at about 2100 nm, fourth order, and so on. Intermediate retardations are manifested in other colors (at least through four orders) in Figure 5.8. Second-order colors are the brightest in this (Newton's) series of interference colors. In the fourth order blues and yellows have faded out. In higher orders than those shown in Figure 5.8, all colors but red have faded, and even red has lightened to pink.

The *order* of retardation colors may be difficult to recognize. In such cases a uniform quartz wedge of three or more regularly increasing orders is useful. The wedge is placed in the slot of the body tube. If this slot is in the NW\SE diagonal, start the test with the fiber oriented in the same direction. If as the wedge is gradually inserted, colors in the fiber increase in order, rotate the fiber 90° to the other direction. Now as colors decrease

in order, count the orders to compensate (to darkness) the color band(s) in question.

Drawn polyester fibers ordinarily exhibit orders higher than the three or four orders of modestly priced quartz wedges. However higher orders may not be needed in this particular test if the third or fourth red band can be recognized so that the higher order reds (pinks) can be counted in the *thickest part* of the fiber.

In addition to bands of different retardation colors due to differences in fiber thickness, some are due to differences in birefringences, for example in the skin-and-core structure of some fibers. These and other radial heterogeneities may be detected in cross sections if they are not recognized in the longitudinal views.

Retardation (polarization) colors and their order of extent (see Figure 5.8) are very determinative once recognized. Granted that thickness varies widely among natural fibers, and purposely in different deniers of man-made fibers, the variation is not too great to spoil the general value of retardation colors and their orders. Low-order grays and colors are especially significant because thickness variations (denier) make so little difference in the order of retardation. Acrylics and cellulose acetates, from fine deniers to carpeting sizes, always show first-order grays or colors, whereas practically all drawn polyesters manifest high-polarization colors. The difference in color order between undrawn and drawn nylon and polypropylene is so great that drawing degrees can be recognized. Immature cotton fibers show yellow or blue on the red background of a first-order red retardation plate. On the other hand mature cotton fibers are distinguished by slightly higher order colors. Recognizing distinctive retardation colors in a mixture of two or more kinds of fibers is not only useful for forming quick qualitative opinions but also for estimating proportions of those kinds of fibers.

The term sign of birefringence (or retardation) is preferred over the term sign of elongation, since the sign of birefringence pertains to all anisotropic fibers whether or not they have been elongated (stretched). Most fibers are positive (+), i.e., they are length-slow: $n_{\parallel} > n_{\perp}$. But some are negative (−), i.e., length-fast: $n_{\parallel} < n_{\perp}$. Those with negative sign at room temperature[7] are acrylics, most cellulose *tri*acetates, and at least some sarans and vinyons. The fact that cellulose triacetate has a negative sign, while cellulose diacetate has a positive one, indicates the ability of the acetyl side group to slow down light. This suggests the best way of differentiating between the two degrees of acetylation.

To determine the sign of fibers showing first-order gray, a first-order red plate is inserted while the fiber is in a position of maximum brightness.

When the slot for the retardation plate is oriented NW\SE, the fiber is also oriented NW\SE (see Figure 6.16); otherwise orientation is SW/NE for both. If the sign of birefringence is positive (e.g., cellulose diacetate), the fiber will be yellow or orange; if the sign is negative (e.g., cellulose triacetate), the fiber will be blue or magenta. Gray on the Michel-Lévy chart (see Figure 5.8) amounts to a retardation of, say, 100 nm. Retardation of the first-order red plate is about 500 nm, and its direction of slow vibration is ordinarily oriented crosswise toward the long plate holder (see Figure 6.16). Thus 500 − 100 = 400 (yellow); 500 + 100 = 600 (blue).

In a fiber of *positive* birefringence, the slower ray vibrates parallel to the fiber's axis. According to one convention, the vibration direction of the *slower* ray is represented by an arrow shorter than the one representing the vibration direction of the faster ray. As shown in Figure 6.16a, in the diagram of a positive fiber, the shorter of the two perpendicular arrows is drawn *parallel* to the fiber's axis and crossed with the (short) arrow of the first-order red plate. The situation can be remembered if we think of the two short arrows making a plus sign, signifying a positive sign of birefringence for the fiber. All this is deduced from observing that the fiber alone was gray between crossed polars and became yellow when the 1° red plate was inserted. Since yellow represents a lower retardation than the 1° red of the plate, there is a *subtraction* (reduction) in retardation of the 1° red plate by the fiber when in the NW\SE position. Hence the vibration direction of the slower fiber rays must have been crossed with that of the retardation plate; the slower rays must be *lengthwise*, and the fiber must be positive.

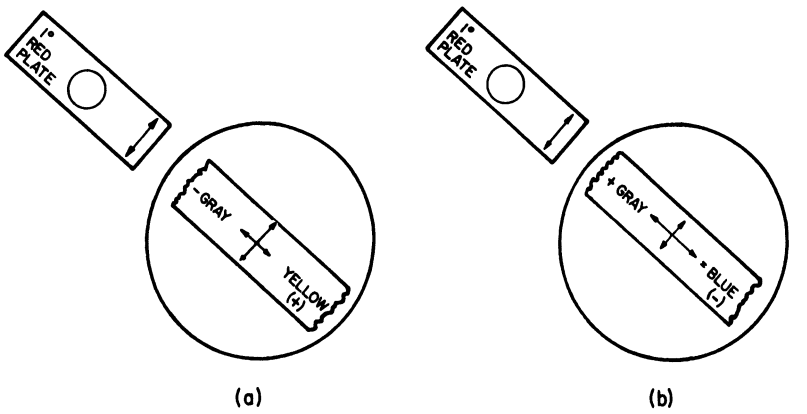


FIGURE 6.16. Determining the sign of birefringence of a fiber manifesting a first-order gray.

In the other case (see Figure 6.16b), the fiber also happens to be gray in position of brightness between crossed polars, but it turns blue in the NW\SE position. Blue represents a *higher* retardation than the red of the plate; that is, the two slow-ray directions of vibration are *parallel,* indicating a negative sign of birefringence.

In some cases of skin-and-core construction,[7–9] such as in some acrylics, the core has a different sign (+) than the skin (–).

With any retardation plate (or compensator),[10] be sure of the vibration direction of the slower ray. If there is only one arrow engraved on the plate mount, it represents the vibration direction of the slower component. But some manufacturers may choose to have that direction oriented lengthwise to the plate's metal mounting; or if the markings are $\uparrow x' \rightarrow z'$, the direction z' is that of the slower ray. Since the retardation disk itself *may become turned in its mount,* check with a known material, such as cellulose diacetate (+) or any acrylic (–) except Orlon® (which may be too weakly negative to use as a strong standard). In cleaning any optical disk mounted in a cylindrical well, be careful not to use sufficient circular pressure to turn the disk. If this should happen, you or a repairman will have to loosen the pressure cap and turn the disk back. Always make sure the pressure cap is tight.

The first-order red retardation (sensitive tint) plate is good for determining the sign of birefringence of any material that shows a retardation (polarization) gray or color (yellow, orange, red) of the first order. The same plate will also indicate certain colors of the second order, such as blue or green, by providing a dark or light gray during subtraction (crossing the slow directions) and a blue or green of third order during addition (parallel slow directions). But for yellow, orange, or red of the second order, subtracting or adding first-order red will merely give the corresponding color of the first or third order. These may be somewhat difficult to tell apart.

The *quarter-wave* (0.25λ) *plate* (light ray) is also good for determining the sign of birefringence of a material showing a first-order gray. Subtracting the two retardations will give a darker gray, and adding will give a brighter gray (or a yellow). With a low-order color, the 0.25λ plate gives a *different* color for subtraction than for addition.

For fibers showing retardation colors (see Figure 6.14), the quartz wedge can be used as a retardation plate by *moving* it between crossed polars over the fibers in a position of brightness. Subtracting results in a succession of colors *downward* in order; adding results in an *upward* succession of colors. With knowledge of the direction of the wedge's slower component, the sign of the fiber's birefringence is determined as indicated by the arrows (not the colors) in Figure 6.16.

Dichroism is the preferential absorption of all* or part of the spectrum in one preferred direction relative to the other preferred direction. The phenomenon is especially visible with deeply dyed fibers. Since dye in the fibers obscures the complementary polarization color, dichroism can substitute its own evidence of anisotropy. Absorption is usually greater in the direction in which the slower ray vibrates. In positive fibers this direction is parallel to the axis of the fiber; in negative fibers it is perpendicular. Therefore dichroism can indicate the sign of retardation (birefringence). The indication can of course be confirmed by using retardation plates with the fiber in a diagonal position (instead of parallel) to the cross lines of the eyepiece.

The arithmetic degree of *birefringence* is the difference between the two principal refractive indices, e.g., $n_{\parallel} - n_{\perp}$[11] Indeed, both the birefringence and its sign can be determined by measuring $n_{\parallel}$ and $n_{\perp}$ and subtracting them. However, the measurements may be long and tedious. Moreover, they are determined only at the surface, which may be different in refractive index from the interior, as in the case of skin-and-core construction.[3] Such radial heterogeneities are actually recognized by *variations* in birefringence; therefore the degree of birefringence is a separate and independent optical property and a very important one.

Birefringence alone allows a broad classification of fibrous types into those of *weak* birefringence, 0.001–0.01 (e.g., natural and regenerated proteins, acrylics, modacrylics, acetates, saran, vinyon); those of *moderate* to *strong* birefringence, 0.01–0.1 (e.g., natural and regenerated cellulose, silk, nylons, polyolefins, vinyl); and those of *intense* birefringence, above 0.1, as in polyesters.

Birefringence can also be defined as the ratio between retardation and thickness of the specimen at a given point. Indeed, the quickest and simplest way estimating birefringence is from the *retardation* color and the *thickness* of the fiber, employing the Michel–Lévy scale of birefringence (see Figure 5.8). If the fiber is practically a cylinder, as in the case of some nylons, polyolefins, polyesters, rayons, acrylics, and modacrylics, the thickness is the same as the *width* of the longitudinal view and can therefore be measured directly with a calibrated ocular scale, as explained in Chapter 5. Fibers of weak birefringence, such as acrylics and modacrylics, usually display first-order gray between crossed polars. Note particularly the shade of gray along the axis of the fiber and match this gray with the likeness on the Michel–Lévy scale. Go up the ordinate until the fiber

*It is interesting to note that dichroism is the fundamental mechanism in polarizing films, such as Polaroid®. In the colorless variety practically all of the visible spectrum is absorbed in one preferred direction but transmitted in the other preferred direction.

thickness is reached, then follow the *diagonal* line to the birefringence and read the result.

Fibers with moderate to intense birefringence and the usual parallel extinction display color bands parallel to the axis. The number of bands (degree of retardation) depends on the fiber's birefringence and path length. With a circular cross section, as shown in Figure 6.17, there is a single central band of colors, and in this particular case, it is flanked by two pairs of color bands of descending order. The colored line congruent with the fiber axis represents the highest retardation of all, and it is the value, together with the diameter of the fiber, used with cylindrical fibers in translating into birefringence by means of the Michel–Lévy scale (see Figure 5.8).

If the cross section is not circular, or even roughly so, it should be studied to anticipate where color band(s) of highest order will appear in the longitudinal view of the fiber. For example in Figure 6.18 the cross section is dumbbell shaped. Such a fiber will most probably lie on its side, which is then seen as the width (*W*) in longitudinal view and measured on the particular fiber in view. The thickness of the particular fiber is estimated from the ratio of the thickest (*T*) part or the center (*c*) to the width (*W*) of a typical cross section of the sample. Then the retardation colors of either the central band or the twin bands are compared with the respective thicknesses to estimate the birefringence from the Michel–Lévy chart (see Figure 5.8).

Likewise cross sections of fibers of some other consistent shapes, such as dogbone, bean, cogwheel, and triangle (trilobal), can be used to estimate what proportion of the width (measured in longitudinal view) is the thickness with respect to a particular retardation color when determining birefringence from the chart. Fibers with tubes are often regular in cross section so thickness in longitudinal view can be estimated from the measured width. Even irregular cross sections, such as those commonly found

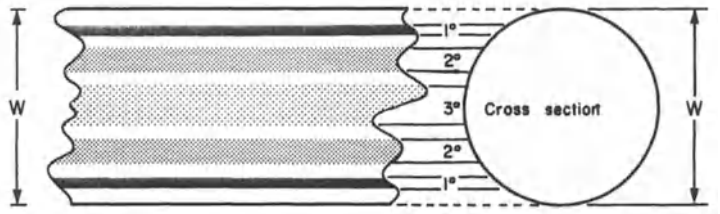


FIGURE 6.17. Diagram of a cylindrical fiber, showing a portion of the longitudinal view between crossed polars. Hypothetically the birefringence and thickness (width *w*) exhibit three orders of interference colors (1°, 2°, and 3°).

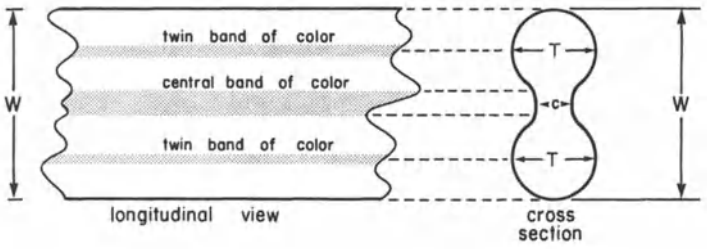


FIGURE 6.18. Diagram of a hypothetical fiber with a cross section like a dumbbell and hypothetically with one set of twin color bands. Considering the shape of the cross section, the twin color bands must be of higher order than the single central band.

in cotton, some viscose and acetate fibers, and some dual-component fibers, may give enough information about the ratio of thickness to width.

Fiber thickness can also be measured directly if it can be turned or twisted to measure it on its edge. Or possibly a microscope is available with a graduated, calibrated fine-adjustment focusing wheel for measuring vertical distance from bottom to top, as explained in Chapter 5. Be sure to multiply the apparent distance by the average refractive index of the fiber. A very rough estimate of the average thickness may be made from the following relation

$$\text{denier} = \pi d^2/4 \times 9000 \times \text{density}$$

Thickness, d, is in millimeters in the preceding formula and in the Michel–Lévy chart (see Figure 5.8). A still rougher estimate can be made by remembering that most fabric-making fibers (except those for carpeting) are between 0.01–0.03 mm in average diameter.

The Michel–Lévy scale is inadequate for polyesters that manifest retardation colors of a higher order than six. For such fibers a high-order compensator is required[10]. This brings up the general subject of measuring birefringence by means of a variable retardation device, the *compensator.*[11] A quartz wedge can be used directly if it is of a sufficient number of orders and graduated. The Babinet compensator is more elaborate; it consists of two opposing quartz wedges. One of them is moved over the other by means of a graduated micrometer screw.[12] The Berek type of compensator employs a different principle: It measures the degree of tilt in an anisotropic plate to the point of compensation.[11] Berek compensators are available in various ranges. For highly drawn polyester fibers a range of 10 orders is needed, and for experimental fibers, 15 or more orders may be required.[8]

Another important point is that exact compensation in white light can be achieved only if the dispersion of birefringence of the fiber and that of the compensator are the same.[8] For path differences under four orders, no difficulty is found, but with higher orders the band of compensation is no longer black but rather unsymmetrically colored and broad. One solution is to establish the color of the compensation band. Another solution is to cut a wedge out of the end of a fiber and examine it in a subtractive position between crossed polars with the Berek compensator in place. A count of the resulting fringes plus any additional fraction gives the retardation.[8] Thickness in millimeters still has to be determined or estimated as previously described.

Determining birefringence has many applications in production and quality control as well as in research. More and more birefringence determination is being used to control the degree of elongation for optimum strength and other desirable properties.

While birefringence alone may be a key to understanding the relationship between elongation and strength, it can also be important to know the actual individual values of the two principal refractive indices of a fiber. The index for light vibrating parallel to the fiber's axis, $n_{\parallel}$, varies from a low of about 1.47 in cellulose acetate to as high as 1.73 in some polyesters. The span for $n_{\perp}$ is not so great, but it is large enough to give a good spread among types; it varies from about 1.47 in acetates to about 1.54 in polyesters and about 1.61 in saran.[13]

The *refractive index* is generally determined by trial and error, and is usually tedious. But experience, technique, cleanliness, and care can make the results very satisfying. To separate $n_{\parallel}$ from $n_{\perp}$, polarized light is supplied by the polarizer only. *The other polar is removed from action.* Figure 6.19 illustrates fiber orientations for determining $n_{\parallel}$ and $n_{\perp}$, respectively, with the polarizer having its direction of vibration ↕, as represented by the respective cross line of the eyepiece. In Figure 6.19a the fiber is aligned parallel to the $\overset{N}{\underset{S}{\updownarrow}}$ cross line, so that $n_{\parallel}$ is being measured. In Figure 6.19b

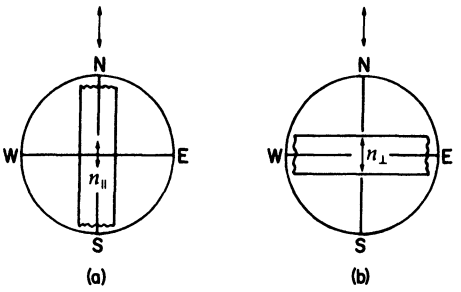


FIGURE 6.19. The alignment of a fiber to determine (a) $n_{\perp}$ and (b) $n_{\perp}$ for a fiber with parallel extinction and positive birefringence. The vibration direction of the polarized light is $\overset{N}{\underset{S}{\updownarrow}}$.

the fiber is aligned parallel to the W↔E cross line, so that $n_\perp$ is being measured. Incidentally the shorter arrow is shown lengthwise; that is, $n_\parallel$ is in this instance greater than $n_\perp$, and the fiber has positive birefringence.

There are two ordinary methods (among special procedures[14]) for determining the refractive indices of a fiber. In both, the fiber is immersed successively in liquids of known refractive index, and a search is made for a match between liquid and fiber for $n_\parallel$ and $n_\perp$. One method for determining whether the fiber index is higher or lower than that of the liquid uses oblique illumination, and the criterion is the position of the resultant shadow cast by the specimen.[6] The other method uses the Becke refraction line and its critical movement during focusing. The Becke method is preferred when the refractive index of the liquid is close to the fiber's. In either case, specifications for illumination are very important, and a series of liquids of known refractive indices is required.[15,16]

The Becke test requires axial transmitted illumination. If the condenser is in place, remove the top lens and nearly close the iris diaphragm, leaving the polarizer in place. Focus up and down and notice the movement of the Becke halo around the fiber. As you focus upward, the halo moves to the medium of higher refractive index (see Figure 6.20). When you are far from a match between fiber and liquid, the over- or under match is very evident because the halo is in bold contrast. As you approach a match, the halo becomes fainter. Darken the room if it helps; concentrate, be patient. The Becke method is very satisfactory to the experienced observer, and, of course, experience always helps. From the first six optical properties and other information, you should have a good idea of what your specimen is and what refractive indices are expected. For your first liquid choose one with a refractive index in the middle of the probabilities. Learn to estimate how much you are over or under the correct index; then take a bold step, trying to bracket the index as soon as possible. Your previous data, especially regarding the sign of birefringence (which is greater, $n_\parallel$ or $n_\perp$?) and the amount of birefringence (difference between $n_\parallel$ and $n_\perp$), will help considerably in your choice of trial liquids. Use short lengths of each of a few fibers to avoid entanglements and bending. Be careful not to contaminate specimens with any other kind of fibers. Use

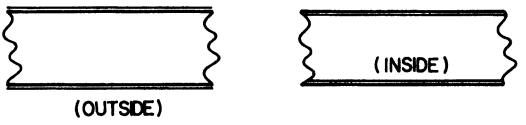


FIGURE 6.20. The Becke line. Simulated fiber is in a liquid of higher index; (left) on focusing upward slightly; (right) on focusing downward slightly.

small cover glasses to save standard liquids and be able to fill the cover glass and slide. When no Becke halo is observable and nothing seems to move as you focus up and down, the fiber and liquid have the same refractive index. Usually, however, the refractive index is an intermediate between the values of those of two successive liquids in the series. Repeat with the fiber in the other orientation, and you will determine both $n_{\perp}$ and $n_{\parallel}$.

The *oblique illumination* test is advantageous when the specific refractive index of the fiber is quite different from the test liquid's. This situation can arise with an unknown fiber. The question is whether the fiber has a much higher or lower index than the liquid. Sometimes it is difficult to tell whether the bold, dark Becke line is moving in or out during focusing, whereas it is easy to tell whether a dark shadow is on one side or the other of the bright fiber illuminated *obliquely* across it. To accomplish this, the *full* condenser is adjusted in focus with its diaphragm wide open. The fiber is oriented $\overset{N}{\underset{S}{\updownarrow}}$ or W↔E, depending on which of the two indices is to be determined. Then a long piece of black cardboard is placed to one side underneath the condenser with its long edge $\overset{N}{\underset{S}{\updownarrow}}$ or W↔E, depending again on which index is being determined. This gives oblique illumination with half a cone of light. If the fiber is of *higher* refractive index than the standard liquid, its dark shadow lies on the same side as the dark side of the field (see Figure 6.21). If the fiber has a *lower* index than the liquid,

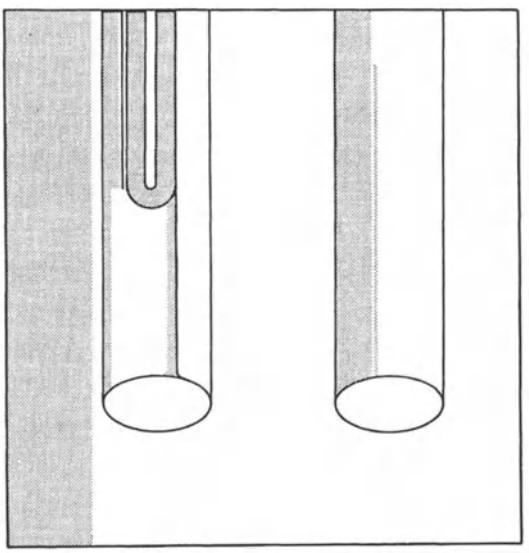


FIGURE 6.21. Oblique transmitted illumination. Glass tube and rod in a liquid of lower refractive index, which has partially filled the tube.

the shadow lies on the side of the fiber that is opposite the dark side of the field.

Sometimes it is important in research to determine $n_{\parallel}$ and $n_{\perp}$ for a series of samples to compare variations in index resulting from some variation in a physical condition, such as percent elongation. Here the birefringence may not vary much, but the precise values of the refractive index may change in some characteristic manner. For example, some acrylic fibers show an increase in both $n_{\parallel}$ and $n_{\perp}$ when stretched, and even though the difference between the two indices (the degree of birefringence) does not noticeably change, the magnitudes of the indices indicate at once that the fibers have been stretched.

The microscopy of fibers is not yet a highly advanced science, but in the relatively short time it has been applied to the technology of textile fibers it has proven to be of great analytical value.[17] It allows the microscopist to distinguish easily between natural and man-made fibers.[18] By comparing results with published values for common fiber classes, some very useful relationships become apparent.[19–21] Properties are found to stem from (1) the chemical (molecular) composition of the fiber, as expected and (2) changes in orientation and spacing of constituent molecules or other structural units during spinning, stretching, or other treatment. This book is not a treatise on fiber chemistry or technology, but some generalizations about structure and composition versus properties will help the microscopist understand how anisotropy comes about, and what governs it.

6.3. ANISOTROPY IN FIBERS

Fibers are usually birefringent due to molecular anisotropy, which originates with electric polarity between adjacent atoms. Figure 6.22 illustrates the three simplest examples:

Figure 6.22a shows a pair of adjacent atoms aligned with the direction of vibration of the polarized light.

Figure 6.22b shows a pair of adjacent atoms aligned at right angles to the vibration direction.

Figure 6.22c shows two atoms relatively far apart.[16]

In all three cases the polarized light waves tend to orient electrons and nuclei of the constituent atoms in the direction of the vibration of the polarized light (e.g., $\overset{N}{\underset{S}{\updownarrow}}$), producing *electric dipoles*. The three kinds of dipole alignments are of different strengths. Each of the *adjacent* dipoles

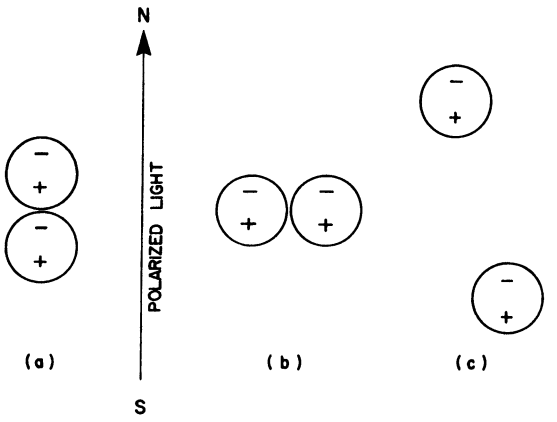


FIGURE 6.22. Effect of polarized light waves on atoms and atom pairs.[16]

aligned parallel to the vibration direction is rendered stronger, and each of the adjacent dipoles aligned perpendicularly is rendered weaker than each of the separated dipoles shown in Figure 6.22c. In long molecules, such as those of cellulose, nylons, polyolefins, and polyesters, atoms are largely chained along the length of the molecule, so that dipoles are strongest when light is vibrating in the lengthwise direction of the molecule.

Molecular birefringence is imparted characteristically to fibers, as suggested in Figure 6.23. The degree with which long molecules grow or come

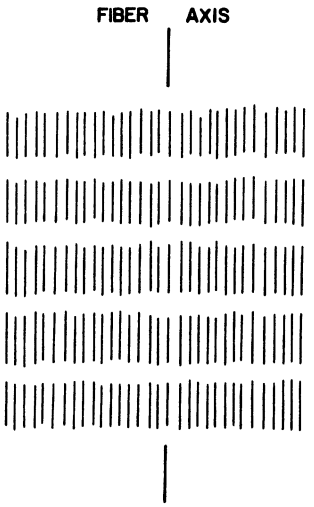


FIGURE 6.23. Idealized alignment of long-chain molecules parallel to the axis of a fiber.[16]

parallel to the axis depends on their formation and subsequent treatment.[17]

With straight-chain molecules substantially like those indicated in Figure 6.23, the refractive index (which is a positive function of the strength of atomic dipoles) is greater for light vibrating parallel to the fiber axis than for light vibrating perpendicular to it; that is, $n_{\perp} > n_{\perp}$, and the fiber is optically positive. For example polyesters are intensely positive; cellulose, nylons, and polyolefins are strongly to moderately positive; but acrylics and cellulose diacetate are only weakly positive[2] because of the opposing polarity of the side groups on each molecule. In fact the third acetyl group of cellulose triacetate has sufficient counterpolarity to turn the positive diacetate into the negative triacetate at room temperatures. As cellulose triacetate fibers are heated, the temperature for *zero* birefringence (T_{ZB}) is reached, above which fibers become optically positive. This phenomenon is explained on the basis of the increasing diameter of the radially polarizing acetyl group as temperature increases. Thus, the dipole movement of the acetyl group decreases to the point where axial polarizability takes over, and the fiber becomes optically positive.[17]

Polymeric fibers are generally assumed to be uniaxial. If so only the lengthwise fiber axis is unique; that is, viewed endwise, i.e., in the cross section, such fibers are *isotropic.* We have just discussed the two characteristic refractive indices $n_{\parallel}$ and $n_{\perp}$; however certain processes (texturizing) involving transverse pressures and heat can lead to a departure from such symmetry and give rise to what may be called a biaxial fiber. Such a fiber, traversely anisotropic, is characterized by three preferred refractive indices—$n_{\perp}$, $n_{\perp 1}$, and $n_{\perp 2}$. Transverse anisotropy has been observed in textured yarns where filaments have been given crimps, loops, coils, or crinkles (to give the yarns properties more like those of wool, for example). Among the texturizing processes, the false-twist process is probably the fastest growing. Its fundamental operations are twisting, heat setting, and untwisting. The result, so delightful to the textile technologist, may appear to the microscopist as a mixed-up mess (see Figure 6.24).[20] The problem, would be relatively simple if we were dealing with one filament at a time (see Figures 6.25 and 6.26); instead filaments are purposely linked. During texturizing the shape of the cross section has been transformed from a circle (see Figure 6.27) to a polygon (see Figure 6.27b).[21,22]

6.4. MOLECULAR ORIENTATION AND ORGANIZATION

Anisotropy due to the orientation and organization of molecules into fibers occurs in many steps of growth or manufacture. Here we confine

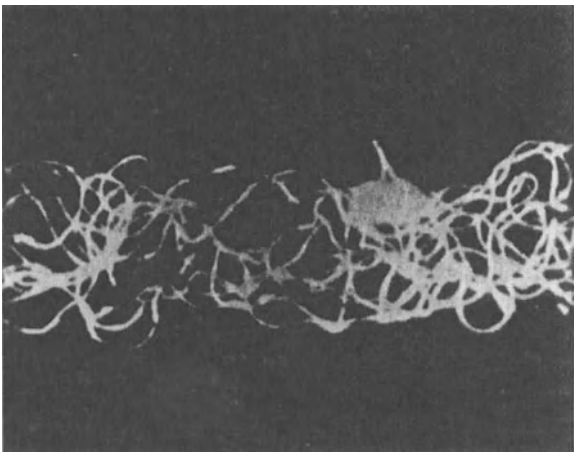


FIGURE 6.24. A typical, false-twist, textured yarn.[21]

ourselves to man-made fibers, since the steps are designed to vary mechanical properties and much can be learned about the process from the various optical properties.

1. *Spinning.* The organization, orientation, and distribution of molecules begin with spinning, either spinning from the melt or spinning from an aqueous (wet) solution or an organic (dry) solvent. Spinnerettes are

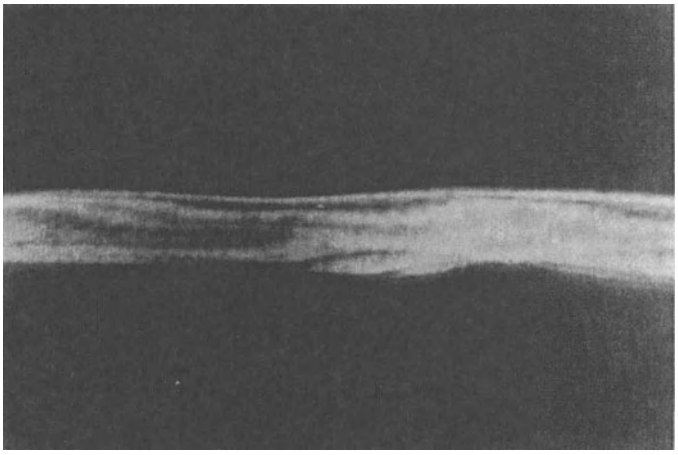


FIGURE 6.25. Textured nylon filament in white light, with crossed polars and universal stage axis A_4 at 45°.[21]

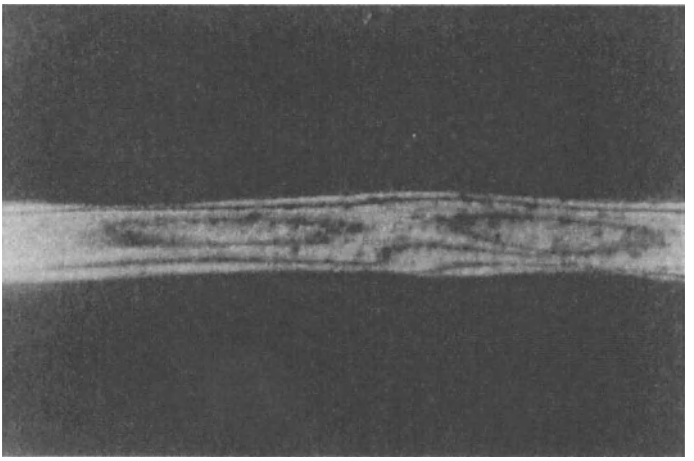


FIGURE 6.26. Same as Figure 6.25, but the universal stage axis A_4 is at 315°.[21]

designed to vary the external shape of the fiber, its internal solidarity or hollowness (number of canals) and by dual spinning, the texture of the fiber. The kind of liquid and design of the spinnerette can affect the orientation of molecules radially (skin-and-core structure) as well as axially (flow birefringence).[9,17]

2. The method and rate of *coagulation* or jelling can affect the optical and mechanical properties of the spun product.[23] The process can be simulated to some extent under the microscope.[24] Postmortems can be performed on commercial lots of dry-spun versus wet-spun fibers.[9] Dry-spun fibers can be coagulated quickly, and wet-spun fibers can be coagulated slowly; fast spinning can favor skin formation over slow spinning.

Scott showed with experimental polyethylene terephthalate ribbons that drawing conditions and treatment can produce a variety of structures

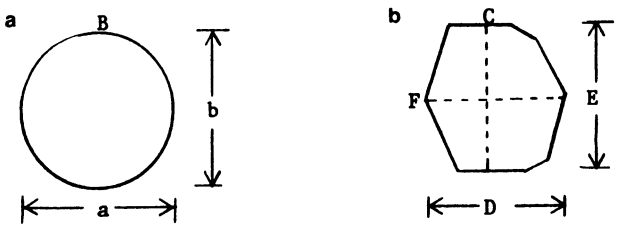


FIGURE 6.27. (a) Diagram of a circular cross-section of an untextured filament.[21] (b) Diagram of a polygonal cross-section of a textured filament.[21]

and corresponding properties.[17] The variation originates with the type of neck produced in the beginning of the stretch-reduction process (necking down). Figure 6.28 gives the shapes of three types of necks. Figure 6.28a shows the usual shape with most plastics; it has sloping shoulders and a concave throat. A core stretches before its skin. Polyester, as spun, draws this way[17] but if the spun polyester is first aged and then drawn, the skin draws before the core, producing the second type (see Figure 6.28b), the lustrous kind. Figure 6.28c shows the third type, produced by drawing the aged as-spun polyester over a knife edge, so that the side away from the edge draws first, producing a clear product of elongation.

Figure 6.29 shows slip planes and lines, the slip front, and filmy luster voids; it also shows the spindle voids typical of polyester aged before drawing, whether or not it also has filmy, luster-producing voids. Both types of voids probably contribute to *form-birefringence.* The degree of contribution can be checked by measuring birefringence immediately after mounting in a penetrating liquid and again after penetration by the liquid, or as Scott did,[17] by measuring birefringence in thick and thin sections.

Aging allows sufficient time (4 days) for crystallization to take place to the extent of about 40%. At the end of 4 days, Scott observed spherulitic nuclei. These nuclei tie molecules together, stiffening the as-spun structure to the extent that slip planes form during deformation, as in metals.

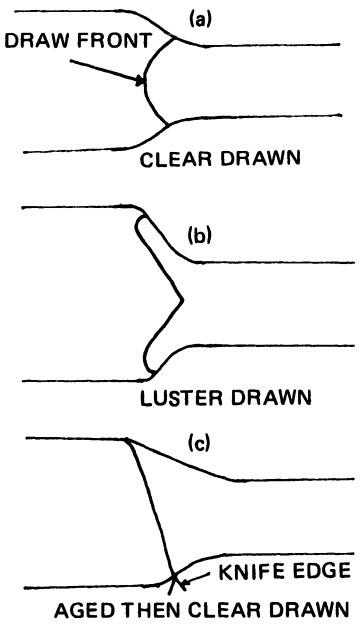


FIGURE 6.28. Scott's three types of reduction in an experimental polyethylene terephthalate ribbon.[17]

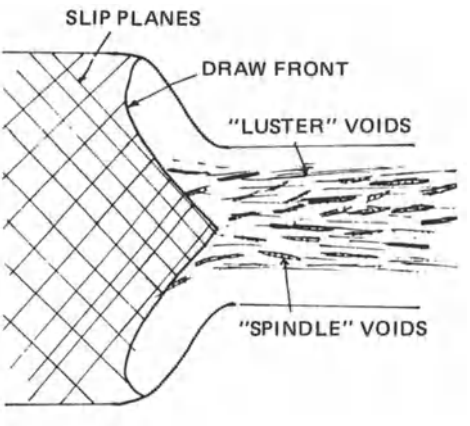


FIGURE 6.29. Another view of Figure 6.26b (Scott's diagram), polyethylene ribbon, showing slip planes, draw front, and voids.[17]

Thus we see that birefringence in fibers is inherent in molecular structure, developed by crystallization, produced by the form orientation of two or more phases, or developed by stress.

6.5. TRANSPARENT SHEETS, FOILS, AND FILMS

Strain as a result of stress in inorganic or organic glass is visible between crossed polars. Indeed organic sheets, such as those of poly(methyl methacrylate), are used in models of structures such as bridges and buildings when studying the location and extent of strain under stress due to the photoelastic effect between crossed polars. Some plastic sheets, such as those used as windows, are prestressed uniaxially or biaxially. Transparent foils and ribbons are often used to study dynamically the effects of tension or compression.

Planar molecules are especially interesting if the molecular planes lie in the plane of sheets or foils, as shown in Figure 6.30, for then a uniaxial (or biaxial) interference figure is visible.[16]

6.6. FIBER IDENTIFICATION

A light-microscopical examination of a longitudinal section mounted in mineral oil of refractive index 1.48 usually identifies wool easily (see Figure 6.1). Should this approach fail, a cross section can be made with a Hardy or another microtome to examine the fiber interior.[1,2] *Natural fibers* can generally be identified by differentiating among their morphologies.

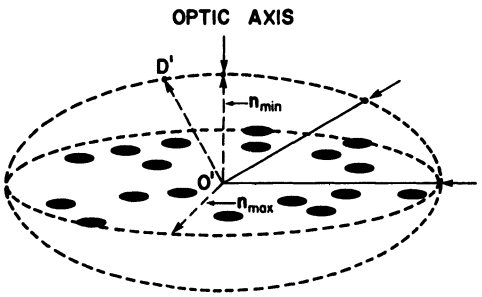


FIGURE 6.30. Planar molecules in sheets or foils.[16]

Man-made fibers, however, vary in morphology within limits only of the shape of the spinnerette's orifice, almost regardless of the constituent polymer's chemical composition. However man-made fibers differ widely in their *optical* properties, particularly in the refractive index parallel to the fiber's axis (ε or $n_{\parallel}$) and perpendicular to the fiber's axis (ω or $n_{\perp}$), thus the difference between the two (birefringence: $\varepsilon - \omega$ or $n_{\parallel} - n_{\perp}$). Some typical values are shown in Table 6.1.[1]

While classifying fibers according to refractive indices appears straightforward (as in Table 6.1), the quantitative determination can be tedious and unnecessary because fibers have a total of eight determinating *optical* properties, which are quite readily obtainable with a microscope made for polarized light. The value of the birefringence is often sufficient to identify most fibers.

TABLE 6.1.
Typical Values of Physical Properties for Identifying Fibers[a]

Fiber	Mp[b] (°C)	Refractive Index[c]		Birefringence[c,d] ($\varepsilon - \omega$)
		Parallel to Fiber Axis (ε)	Perpendicular to Fiber Axis (ω)	
Acetate	260	1.479	1.477	0.002
Acrylic	dnm[b]	1.520	1.524	-0.004[e]
Anidex	s 190	*f*	*f*	*f*
Aramid				
Nomex®	371	1.790	1.662	0.128
Kevlar®	425	2.322	1.637	0.685
Asbestos		1.5–1.57	1.49	0.01–0.08
Cellulosic				
Flax	dnm	1.596	1.528	0.068
Cotton	dnm	1.580	1.533	0.047

Fiber	Mp[b] (°C)	Refractive Index[c] Parallel to Fiber Axis (ε)	Refractive Index[c] Perpendicular to Fiber Axis (ω)	Birefringence[c,d] (ε − ω)
Fluorocarbon	288	1.37		
Glass (inorganic)	s 570	1.547	1.547	0.000
Modacrylic	dnm	1.536	1.531	0.005
Novoloid	dnm	1.650	1.648	0.002
Nylon				
Nylon-6	219	1.568	1.515	0.053
Nylon-6,6	254	1.582	1.519	0.063
Qiana®	275	1.554	1.510	0.044
Nylon-4	265	1.550		
Nylon-11	185	1.55	1.51	0.04
Nytril	176	1.484	1.476	0.008
Olefin				
Polyethylene	135	1.556	1.512	0.044
Polypropylene	170	1.530	1.496	0.034
Polycarbonate	294	1.626	1.566	0.060
Polyester				
2GT[g]	256	1.710	1.535	0.175[h]
4 GT[i]	227	1.690	1.524	0.166
CHDM-T[j]	283	1.632	1.534	0.098
Oxybenzoate[k]	225	1.662	1.568	0.094
Rayon				
Cuprammonium	dnm	1.548	1.527	0.021
Viscose	dnm	1.547	1.521	0.026
Saran	170	1.603	1.611	−0.008
Silk	dnm	1.591	1.538	0.053
Spandex	230	1.5		
Triacetate	288	1.472	1.471	0.001
Vinal	dnm	1.543	1.513	0.030
Vinyon (PVC)	dnm	1.541	1.536	0.005[e]
Wool	dnm	1.556	1.547	0.009

[a]Courtesy of the American Society for Testing and Materials.
[b]dnm indicates the fiber does not melt; s indicates softening point.
[c]The listed values are for specific fibers that warrant the highly precise values given. For identification purposes, these values should be regarded as indicating only the relative values of the properties.
[d]Approximate values.
[e]Varies; always weak; sometimes negative.
[f]The fiber is opaque.
[g]ethylene glycol type.
[h]Staple and fully oriented filament yarns (FOY), partially oriented yarns (POY), and undrawn yarns may have much lower values of birefringence and refractive index.
[i]1,4-butanediol type.
[j]1,4-cyclohexanedimethanol type.
[k]*p*-ethylene oxybenzoate type.

6.7. SUMMARY

Microscopy is necessary for studying the *morphology* and *structure* of fibers. Microscopic sizes of fibers and their parts are resolved in terms of the metric system unit, the micrometer (μm), formerly called micron (μm), for one-millionth of a meter. The angstrom (Å) (0.1 nm) remains in the literature, although it is not an official unit. The theoretical and practical limits of fiber morphology were first determined by means of light microscopy. Transmission electron microscopy added its advantages of resolving power and resolution about the same time that various properties of man-made fibers were being modified by varying manufacturing conditions. Scanning electron microscopy added its own advantages, especially the contrast and realistic, three-dimensional aspects; accordingly some 13 scanning electron micrographs introduce this chapter to illustrate variations in morphology. Such variations serve especially well in *natural* fibers to describe and therefore identify them. Among *man-made* fibers, however, variations in *optical properties* are more important for identification, since they vary more than morphology. Such fibers are made of the melt or solution of high-polymer extruded through a spinnerette of a certain size and shape. During and/or after spinning, filaments are subjected to a certain kind and degree of stress. Such treatment results in a distinct structure, composed of macromolecular domains, spherulites, and/or stress patterns. These are specifically manifested in reaction to *polarized* light; indeed about eight different optical properties are determinable by means of a polarizer, analyzer, Michel–Lévy interference color chart, and rotatable stage. Furthermore polarized light microscopy is both descriptive and determinative with polymeric sheets, foils, moldments, castings, and any other sufficiently transparent anisotropic material. Thus polarized light microscopy is unique among all other kinds of microscopy.

Natural fibers are named in terms of their origin: animal, vegetable, or mineral, such as wool, cotton, or asbestos. Man-made fibers are named for regenerated material or its treatment. Examples of a trade name (two words) are nylon and acrylic (not capitalized). Orlon® and Acrilan® are trademarks (one word) that are registered in the U.S. Patent Office for exclusive use by the sole owners.

7

Microscopical Properties of Crystals

7.1. STRUCTURAL CLASSIFICATIONS

A solid *crystal* is composed of atoms, ions, or molecules arranged in a pattern that is periodic in three dimensions.[1] There are six *structural*[2] classifications: *isometric, tetragonal, hexagonal,*[1] *orthorhombic, monoclinic,* and *triclinic* (see Figure 7.1a).[2]

Bravais lattices for the 14 possible unit cells are shown in Figure 7.1b.[2a] In a crystal unit cells are the basic space-filling units. They can be repeated in the three dimensions and in this way generate the total crystal. The uniqueness of the basic six crystal systems depends on their axial dimensions and internal structural angles. Geometries of the crystal systems are given in Table 7.1.[2a]

The rhombohedral class (of which the calcite phase of $CaCO_3$ is a typical example) can be considered in the hemihedral class of the hexagonal system, since it is made up of alternate faces of a hexagonal bipyramid.[2a] Structure determines the inherent properties of a crystalline compound; for example the three crystalline phases of $CaCO_3$, calcite, aragonite, and vaterite, all have different crystalline structures; different solubilities; and different melting points, hardnesses, and optical properties.

7.2. MORPHOLOGY

Crystals are also characterized by their *morphology,*[1] that is, their sizes and shapes, as grown from solution, melt, vapor, or by altering other crystals. The morphology of a crystal determines its rate of change, that is, its rate of dissolution, melting, or other phase transformation, because

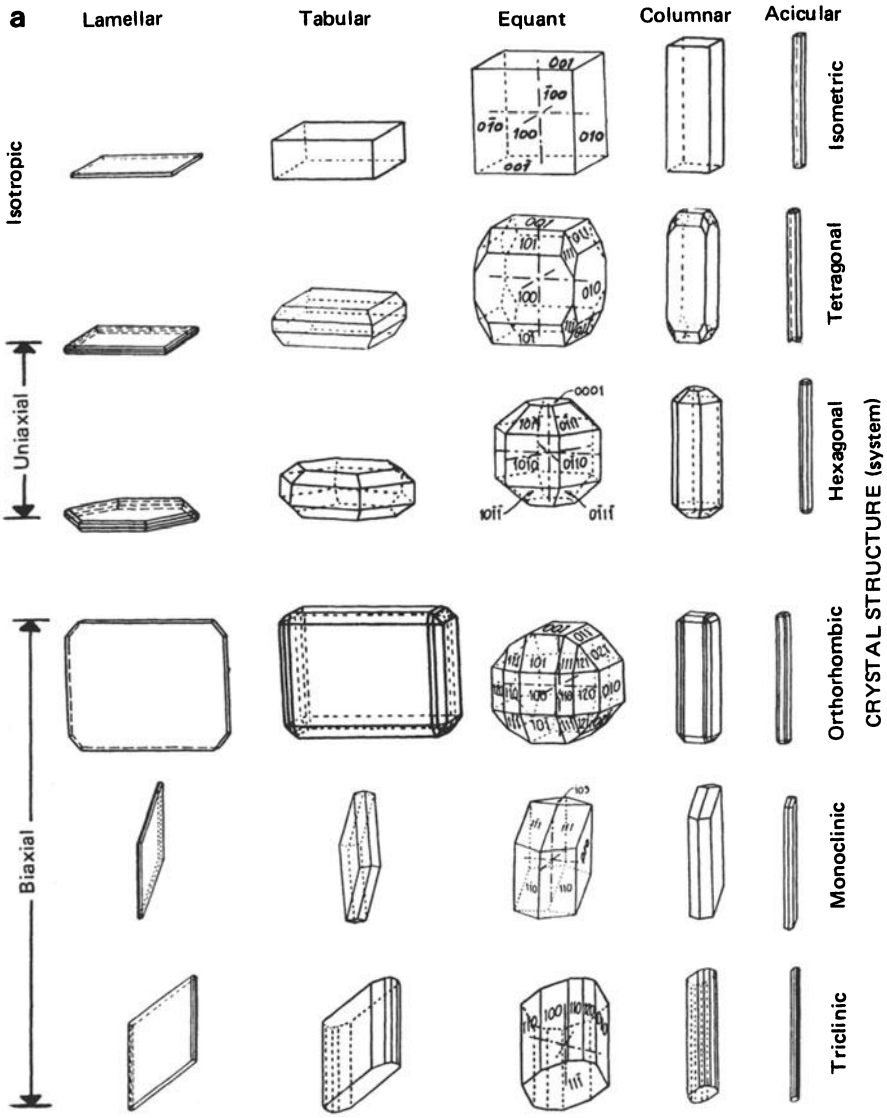


FIGURE 7.1. (a) Crystal structure and morphology.[2] The trigonal (rhombohedral) system is not represented here, but see Figure 1a. (b) The 14 Bravais lattices, representing the fundamental, three-dimensional, space-filling patterns.[2a]

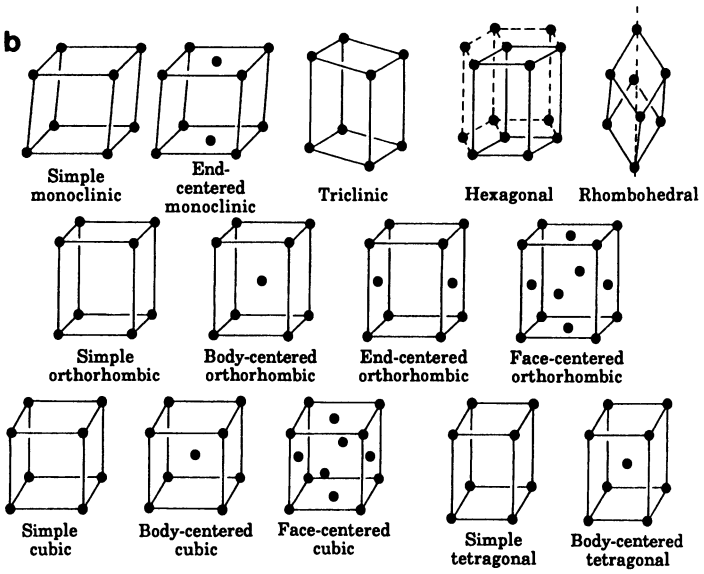


FIGURE 7.1. (*Continued*)

morphology controls its specific surface. A crystal grown slowly from solution, manifests characteristic *faces* related to the structure. Thus one kind of face, called a *form*,[1] has constant angles, whether the crystal is equally developed (equant), long (columnar or acicular), or flat (tabular or lamellar).[2] Therefore *each* of the six or seven kinds of structure has *five* kinds of naturally polygonal morphology, called *habit*, as illustrated in Figure 7.1.[2]

Crystals grown from solution[3] or by precipitation[4,5] on a microscopical slide are seldom equant because the drop of mother liquor is

TABLE 7.1.
Axes and Axial Angles of Crystal Systems.[2a]

System	Axes	Axial angles
Cubic	$a_1 = a_2 = a_3$	All angles = 90°
Tetragonal	$a_1 = a_2 \neq c$	All angles = 90°
Orthorhombic	$a \neq b \neq c$	All angles = 90°
Monoclinic	$a \neq b \neq c$	Two angles = 90°; 1 angle ≠ 90°
Triclinic	$a \neq b \neq c$	All angles are different; none equal 90°
Hexagonal	$a_1 = a_2 (= a_3) \neq c$	Angles = 90° and 120° (or 60°)
Rhombohedral	$a_1 = a_2 = a_3$	All angles are equal, but not 90°

shallow and difficult to stir while under the objective. Common salt (NaCl) for example recrystallizes from a drop of solution on a slide not as equant cubes but as long, flat rectangular shapes (see Figure 7.1a), sometimes tipped to make interpretation all the more challenging. The terms for habit vary in the literature,[3–5] but the structure remains constant. For this reason face angles and optical properties are also constant, no matter what the habit.

7.3. MILLER INDICES

Figure 7.1a shows the *Miller indices* for typical faces of equant habits. These indices are reciprocals of the fractional intercepts that a face makes on crystallographic axes. The axes are graphic (typical spacing directions of the structural units, whether atoms, ions, or molecules). The form representing the isometric system in Figure 7.1a is the cube, a special hexahedron whose face angles are all right angles. It is a closed form; that is, all of the faces are structurally alike (regardless of the dimensions of the edges in the long flat habits). As the axes are set up in the equant habit in Figure 7.1a, the front face cuts one axis by one unit (1) and the other two axes at infinity (∞), so the Miller indices are $1/1:1/\infty:1/\infty = 100$. The back face is the same, but to show that it is cut in back by one unit, its specific Miller index is $\{\bar{1}00\}$. The right side is written {010} and the left $\{0\bar{1}0\}$, the top {001}, and the bottom $\{00\bar{1}\}$. The typical index for the cube is simply written {100}. If we set up the axes diagonally, then the typical index would be {110}, but in this instance nothing is to be gained by doing so. The Miller indicesof the lamellar, tabular, columnar, and acicular habits are exactly the same as those of the equant habit (cube). It does not matter that the edges are of various lengths except that there are more unit cells (all isometric) attached along the longer edges. How do we prove isometry microscopically? If the crystals are isotropic (black) in every orientation as observed between crossed polars, they are isometric.

It is easy to see why a cubic crystal is isotropic. Since unit spacing is the same along the three axes, the velocity of light (and refractive index) for a particular wavelength is the same in any direction at any one temperature. For sodium chloride, NaCl, the refractive index, $n_D^{20°C}$, is 1.544; for potassium chloride, KCl, $n_D^{20°C}$, is 1.490. While NaCl and KCl are both isometric and isotropic, they are *not isomorphous*[1]; that is, they do not crystallize together as mixed crystals in a *solid* solution. Sodium and potassium ions are not interchangeable in the crystal structure (see Figure 7.2), but ammonium ions are interchangeable with potassium ions in crystals of their chlorides to make a continuous isomorphous[1] series with

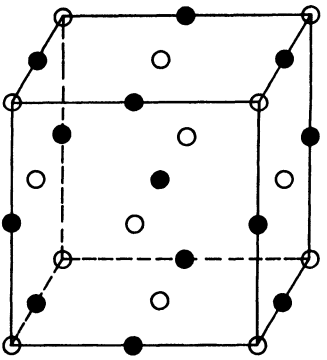


FIGURE 7.2. Isometric ionic lattice. ● represents positive ion, such as K^+ or NH_4^+, interchangeably. ○ represents negative ion, such as Cl^-, Br^-, or I^- (interchangeably).

refractive indices varying from $n_D^{20°C} = 1.49$ for pure KCl to 1.64 for pure NH_4Cl, in direct proportion to the potassium and ammonium content.[6] Hence the microscopical refractive index of a mixed crystal coupled with the isotropy is very descriptive and determinative once the presence of NH_4^+ and K^+ is established.[4] Rubidium and cesium ions are also interchangeable with K^+ and NH_4^+ and thus could influence the optical properties, but their absence or presence can be detected by other tests.[4]

If NaCl in solution contains a small amount of OH^- ion, such as from urea, *octahedral* faces appear on its crystals with or without cubic faces.[7] Miller indices of the octahedral faces of NaCl are all {111}; that is, all three axes are intercepted equally. The structure of the NaCl remains the same: isometric; therefore octahedral NaCl remains isotropic, with the same refractive index, $n_D^{20°C} = 1.544$, as cubic NaCl.

7.4. ISOMORPHISM

The alums are a remarkable set of isometric–isotropic crystals with interchangeable ions, all patterned after their namesake, potassium aluminum sulfate dodecahydrate, $K_2SO_4 \cdot Al_2(SO_4)_3 \cdot 24H_2O$. The *water of hydration* of an alum, too, is in the space lattice[8] because it is in the ions' coordination sphere. The K^+ may be replaced by NH_4^+, Na^+, Cs^+, Rb^+, or Ag^+; the Al^{3+} may be replaced by Cr^{3+}, Fe^{3+}, Mn^{3+}, In^{3+}, Ga^{3+}, or Tl^{3+}; the SO_4^{2-} may be replaced by SeO_4^{2-}.[3] Moreover a crystal of one pure alum (e.g., a colored one) acts as a foundation for an overgrowth of another (e.g., a colorless one). The substitution of various ions for one another and the overgrowth of one alum on another are both manifestations of isomorphism.[3] All interchangeable ions fit into specific places in a single crystalline pattern that is *isometric* in structure and *octahedral* in habit. True the re-

fractive index varies with composition, but it varies directly with the proportional content of substituted ion. This is an analytical advantage, for it allows rapid determination of composition once constituent ions are known. Some other microscopical tests often identify ions, too: Cesium alum for example is much less soluble than other alums, and it can be detected as it precipitates at low concentration from a drop of solution on a slide.[4]

7.5. SKELETAL MORPHOLOGY

The morphology of mature euhedral[6–9] crystals is illustrated in Figure 7.1a; no matter how small, they are filled out to the full extent shown. Immature crystals can look different and be misinterpreted. If for example NaCl crystals are grown from a very shallow drop (one spread over too much of the microscopical slide), *hopper-shaped* crystals may develop. Because the drop is so shallow, the top face has run out of solute while the meniscus of mother liquor attached to each vertical wall has supplied solute to build the walls higher than the top surface. As such a crystal grows, it becomes dished like a rectangular hopper. Unless we focus up and down with an objective of short depth of focus (high NA) or use oblique illumination, we may misinterpret the depression for an elevation—indeed for a set of faces.

Dendrites are even less filled-out crystals. They are fernlike, treelike, branchlike skeletal crystals that have been grown so fast that they have not had time to form faces and are therefore *anhedral.*[9]

Crystals grown from the melt[3,10,11] usually meet their neighbors in more or less straight polygonal boundaries of crystalline *grains,* as in igneous rocks, cast metals, frozen water, and other solidified liquids. This action may be observed by watching water or other liquids freeze on a cold stage.[4] Many organic and some inorganic solids can also be observed during melting and freezing on a hot stage.[3,10,11] Thus dynamic processes can include changes in temperature gradients, annealing and quenching, concentration and distribution of components and impurities, changes in morphology (sizes and shapes of grains), and variations in structure (polymorphic transformations). Such knowledge can lead to a better understanding of materials as they undergo changes during manufacture, bulk-testing, use, storage, weathering, or failure. The experience is also very helpful in forensic investigations.[12] Usually metals, ceramics, and other inorganic systems melt at too high a temperature to be studied directly on the hot stage of a microscope. In such cases experiments are conducted elsewhere, and samples are taken and studied under the microscope. Here

experience with low-melting-point materials as they undergo dynamic changes aids in interpreting appearances of high-melting-point or opaque materials.[10]

The polyhedral boundaries of crystalline grains, such as those grown from the melt in igneous rocks and certain ceramics, are the result of close packing, like crowded soap bubbles. A crystalline grain can be a single crystal, a twinned crystal, or a spherulite. Each has its characteristic optical properties. Transparent single crystals grown from the melt can be studied microscopically like euhedral[9] ones except that optical properties cannot usually be related to grain boundaries because they do not consistently represent crystallographic faces. Yet certain optical properties of anhedral[9] crystalline grains can be interpreted in terms of the crystallographic axes, and these are worth noting. For example (see Figure 7.1), in the *tetragonal* and *hexagonal* (including trigonal) systems, there is only *one unique axis.* Such crystals are *uniaxial* because the unique axis represents the only direction down which the perpendicular field of view appears to be isotropic. That is, viewed along that axis the field is dark throughout 360° of rotation between crossed polars. This occurs because the unique axis (the *c*-axis) is at right angles to the plane of the other two (tetragonal, *a, a*) or three (hexagonal, *a, a, a*) along which the spacing is the same. Therefore looking down the *c*-axis, the isometric plane likewise is perpendicular to the axis of the microscope, and we see the *one plane of isotropy.*

In the *orthorhombic, monoclinic,* and *triclinic* systems (see Figure 7.1a), spacings are different in all three crystallographic directions, so there is no plane of equal spacing. However there are two directions in each whose field of view is isotropic (dark throughout 360° of rotation of the specimen between crossed polars). These, the two *optic axes,* are the directions in which light travels with no apparent birefringence.[3] A bisectorial view includes both optical axes *if* the angular apertures of the condenser and the objective are wide enough. It follows that there is an *acute bisectrix*[1] and an *obtuse bisectrix,*[1] and each is recognized by its characteristic interference figure (see Chapter 5).

These and some other crystallographic and optical properties may be determined on unicrystalline grains in thin sections (≈30 μm) of rocks, minerals, and man-made products[6,13] within a wide range of grain sizes. Searching for grains of adequate orientation[14–16] may be time consuming. If so, a spindle[15] or a universal stage[17] to orient the specimen conveniently may be desirable.

If it can be determined whether a crystal (euhedral or anhedral) is isometric, uniaxial, or biaxial, a classification of sorts can be made on this basis alone. Thus Table 7.2 shows the distribution of 935 frequently encountered crystalline phases, data compiled by Winchell[6,13] and classified

TABLE 7.2.
Distribution of 935 Crystalline Phases among Isotropic, Uniaxial, and Biaxial Crystals[6,18]

System	Number of phases		Percentage Total Inorganic Organic
	Inorganic[a]	Plus Organic[b]	
Isotropic	86 (17.0, 9.2)	7 (1.6, 0.8)	10.0
Uniaxial	101 (19.9, 10.8)	49 (11.5, 5.2)	16.0
Biaxial	315 (62.1, 33.7)	359 (83.9, 38.4)	72.1
Intermediate	5 (1.0, 0.5)	13 (3.0, 1.4)	1.9
Totals	507 (100.0, 54.2)	428 (100.0, 45.8)	100.0
Grand total,[3] number of phases = 935			

[a]Values in parentheses represent, respectively, the percentage of the total number of inorganic phases and the percentage of the total number of inorganic plus organic phases.
[b]Values in parentheses represent, respectively, the percentage of the total number of organic phases and the percentage of the total number of inorganic plus organic phases.

by Kirkpatrick.[18] Only 10% of the 935 are isotropic, and most of these are inorganic (probably because the coordination spheres of the constituent ions require such a high degree of symmetry). Sixteen percent of the 935 phases crystallize in the uniaxial systems, one-third of them are organic compounds. And 72.1% of the 935 phases are biaxial, more than half of them organic compounds, showing how relatively large molecular units of organic compounds pattern themselves.

7.6. ISOTROPIC SYSTEMS

For a given wavelength and a given temperature, there is only one value for the refractive index of an isometric crystal, since it is isotropic. Figure 7.3[18] shows the distribution of the approximate refractive indices (with white light at room temperatures) for inorganic isotropic substances. The peak value is about 1.45 (25% of the total) for values falling between 1.33–2.0.[3] For each species of isometric crystal, there is some variation in refractive index with respect to the wavelength, called *optical dispersion.*[1] For example the refractive index of $2Na_3PO_4 \cdot NaF \cdot 19H_2O$ for the sodium D line is 1.452, and the refractive index for the sodium C line is 1.451. Optical dispersion is

$$n_C^{25°C} - n_D^{25°C} = 1.451 - 1.452 = -0.001.^{[19]}$$

Dispersion is just as much a determinative optical property as any other. The degree of dispersion is also important in *dispersion staining,*[20,21] in

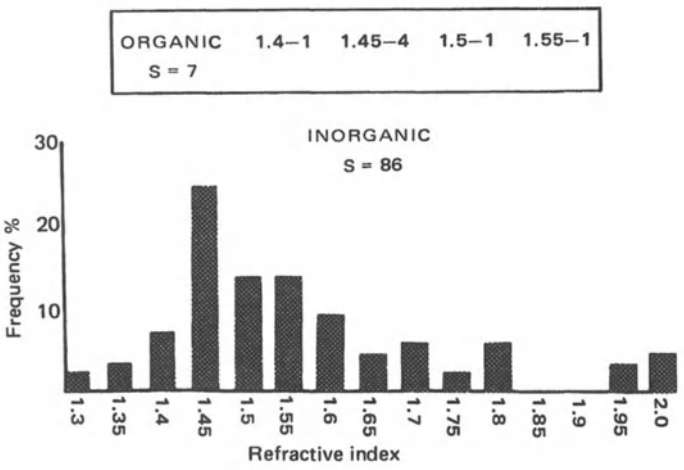


FIGURE 7.3. Distribution of the approximate refractive indices (with white light at room temperature) for inorganic isotropic substances.

which differences in refractive index for some wavelengths (but not others) between crystals and mounting liquid lead to colored outlines (see Chapter 9).

It is relatively easy to recognize octahedra {111} when they are equant; see Figure 7.4a. The octahedron is lying on one of its equilateral triangles (dotted). The opposite one is shown in Figure 7.4a (solid outline), pointed the opposite way. The other six sides are *alternately* pointed *up* or *down*, connected by edges that alternately go from top to bottom or vice versa. These six zigzag edges, *in perspective*, make an equilateral hexagon, which is not to be confused with a truncated bipyramid in the hexagonal system

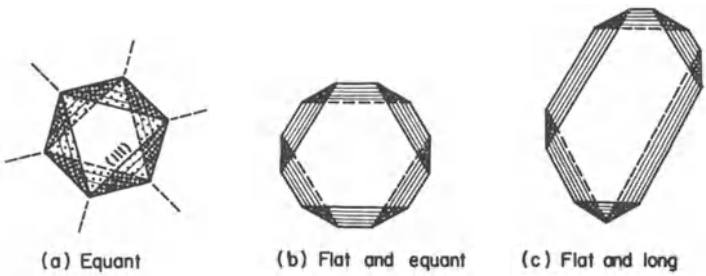


FIGURE 7.4. Drawings[3] of octahedra, each lying on an octahedral face and all three showing the same angular symmetry.

(or anything else), since there are the *doubly shadowed* up *and* down portions that look like smaller, darker triangles in perspective. These small dark triangles are also shown in the *flat* octahedra (see Figures 7.4b and 7.4c), but the equant and flat octahedron (see Figure 7.4b) shows in perspective 12 side lines instead of six, as shown in Figure 7.4a. The long, flat octahedron (see Figure 7.4c) is most difficult to interpret because the side lines are not of equal length. However the *angular* symmetry is still the same, and that is what indicates the *isometry;* the isotropy clinches the case. Incidentally the shadows (shading) in all three drawings (see Figure 7.4) represent the partial reflection of incident light away from the microscopical objective. The degree of shading represents the obliquity of the side faces.[3]

In addition to the cube and octahedron, the isometric system is represented (less frequently) by the rhombic dodecahedron (as of hexamethylene tetramine) and pentagonal dodecahedron (as of cerium formate). There is also the hemihedral class of the octahedron, i.e., the tetrahedron (as of sodium uranyl acetate).[4]

7.7. UNIAXIAL CRYSTALS

In the *tetragonal* and *hexagonal* systems (see Figure 7.1), there is one unique axis, namely, the *c*-axis, along which spacing of the structural units is shorter or longer than along the *a*-axes, which lie in a plane perpendicular to the *c*-axis. In the tetragonal system there are two *a*-axes at 90° to each other; in the hexagonal system there are three *a*-axes at 120° to each other. In either case in any plane, light vibrates in one direction at a constant velocity, and therefore the ordinary refractive index ω is always "seen." On the other hand the extraordinary refractive index ε is seen only if the crystal is lying on a prism face, such as {100} or {110}. On an inclined face (*e.g.*, pyramidal or rhombohedral), the refractive index for light vibrating at right angles to the always-seen ω is somewhere between the values for ω and ε; it is represented by the symbol ε'. For crystals lying on a definite face inclined to the *c*-axis (such as a pyramidal or rhombohedral face), the value for ε' is *constant* for that *particular form* (kind of face). Values for ε' are not generally given in compilations,[2,6,13] so the reader should start a collection of ε' values in some retrievable manner. We emphasize that the values ω, ε, and ε' are for crystallographically oriented vibrations. If there is any doubt about to the orientation of the axes, the more abstract and general symbols,[9] such as n_1 and n_2, should be used.

The plane of the *a*-axes is the basal pinacoid. Crystals resting on this face appear dark in every position of retardation between crossed polars.

Most uniaxial crystals recrystallized on a microscopical slide do not rest on a basal pinacoid, but some species do (for example iodoform, lead iodide, cadmium iodide, and Na_2SiF_6). Uniaxial crystals with low-enough melting points, such as sodium nitrate, may be melted on a slide, covered with a cover glass, and recrystallized from the melt. The resultant crystalline *grains* can be examined for the isotropic view. If either of these two methods fails to produce an isotropic view and if a universal stage[17] is available, it can be employed to rotate the crystal 180° in three perpendicular directions. Such a stage can be used with almost any small single crystal, whether it is a polygon, fragment, sand grain, sawed section, or product of fusion.

Once an isotropic view is sighted, the top lens of the condenser is put in place and the diaphragm is opened all the way to give *conoscopic* illumination and an interference figure.[3] (An objective of NA = 0.85 is preferred if its presence is assumed in tables of computation.)[14] Next the Bertrand lens (see Chapter 5) is put into place in the microscope's tube below the ocular to provide a compound microscope focused on the *back aperture* of the first objective. If no Bertrand lens is provided, the ocular is removed and the back aperture of the objective is viewed directly, i.e., without magnification. If the crystal is uniaxial and the microscope is properly aligned, the isotropic view provides a *centered uniaxial interference figure* or pattern. As shown in Figure 7.5, there is a dark Maltese cross ringed with interference colors of numerous orders, depending on the degree of birefringence. The rings of interference colors of Newton's series and the Michel–Lévy's chart represent the increased retardation derived from increased angularity from the axis to the periphery of the cone of

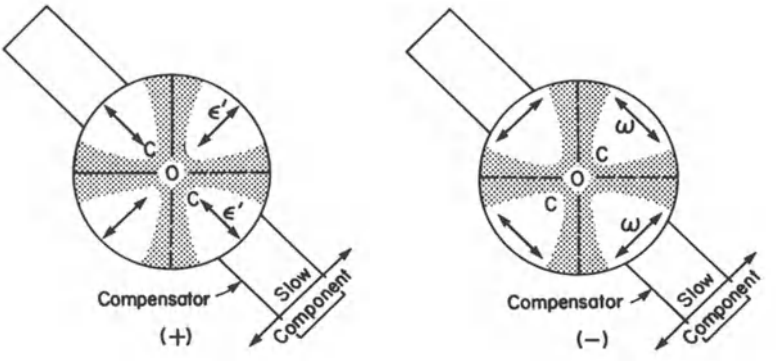


FIGURE 7.5. Diagram of uniaxial interference figures, positive (left) and negative (right). Vibration direction of slower components are indicated by arrows, *c*, *c* represent quadrants in which compensation occurs. *o* represents the optic axis.[3]

illuminating rays. In other words rings of *isochromes* are summations of retardations corresponding to variations in incident ray orientation, with reference to the crystal's *c*-axis. The black arms of the Maltese cross are called *isogyres* of the interference figure.[3] The cross is similar to the one shown by spherulites, such as starch grains, and for the same reason. They represent positions of extinction $\overset{N}{\underset{S}{|}}$ and W↔E. Instead of a sphere of crystallites, we have a sphere of directions. Indeed we can obtain the same effect by illuminating a sphere made of the uniaxial crystal with axial light, employing crossed polars.

What good is an interference figure? If it indicates that the crystal is *uniaxial*, we immediately know that we are dealing with a species that represents only 16% of a total of 935 crystalline species (see Table 7.1). If the substance is *organic*, it is among only 5%; if it is *inorganic*, the probability is 11%. Thus uniaxiality alone is distinctive and determinative.

Moreover, the optic sign is easily obtained from an interference figure.[3,15] If a first-order (1°) red retardation plate is inserted into the NW\SE diagonal with its slower component vibrating SW/NE, the color near the center of the (uniaxial) black cross is yellow (subtraction) in the NW and SE quadrants, and the crystal is optically positive. If instead the color is blue (addition), the crystal is negative. Or the quarter-wave (0.25λ) plate can be used. Then if a black dot is seen NW and another SE of the center of the black cross, the crystal is positive; if NE and SW, it is negative. Otherwise a quartz wedge can be moved in place with increasing order of interference color. If the crystal's colors move *outward*, it is positive; if *inward*, it is negative.[15] From Figures 7.6 and 7.7 we obtain a distribution (percentage of probability) along the ordinates for uniaxial positive and negative crystals among *inorganic* and *organic* species with regard to ω (abscissa). This is the sole value seen down the unique axis (*c*-axis). Thus the combination of uniaxiality and quantitative value for ε′ is very descriptive. The combined data placed on punched cards or fed into a computer become very selective, especially at the extremes of the distributions.

Off-centered figures can be used not only to obtain the optic sign but also to show the vibration direction for ε′.[3] For a given path length, the more rings in the off-centered figure, the closer ε′ approaches ε in value. Therefore measuring the tilt angle of the optic axis (*c*-axis) and adding the value for ω provide an estimate of ε.[15] Determining values for ω and ε for different wavelengths of light establishes the degree of *dispersion*; in very distinctive rare cases, the optic sign may even change.

A *euhedral crystal* resting on a prismatic face {100}, {110}, {1010}, or {0100} presents ω and pure ε. These may be measured directly by any of the methods discussed in Chapter 6, or elsewhere.[3,15] The difference gives

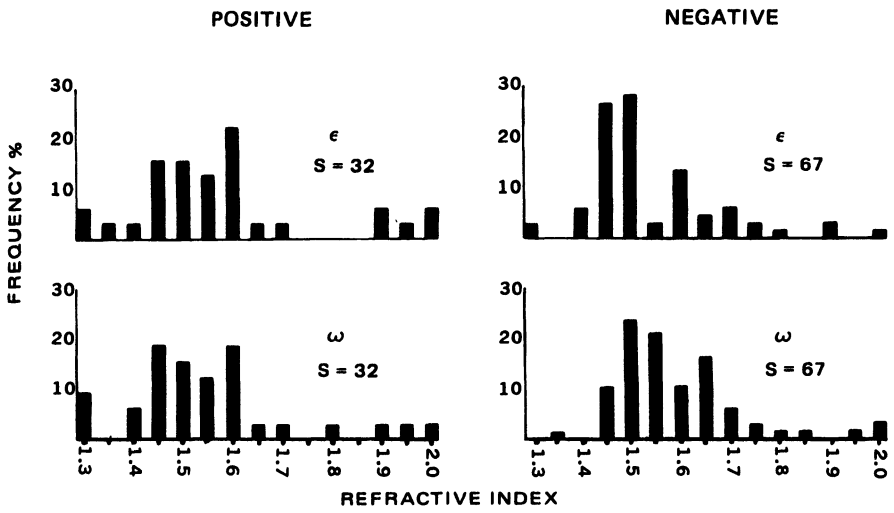


FIGURE 7.6. Refractive index distribution for uniaxial positive and negative crystals among norganic species with regard to ω and ε.

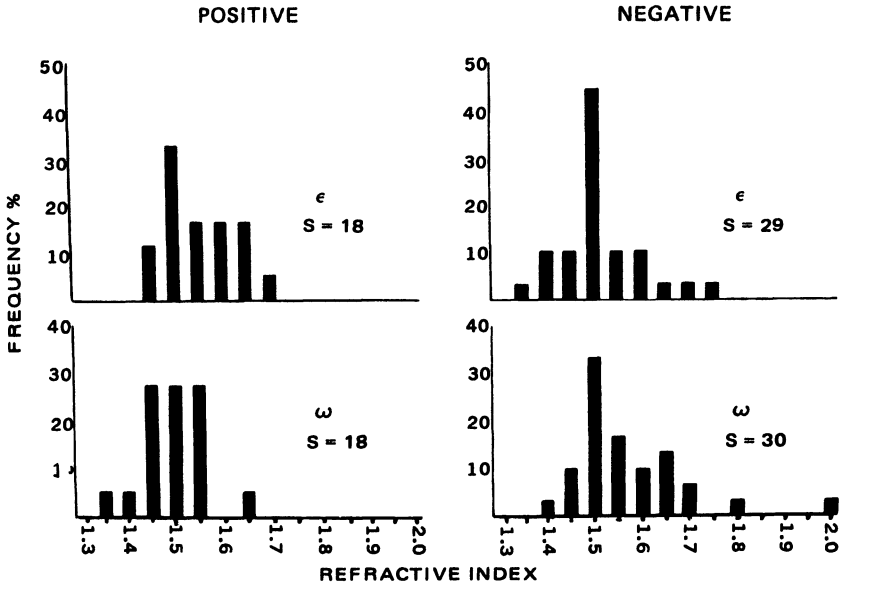


FIGURE 7.7. Refractive index distribution for uniaxial positive and negative crystals among organic species with regard to ω and ε.

the birefringence and optic sign. If $\varepsilon > \omega$, the crystal has a positive birefringence; if $\varepsilon < \omega$, a *negative* birefringence. Alternatively the birefringence and optic sign can be determined directly, as mentioned earlier. Moreover a prismatic view best presents the phenomena of dispersion and pleochroism.*

A pyramidal, tetrahedral, or rhombohedral face presents ω of course but also a special and constant value for ε', which is therefore descriptive and determinative. Furthermore if the face angle is known, data depending on true ε can be determined; traces of the uniaxial interference figure may also be visible.

It is now obvious that attempts should be made to obtain crystal faces for observation. While crystals as commercially received are not always good enough for crystallographic and optical purposes, recrystallization from a drop of solvent on a microscopical slide[3,4] is generally fruitful.

7.8. BIAXIAL CRYSTALS

Table 7.1 shows that about 62% of inorganic crystals are biaxial, and even more organic crystals, 84%, are in this category. Figures 7.8 and 7.9 show distributions according to optic sign and the *three* principal refractive indices n_x, n_y, and n_z.[18] Since there are two *optic axes*, the angle between them ($2V$) is also descriptive and determinative (see Figure 7.10). This value is measurable or estimatable directly from the adequately centered biaxial figures (see Figures 7.11a and 7.11b) whether the bisectrix is acute or obtuse. Measurement is made easier by standardizing on a numerical aperture of 0.85.[14] Then use a standard chart for $2V$ versus δ, the angle of rotation of the stage necessary to cause the isogyres (curves around the optic axes) to move from the center to the edge of the microscopical field, to convert from $2E$ (the optic axial angle in air).[15]

It is probably already apparent that biaxial crystals are optically and crystallographically complicated (see Figure 7.12).[22] This means that identifying the three principal refractive indices qualitatively and quantitatively may be difficult. But some values are always visible and capable of measurement however vague their specific identity may be at the time of observation. The important thing is to record them and to recognize them on every repeated occasion, so that a pattern appears.[23]

Pleochroism is the general term for absorption of different colors as light passes in different directions through a crystal. If crystal exhibits two distinctive colors when viewed in two unique directions in white light, it is said to be *dichroic* (see Chapter 6). If there are three directions and three colors, it is *trichroic*.

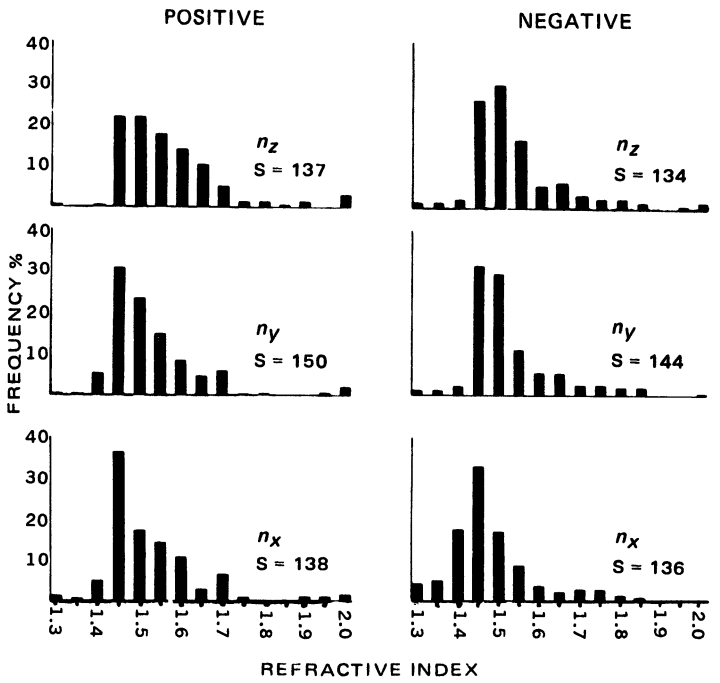


FIGURE 7.8. Refractive index distribution for biaxial positive and negative crystals among inorganic species.

7.9. OPTICAL PROPERTIES OF THE LIQUID CRYSTALLINE OR MESOMORPHIC STATE

Some solid organic compounds assume the *mesomorphic* state, which is intermediate between that of a solid crystal and a true liquid. Mesomorphism that sometimes occurs when a solid crystal is heated is termed *thermotropism;* what occurs when a solvent is added is called *lyotropism.*[24]

Most compounds that manifest mesotropism have long molecules, sometimes also flat, such as those containing *para*-substituted benzene rings. Mesomorphic substances also contain one or more polar groups. In the solid crystalline state, the long and/or flat molecules are arranged in parallel positions, holding together by means of the polar groups and van der Waals forces. When such crystals are heated or loosened with solvent, if the polar forces are not too strong, the molecules find some freedom of movement while retaining some degree of order.[24]

Mesophases are birefringent. In lyotropic systems form birefringence

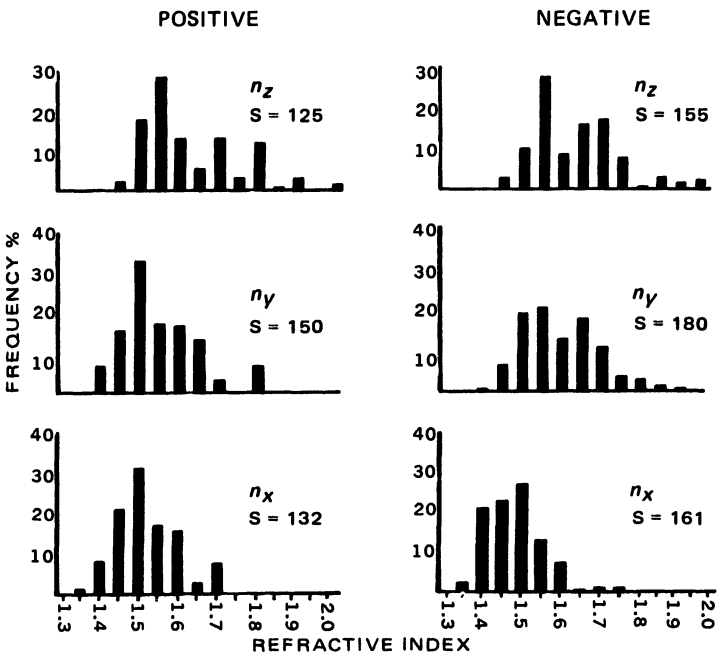


FIGURE 7.9. Refractive index distribution for biaxial positive and negative crystals among organic species.

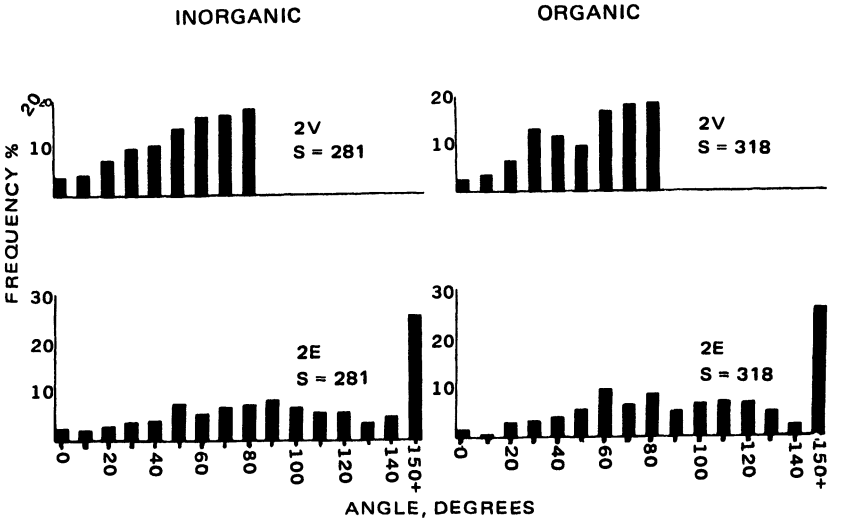


FIGURE 7.10. Optic axial angle distribution for biaxial positive and negative crystals among inorganic and organic species.

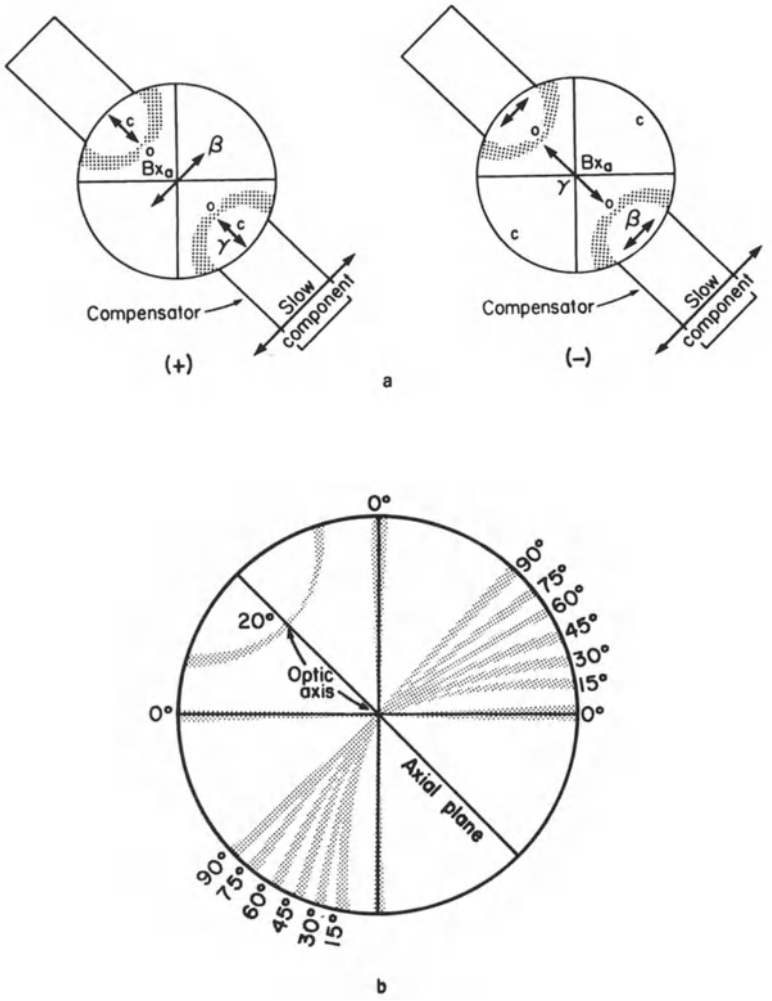


FIGURE 7.11. Vibration directions of slower components is indicated by arrows; *c*, *c*, quadrants in which compensation occurs; *o*, *o*, optic axes; B_{xa}, acute bisectrix.[3] (b) Estimation of $2V$ from curvature of isogyre, in optic axis interference figure. After F. E. Wright.[3]

(see Chapter 6), pertaining to the difference in refractive index between liquid solvent and the long or flat solute molecules, may account for some of the birefringence, but not all of it.[24] The fundamental cause of birefringence in mesophases lies in the activity of polarized light waves as they displace electrons in atoms in the vibration direction of polarized light, producing electric dipoles oriented toward polarization. When the

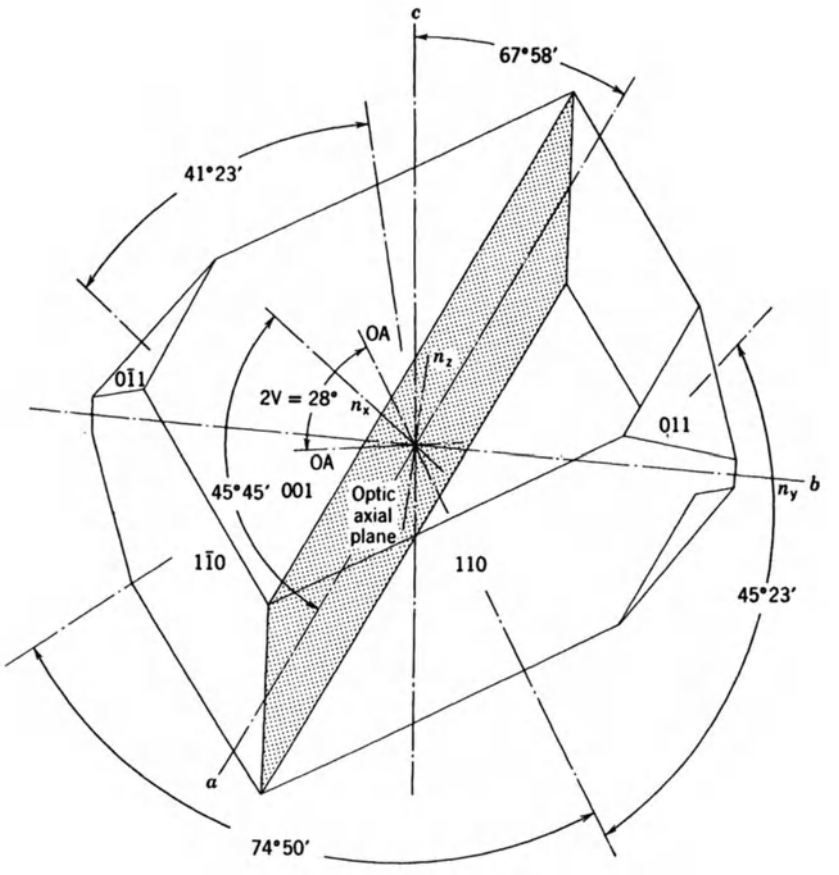


FIGURE 7.12. Crystallographic properties of melamine.[22]

System	monoclinic	Refractive indexes	n_x = 1.487
Class	prismatic		n_y = 1.846
Axial elements	$a:b:c$ = 1.4121:1:0.9728B		n_z = 1.879
	= 112°2′	Optic sign	(−)
Birefringence	0.392	Optic axial angles	$2E$ = 53°30′
			$2V$ = 28°38′

vibration direction is parallel (→) to the lineup of the dipoles (+−) (+−) (+−), the total electric field is *greater* than if the dipoles were widely separated. When the light is vibrating at right angles to dipoles that are side by side (+−), the total electric field is *less* than for widely scattered atoms. When light is vibrating parallel to the long axis, the average strength of the dipoles of long molecules, is greater (the refractive index higher) than when light is vibrating crosswise.[24]

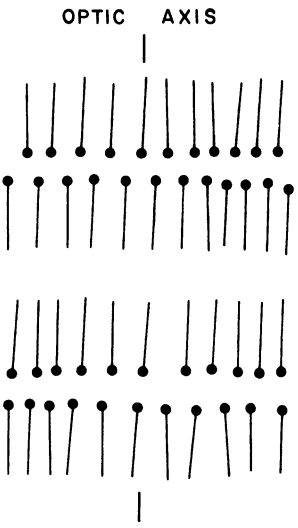


FIGURE 7.13. Smectic structures (schematic): Double layers in soaps. • represents terminal polar groups, e.g., —COONa.[24] Courtesy of Microscope Publications.

7.10. THERMOTROPIC, MESOMORPHIC, SINGLE COMPOUNDS

Generally thermotropic mesophases of single compounds having long molecules belong to one of the following three structure types:

1. *Smectic* mesophases is the type to which soaps belong (the Greek word for soap is *smega*). Soaps have a polar group, such as—COONa at the end of a long hydrocarbon chain. The polar group of one long molecule

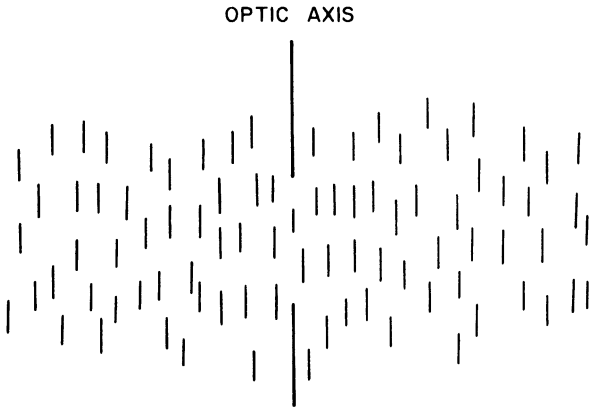


FIGURE 7.14. Nematic structure (schematic).[24] Courtesy of Microscope Publications.

attracts that of another molecule head-to-head, forming a double layer in the smectic phase, as illustrated in Figure 7.13. The layers are flexible and glide over one another. Most smectic phases are optically *positive* and uniaxial.[24]

2. *Nematic* mesophases are named for the Greek word for thread, *nema*, because some members of this type manifest thready lines. All members of this type are composed of single layers of more or less parallel lengthy molecules, as shown in Figure 7.14. Nematic phases are optically *positive* and uniaxial.[24]

3. *Cholesteric* mesophases are named for *cholesterol* and its derivatives because they are in the majority of members of this type. Theirs is a spiral arrangement of nematic layers, as shown in Figure 7.15. The pitch of the spiral is about half that of a wavelength of visible light, giving the cholesteric structure very special optical properties, including strong rotation of the plane of polarized light around the axis of the spiral (which is also the single optic axis). Cholesteric mesophases are optically *negative* because long strands of such molecules are oriented in various directions around the axis. Since these molecules are also flat and spaced close together, their optical properties resemble those of planar molecules. When the layers are parallel to the slide, they may show an off-center biaxial interference figure with an optic axial angle of about 20°.[24]

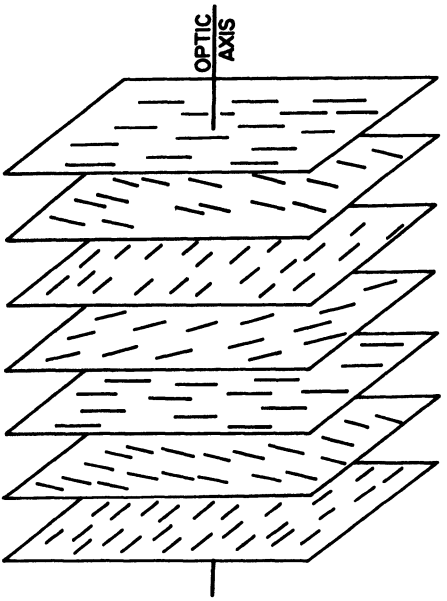


FIGURE 7.15. Cholesteric structure (schematic), with right-hand twist. The planes mark levels in the structure between which a 45° rotation of the molecular axes occurs.[24] Courtesy of Microscope Publications.

Some smectic types show polymorphism, believed to be due primarily to different lateral arrangement of the molecules. A smectic phase or phases can also manifest either a nematic or a cholesteric phase, but not both. The smectic phase always occurs at lower temperature than the nematic or cholesteric phase, since the smectic phase is more ordered. Because the crystalline phase of the same compound is still more ordered than its smectic phase, the crystalline phase is stable at a lower temperature.[24]

7.11. MORPHOLOGY TYPES

The *morphology* of mesophases is called *texture* by Hartshorne.[24] There are several kinds of texture:

7.11.1. Homeotropic Textures

Homeotropic textures (named by Lehman and Friedel) refer to smectic and nematic mesophases with the optical axis perpendicular to the glass surfaces confining them. Hartshorne extends the definition to include also cholesteric phases.[24] If for example a little (smectic) ammonium oleate is pressed into a thick layer between a glass slide and cover glass, a confused texture is seen between crossed polars because the specimen is too viscous for the layers to flatten out. If the glass surfaces are cleaned with hot soapy water without an etching agent, then rinsed with hot water, dried with a clean cloth, and not handled thereafter and if the cover glass is gently moved round and round by means of a rubber-tipped pencil, inspection between crossed polars reveals a gradual disappearance of the polarization interference colors. When the field becomes quite dark and the illumination is conoscopic, a uniaxial interference figure appears, as shown in Figure 7.16. By means of a first-order red plate, the optical sign is shown to be positive in the case of ammonium oleate because it is typically smectic. Typical nematic mesophases are also optically positive, but cholesteric mesophases are generally negative.[24]

7.11.2. Focal Conic Textures

Focal *conic textures* arise when a mesophase is confined to surfaces with which it forms strong local attachments, for example to a glass slide and cover glass etched with hydrofluoric acid. Long molecules around

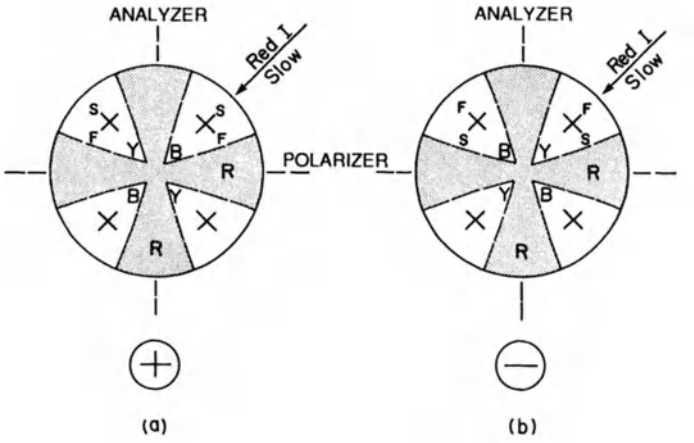


FIGURE 7.16. Determining the optical sign on an optic axial interference figure by using Red 1 plate. (*B* = blue; *Y* = yellow; *R* = red.)[24] Courtesy of Microscope Publications.

each center of attachment are required to adopt a radiating arrangement resembling a tipped hollow ring, known as a Dupin cyclide, as shown in Figure 7.17. The Dupin cyclide is truncated by the glass slide and cover glass. If the preparation is fairly thick and not too viscous and heated between a slide and cover glass (both slightly etched) until the liquid phase begins to appear, on lowering the temperature and agitating the cover glass, a number of *polygonal* areas appear. In each polygonal area there is a family of ellipses consisting of large ellipses with smaller ones in the

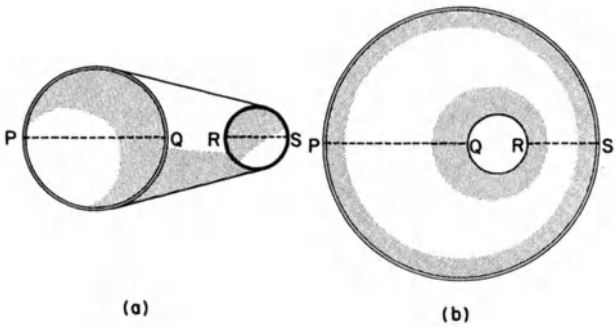


FIGURE 7.17. Dupin cyclide. (a) Half-cyclide showing principal sections, (b) plan view.[24] After Hartshorne and *Stuart, Crystals and the Polarising Microscope*, Arnold, 1970. Courtesy of Microscope Publications.

interstices. The major principal axes of the ellipses in any one polygon *meet at one point* within the polygon, as shown in Figure 7.18. If the upper surface of the preparation is in focus and the analyzer is in place (without the polarizer), each ellipse is crossed by a straight isogyre, whose narrowest point coincides with one of the foci of the ellipse. If the *polarizer* is employed alone, a similar pattern is in focus on the lower surface of the preparation. If the focus is gradually lowered from the upper surface to the lower surface of the preparation, each ellipse is joined by one branch of a hyperbola that starts at the isogyre. All of the hyperbolas belonging to the ellipses of one polygon meet at the lower surface at one point below the intersection of the major axes of the ellipses. This point is a common corner in a system of polygonal areas in the lower surface. Hartshorne explains these phenomena.[24]

7.11.3. Other Smectic Textures

Other textures of smectic substances are

Bâtonnets: separate little images of focal conic textures, often highly ornamented; they may form on cooling the melt.

Fanlike textures: revealed by crossing the polars, successive radial bands extinguishing at slightly different positions of orientation of the stage.

Oily streaks: observed between crossed polars when a focal conic texture is destroyed by shifting the cover glass on a preparation; the streaks are birefringent *bands* having *transverse striations.* At sufficient magnifica-

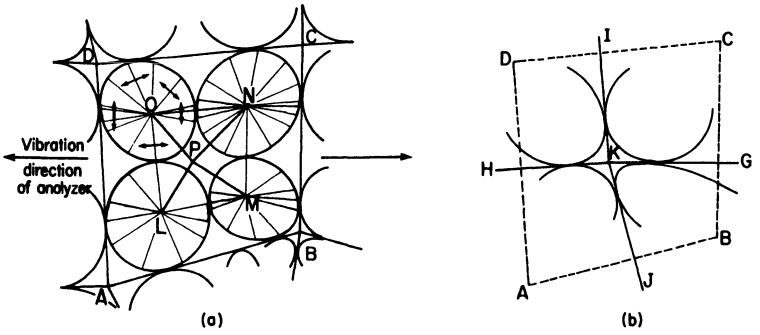


FIGURE 7.18. Polygonal texture. (a) Upper surface focused (analyzer, no polarizer), (b) lower surface focused.[24] After Hartshorne and Stuart, *Crystals and the Polarising Microscope,* Arnold, 1970. Courtesy of Microscope Publications.

tion the streaks' focal conic groups can be seen with the hyperbolas parallel to the striae. The bands are length fast ($n_{\parallel} < n_{\perp}$).

7.11.4. Nematic Textures

The *texture of nematic* mesophases is typically threadlike, formed by rapid cooling the isotropic melt to nematic temperatures. These wormlike textures mark structural discontinuities, analogous to ellipses and hyperbolas in smectic phases. However the nematic discontinuities do not conform to any definite geometrical plan because there is no stratification. Approximately centered optic axis figures (see Figure 7.16) or optic normal figures (see Figure 7.19) are usually found with which to determine the optical sign (+). Incidentally the initial morphology of the nematic phase from the melt is spherical droplets, not bâtonnets as from a smectic phase.[24]

7.11.5. Cholesteric Textures

The *texture of a cholesteric* phase, produced by cooling an isotropic melt, resembles a cloud of very fine particles. When the cloud is disturbed by moving the cover glass, a homeotropic texture is produced, with the optic axis normal to the plane of the slide. The *vivid colors* in the specimen held between crossed polars are very different from those of homeotropic smectic and nematic phases. The specific color (or occasionally invisible

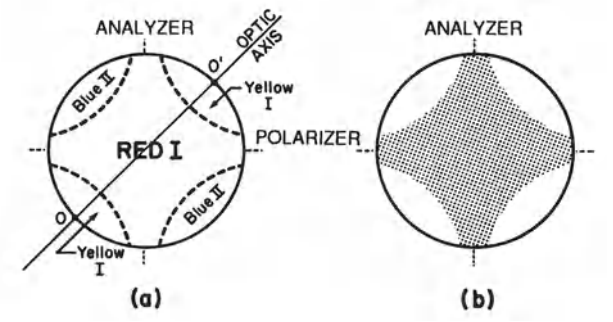


FIGURE 7.19. Optic normal figure ("flash" figure). (a) 45° position showing example of color distribution. In a positive mesophase, light vibrating along *O–O'* (direction of the optic axis) is slow. In a negative mesophase it is fast. (b) Extinction position.[24] Courtesy of Microscope Publications.

wavelengths) depends on the temperature and angle of observation. The changes in color with temperature have been developed commercially to register temperatures and temperature gradients.[24]

The second characteristic of cholesteric phases is the *rotation* of the plane of polarized light. This phenomenon is manifested qualitatively under the microscope by a lack of extinction between crossed polars. Instead of darkness there is a color that changes as the analyzer is rotated, due to the dispersion of optical rotation. The optical activity of most cholestive substances is so great that *several turns* of the rotating stage are necessary to restore extinction. Therefore a circularly wedged preparation is used (see Figure 7.20). The number of rings indicates the orders of 180° rotation by the specimen. A slight rotation of the analyzer to the right, noticing whether rings go in or out, indicates whether the specimen is levo- or dextrorotatory. A very low-power objective (≈48 nm) and eyepiece should be used. Since most cholesteric phases of single compounds are stable above room temperature, a hot stage should be used, with the temperature of the test recorded as a factor in the degree of rotation by the specimen. Of course a plane wedge can be made from a large cover glass propped up at one edge by another piece of cover glass. The specimen is then run in between the sloping cover and a slide.[24]

7.12. LYOTROPIC PHASES

Solutions of mesomorphic phases behave like cholesteric thermotropic phases.[24] Aerosol® OT, a substance with peglike molecules of sodium-di-2-ethylhexyl sulfosuccinate (a waxy smectic phase, difficult to handle) is commercially liquified to a pourable lyotropic phase by adding about

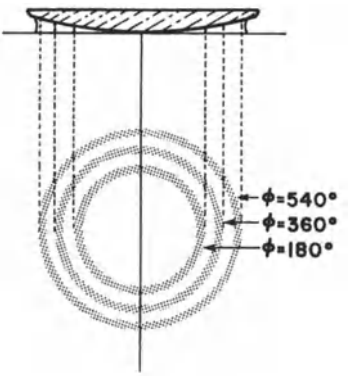


FIGURE 7.20. Principle of determining optical rotation of a cholesteric phase.[24] Courtesy of Microscope Publications.

25% of an alcohol–water solution. Aerosol® OT is a typical amphiphile; that is, the COONa group is strongly polar and *hydrophilic* (lypophobic), while the double peglike chain is strongly lipophilic (hydrophobic), as shown in Figure 7.21b).[24]

The basic unit of amphiphile is the *micelle*, a cluster of polar molecules with single or double chains with their polar groups oriented in the water. Figure 7.22a shows a micelle in the water of a dilute, isotropic, colloidal solution. Figures 7.22b–7.22d illustrate the chief kinds of birefringent lyotropic mesophases. In Figure 7.22b the term *neat* comes from soap technology referring to smectic molecules structured in a lamellar phase as a double layer tail-to-tail with the polar groups facing the layers of water. The resulting birefringence is positive, but the strength may be weakened by some negative form of birefringence.[24]

In the soap industry middle phase (M_1) pertains to single-chain molecules grouped into rodlike micelles of indefinite length arranged side by side in a hexagonal pattern, as shown in Figure 7.22c. In each rod the average orientation is radial, with the polar groups outside toward the water between the rods. The optical sign is *negative* (in accordance with a planar structure), but the crystal birefringence is probably lessened by some degree of form birefringence.[24]

The *inverse* (M_2) phase is in response to a higher concentration of the long molecules, which then turn around so that their polar groups face water now contained in hollow rods, as shown in Figure 7.22d. This phase is also optically *negative*.[24]

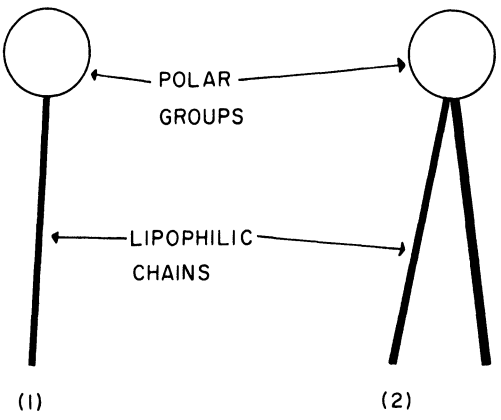


FIGURE 7.21. Types of amphiphillic molecules (schematic).[24] After Hartshorne and Stuart, *Crystals and the Polarising Microscope*, Arnold, 1970. Courtesy of Microscope Publications.

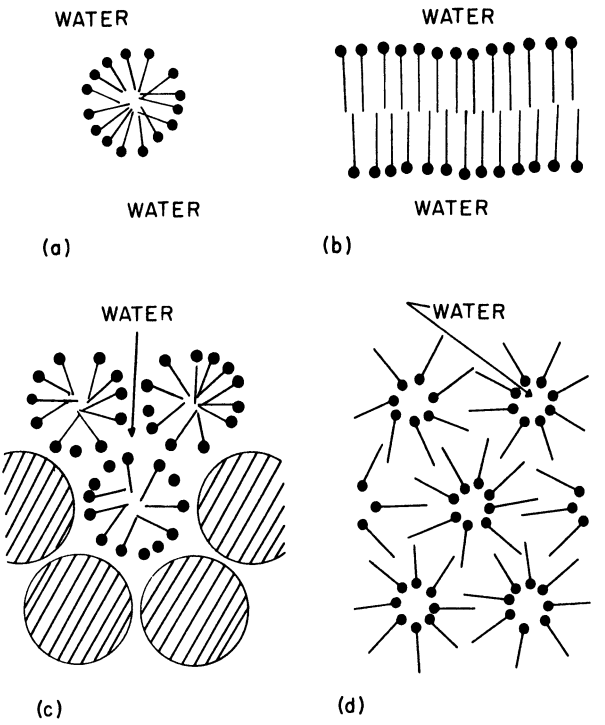


FIGURE 7.22. Main types of micelles in amphiphile–water systems. Only molecules with single lipophilic chains are shown, but peg-shaped molecules would be arranged similarly, though such molecules are not known to form M_1 phases: (a) diametral section of spherical micelle, (b) smectic layer in neat phase *G*, (c) middle phase M_1 showing cross section of micellar rods (according to Luzatti), (d) inverse middle phase M_2 showing cross section of micellar rods (according to Luzatti).[24] After Hartshorne and Stuart, *Crystals and the Polarising Microscope*, Arnold, 1970. Courtesy of Microscope Publications.

Lyotropic phases can display spherulites and fan shapes. They are usually *positive*; that is, the slow component is radial. However in neat phases isogyres are merged as in Figure 7.23a, four of them producing a larger, *negative* (radially fast) spherulite. A pinwheel effect (see Figure 7.23b) is apparent when the stage is rotated. These effects were not noticed by Rosevear (1954) in spherulites of the middle phases.[25] He noted that the neat phase had a greater birefringence than the middle phases. In applying Rosevear's observations as a test of amphilic types, retardation must be reduced to birefringence by taking thickness and orientation into consideration. The Rosevear test does not apply to peg-shaped (double-chain) molecules. Incidentally the sign of a spherulite is not necessarily the same as the intrinsic optical sign of the mesomorphic structure; the sign of a

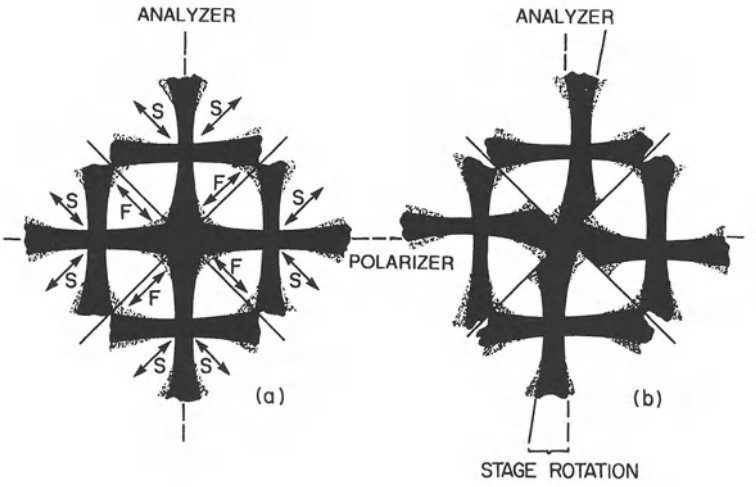


FIGURE 7.23. Merging of positive spherulites in neat phases of soaps and detergents. (a) the four positive spherulites combine to give an apparently negative spherulitic region in the center (radial direction "fast"). (b) On rotating the stage from the position in (a), a pinwheel effect is obtained at the center.[24] Courtesy of Microscope Publications.

spherulite is merely an indication of the arrangement of units in the sphere or disk.[24]

There are *apparently* isotropic phases in some lyotropic systems. For example Winsor and Rogers[26] evaporated a drop of a 30% solution of Aerosol® OT in water and observed an isotropic phase between an M_2 (−) and a G (+) phase. While such a phase may not have an ordered structure, it may have exactly compensating positive and negative structures, possibly including some form birefringence.[24,25]

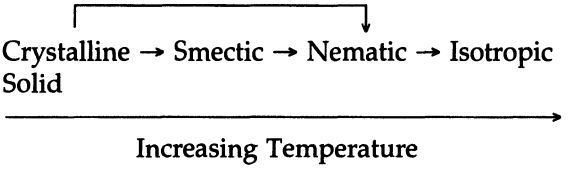
When emulsions of water, soap, and a hydrophobic phase are frozen, they sometimes break,[27] depending on whether or not the wall of third phase[1] around each droplet is broken by the growing crystals of either major phase. On the basis of current knowledge of mesophases of soap and other emulsifying agents, breaking such emulsion on freezing should be studied for their mesomorphic structure and habit at prevailing temperatures and gradients.

7.13. LIQUID CRYSTALLINE POLYMERS

Development of polymers capable of producing liquid crystalline structures has greatly expanded in recent years; they are of both thermo-

tropic and lyotropic types. The three most important groups are (1) aromatic polyamides, (2) rigid-rod polymers, and (3) aromatic copolyesters. An atlas of photomicrographs preceded by a survey introduction has been presented by Woodward.[28] Liquid crystalline phases are normally birefringent and often show one or more of the textures discussed in Chapter 7.

Sawyer and Grubb state that essentially all microscopical techniques have been used in characterizing these structures, including etching, sonication (for fibrillation), ultramicrotomy, fractography, and peeling to expose the internal structures.[29] They note that many thermotropics can be transformed from one polymorphic configuration to another if the temperature is changed.



It is important to note that surfaces, as with glass slides, often have a strong influence on the preparation's observed order and the initially observed structure ultimately relaxes to another structure with time: it may take several days or longer for this relaxation to occur.

As discussed in Chapter 13, Young considers measurements of molecular orientation on aramid polymers and shows these have superior sensitivity to those of X-ray diffraction.[30] He also shows the molecular structures of Kevlar® and Nomex® polymers.

7.14. SUMMARY

Microscopists often use crystallography because crystals are distinctive and have many precise characteristics, which are of enormous help in identifying and studying substances. Crystallography is an extensive subject with a formidable vocabulary, but students can take comfort from the fact that only six crystal systems are needed to embrace the (literally) millions of known pure solid phases and their descriptions are logical and orderly, although an ability to visualize solid geometric shapes helps.

Crystal axes are imaginary lines employed to describe the structure and symmetry of crystals. The simplest crystal system, the isometric, is represented by three mutually perpendicular axes, exactly like the familiar *x-y-z* Cartesian coordinates. The structural units of such a crystal, whether

ions, atoms, or molecules, are arranged in equidistant fashion along these three equivalent axes. Hence the optical (and other physical) properties are the same in any direction within the crystal, which is isotropic as well as isometric. Such a crystal appears dark in all orientations when viewed between the crossed polars of a polarizing microscope. In Winchell's very useful lists of 935 commonly encountered crystalline solids, only 1.6% of the organic and 17% of the inorganic substances are isometric, representing 94 of the 935 solids. Hence the fact that a crystalline solid is isometric immediately limits the possibilities. If the index of refraction is then determined, this further limits the choices within Winchell's list. Some judicious chemical tests under the microscope then lead to a tentative identification of the phase, and the choice can be verified by preparing a few characteristic derivatives under the microscope and checking their optical properties.

The second crystal system is the tetragonal, which also has three axes at right angles, but one of them has different spacings along itself, and it is considered to be of different unit length. Viewed along the unique axis (the *c*-axis), a tetragonal crystal appears to be isometric because its other two axes (the *a*-axes) are equivalent, but viewed along an *a*-axis, such a crystal is birefringent because there are different velocities of light along the two monequivalent axes. The third system, the hexagonal, has three equivalent axes at 60° to each other, all perpendicular to the longer (or shorter) *c*-axis. Hence a hexagonal crystal is also isometric when viewed along the *c*-axis and birefringent in any other position. Tetragonal and hexagonal crystals together are called *uniaxial* because they have only one unique axis. These crystals account for 19.9% of the inorganic substances in Winchell's lists and 11.5% of the organic substances. In appropriate orientations and with conoscopic polarized light, uniaxial crystals produce a characteristic interference figure (a Maltese cross centered on a series of colored diffraction rings) that can be observed at the back of the objective.

The remaining crystal systems have more than one unique axis. The orthorhombic system has three axes (*a, b, c*) all at right angles but all of different length, so that its crystals are always birefringent in all orientations. The monoclinic system has two axes (*a, b*) at right angles, but the third (*C*) is at some other angle to the plane of the first two. The last system, the triclinic, has three unequal axes all at angles other than 90° to each other. These three systems together make up 62.1% of the inorganic and 83.9% of the organic substances in Winchell's lists. Their identification is more difficult than identifying isotropic and uniaxial crystals, but determining the angle between any two axes helps, as do the characteristic interference figures. The refractive indices are determinative, likewise for the *sign* of the birefringence. Even without quantitatively determining

refractive indices, the sign can be determined by using a retardation plate or compensator.

In addition to six kinds of geometric structures, crystals have characteristic morphologies by which they are recognized under the microscope. Within each system crystals may be in the shape of flat plates (lamellar), thicker tablets (tabular), more regular solids with edges more or less equal (equant), elongated prisms (columnar), or tiny needles (acicular). No matter what the morphological type, a given pure phase always has the same refractive index (or indices) and the same optical sign because its internal structure remains constant. In addition to the five general morphological types within each system, there are characteristic shapes and faces (forms) by which the microscopist recognizes crystals. Thus isometric crystals often show up as cubes, octahedra, or tetrahedra if they have had an opportunity to grow regular faces and sides. (If crowded together, as when crystallized from a melt or crushed or powdered, pieces of any crystalline type are *grains* rather than regularly shaped units, but they have the same optical properties as the beautiful equant shapes.) Well-developed tetragonal crystals are rectangular prisms and tablets, sometimes with sloping end faces. Hexagonal crystals may be the familiar hexagonal prisms, as in quartz, or the equally familiar rhombohedra (hemihedral hexagonal bipyramids), as in calcite. Some crystallographers prefer to call the rhombohedral class of the hexagonal system a separate (seventh) system. Orthorhombic crystals are often tabular, as in the familiar form of sulfur. Triclinic crystals have the "leaning prism" look, as in the familiar bright blue pentahydrate of copper sulfate.

The faces (forms) of any crystal habit are best defined in terms of the *Miller indices*, which are reciprocals of intercepts of a particular face with the crystallographic axes. Thus the faces of a cube are represented by the indices 100, 010, and 001, while any face of an octahedron is designated 111. Miller indices of the equant habits of all seven crystal systems are given in Table 7.1, and the indices of lamellar, tabular, columnar, and acicular crystals of the same system are equal to those shown there.

Most of the usefulness of crystallography to the microscopist comes from long experience in recognizing particular systems from the way their crystals of characteristic habit appear as they lie on this face or that or as they develop from solution on a slide or freeze from a sample held on a hot stage or cold stage. Dozens of tips on how to do this are illustrated with many examples, but the microscopist must be familiar with all of the techniques and endure all the pitfalls before gaining the facility that comes with that experience. The systematic study of pure compounds, and especially faithfully recording all optical properties of every substance that comes to the microscopist's attention (under some

system that allows rapid retrieval of the information), will do wonders for reaching that state.

Liquid crystals, which are manifestations of residual order held over as a solid begins to melt or to dissolve in a solvent are discussed. The order usually arises from the persistence of oriented electric dipoles, which is why liquid crystals can be manipulated by electric fields to display digital readouts in calculators and watches. The narrowly limited temperature range of thermotropic liquid crystals is a reason why they are used also in digital thermometers.

8

Confocal Scanning Light Microscopy

8.1. INTRODUCTION

Confocal scanning light microscopy, a new type of microscopy, has generated considerable excitement. It gives higher resolution and thinner noninvasive optical sections, or planar views, than those obtained by classical bright-field or dark-field microscopy, and increased contrast is another major advantage. Fluorescence microscopy[1] is also greatly improved by using confocal scanning light microscopy, since three-dimensional views can be generated, which lend themselves well to digital image processing and possibly holography. In an image-processing system, a hundred or more very thin optical sections can be stored and combined into a composite, three-dimensional image. Then a display of the total three-dimensional view, or selected parts, can be generated. The composite three-dimensional views can resemble those from scanning electron microscopy, but the specimen does not have to be in a vacuum, as with normal scanning electron microscopy. Currently the technique is used only for reflected light, bright-field and fluorescent microscopies, but additional techniques are under development. Transmission and polarized light microscopes should be available in the future. The confocal microscopical concept was patented in 1957, but the technique has only recently become more common. Delay in its development is probably due to the only recent common application of lasers and scanning techniques. Currently there are some complications with all varieties of these instruments, and efforts are continuing to decrease these.

Confocal means having the same foci. In general two lenses are arranged to focus on the same point. Because of pinholes, or apertures, on each side of the specimen, as shown in Figures 8.1 and 8.2, only a very

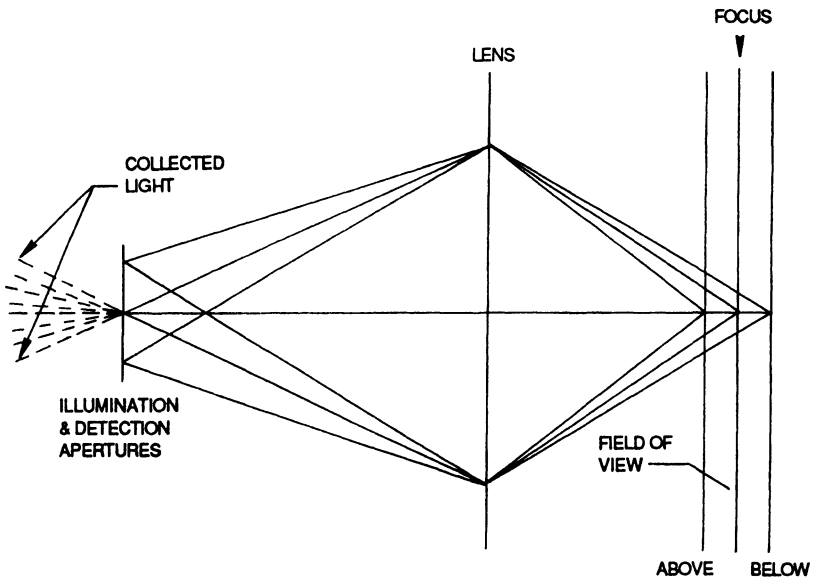


FIGURE 8.1. Diagram illustrating the confocal principle. An important point is the aperture labeled illumination and detection apertures. Lines emanating from the plane of focus pass through this aperture or the confocal pinhole, which is at the center. Rays from above or below the plane of focus are intercepted by the solid material surrounding the aperture.[2]

small volume is focused at any time. Light from only the actual plane of focus enters the eyepiece, which eliminates practically all of glare and scattered light. Fruition of the technique depends on recent developments in many fields, mainly light microscopy, confocal imaging, scanning and video microscopy, laser illumination, and computerized image analysis.[1] The small volume of light entering at an instant does however create problems in real-time imaging, as it does with photomicrography in scanning electron microscopy.

The main difference between confocal and conventional microscopy is that at any instant, the confocal microscope images points of light rather than large volumes of light.[1-3] As shown in Figures 8.1 and 8.2, a second pinhole at the back focal plane of the objective acts as a spacial filter. This second pinhole combined with the one in the front focal plane of the objective permits the special features of confocal imaging.[2]

In theory the technique works in both transmission and reflection modes, but back reflection is currently the only practical technique because of alignment difficulties in matching conjugate components and the lack of precision in the transmission mode. Most of the instruments are the epi-

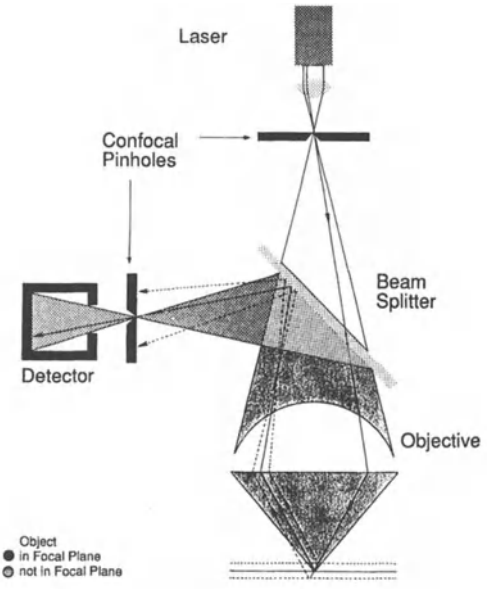


FIGURE 8.2. Schematic illustrating the normal arrangement of the confocal pinholes in a microscope. Courtesy of Leica.[2a]

illumination type, where epi-illumination means that a single objective serves as both condenser and objective. Practical techniques have not yet been developed to generate results of the type generated by polarized light microscopy.

The principal difference between confocal- and conventional scanning light microscopy is that the confocal uses a detector for points of light rather than the traditional detector, which collects a much larger three-dimensional volume of light. All of the unique features of confocal microscopy stem from this difference.[1–3] Some confocal microscopes use conventional illumination sources, while others use laser illumination. Each technique has certain advantages over the other one which are described in recently published surveys.[1–10]

8.2. SCANNING MODES

Scanning is required because only a small volume is illuminated at any instant, but a signal from a larger area or volume must be collected for a generally meaningful image. Scanning is accomplished by either beam

scanning or stage scanning. Other techniques exist, but the three most common scanning techniques are beam scanning by Nipkow disk scanning,[1] illumination or mirror scanning, and stage scanning.[2] Relative advantages and disadvantages of the different scanning techniques are listed in Table 8.1.[10]

8.2.1. Nipkow Disk Scanning

Paul Nipkow (1884) discovered how to dissect an image by scanning it in a raster pattern by using a rotating opaque disk perforated by a series of rectangular holes arranged in a spiral. This discovery inspired early attempts at television for the next 40 years, culminating in Jenkins's mechanical receiver in 1925.[1,4]

At any instant apertures on one side of the disk are illuminated and

TABLE 8.1.
Features of the Three Types of Confocal Microscopes[10]

Feature	Stage Scanning	(Nipkow) Disk Scanning (Tandem scanning)	(Mirror) Beam Scanning
Light source	Laser	Hg Arc white light	Laser
Beams	One	100–500	One
Scan speed (limited by)	Slow (specimen mass)	Very fast (Disk rotational speed)	Medium (galvanometer response)
Optimal Photo Detector:			
Present	Photomultiplier tube	Cooled charge coupled device	Photomultiplier tube
Limitation	Low quantum efficiency	Leakage and electrical noise	Low quantum efficiency
Future	Cooled or avalanche photodiodes	Same	Same
Limitation	Max. countrate = 10^6–10^7/sec	Same	Same
Advantages	On-axis optics transmission mode	Real-time image White light High detector quantum efficiency	Best for quantitative analysis Suitable data for digitizing
Disadvantages	Slow Vibrates specimen Difficult to locate correct field of view	Lacks sufficient illumination High detector noise Non variable pinhole diameter	High saturation and bleaching Slow scanning speed

act as point light sources, while conjugate apertures on the disk's other side serve as point detectors. Spiraling receiving perforations exclude light emanating from points in the specimen not illuminated in the first set of pinholes; this results in confocal illumination. This technique tends to be limited by the relatively small amount of the light that is collected through the pinholes, and precise mechanical alignment is necessary for relative juxtaposition of all components and pinholes.

Advantages of disk scanning, as well as beam scanning, over stage scanning involve being able to generate the total image from the small, discrete volumes of light without physically moving the specimen. Instead, the stationary specimen is scanned by flying spots of light, so that scanning speed is not limited by the movement of the specimen. Hence the specimen can be observed in real time through an eyepiece,[1] which is difficult with beam and stage scannings.

8.2.2. Beam Scanning

The stage scanning system has certain advantages over beam scanning where the beam is scanned or deflected, since it is perhaps simpler to translate the stage and all optical measurements are symmetrical about the optic axis.[2,4] If however the field of view is illuminated by beam deflection rather than stage movement, faster scan rates can occur and specimen preparation can be stationary. Such stability may be necessary to facilitate specimen manipulation.

8.2.3. Stage Scanning

A major advantage of stage scanning where the specimen, and not the light moves (as in beam scanning), is that total light intensity is much greater and the necessary mechanical precision is not so great as with Nipkow disk scanning.[1] Stage scanning is generally used with laser illumination, but theoretically this is not necessary. However the image is not actually generated in true real time, since the stage and specimen are physically translated, and too much time is required for this movement to permit real-time imaging.

8.3. ILLUMINATION

In theory any type of illumination source can be used. A commercial microscope with illumination for both normal and confocal viewing is

illustrated in Figure 8.3. Conventional sources provide both color and contrast as the viewer is traditionally accustomed to them, but with a conventional illumination source, it may be difficult to obtain sufficient illumination without switching to a monochromatic laser source. If a laser is used, artificial color contrast can be generated, as can be done for scanning electron microscopy photomicrographs. Fluorescent microscopy generally requires the use of higher intensity laser illumination than is possible with white light because of illumination loss in exciting the fluorescence. Confocal scanning fluorescence microscopy (CSFM) has produced a renaissance in fluorescence studies, since it essentially eliminates out-of-focus blur.[5]

In normal microscopy scattered light is collected from both above and below the plane of focus. This generates a fog or halo of nonfocused rays around the true image. Such a halo does not occur with confocal microscopy because of the two small apertures, and the resulting clarity of the images is dramatic in comparison with conventional microscopy. In-

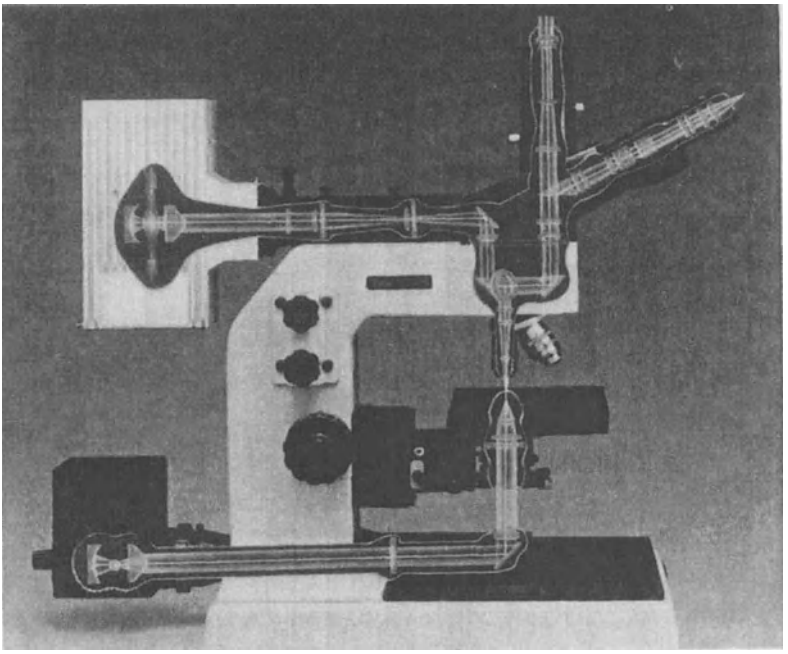


FIGURE 8.3. Illustration of a commercial confocal microscope. This model from Tracor Northern permits the viewer to combine both confocal reflected light viewing and conventional transmitted light viewing. Courtesy of Tracor Northern.[12]

creased contrast and resolving power, and decrease in glare, are especially useful in high-resolution, low-light intensity fluorescent microscopy. These advantages are illustrated in Figures 8.4 and 8.5.

With UV or short wavelength light, epi-illumination is termed epifluorescence. In conventional epifluorescent microscopy, the imaged volume includes both considerable out-of-focus image as well as the in-focus image. However with CSFM out-of-focus signals are nearly completely removed, as described earlier. This results in an image with very high contrast, and such a possibility has generated a renewal of interest in fluorescent microscopy, especially for biological studies. Optimized reflection imaging in laser confocal microscopy has recently been reviewed.[7] Here we discussed how to decrease image degradation due to internal microscopical reflections by using critically rotated polarizing components in the microscope to eliminate or decrease internal, non-image-forming reflections within the microscope. Custom selecting different wavelengths of laser light, i.e., red, green, or blue, for different stains is shown to have a significant influence on image quality.

8.4. COMPARING THREE TYPES OF CONFOCAL MICROSCOPY

No one type of the three practical varieties of confocal microscopes has all of the desired features. Table 8.1 presents most of the individual

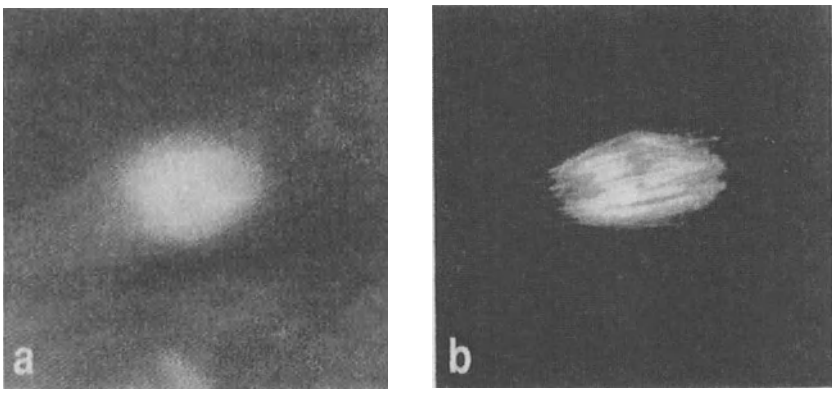


FIGURE 8.4. Mitotic in a moss protonema cell, microtubuli image. (a) Conventional epifluorescence and (b) confocal laser-scanning image. In conventional epifluorescence microscopy, stray light causes considerable distortion of fine details (especially with thick samples) as shown in (a). As shown in (b) stray light is almost completely eliminated by the confocal microscope, so that fine details become visible. Courtesy of Leica.[2a]

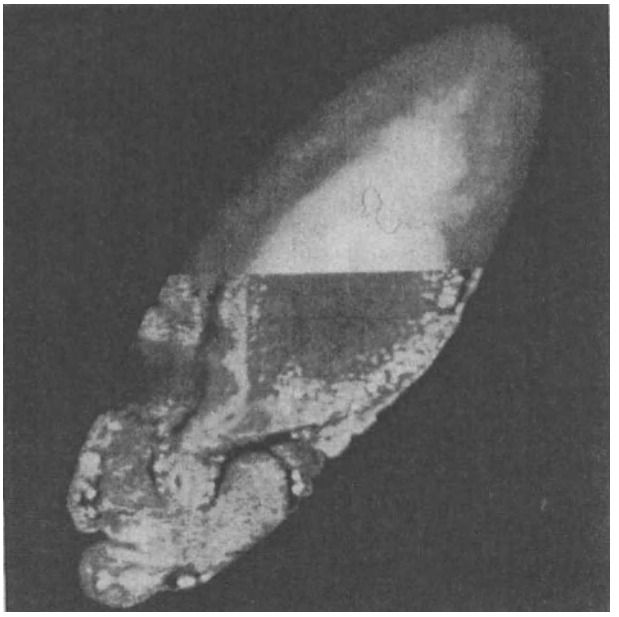


FIGURE 8.5. Photomicrograph of drosophila embryo obtained by both confocal imaging (bottom) and nonconfocal imaging (top). The photomicrograph shows dramatically the power of confocal laser scanning to reject out-of-focus light. The specimen is a whole drosophila embryo. This is a rather thick object, roughly shaped like a football—1.5 mm by 1 mm. The upper-half of the image is scanned nonconfocally, whereas the lower-half is in confocal mode. It would be very difficult to quantitate the degree of improvement, since there is almost no detail visible in the nonconfocal image. Courtesy of Leica.[2a]

features. Because of low final-light collection efficiency and relative speed of operation, most practical fluorescent microscopy is done by mirror beam scanning. Confocal microscopy is very useful, but feasible techniques are fairly new and will remain in a state of rapid evolution for a period of time.

8.5. CONVENTIONAL, CONFOCAL, AND AXIAL RESOLUTIONS

Both lateral or horizontal and axial or vertical resolutions can be increased with correctly used confocal microscopy, as shown in Table 8.2. It is well known that lateral resolution increases as a function of the first power of the NA. What is not generally known however is that resolution differs when perpendicular to the plane of focus rather than in the plane

TABLE 8.2.
Practical Resolution Limits[9]

Numerical Aperture	Conventional Microscopical Horizontal Resolution (μm)	Confocal Microscopical Horizontal Resolution (μm)	Axial Resolution (μm)
0.98	0.39	0.30	0.92
0.64	0.60	0.45	2.16
0.45	0.90	0.70	4.40
0.24	1.60	1.20	15.10
0.10	3.90	2.90	89.5

of focus. Along the axis of the microscope, above and below the focal plane of a point source, the image resolution with a large NA is only one-half as great as the lateral resolution.[11] In other words at high resolution theoretically, the axial resolution is only one-half the lateral resolution. This is an important concept to remember when collecting vertically sequential high-resolution images that may have to be as thin as possible.

Field depth in the specimen is also an important consideration, especially for confocal microscopy. Field depth is influenced by the light beam spreading above and below the plane of focus, the microscopist's eye accommodation, and magnification of the image.[11] In conventional fluorescent and dark-field microscopy, areas above and below the sharp plane or focus contribute light to the image. Fluorescent and light-scattering points also contribute light to a generally collected image. The ability to reject much of this undesired light is a possible advantage of confocal microscopy, which collects only a very thin planar image at any instant.[11]

In the 1950s it was shown from information theory that lateral resolution is improved by $\sqrt{2}$ over the classical value when the field of view becomes very small.[11] All of the preceding reasons contribute to why the resolution of confocal microscopy is greater than that of conventional microscopy. In addition to potential resolution, increased contrast, specimen transparency, and ease of three-dimensional optical sectioning are also potential advantages. Total theoretical understanding however of confocal microscopy does not yet exist.[11]

8.6. DATA ACQUISITION AND PROCESSING

Powerful microcomputers, modern photomultiplier tubes, and image-digitizing systems are useful combinations with the unprecedented serial or optical sections provided by the confocal light microscope.[1,6]

Digitized image manipulation has expanded tremendously in recent years, so that now a series of digitized planar images can be collected with a video camera and a frame grabber (see Figure 8.6). In Figure 8.6 a set of two-dimensional non invasive slices enters a digitized volume set called a voxel.[6] The set requires considerable storage space by either magnetic tape or high-capacity hard disk, and it is generally possible to exceed storage capability with voxel data (a three-dimensional array).

8.7. SUMMARY

The confocal concept can be thought of as two microscopes with a common optical axis, where both microscopes are focused on a common focal plane.[8] The focused field of view is imaged in the optical viewing tube at a second pinhole at the back focal plane of the objective. This pinhole acts as a spatial filter. To have practical value and provide a meaningful field of view, confocal light microscopes are of the scanning type; that is, either the specimen is scanned or the illuminating beam is scanned across the specimen. To generate a total image of the specimen, the very small volume of light, termed *voxel*, is scanned across the specimen by either deflecting the spot of light or scanning the specimen through the illuminated volume.

Optical sectioning is now commonly used in confocal microscopes to read compact disks.[9] It is not generally recognized that this operation is essentially the same as that of a confocal microscope; however the stylus on such monitors is a confocal microscope that collects information at only a single axial point inside the plastic sheet and ignores covering surface

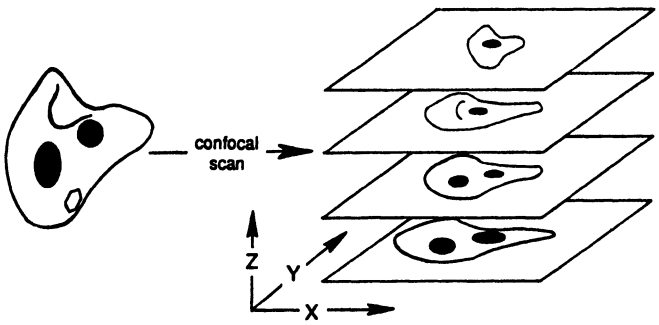


FIGURE 8.6. Series of confocal scans of a cell. Reprinted by permission from *American Laboratory* **22**, no. 11 (1990), p. 19. Copyright 1990 by International Scientific Communications.[6]

scratches and dust because of very thin planar images that are collected.[9] In this manner a scanned image can be constructed that is an optical section with very little noise outside the focused plane. Resolution is controlled by the illumination wavelength and the NA of the objective and the areas of the confocal pinholes.[9,10]

The scanning technique and confocal principles will theoretically function for all standard light microscopical techniques, but they have not yet been generally applied to more than bright-field and fluorescent microscopies.[13] Series of two-dimensional images can be collected into a three-dimensional array, or voxel, which can be electronically acquired and processed for three-dimensional viewing and manipulation.

9

Micrography

9.1. MICROGRAPH: IMAGE PRODUCED BY LIGHT, ELECTRONS, OR X RAYS

A *micrograph* is a reproduction of an image of an object formed by a particular kind of microscope[1]; thus a *photomicrograph* is an image taken by a light microscope and a light-sensitive material.[1,2] An *electron micrograph* is a photographic reproduction of an image formed by the action of an electron beam.[1] Likewise an *X-ray micrograph* involves any kind of X-ray microscopy.[3] Alternatively a microphotograph is a small, microscopic photograph, requiring a lens system to view its details.[1]

There are at least two approaches to photomicrography: the *artistic* approach and the *scientific* one. The artistic (subjective) mode is *imaginative*, whereas the scientific (objective) mode is based on *thought* and *memory*. Sometimes the purpose of a photomicrograph changes from scientific to artistic; for instance photomicrographs produced by research and development scientists and technologists can be exhibited and judged competitively on their artistic appeal.[4] Furthermore scientific micrographs can be used as advertisements,[5–7] greeting cards,[8] or strictly art,[8a] such as "microscapes" (imaginative landscapes or seascapes).[9] Much if not all of the artistic appeal involves color; accordingly color is sometimes added to scientific micrographs simply for artistic effects.[9]

However in science and technology the approach must be objective. Photographs vividly describe objects and observations (see Figure 9.1) and are relied on implicitly by the observer. Generally graphic descriptions of these are interpretive illustrations of what has been repeatedly or characteristically observed.

Another purpose of photography is to record a series of changes in a specimen, such as those that occur during cooling, heating, extruding,

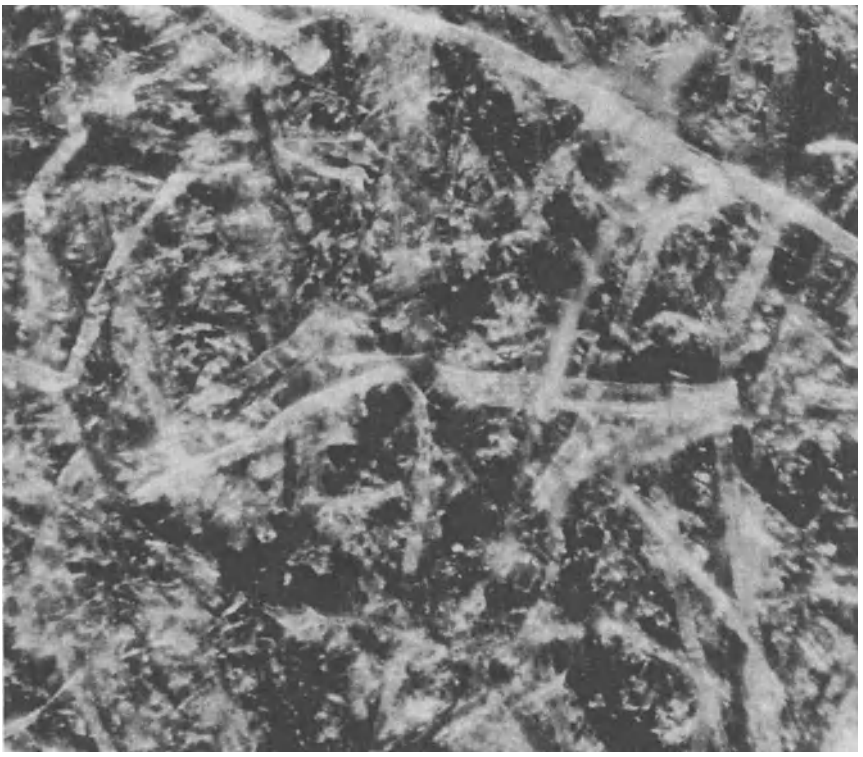


FIGURE 9.1. Surface of wallpaper stained so that chemical pulp appears white and ground wood appears dark. The photomicrograph tells a story.

stretching, relaxing, or immersing.[10] The action can be moderate, allowing for snapshots[11]; fast,[12] requiring high-speed techniques; or slow, using time-lapse techniques.[13]

In electron microscopy, especially transmitting (rather than reflecting) electrons, the specimen is heated by the electron beam and may change in appearance. To show this or obtain the most accurate change as many photomicrographs as possible are usually taken in a short time and studied rather than studying the specimen visually (see Chapters 13–15). In any event the story in a photomicrograph is told by the photomicrographer—not by an armchair microscopist.

9.2. EXPERIENCE: RECORDS OF NEGATIVES

Experience is the only way of achieving satisfactory results in photomicrography. Keep a good record of each photographic exposure on a data sheet, such as the one in Table 9.1.[6] It is especially important to record readings of the exposure meter and the time of exposure. By correlating these with results (to be entered in the Remarks column in Table 9.1), you will gain enough experience to avoid having to take more than one frame per specimen to ensure success.

9.3. IMAGINATION

Imagination helps in selecting the best combination of attributes contributing to visibility: the best conditions, equipment, techniques, and photosensitive materials to record the image both artistically and scientifically. Above all, imagination helps combine the best of the old and the most promising of the new.

9.4. RESOLVING POWER

Resolving power in photomicrography, as in visual work, is expressed by NA. In both photomacrography and photography, resolving power is expressed by f (focal length divided by the actual opening in the lens). By neglecting the difference between the tangent, i.e., f and the sine of half the angular aperture, the practical conversion is $f = 1/2\text{NA}$. This estimate is adequate for a compound microscope whose tube length is fixed. But when photographic single-lens systems (simple microscopes) are used with a bellows (see Figure 9.2), f varies with the ratio of image distance to the object distance (magnification), as shown in Figure 9.3. As the object distance (D_o) becomes smaller in focusing on a larger image (I), the angular aperture (α) becomes greater; therefore NA becomes larger.

9.5. RESOLUTION WITH PHOTOMACROGRAPHIC LENSES

Resolution by means of photo*macro*graphic lenses[14a] is as good or better than with compound microscopical objectives because the *macro*-lenses[14,16] are highly corrected for a wide field. They are particularly good at bellows extensions of 250 mm or more, provided they have been

TABLE 9.1.
Example of a Record of Photomicrography on Roll Film[6]

Purpose _____ Film _____ Date _____

No.	Objective	Photo Eyepiece	Specimen	Filter	Voltage	Aperture Iris Diaphragm	Exposure Adjustment	Remarks
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
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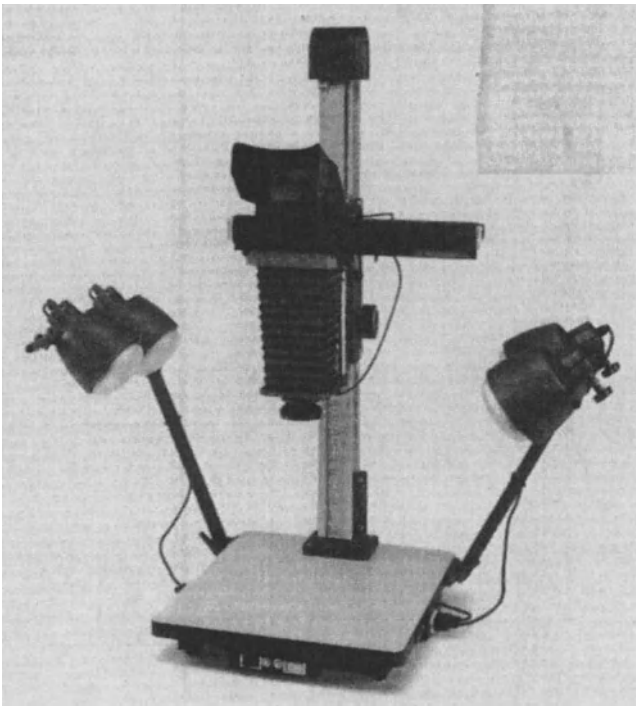


FIGURE 9.2. The Polaroid MP-4 multipurpose camera system. Courtesy of Polaroid.[15]

mounted for close-up objects (the reverse of the situation where cameras are focused on relatively distant objects). For close-up work the more convex of the two outer glass surfaces is mounted toward the object; for distant objects the more convex glass surface is mounted toward the photographic plane.[14] Macroobjectives of focal lengths 32 mm or shorter are usually mounted with the standard microscopical thread.[17] If not, an adapter is usually available for the lens board shown in Figure 9.2.

Because macrolenses are so well corrected to their extremities and their characteristics are not modified by an eyepiece or projection piece, they can be used with a very long bellows or even a dark room. Indeed pictures taken this way represent a kind of photographic art.

Photomacrography with a bellows camera (see Figure 9.2) continues to be a simple and inexpensive way of picturing macrosamples, such as those shown in Figure 9.4. However shadows and other lighting problems may be present; equipment and photographic films manufacturers[15] (see Figure 3.10 for a fiber-optic cable illuminator).

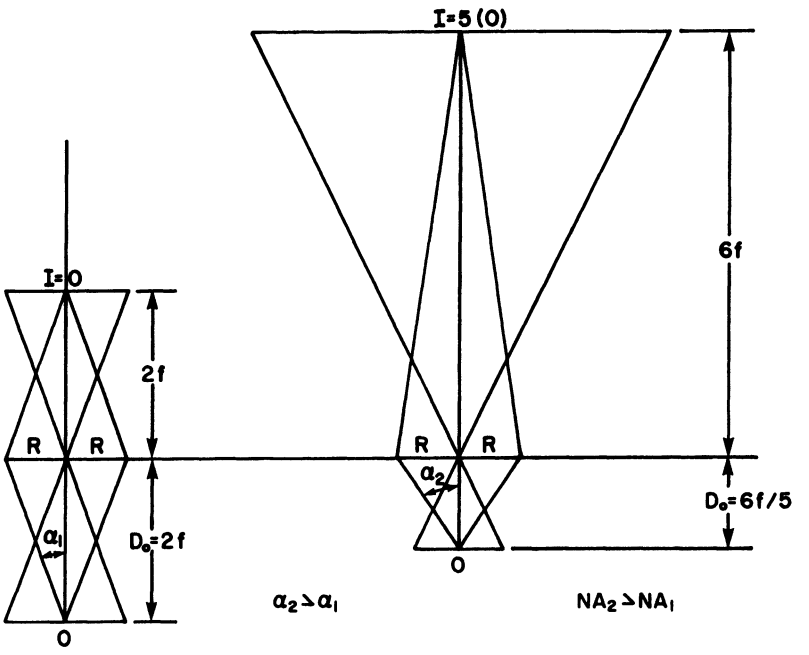


FIGURE 9.3. The NA of a macrolens system increases with magnification.[15] Courtesy of Microscope Publications.

Photomicrography is a broad and detailed subject.[18] In the 1970s photomicrographs were still being taken by somehow assembling a visual microscope, camera or camera back, light source, and various accessories. The literature reveals the high degree of microscopical success in various scientific and technological areas; the degree and extent of failures are just as well forgotten. Charge them to makeshift assemblies that invited vibrations in the optical system, lengthy photographic exposures to slow photographic emulsions, breakable glass plates, complicated darkroom procedures, *etc.* In the meantime various improvements were made along these and other avenues. So-called students' microscopes, stable camera backs, fast, roll, photographic film, or Polaroid® self-processing photographic film are currently available.[19,20] However professional microscopes now being manufactured,[21–25] are or can be converted into *photomicroscopes.*[5] Figure 9.5 shows a photomicroscope fitted with a camera for 35-mm roll film[6]; Figure 9.6 illustrates the addition of a camera back for 4 × 5 cut film.[22] Photomicroscopes fitted for Polaroid® self-developing, positive photomicrographs are available.[6]

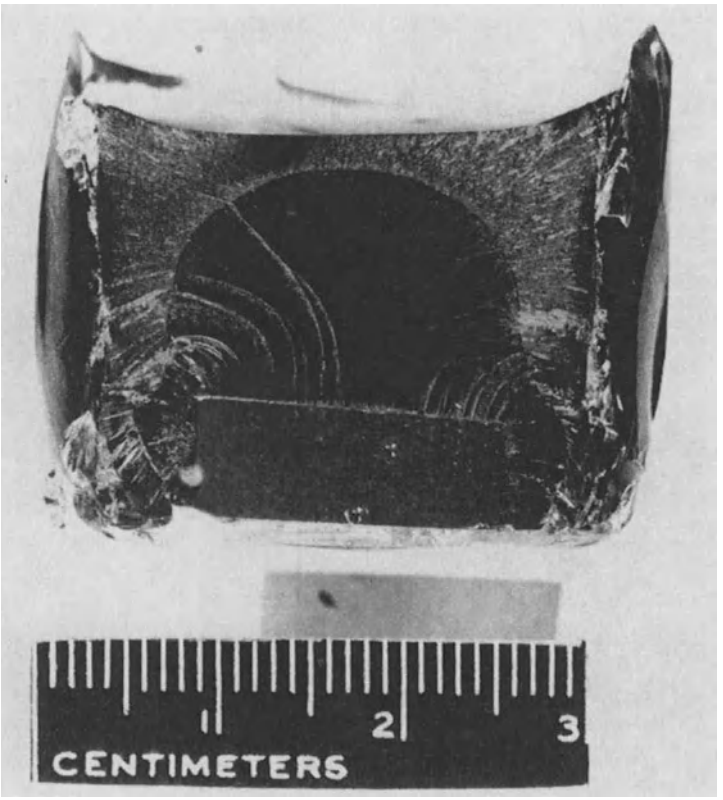


FIGURE 9.4. Photomicrograph taken simultaneously of a piece of plastic and a centimeter scale recording the magnification.

9.6. SUMMARY

Photomicrography is the art of photographing images produced by a microscope to provide a record for display, measurement, publication, or future reference. Photomicrography combines aspects of photography with the principles of microscopy with regard to both equipment and techniques employed. The goal is a clear, bright picture of details chosen by the microscopist that is large enough to serve its intended purpose but has sufficient resolution to show the desired detail and in focus out to the edges. If the photomicrograph is in black and white, proper contrast is necessary to reveal that detail; if it is in color, there must be due regard for correct color balance and density. Since a camera does not have the human

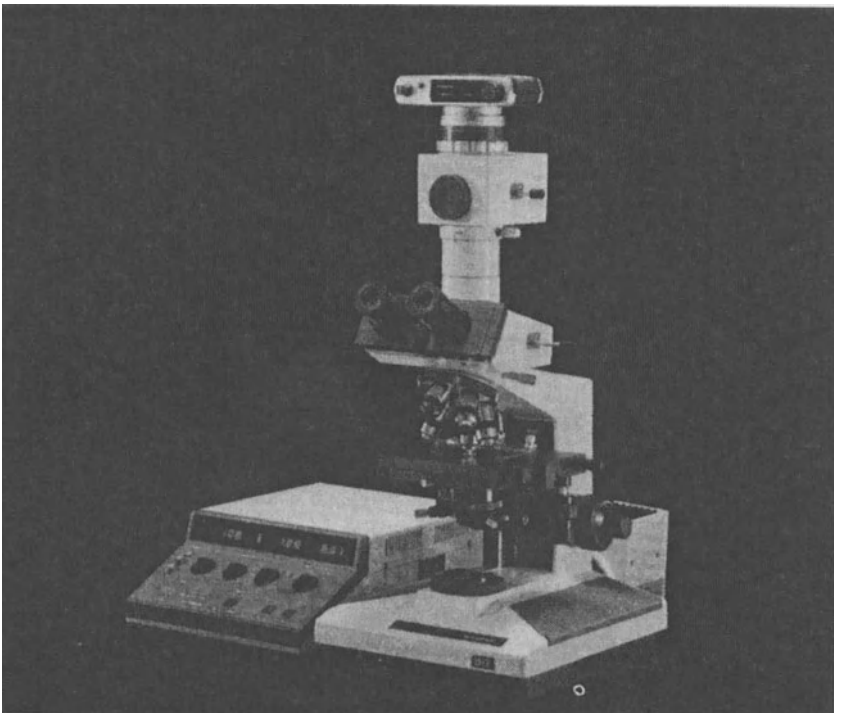


FIGURE 9.5. Binocular, monobjective microscope, with camera for 35-mm roll of film and cabinet of controls. Courtesy of M. Abramowitz and Olympus Corporation.[6]

eye's depth of focus and cannot accommodate itself, extreme measures must be taken to obtain a flat field in the plane of the film. All this makes photomicrography more exacting than visual microscopy, but results are worth the additional effort; a good picture can convey more information than thousands of words and can provide a compact way of storing results. Photomicrographs can also be beautiful in their own right, quite apart from their scientific content, and many have won prizes in art shows.

In the past several decades of photomicrography, camera and microscope have become one: the photomicroscope. Among the advantages are instantly switching from visual observation to photographic recording, precise if not permanent design, mechanical stability and relief from vibrations, standardized operation, and reduced training time for a technician. Among the disadvantages are the cost of the purchase (and perhaps maintenance); reduced experimentation with technique by the technologist; restricted availability for college graduate and undergraduate students

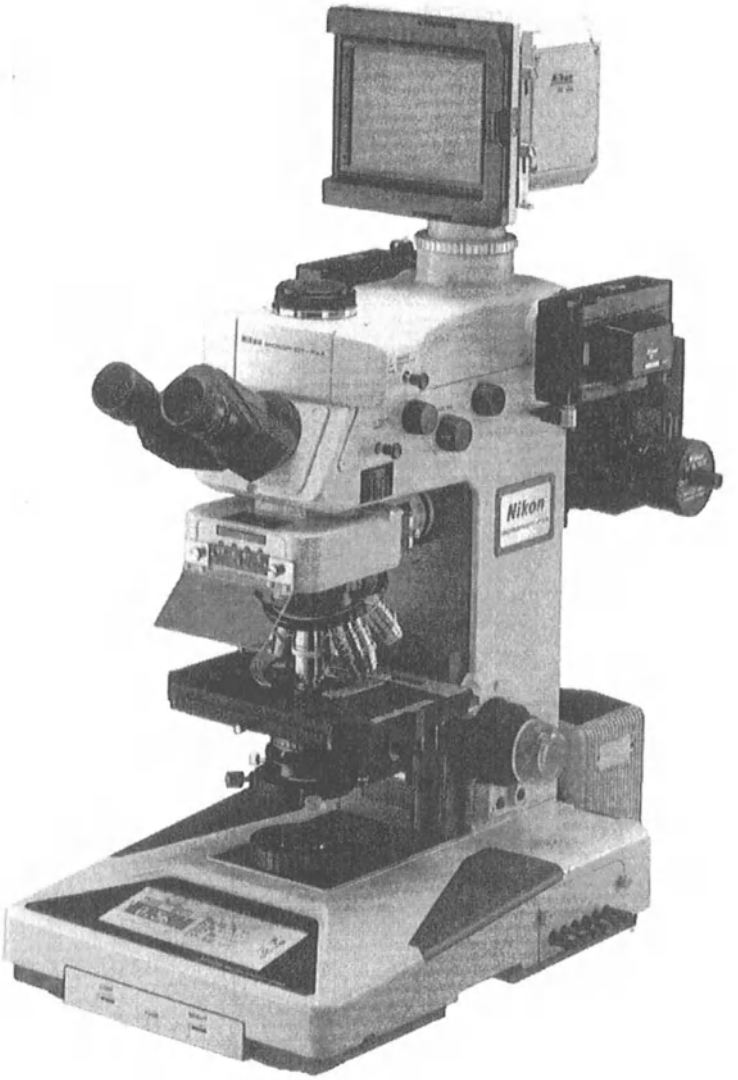


FIGURE 9.6. Binocular monobjective photomicroscope, with two different camera backs: one for "4 × 5" cut film and one for a 35-mm roll of film. Courtesy of Nikon Inc. Instrument Group.[22]

and certainly high school students, since the photomicroscope is used more for industrial and subsidized research than academic research.

The photomicroscope has brought corresponding changes in photographic areas. It is designed to take 35-mm roll film and/or Polaroid® self-processing film. Some photomicroscopes accommodate 4 × 5 inch sheet film and perhaps larger. Such sheet film as well as rolls can be processed in commercial or specialized dark rooms rather than within the microscopical realm.

10

Contrast: Phase, Amplitude, and Color

10.1. CONTRAST: COLORLESS AND COLOR

Contrast, as pointed out in Chapter 2, contributes to visibility, which is next to resolution in importance. Indeed two parts of an object that are resolved separately are not seen separately unless their images are contrasted against what is between them. In light microscopy we are concerned with two kinds of contrast: colorless and color. In both kinds we are also concerned with intensity, the *amplitude* of light waves. Intensity of colorless contrast is in terms of black, white, and intermediate grays. This kind of contrast comes from interference and reinforcement of light waves originating at each point in the object but traveling different paths and lengths through the optical system to form the final image.

10.2. INTERFERENCE: DESTRUCTIVE AND CONSTRUCTIVE

Destructive interference occurs when two waves are *out of phase.*[1] In Figure 10.1 waves *b* and *c* have the same length λ, but they are exactly one-half wavelength out of phase. Since they happen to have the same amplitude, the net result is complete interference (zero intensity, i.e., darkness). *Constructive interference* occurs when two waves are *in phase.* In Figure 10.2, waves *b* and *c* still have the same length λ, but they are exactly in phase. Since they also happen to have the same amplitude, the net result is constructive interference[2] to the extent of twice the original amplitude. Intensity is proportional to the square of the amplitude.

A diffraction image is produced by interference and reinforcement from various light waves from all points in the object (of specified thick-

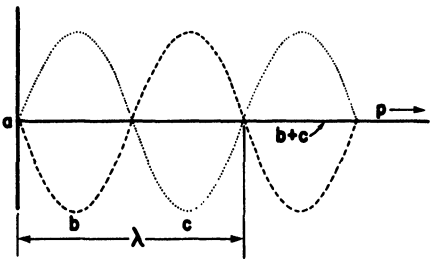


FIGURE 10.1. Two light waves *b* and *c* of the same length λ being propagated in the same direction *p* and having started exactly *one-half* wavelength out of phase. At any point along the path *p* the net result is complete cancellation (zero amplitude, 0*a*).

ness) along different paths within the angular aperture of the objective. Thus by controlling angular apertures in the condenser and objective, the microscopist can go a long way toward controlling the kind and extent of contrast obtained in the image of a given object. In bright-field illumination[1] (see Figure 2.4) by transmitted light, control of the angular aperture of the condenser is by means of a diaphragm, such as the well-known iris diaphragm in Figure 10.3. It provides a variable annular stop, as shown in Figure 10.4a.[3] With the iris diaphragm, nonscattering and poorly scattering parts or particles tend to appear brighter than highly scattering ones. The reverse is true with a central stop, which eliminates some but not all direct rays from the condenser into the objective, as indicated in Figure 10.4b. With this differential stop more highly scattering parts tend to appear brighter than lesser scattering ones. This result is accomplished most simply by inserting into the filter rack of the condenser a diaphragm with an opaque central disk (Figure 10.5) just large enough to screen out all direct rays to the objective when the condenser iris diaphragm is wide open.[4] With this arrangement the field appears black, but those compo-

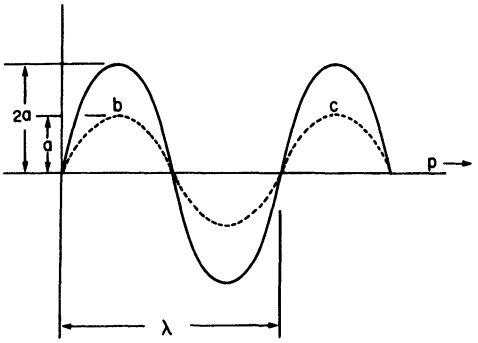


FIGURE 10.2. Two light waves *b* and *c* of the same length λ being propagated in the same direction, but having started exactly in phase. At any point along the path *p*, the net result is constructive interference (double amplitude, 2*a*).

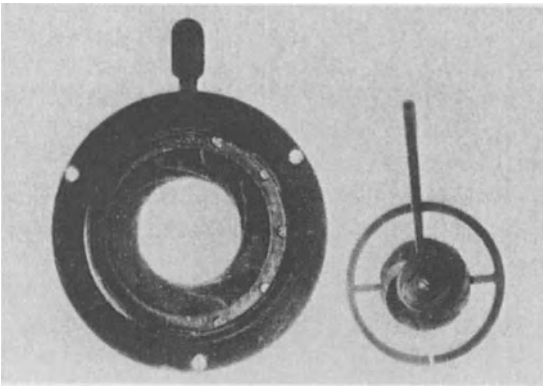


FIGURE 10.3. Iris diaphragm (left) (variable annular stop). Traviss type of expandable central stop (right).

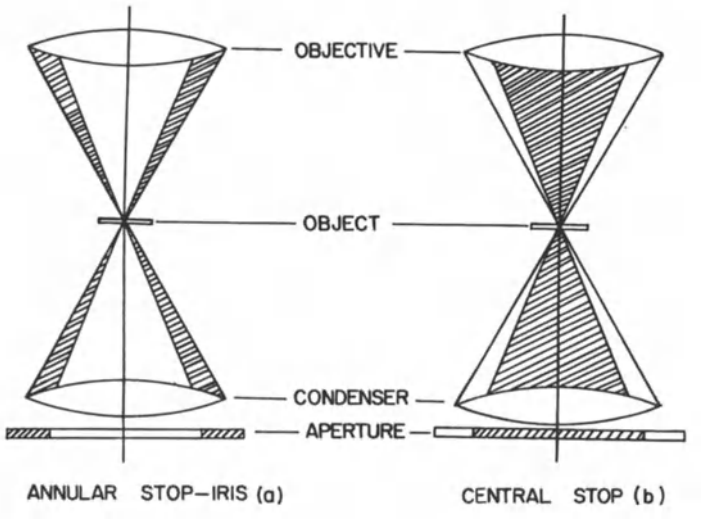


FIGURE 10.4. Reciprocal cones of bright-field illumination, illustrating the use of *annular* (iris) stop (left) and complementary *central* stop (right). The shaded areas indicate portions cut off by the particular variable stops.

nents of the object that scatter light appear as bright spots against the dark background.[5] As a consequence dark-field illumination is extremely contrasty and often very helpful in improving visibility.

If however the central disk of the diaphragm shown in Figure 10.5 is transparent and colored, say, red and the annulus is blue, the background appears red instead of black, and parts or particles that scatter light appear blue instead of white. Such an arrangement is called a Rheinberg filter.[6,6a] It produces a kind of optical staining that improves the visibility of many transparent objects.

These are some of the many ways of obtaining contrast; they should all be tried on any unfamiliar specimen rather than going directly to the phase-amplitude-contrast method, which has its own problems of interpretation. There are however times and circumstances when contrast by earlier methods is inadequate, and staining or treating the specimen is impossible or undesirable.

10.3. PHASE-AMPLITUDE CONTRAST

The phase-amplitude method of obtaining contrast builds on the use of diaphragms for separating and recombining direct versus diffracted rays.[5,7] Figure 10.6[5] illustrates a typical phase-amplitude system for *transmitted* light. Köhler illumination is incident on the lower focal plane of the condenser, where there is an annular diaphragm with an opaque central stop. Rays through this diaphragm are focused as a hollow cone onto the specimen.

In the back focal plane of the objective, there is a conjugate annular diaphragm called a *diffraction plate*, because diffracted and undiffracted rays strike different parts of it (see Figure 10.6). It is also called a *phase plate*

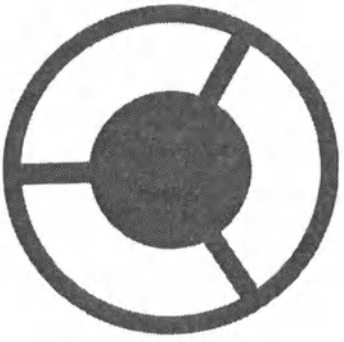


FIGURE 10.5. Fixed central stop, which fits under the substage condenser.[4] The diameter of the permanent stop is selected according to the numerical aperture of the objective and whether bright-field or dark-field effects are desired.

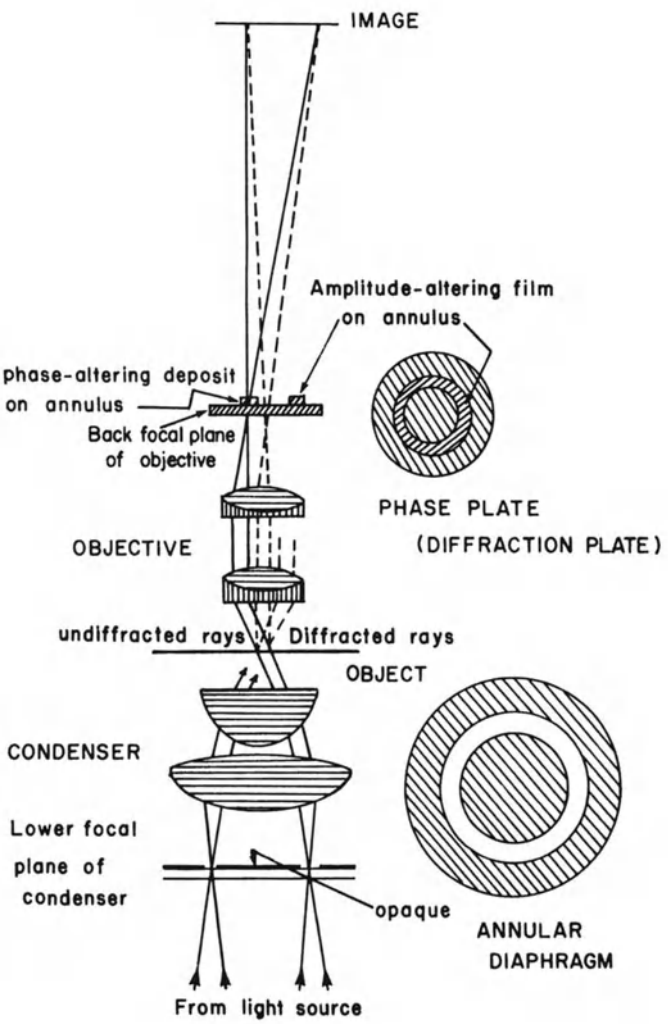


FIGURE 10.6. Typical phase-amplitude microscopical system by transmitted light. In this case the phase-altering material has been deposited in the annulus.[5]

because the phase relationship is altered here. If for example undeviated rays are retarded by a transparent film of proper thickness, *bright* contrast results (see Figures 10.7 and 10.8a).[7] If the phase-delay film is on the center instead of on the annulus, dark contrast results (see Figures 10.7b, 10.8, and 10.9).[7] The actual degree of darkening or lightening depends on the retardation of the light waves from the object. In Figure 10.7c retardation is

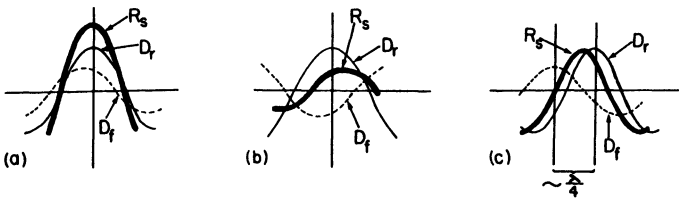


FIGURE 10.7. Principle of phase-contrast illumination.[6] (a) Bright-phase contrast. (b) Dark-phase contrast. D_r = direct (zero-order) wave, D_r = resultant wave. (c) Phase object (with refractive index slightly greater than that of surroundings). Courtesy of Microscope Publications.

assumed to be 0.25λ and the refractive index of the object is assumed to be slightly greater than that of the medium.[7] If instead the refractive index of the object is slightly lower than that of the medium, the results are reversed. With either a bright- or a dark-contrast phase plate, the annulus is usually coated with a partially absorbing (very thin) film, such as silver (Zernike) or carbon soot (Wilska),[5,7] to reduce the amplitude (intensity) of the undiffracted direct rays, so they are commensurate with the low intensity of the diffracted rays.

From the foregoing we see that a great variety of accessories and their combinations is possible, and many are commercially available. The trend of manufacturers has been to develop a wide variety of combinations with interchangeable annuli on one condenser and phase- and amplitude-modification plates built into the many objectives of various contrasts and numerical apertures. Annuli are interchanged in the condenser by rotating them in a turret fitted with labeled click stops. The turret must be "centerable," so that a particular annulus can be made conjugate with the corresponding objective's ringed phase plate. Coincidence may be observed by means of a special compound microscope (telescope) in place of the eyepiece. It your microscope is already fitted with a Bertrand lens (see Chapter 7), use it instead.

10.4. PHASE-AMPLITUDE CONTRAST IN DETERMINING REFRACTIVE INDEX

Figures 10.8a–c indicate that phase-amplitude contrast may be helpful in determining refractive index by immersion methods, especially as matching refractive indices between specimen and immersion medium becomes close, as in Figure 10.8c. Reportedly precision and accuracy in determining refractive index by the Becke test have advanced an additional decimal point by means of phase-amplitude contrast.[5] The commercial

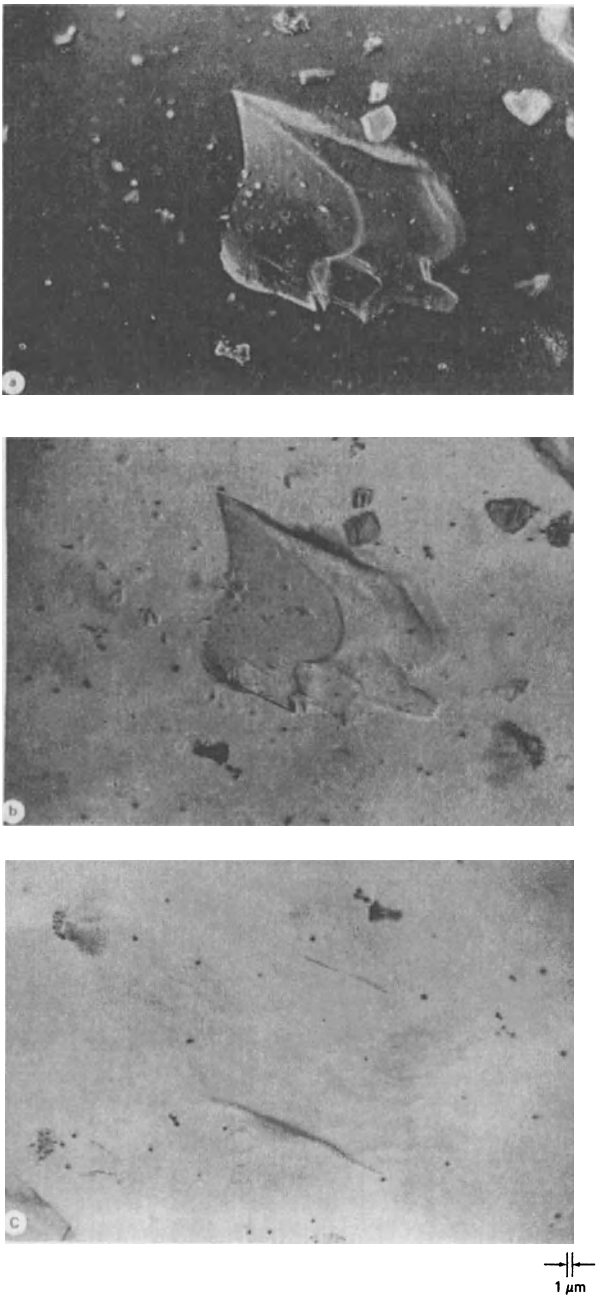


FIGURE 10.8. Phase contrast in practice. (a) Glass particles, bright-contrast phase.[7] (b) Glass particles, dark-contrast phase.[7] (c) Glass particles, bright-field. Same field, same mounting medium (Clarite) as Figures 7a and 7b.[7] Poor contrast.

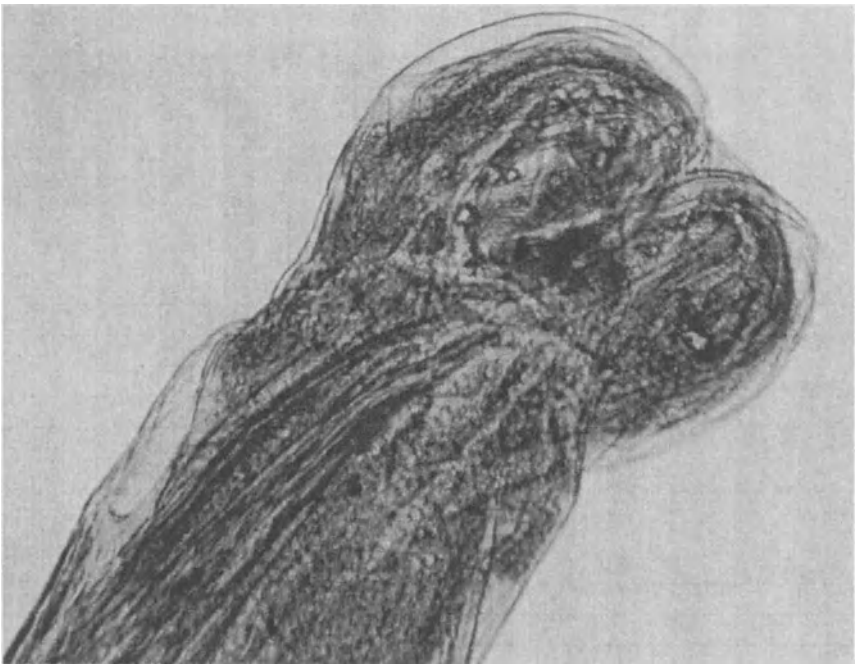


FIGURE 10.9. Head of unstained nematode worm; dark-contrast phase. Electronic flash.[7]

equipment just described is useful only with isotropic or weakly anisotropic specimens however, since the illumination is conoscopic. As discussed in Chapter 7, the Becke test depends on strictly unidirectional rays passing through the crystal,[7a] and using conoscopic illumination violates this principle. Figure 10.9 indicates one way out of the difficulty: (a) a clear slot for the condenser and (b) a groove for dark contrast or a ridge (for bright contrast) inserted in the objective. Both the slot and the groove or ridge are at right angles to the vibration direction of the polarized light.

The slot provides the *plane* that contains the vibration direction and the (perpendicular) propagation direction of the incident light. The vibration plane *is parallel* to only one axis of the crystal at a time. In the one view of the crystal mounted on the microscopical slide, we are interested in only two axes of the crystal; they correspond to the two perpendicular positions of extinction when the crystal is rotated between crossed polars. Therefore the procedure is to rotate the mounted crystal between crossed polars until one of the two perpendicular positions of extinction is reached. Remove the analyzer and match the refractive index for light vibrating from the

polarizer (see Figure 10.10a) with a standard liquid (i.e., of known refractive index). After this is done, turn the mounted crystal to the other (perpendicular) position of extinction, then determine the other principal refractive index for this particular view of the crystal. This method is more accurate than the ordinary Becke method because the accessories shown in Figure 10.10 put more contrast into the Becke band.[7a]

Unfortunately, such accessories may not be commercially available for the particular microscope being used. Saylor[8] describes how to make a negative phase strip in the laboratory; Hartshorne and Stuart[7a] show how to make a corresponding substage slot. An alternative method involves using regular phase-amplitude equipment (see Figure 10.6) but places a slot diaphragm perpendicular to the vibration direction of the polarizer *over* the substage annulus. The result is the same as before but with much reduced intensity.[7a]

Phase-amplitude contrast is especially applicable when examining parts or particles that differ little in refractive index from their natural or designed mounting medium; it is also useful when examining very thin sections. One application is sampling ultrathin sections for transmission electron microscopy (see Chapter 14). Another application is deliberate thinning sections to bring them within the prevailing depth of focus and thereby improve the sharpness of the image.[9]

To examine dry ultrathin sections and other uncovered specimens in air, objectives corrected for use without a cover glass are used, with transmitted phase-contrast illumination provided by a properly diaphragmed condenser.[9]

10.5. VARIABLE PHASE-AMPLITUDE MICROSCOPY

Microscopists employing fixed phase-amplitude plates sometimes need bright contrast on a darker background or dark contrast on a brighter

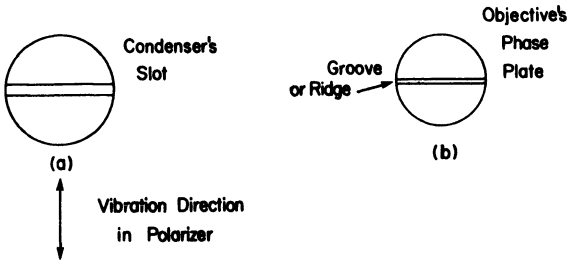


FIGURE 10.10. Special phase-amplitude plates. Substage slot (a) and directional phase plate (b) for determining a specific refractive index of an anisotropic specimen by the Becke test.[7a]

background, with increased or decreased contrast within a specimen. Such specimens include unstained cells and tissues, biopsy tissues, ultrathin sections, and any other unalterable system of poor refractive contrast. The Polanret™ microscope provides continuously variable phase and amplitude alterations. Polanret™ is coined from *pol*arizing, *an*alyzing, and *re*tarding. As Figure 10.11 indicates there is a polarizer and analyzer and a quarter-wave retardation plate. The diagram also shows a turret of four Polazone™ plates, one for each objective: NA = 0.25, NA = 0.50, NA = 0.66, and NA = 1.25 (oil). Each Polazone™ plate has the conjugate area and the complementary area polarized at right angles to each other. The upper right insert in Figure 10.11 indicates that there are four types of phase plates (*A, B, C,* and *D*): solid area, absorbing film, stippled area, and dielectric film. Figure 10.12 shows a pair of phase-amplitude photomicrographs taken at optimum selective settings on the Polanret™ microscope.[10,11]

Figure 10.13 shows another pair of phase-amplitude photomicrographs: protoplasmic bridges, *Diospyros discolor*, taken at optimum selective settings on the Polanret™ microscope.[10]

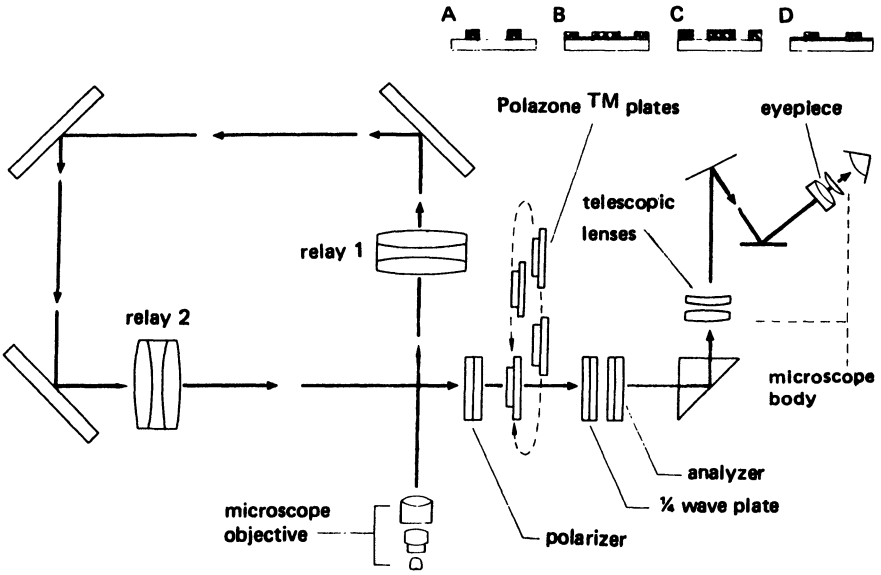


FIGURE 10.11. Diagram of the Polanret™ type system. Upper right insert: Cross-section diagrams of the four types of diffraction plates. Solid area, absorbing film; cross hatched area, dielectric film.[10]

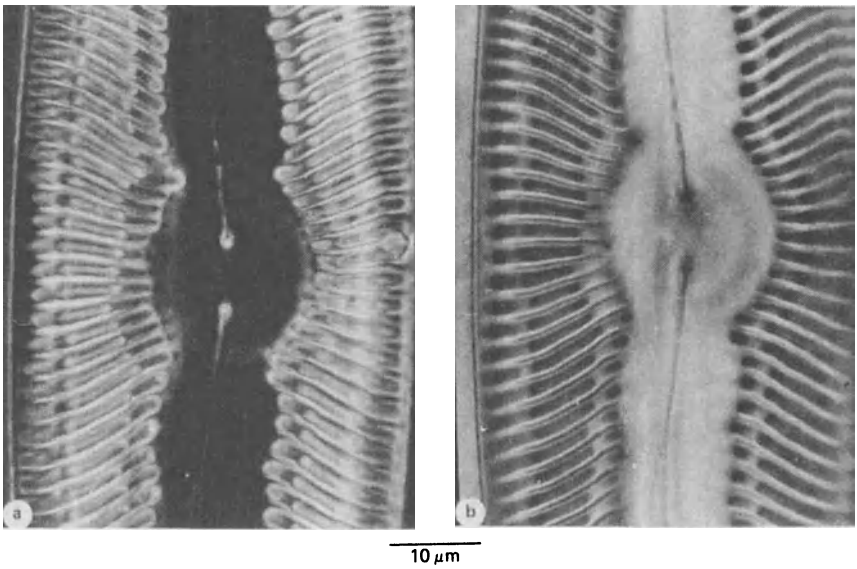


FIGURE 10.12. Diatom, *Pinnularia* sp., photomicrographed with Polanret™ variable phase-amplitude microscope.[10] (a) Bright-contrast plate *A*, dial setting 0.3; retardation setting 9.25 λ. (b) Dark-contrast plate *B*, dial setting 0.2; retardation setting 0.25 λ.[10]

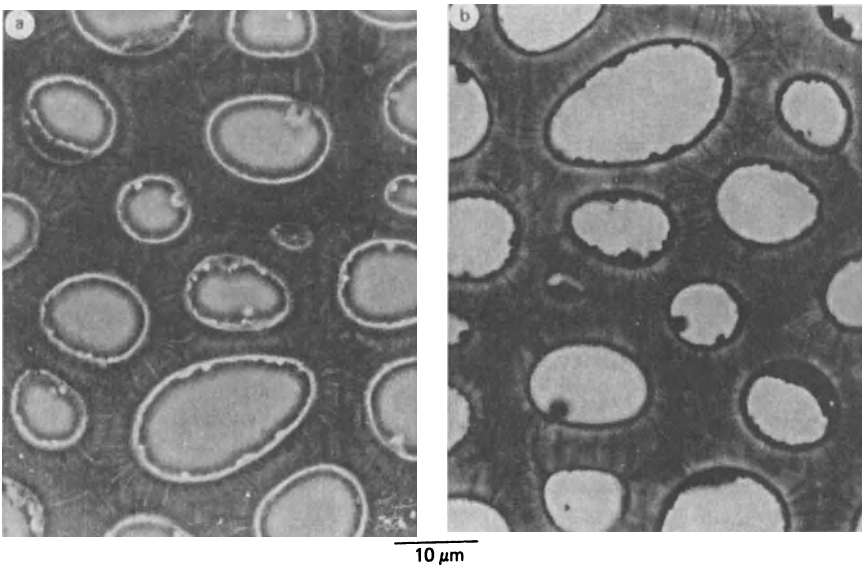


FIGURE 10.13. Protoplasmic bridges, *Diospyros discolor*, photomicrographed with Polanret™ variable phase-amplitude microscope.[10] (a) Bright-contrast plate *A*, dial setting 0.1; retardation setting 0.5 λ. (b) Dark-contrast plate *B*, dial setting 0.55; retardation setting 0.5 λ.[10]

10.6. MODULATION-CONTRAST MICROSCOPY

Chapter 2 discusses the use of oblique illumination to increase resolution and unidirectional oblique illumination to increase contrast. In our present discussion of the Polanret™ phase-amplitude microscope, variably crossing polars[1] is mentioned as a means of modulating the intensity of a light beam.

The novelty of Hoffman's system[12,13] for modulating contrast lies in the variety of effects obtainable by using a sliding, rotatable, slit diaphragm of variable width, all fitted under the condenser. The variable slit is imaged in the back focal plane of the objective, where the modulator is located with its permanently dark, gray, and bright segments. Positioning and adjusting these components must be carefully controlled if optimum visibility is to be achieved.[12] Indeed the components must be adapted and especially fitted to the particular manufacturer's model.[13]

Figure 10.14[13] illustrates the general principles of modulation contrast: P_1 represents a rotatable polarizer of incident light; behind the po-

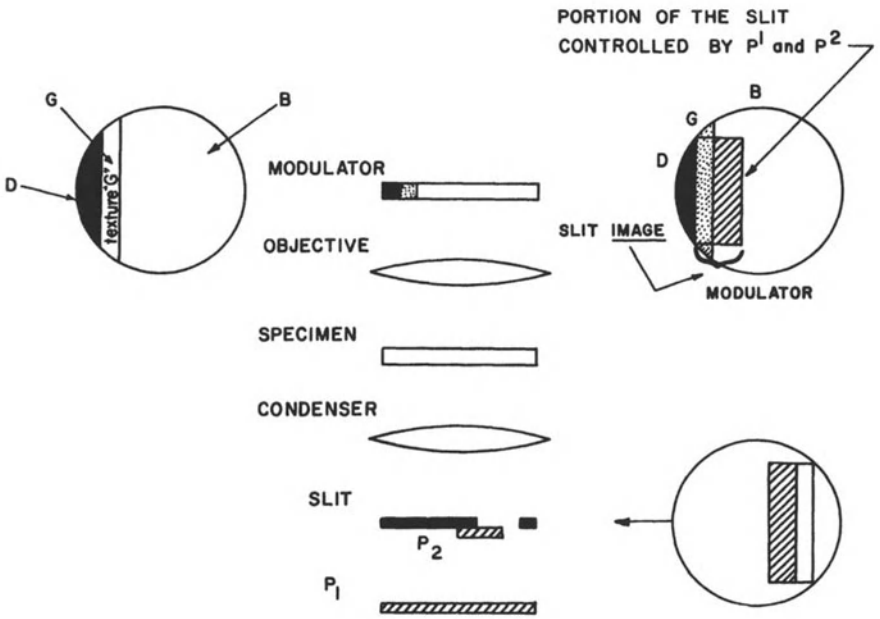


FIGURE 10.14. A diagram showing components for converting a bright-field microscope to a modulation-contrast microscope. The left side shows the modulator regions; dark (*D*), gray (*G*), and bright (*B*). The right side shows the slit image correctly registered and superimposed on the modulator. P_1 and P_2 are polars.[13]

larizer P_1 and in front of the condenser is a sliding slit aperture with a second polar P_2 covering a variable part of the slit. The combination of the slit and P_1 is a rotatable slide. The modulator with its dark region (*D*), gray region (*G*), and bright region (*B*) is placed in the back focal plane of the objective.

Centered Köhler's illumination (see Chapter 2) is used in the system. The modulator (see Figure 10.14) is viewed through a telescope or eyepiece and a Bertrand lens (see Chapter 5). The slit plus polarizer P_2 are slid into place and oriented so that their *image* is superimposed on the modulator, as shown in Figure 10.14. The width of the P_2 area (cross-hatched) is controlled by the degree to which the operator slides P_2. The darkness of the P_2 area is controlled by rotating the polarizer P_2. Focusing the slit and P_2 is performed with the condenser. Finally, the telescope (or Bertrand lens) is removed, and the eyepiece is replaced (or left in).[13]

The following figures show what can be done with modulated contrast. Figure 10.15 is a fresh human cheek cell, showing the three-dimen-

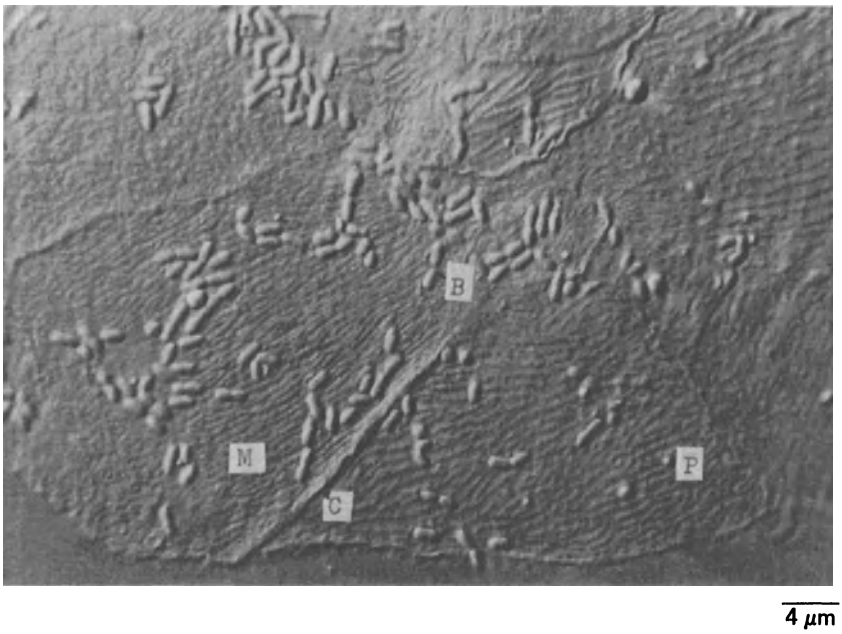


FIGURE 10.15. Modulation-contrast image of the surface of a fresh human cheek cell showing the three-dimensional appearance of bacteria (*B*), membrane folds (M), cell folds (C), and small particles (P). Taken with a 100× Neofluar objective.[13] Courtesy of Robert Hoffman (1977).

sional appearance manifested by the shadows alongside the bacteria and the cell's folds.[13] Figure 10.16 shows carcinoma cells from a mouse, comparing (a) modulation contrast with (b) phase contrast. Figure 10.17 shows that modulated contrast can be used on a stained section to give more detail than that manifested by a bright field. Figure 10.18 compares photomicrographs taken by modulation contrast (a), phase contrast (b), and bright field (c). The indefinite halo (H) around the phase-contrast image (b) presents difficulty in locating the edge when measuring the diameter, area, or volume. The bright-field image (c) shows a sharp circumference but with little contrast for micrometry. Micrograph (a), taken by modulated contrast, gives a good sharp edge and minute detail.[13]

10.7. DISPERSION STAINING

Optical dispersion is the variation of refractive index n with wavelength λ.[1] One way of expressing the dispersion of a particular substance is by the nu value, ν.[14]

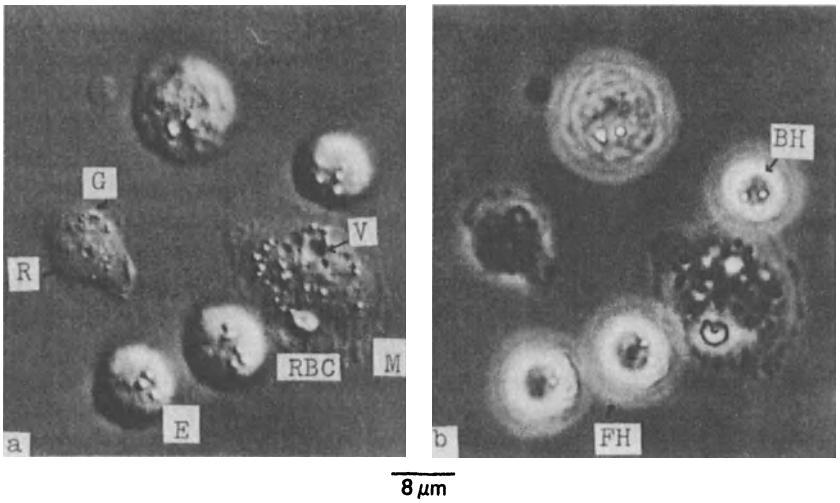


FIGURE 10.16. Comparison photomicrographs using modulation contrast (a) and phase contrast (b) to view mouse peritoneal exudate containing Ehrlich carcinoma cells (*E*) and a relatively flat macrophage (M). Red blood cell (RBC), vacuole (V), and granules (G) are more apparent in the modulation contrast view. Cell R shows multidirectional resolution of the granules. The bright halo (BH) in phase contrast corresponds to position of optical gradients revealed by modulation contrast. The faint halo (FH) in phase contrast is an artifact.[3] Courtesy of M. Padnos and R. Hoffman.

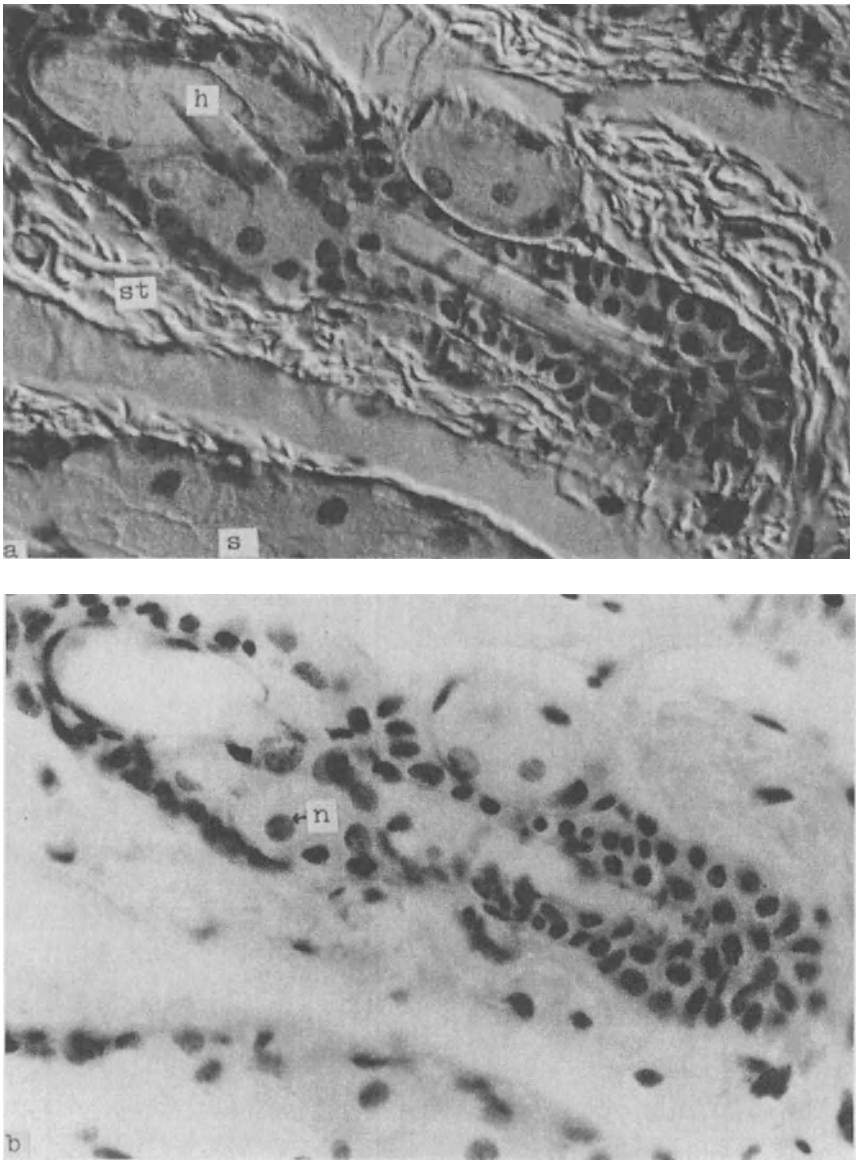


FIGURE 10.17. Comparison of photomicrographs using modulation, contrast (a) and bright field (b) to view a cross section of a mouse skin stained with hematoxalin and eosin.[13] The granular nature of the secretory cells (s), hair shaft (h), and stroma (st) are clearly revealed by modulation contrast. The nucleus (n) is clearly revealed by both systems. Courtesy of Robert Hoffman (1977).

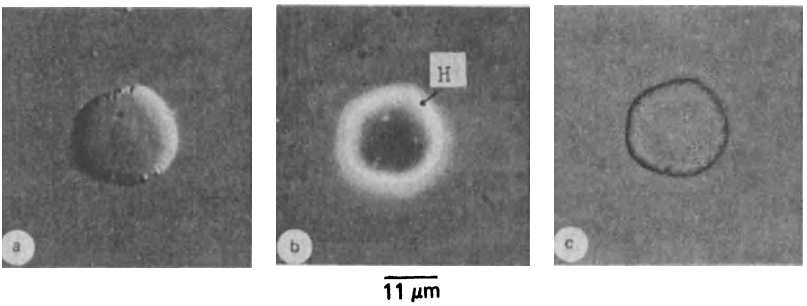


FIGURE 10.18. Photomicrographs comparing the image of a rounded phase object by modulation contrast (a), phase contrast (b), and bright field (c).[13] In (a) note the three-dimensional appearance, image dimension, and edge detail. In (b) the edge detail is only partially visible. The image dimension is indefinite if the bright halo (H) is considered to be an artifact. The halo however represents structure and corresponds to optical gradients. The bright-field image (c) is barely visible except for its extent and some edge detail. Courtesy of M. Padnos.

$$\nu = \frac{n_D - 1}{n_F - n_C}$$

wherein D, C, and F, are particular wavelengths λ in the visible spectrum, usually chosen as follows:

D = 589 nm C = 656 nm F = 486 nm

If a specimen is immersed in a fluid of the same refractive index as its own for a single wavelength λ and the dispersion is quite *different* for the two media, either the specimen or the mounting medium will show color contrast, depending on the position of focus. The phenomenon is called the Christiansen effect,[5] and it is often noticed when determining the refractive index by the Becke method. The Christiansen color may be natural or fortuitous; it can also be produced purposely, and then the process becomes a kind of *optical staining.* Crossman, who advocated *dispersion staining* in 1949,[15] used mixtures of cinnamic aldehyde with butyl carbitol[16] to vary the refractive indices to suit the specimen being optically stained. Various other mixtures are suggested by Hartshorne and Stuart,[7a] including a commercially available[17] series of miscible liquids. Each bottle of liquid is labeled with the refractive index for the D line, its temperature coefficient, and its dispersion.

McCrone and his colleagues have published data for the dispersion staining of hundreds of minerals, chemicals, and other materials.[18,19] In each case at a given temperature (25° C), values are given for n_F (486 nm),

n_D (589 nm), and n_C (656 nm). NOTE: These refractive indices are for *three different liquids* measured for the *D line* at 25° C.[7a,20] The liquids are those whose refractive indices *match* that of the specimen at the specific wavelength (color). While McCrone's tables are primarily for determinative purposes, they also give information about optical staining to improve contrast.

Generally speaking, liquids have greater optical dispersion than solids, as shown in Figure 10.19.[14] Therefore if a particle of a typical solid is microscopically mounted in a typical liquid whose refractive index of the solid in red light (n_C) is *greater* than the (n_C) for the liquid and whose refractive index of the solid in blue light (n_F) is *less* than the n_F of the liquid, as the objective is focused *upward*, the particle will appear red and the liquid appears blue, as indicated in Figure 10.20.[14] The light (λ_0) of matching refractive index (say, n_{25}^D) will not be refracted but will be transmitted parallel to the optical axis of the microscope. The corresponding analytical dispersion staining "curve" (*A*) is shown in Figure 10.20. The abscissa is $1/\lambda^2$ instead of λ (the wavelength for the matching refractive index of liquid and solid) to straighten out the curves. In curve *B* in Figure 10.20, the difference in dispersion between liquid and solid is much less than in *A*. In *C* there is no difference. In *D* the solid has greater dispersion than the liquid. The corresponding spectra seen in the back aperture of the objective are indicated in Figure 10.21.[14]

10.8. SPECIAL ACCESSORIES

For situations *A* and *B* in Figure 10.21, a large annular stop transmits the central rays *Y*, whereas a large central stop shuts them off. In situation *C* in Figure 10.21 (rare), no stop can help the color contrast significantly. In

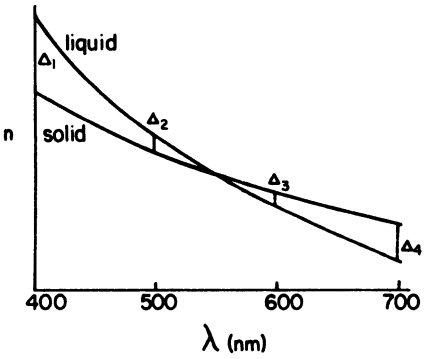


FIGURE 10.19. Dispersion curves.[14]

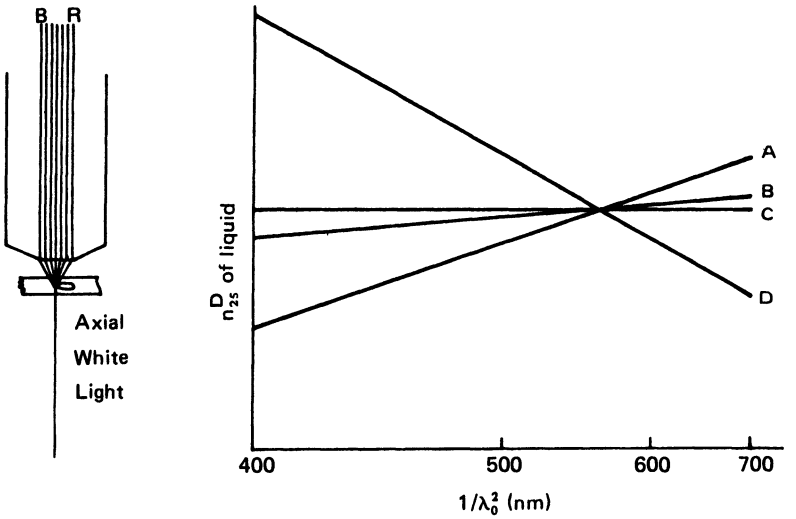


FIGURE 10.20. Refraction occuring at particle liquid interface (left) corresponding to dispersion staining curve *A* (right); other possible dispersion staining curves are also shown (right).[14]

situation *D* in Figure 10.21 (unusual), the coloration is the reverse of situations *A* and *B*.[14] In 1962 Malies designed a turret with two stops that is fitted with the standard objective thread.[21] The housing screws into a standard[22] microscope and carries an objective of NA = 0.25 (see Figure 10.22). The turret in the housing has three openings: plain, annular stop (0.2-nm hole), and central stop.[21] These produce the three effects just noted.

10.9. SCHLIEREN MICROSCOPE

Schlieren are regions of varying refraction in a transparent medium, often caused by differences in temperature or pressure, and detectable

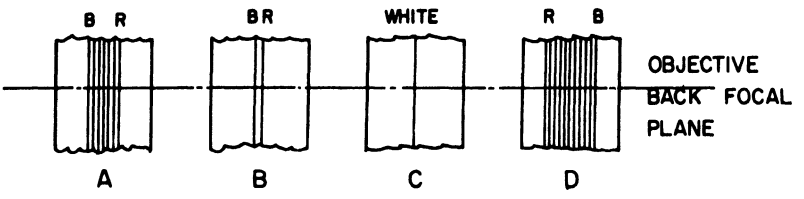


FIGURE 10.21. Objective back focal planes corresponding to dispersion staining curves in Figure 10.20 (right). The specimen is assumed to be the one shown in Figure 10.20 (left).[14]

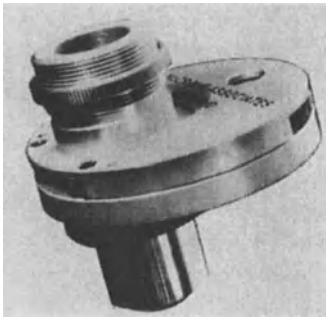


FIGURE 10.22. Turret carrying three openings (plain, annular stop, and central stop) over the objective, NA = 0.25.[21] Courtesy of Microscope Publications.

especially by photographing the passage of a beam of light.[1] The schlieren microscope[23] uses stops only 10–12 μm in diameter, which makes it the most sensitive dispersion-staining system.[14] Such small stops cannot be used with objectives of high NA, wherein they would do the most good, because the back focal plane of such objectives is inside the objectives. Instead the stop is placed at the eye point of an ordinary eyepiece with an auxiliary eyepiece placed above, as shown in Figure 10.23. The original eyepiece now becomes a transfer lens; the new eyepiece should be a 1× telescope. A well-corrected, high-aperture (f = 1.5), short-focal-length (50-mm) camera lens is adequate. Its mount should be 'centerable' with the condenser's iris diaphragm. The overall tube length is 200 mm (instead of 160–170 mm). Köhler's illumination is used. After the proper schlieren stop is placed at the eye point, the condenser's iris diaphragm is opened as far as possible while maintaining a dark field. A high-NA objective may be used, even an oil-immersion objective.[24]

Using dispersion staining to determine specific refractive indices of an anisotropic substance involves no monochromator, special illuminators, or filters, no index match with a specific liquid, and no problem with ambiguous movement of the Becke line.[23] It does require determining the dispersion-staining curve by noting the matching wavelength (λ_0) for several different liquids in the matching range. The intersection of the best line through λ = 589.3 nm is n_D. The index n_F is given on the bottle for λ_0 at 486.1 nm, and n_C is given on the bottle for λ_0 at 656.3 nm.[14]

Alternatively the three λ_0 can be determined by mounting the specimen in a Cargille liquid showing λ_0 near 700 nm at room temperature. Using the temperature coefficient of the refractive index for that liquid enables us to calculate the index for each λ_0 as the temperature is raised on a hot stage (see Chapter 12). A quantitative schlieren method is described by Kafi and Glatt, who give a theoretical explanation of the schlieren method.[25]

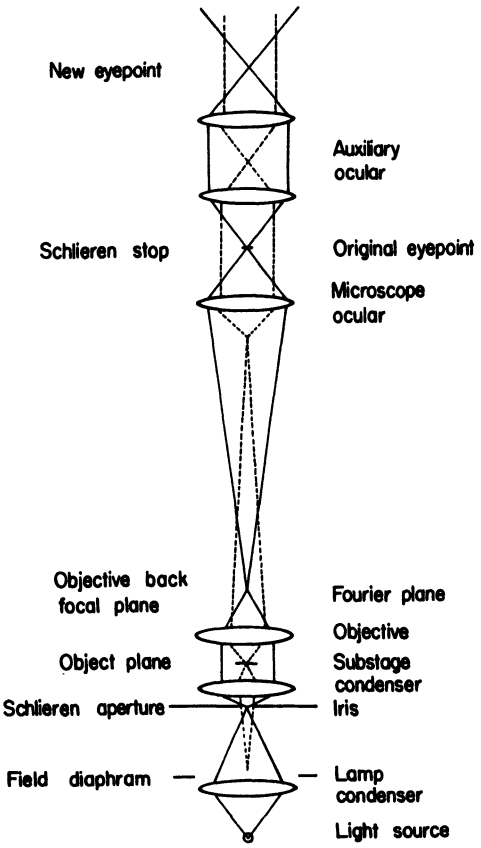


FIGURE 10.23. Light path through a microscope with a schlieren eyepiece.[23]

10.10. SUMMARY

If two adjacent particles of a microscopical specimen are not resolved by the physical quality of the optical system (as explained in Chapter 2), then they cannot be distinguished, no matter what the magnification. And if they are resolved but do not stand out against the background with sufficient contrast, they still cannot be seen. This second situation arises frequently when examining biological tissue if the watery material differs very little in refractive index from the watery medium in which it is suspended. In such instances the microscopist can gain the necessary contrast by resorting to phase contrast, phase-amplitude modulation, color contrast, continuously variable contrast modulation, or dispersion staining.

Since the diffraction image of an object is produced by various degrees of destructive interference and reinforcement of the light waves from all

parts of the object included in the angular aperture of the objective, one way of varying the contrast is to vary the paths of light within that cone. Closing down the iris diaphragm of the condenser reduces the prominence of highly scattering parts of the object and thus emphasizes nonscattering transparent parts. Conversely inserting a diaphragm with a small opaque disk into the condenser (with a wide-open iris diaphragm) cuts off some direct rays and emphasizes wide-angle rays from highly scattering parts. The extreme of this technique involves cutting off all direct rays and producing dark-field illumination, so that the opaque or highly scattering particles in the object appear bright against a black background.

A variation of this technique uses multicolored diaphragms (Rheinberg filters) instead of opaque stops. For example a condenser diaphragm with a red transparent center and a blue annulus around it produces a red background against which any highly scattering particles appear to be blue dots. *Phase-amplitude* contrast is achieved by a more elaborate arrangement in which a condenser diaphragm with an opaque central stop is complemented by a phase plate, a small diaphragm inserted in the back focal plane of the objective. The phase plate has a film of material deposited on it that delays light rays passing through it, e.g., by one-quarter of a cycle, so that the delayed rays interfere with the direct rays and enhance the contrast. If the phase-delay film is deposited on the annulus of the phase plate (and the refractive index of the object is greater than that of the medium), the object appears brighter than its background (the mounting medium). If the phase-delay film is deposited on the central portion of the phase plate instead, then the object appears darker than its background. In either case interference caused by phase delay reduces intensity, so a compensating reduction of intensity in the oblique rays is achieved by depositing a very thin film of silver or carbon on the annulus film. The combined effect is then phase-amplitude contrast.

There are many devices and accessories designed to achieve phase contrast conveniently, all of which involve modifying the condenser and the objective to include the necessary diaphragms. An arrangement that allows quick change from bright-field to dark-field to phase-contrast illumination with parfocal objectives is preferred. All devices require provision for centering the complementary diaphragms. Once the chosen equipment is installed, phase-amplitude contrast can be used to improve the accuracy and precision of determining refractive indices.

There is call for continuously variable alteration of phase and amplitude. The Polanret™-type microscope obtains the desired variability by a system of regular polars, a turret of Polazone™ phase-amplitude polar plates, a turret of objectives, and dial settings on a specialized microscope for varying either bright or dark contrast.

The Hoffman system for modulating contrast custom fits a set of only three added units: a polarizer for the incident light; a rotatable holder for a sliding slit with a partial polar to fit under the condenser; and a modulator disk with dark, gray, and bright areas to fit in the back focal plane of the objective. The result is directional oblique illumination with modulated contrast between image and background. Such an arrangement provides modulation shadows and sharp outlines without halos, a very three-dimensional effect. Comparing the modulated contrast image with that of a bright field or phase amplitude is easy.

Dispersion staining takes advantage of the Christiansen color effect that is sometimes noticed when determining the refractive index for white light by the Becke method (see Chapter 6). The Christiansen color is manifested when the variation of the refractive index with the wavelength in white light is different (usually greater) for the immersion liquid than for the immersed solid. If for a typical example the refractive index in red light is *greater* for the solid than for the liquid and if the index in blue light is *less* for the solid than for the liquid and the microscopical objective is focused *upward,* the solid will appear *red* and the liquid *blue.* In recent years dispersion staining has gained usage for determinative purposes by virtue of the commercial appearance of the required microscopical accessories and standardized immersion liquids of high dispersive power.

11

Interference Microscopy

11.1. INTERFERENCE OF TWO WHOLE BEAMS

In Chapter 11 we are essentially concerned with *two whole microscopical beams* (rather than individual rays) that are deliberately caused to interfere with each other. The graphic result is a pattern of *interference fringes* analogous to Newton's rings.[1] With incident white light, the fringes are from Newton's series of color bands more or less superimposed on the pictorial image. Figure 11.1 for example shows three different micrographs of the same surface area of crystalline grains.[2] All three micrographs were taken on the same simple microscopical interferometer, shown schematically in Figure 2. Micrograph (a) in Figure 11.1 was taken with practically no tilting angle α to the reference surface [see reference reflector (4) in Figure 11.2]; hence there was practically no interference. Incidentally the reference beam was sufficiently out of phase with the specimen's beam to produce *interference contrast*. Thus interference microscopy is related to phase-amplitude contrast (see Chapter 10).

Photomicrographs (b) and (c) in Figure 11.1 show definite interference bands. They are contour bands, parallel within grain boundaries but disjointed at the boundaries because the grain surfaces are at uneven levels due to etching rates corresponding to crystallographic orientations of the three-dimensional grains. Differences in detectable surface level among grains are slight, varying from a fraction of a wavelength to 10 μm (1958)[1] to 0.5 μm (1970).[3] While interferometry is a delicate kind of vertical micrometry, for purposes of explaining the significance of interference fringes as contour lines or bands, our model (see Figure 11.3) is crudely made for the sake of simplicity. Nevertheless the stepwise elevation indicated in the side view (a) of Figure 11.3 is suggested by the microsteps on the faces of some crystals, which show a plan view similar to that of the model (b).[4]

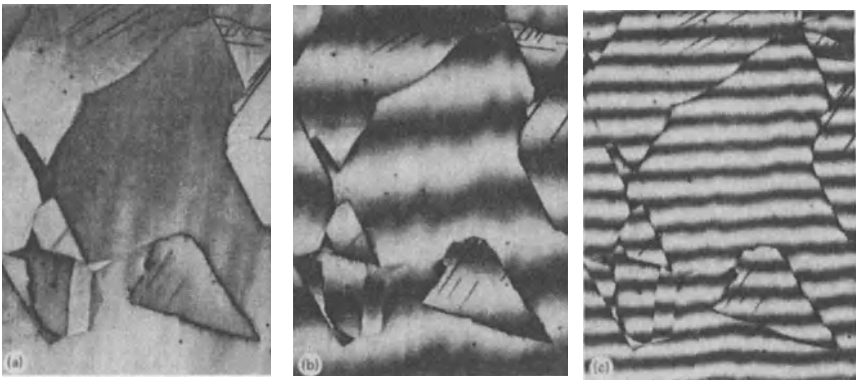


FIGURE 11.1. Three micrographs of the same surface area of crystalline grains, (a) interference contrast, (b) broad bands, and (c) narrow bands, taken on the same interference microscope (see Figure 11.2) but with the interfering reference beam at a tilt angle α varying from zero for (a) to a larger angle in (b) and largest in (c), so that band width varies from infinity in (a) to narrowest in (c).[2] Taken through Zeiss optics. Courtesy of Carl Zeiss.

11.2. TYPES OF INTERFERENCE MICROSCOPES

11.2.1. Single Microscopes

In Figure 11.2,[2] light from the source (1) is split into two beams by the semitransparent mirror (2). One beam is reflected from a reference reflector (4) and a semitransparent reflector (2) into the simple or compound microscope (5). Along the way to the screen (6) (or eye), the reference beam

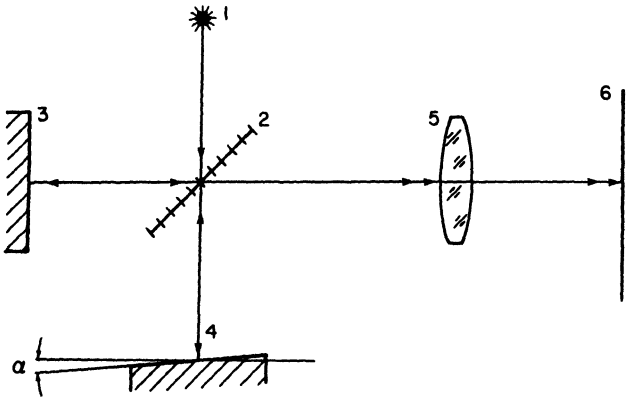


FIGURE 11.2. Schematic diagram of a typical beam-splitting interference system.[2]

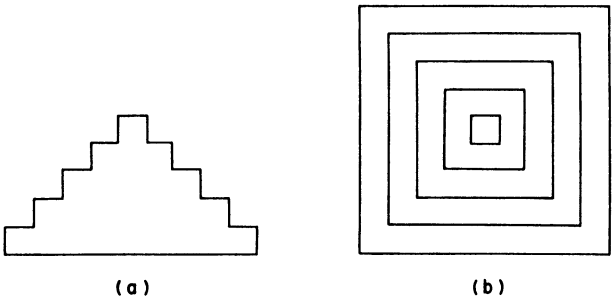


FIGURE 11.3. Model of a stepwise pyramidal elevation: (a) side view showing elevation and (b) plan view showing contour lines.[4]

interferes with the beam from the specimen (3), producing interference images, such as are shown in Figure 11.1.[2]

While the single-lens system in Figure 11.2 belongs to a simple microscope with the semitransparent reflector (2) in front of the lens system, a prototype interference microscope is based on a microscope fitted with a vertical illuminator (see Chapter 4) and an optically flat glass plate in close contact with the specimen surface. The surface of a metallic specimen is too reflecting when compared with the surface of the glass plate, so the glass plate must be given a semireflecting coating of commensurate reflectance with silver or other metal. With monochromatic light, adjacent fringes correspond to differences in depth of half a wavelength, so that even shallower depths can be measured. This principle has been modified by Mirau, who converted the glass plate into a beam splitter, so that one beam is directed to a separate metal-coated reference surface while the second beam is reflected from the surface under examination, as shown in Figure 11.4[5] The Watson manufacturers have converted the system into a *unit objective* by bringing the illumination into the side of the objective instead of the side of the microscope's tube. Therefore the semireflecting surface in Figure 11.4, or the equivalent, must be removed if the microscope is fitted with a vertical illuminator. As shown in Figure 11.5, the Watson interference objective fits on almost any microscope equipped with a standard thread.[6] The light from a sodium lamp (no filter) or mercury lamp (plus *D* filter) enters the collimating condenser *C* on the side of the objective. The beam splitter *E* sends some light down to the specimen (and then through the objective system *O*). The beam splitter *E* sends its second beam to the reference mirror *L*. The reference beam from *L* is aimed back to the beam splitter *E* by adjusting screws *G*, *H*, and *K*, so that the reference beam is reflected into the objective system *O*. There the reference beam interferes

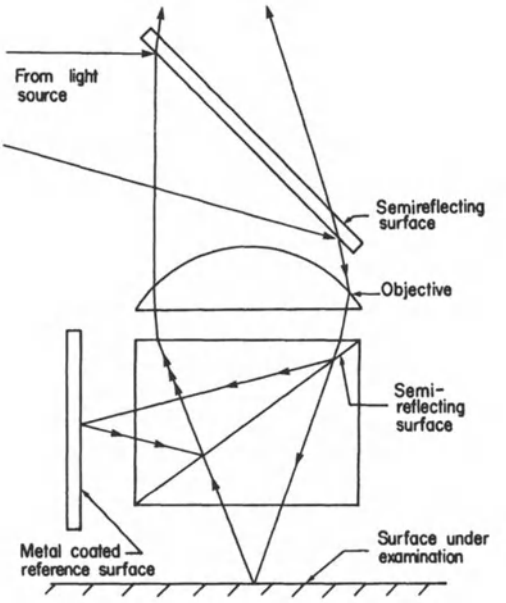


FIGURE 11.4. Mirau interference objective principle.[5] Courtesy of Microscope Publications.

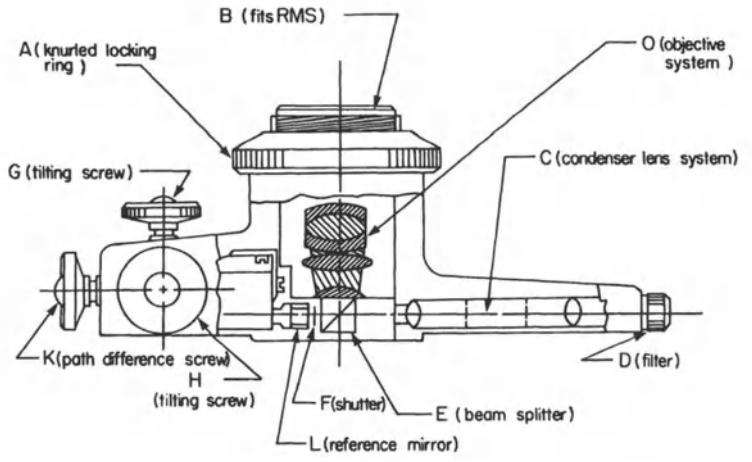


FIGURE 11.5. Watson interference objective. (Drawing adapted from illustration courtesy the Watson Microscopy Division of M. E. L. Equipment Company.)[5] Courtesy of Microscope Publications.

with the beam from the object to produce tell-tale fringes depicting the specimen's surface. Shutter *F* shuts off the interfering beam when the specimen is to be observed without the interference fringes.[5]

These single beam-splitting interference microscopes (see Figures 11.2, 11.4, and 11.5) have been used in examining metallic surfaces and edges (such as razor blades) and thin films (such as lacquers and electron-microscopical substrata). Thin biological specimens can be studied in similar fashion by mounting them between half-metallized optical flats. In this case internal structural features of the specimen determine local resistance to cutting, and so features are reflected in the surface topography of the thin section.[1]

In the interference microscopes illustrated in Figures 11.2 and 11.4, the specimen surface and reference surface are separated by a few centimeters so the two wavefronts can be separated by as much. However another way of separating the two wavefronts employs a doubly refracting substance, such as calcite (see Chapter 5). Then separating the two wavefronts is reduced to as little as the resolving power of the light microscope (~0.2 μm; see Chapter 2). Such fine beam splitting is called *differential* interference contrast or differential separation of the wavefronts.[2] Figure 11.6 shows a

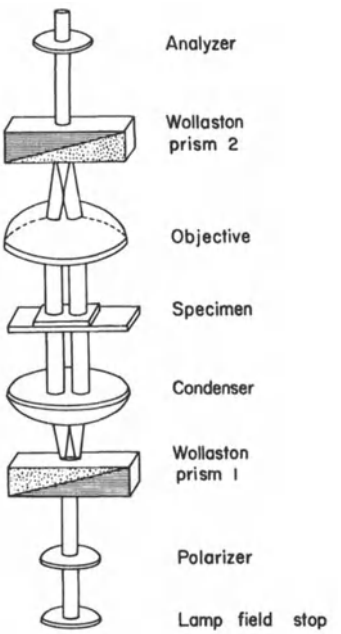


FIGURE 11.6. Schematic diagram of a two-beam interference microscope for differential interference contrast in transmitted light.[2] (Separation between the two wavefronts is exaggerated.)

schematic diagram of differential beam splitting by *transmitted* light, and Figure 11.7 shows beam splitting by *reflected* light.[2]

Both microscopes (see Figures 11.6 and 11.7) feature the Wollaston prism as modified by Nomarski.[2] This prism receives polarized light, then splits it into two wavefronts, separated by only 1 μm (exaggerated in Figures 11.6 and 11.7). The Zeiss–Nomarski equipment is designed to complement the Zeiss phase-amplitude contrast equipment. This interchangeability is in itself an advantage; at the same time it emphasizes that the chief purpose of this type of interference equipment is to gain contrast. Such contrast is gained without loss in resolution or optical sectionability; at the same time there are no troublesome halos and no loss in path length. The brilliance of colors is especially striking by reflected light.[2] The Nomarski method is also recommended for examining polymer crystals, which are otherwise low in contrast[4] because they differ so little from their surroundings in refractive index.

The Baker interference microscope incorporates a continuously variable-phase difference between images in and out of focus, as produced by doubly refractive rotatable plates. The image is in color contrast, which shows very small differences in thickness and refractive index. Like the Nomarski microscope, the American Optical Baker interference microscope is recommended for the contrast it provides to specimens low in contrast[4] (see Figure 11.8).

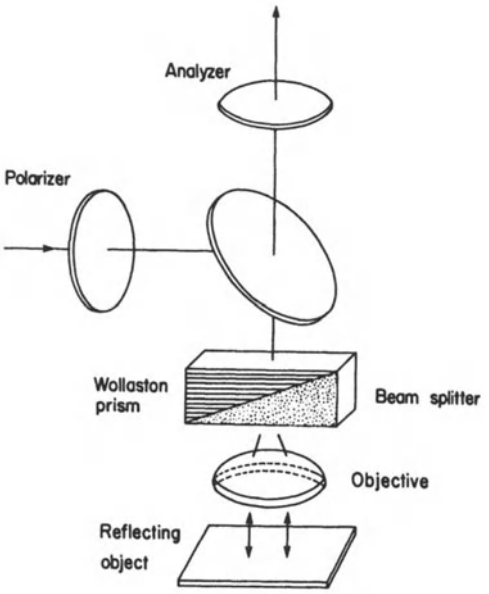


FIGURE 11.7. Schematic diagram of a reflected light system for differential interference contrast microscopy.[2] (Separation between the two wavefronts is exaggerated.)

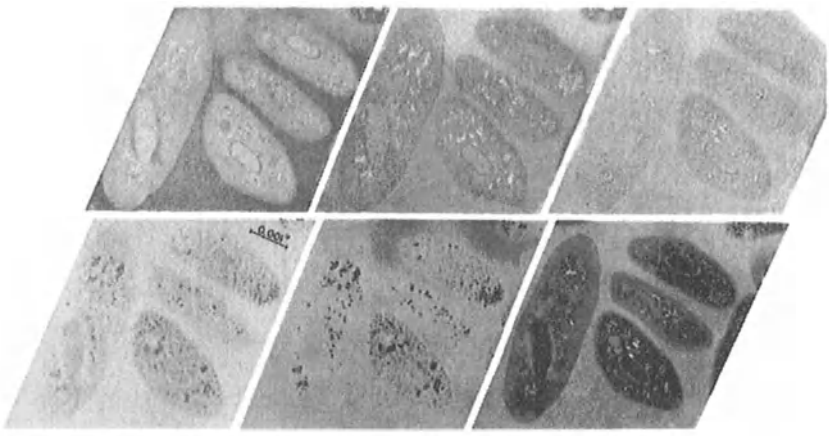


FIGURE 11.8. Taken with the A-O Baker interference microscope. Contrast can be changed to show desired detail, as in these photomicrographs of unstained protozoa—part of a series used in the *Scientific American* and on educational television. Courtesy of American Optical Co. and R. W. Richards.

11.2.2. Mach–Zehnder Systems

The double interference-contrast microscope, devised by Linnik, is built like Michelson's interferometer.[1] The illuminating beam is split and sent into *two* microscopes: one for the specimen and the duplicate for the reference beam. In the Leitz interference microscope designed by Mach and Zehnder, the two beams are separated by 62 mm.[7] The isolation of the two beams removes all concern about the identity of the specimen area and the reference area. An anisotropic specimen produces a fringe shift of a particular magnitude and direction. If the same specimen is placed under the *other* microscope, the fringe shaft is equal but *opposite*.[7]

An important use of this type of interference microscope is in determining refractive indices.[7,8] Since most specimens are anisotropic and therefore have two or more specific indices, the instrument uses polarized incident light. Part of the polarized light passes through the specimen, which is mounted in a liquid of known refractive index and oriented at one of the two positions of extinction. The other part of the polarized light goes through only the standard mountant. The two beams are then combined, and the resultant retardations appear as amplitude differences in the image. These are measured by means of a built-in compensator, such as the Sénarmont type. If the thickness at one given point in the specimen is known, the refractive index can be calculated. Or the specimen can be

mounted in a second standard liquid and the second retardation can be observed as before. Then it becomes possible to calculate the refractive index without knowing the thickness. This method gives a more accurate value for the refractive index than the Becke method. Besides interferometry gives the *average* value through the cross section of the specimen, while the Becke method gives the refractive index of the superficial layer. Therefore if the specimen is a coated (e.g., weathered) crystal or a fiber with a characteristic skin, values from the two different methods are different and thereby determinative.

Another brand of interference microscope that has had wide acceptance in recent years is manufactured by aus Jena. Its split beams do not go through two essentially structurally independent microscopes as does the Leitz interference microscope previously described. This means that the aus Jena model cannot be used for examining large samples as can the Leitz, but the aus Jus model is simpler, easier to maintain, and less expensive. It is effectively used for fields of view of 200 μm or less. The aus Jena model does use a Mach–Zehnder interferometer, as does the Leitz model, which has not been manufactured for a considerable number of years.

The aus Jena model uses a shearing method to separate the two mutually polarized beams to generate two images of the same object, and a small Mach–Zehnder interferometer is located on the image side of the optical path. Like most true interference microscopes, this microscope determines refractive indices to an accuracy of 2×10^{-4}, path lengths of a few nm up to 5 μm, as well as precise measurement of contact angles.[9] The initial beam into the specimen is generally polarized by diffraction from a slit in the front focal plane of the microscope.[9,10] This instrument functions through a Mach–Zehnder interferometer composed of a pair of Wollaston prisms on the image side of the objective, and here the beam is converted into two beams that are mutually polarized by the first of these two prisms. In the rear focal plane, after the first Wollaston prism, one of the two beams goes through a shearing prism to generate two images and then through a wedge compensator to set the phase difference between two wavefronts; the other beam goes through adjustable compensator plates. A diagram of the light raypath for the aus Jena interference microscope is shown in Figure 11.9.[9] Similar microscopes for reflected light also are produced.[9]

Basic concepts of the aus Jena microscope can be somewhat complicated, but its use is straightforward after a procedure is established. In addition to the basic reference of Krug *et al.*,[10] additional references can provide further background material.[11,12]

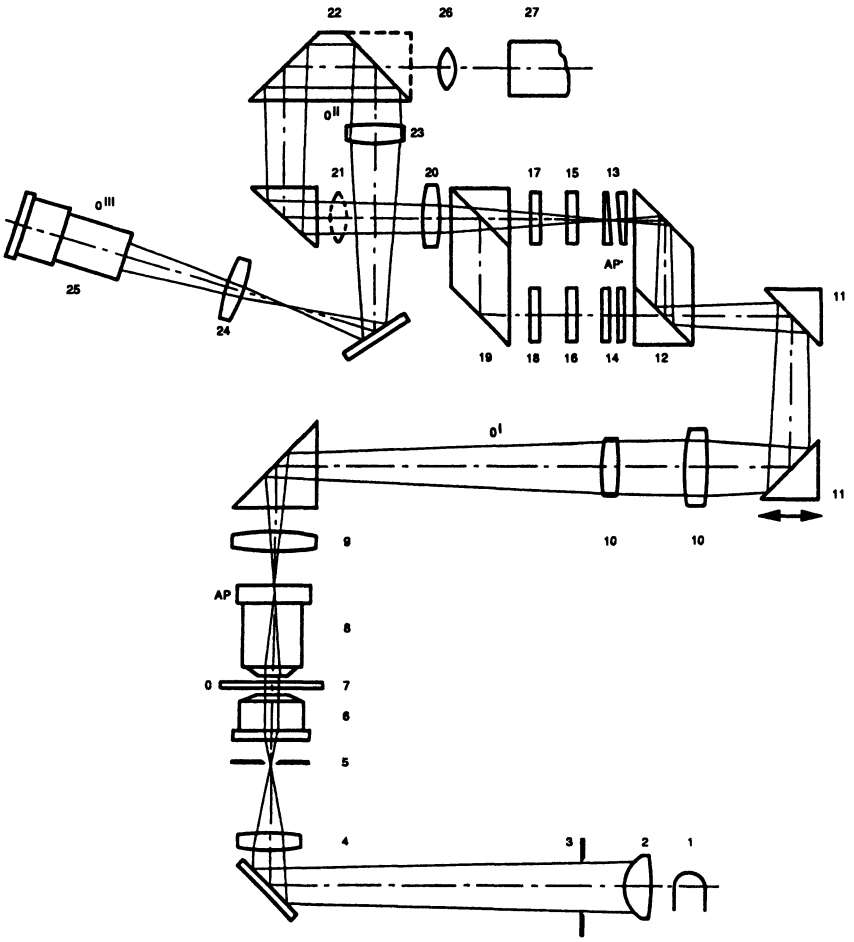


FIGURE 11.9. Optical diagram of JENAVAL-interphako. *O*-object; *O'* . . . *O'''*-intermediate images of the object; *AP'*-exit pupil; *AP*-image of exit pupil; 1-light source; 2-collector; 3-field iris; 4-collimator; 5-slit diaphragm; 6-condenser; 7-specimen; 8-objective; 9-first tube lens; 10-relay system; 11-angled mirror; 12-interferometer prism; 13-shearing wedges; 14-compensation wedges; 15,16-adjusting wedges; 17-compensating wedge; 18-measuring wedge; 19-interferometer prism; 20-second tube lens; 21-Bertrand lens; 22-deflecting prism; 23-field lens; 24-imaging system; 25-eyepiece; 26-relay system; 27-photomultiplier.[9]

11.3. APPLICATIONS TO HIGHLY BIREFRINGENT SPECIMENS

If the birefringence of a man-made fiber is low, the fringe shifts for $n_{\parallel}$ and $n_{\perp}$ are easily determined. Then if the thickness of the fiber is known, the actual values for $n_{\parallel}$ and $n_{\perp}$ can also be determined (see Chapter 6). However when the fringe shift is more than three orders, the thickness of the sample changes so rapidly at the edge of the fiber that it becomes difficult to assign an exact order number to a fringe. To solve such a problem, Scott[7] mounted highly birefringent fibers in a liquid of refractive index near $n_{\parallel}$ or $n_{\perp}$. Using unpolarized light he obtained the Moiré effect of superimposing the interference pattern for $n_{\parallel}$ in the fiber on the interference pattern for $n_{\perp}$ in the fiber. The resulting dark regions correspond to places where X orders of interference at $n_{\parallel}$ overlap Y orders of interference at $n_{\perp}$, X and Y being whole numbers.

Considerable advances have been made in computer processing these data in recent years.[13,14] In addition to the more common need to measure radial birefringent gradients in optical fibers and textile fibers, a difficult problem has recently occurred with high-speed production of fibers in excess of 6000 m/min and possible void formation under some conditions. Voids generate form (two-phase) birefringence, which, if confounded with general (one-phase) birefringence, can make the calculated molecular birefringence invalid. This possible evaluation problem is somewhat negated by calculating the independent Lorentz density or optical density.[14,15]

The refractive index of a material through the Lorenz–Lorentz relationship depends on the polarizability of all molecules in the field.[15] Optical density is also an independent way of calculating the material's internal order or density if the polymer contains voids.[16]

11.4. SUMMARY

Interference microscopy provides a way of gaining contrast by superimposing the image of a specimen and interference rings or bands of controllable width and density. This is accomplished by splitting the illuminating radiation into two separate beams, putting one beam through the sample, introducing a controlled delay or phase difference into the reference beam, and then combining the two beams so that they interfere with each other and produce the rings. If white light is used, the rings are like the familiar Newton's rings in color. When monochromatic light from a sodium lamp or a filtered mercury arc is used, the image is sharper and more easily interpreted. Interference microscopy also provides clear per-

ception of very small changes in depth or elevation in the sample, and such changes can be measured by counting the successive interference rings. Still further refinement of the method, using polarized light, allows precise calculation of the refractive indices of birefringent fibers and crystals.

An interference microscope can be quite simple or extremely complex, depending on what is required of it. The simplest arrangement splits the illuminating beam by means of a lightly silvered semireflecting glass plate placed at an angle of 45°, then reflects one beam from the sample while the other beam is reflected from a reference surface that may be tilted through small angles to introduce a phase difference. The two beams are then recombined within the optical system of a simple or compound microscope to produce the interference rings or bands in the image. Alternatively the semireflector and its associated illuminating system can be converted into an objective designed for vertically illuminating opaque objects. Thin transparent objects, such as biological specimens, can be mounted between half-silvered optical flats.

Another way of splitting the illuminating beam involves using a prism made of a doubly refracting crystal, such as calcite (the Wollaston or Nomarski prism). The Baker system uses doubly refractive rotatable plates to achieve a continuously variable phase difference. It is especially useful for examining thin crystals or thin sections of minerals.

The greatest flexibility and capability in an interference microscope is achieved by using two separate compound microscopes, one for the object beam and the other for the reference beam. The Leitz interference microscope is an advanced example: It splits the illuminating beam into two parallel beams by means of a prism, sends one beam through the sample via a fully equipped compound microscope, sends the other beam through a duplicate optical system fitted with tiltable-plate phase-delay devices and a wedge compensator, and then combines the two beams in a duplicate prism before sending them through the eyepiece. Both optical systems are fitted with polarizers, analyzers, and all appropriate diaphragms and stops. This arrangement allows the specimen to be moved about at will and keeps the specimen area separate from the reference area. Furthermore the specimen can be shifted from one stage to the other, so any fringe shift of a particular magnitude or direction caused by the sample is reversed when the sample is shifted to the other stage. Thus all confusion is removed from the interpretation. A simpler microscope that provides similar information for small samples is the aus Jena, which also uses a Mach–Zehnder interferometer.

12

Microscopical Stages

12.1. INTRODUCTION

Practically all of microscopy is staged in some way and to some degree. In its simplest state a microscopical *stage* is a device used to hold the specimen in a desired position in the optical path.[1] On stereoscopic, biological, and metallographic microscopes, the fundamental stage is usually rectangular, flat, and drilled to take clamps to hold the microscopical slide or the specimen itself. Some rectangular stages are made with built-on *mechanical stages* (see Figure 12.1).[2] Otherwise there are usually tapped holes to take the fastening screws of a mechanical stage[2,3] to move the specimen in directions X and Y. Graduations plus their vernier scale on *rack-and-pinion scales* usually allow measurements in tenths of a millimeter.[3] Some specialized mechanical stages[2–6] operate with *micrometer screws* with vernier measurements to within hundredths of a millimeter (see Figure 11.2). Micrometer screws can be integrated into a single mechanical stage so that in a single traverse of a composite specimen, paths over separate constituents can be measured separately (see Figure 12.3).[2] This mechanical method of areal analysis has been automated by means of motors and counters.

All stages should be waterproof and oilproof; they should also be reasonably resistant to solvent, chemicals, and heat. The plane of the main stage must be exactly perpendicular to the axis of the microscope, otherwise the optical quality is set back hundreds of years. If the microscope is ever dropped or the stage is struck in any way, the microscope should be carefully examined, and if damaged, it must be repaired. The central opening should be at least 25 mm in diameter so that the condenser can be raised level with the upper surface of the stage.[7]

Metallographic and other microscopes for thick specimens should provide movement in the third *(Z)* direction (see Figures 12.3 and 12.4).

FIGURE 12.1. This biological microscope has a built-on, graduated mechanical stage.[2]

Polarizing microscopes should be provided with a *rotatable stage,* graduated in degrees (and a vernier in tenths) at the circumference (see Figure 12.4).[2] Centering screws should be provided unless the objectives and condenser are "centerable." Ball bearings ensure against wear and keep the stage from wobbling when turned. A rotatable stage should be large enough so that the central 40 mm of a 75-mm slide can be explored and rotated without having the slide hang over the edge of the stage. If the rotatable shape is easily removed, a hot stage or other accessory can be inserted in its place. A removable rotatable stage is also easily lubricated and otherwise serviced.[7] A locking screw or other device will prevent rotation whenever a small attached mechanical stage is used (see Figure 12.4).[2]

The *glide stage* is relatively new (see Figure 12.5).[3] It is designed to speed up scanning or searching an entire large specimen, such as a biolog-

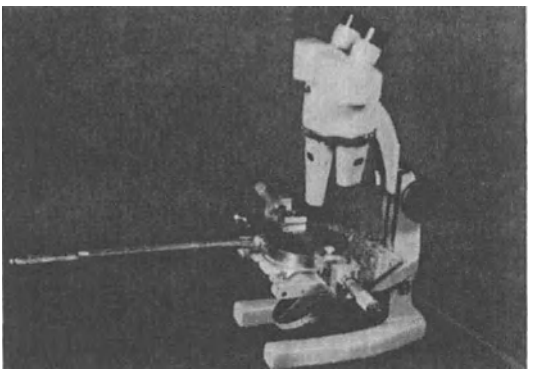


FIGURE 12.2. Film stretcher equipped with graduated micrometer screws and Köfler hot stage.[4]

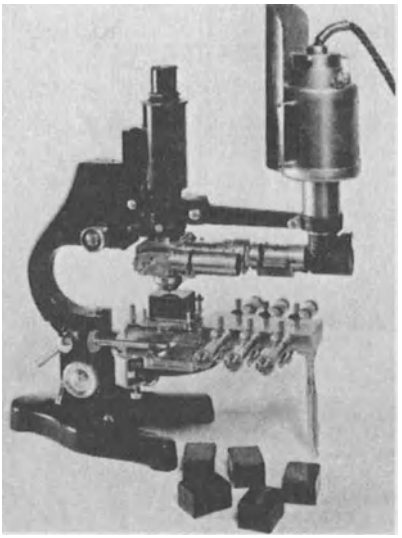


FIGURE 12.3. Integrating stage (Leitz). Its six micrometer screws permit the quantitative approximation of six constituents or groups of constituents in a single traverse of the specimen.[2]

ical or crystalline culture in a Petri dish. Glide stages should provide ease of motion on the glide plane and rotational bearings and yet be resistant to slight unintentional pressures.

There are many special stages and holders[3,7]; some are designed to level a polished section to be perpendicular to vertical illumination. Other stages and holders are intended for special objects, such as a chuck for

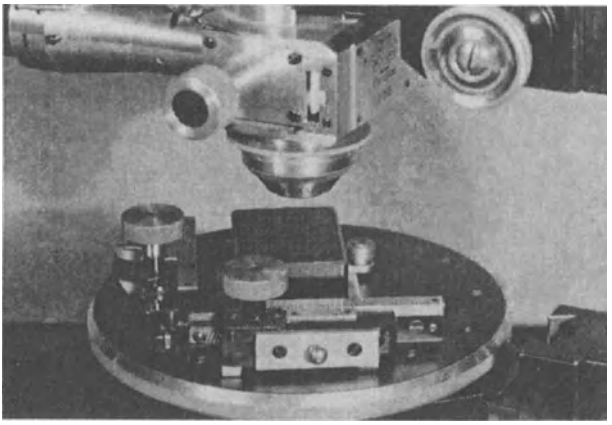


FIGURE 12.4. Rectangular briquet fitted in a mechanical stage (Leitz) on a rotatable stage (Leitz).[2]

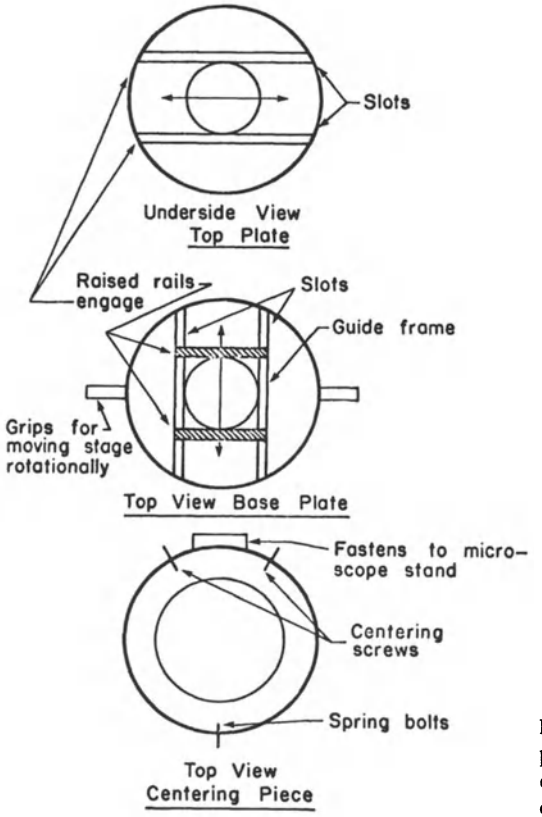


FIGURE 12.5. Glide stage principles. (After photographs courtesy of Carl Zeiss.)[3] Courtesy of Microscope Publications.

bullets.[3] Chucks can be used for a variety of purposes; Figure 12.6, for example, shows a chuck used to hold a dental drill for removing a microsample from an ore fraction.[2] The chuck in this case is on a separate stand (coarse micromanipulator) because the rotating cable and its sheath are too stiff to be mounted directly on the microscopical stage.

Another important use for the mechanical stage is in locating and recording a certain position on a specimen by means of numerical coordinates x and y. To return to a position, the specimen must be put back into the mechanical stage in its original orientation of front versus back and left versus right. A rectangular specimen, such as a microscopical slide or a rectangular briquet (see Figure 12.3), properly labeled, presents no orientation problem. Circular specimens need an index such as a notch engaged by a lug. The return to a location is initiated with a low-power objective.[2,3]

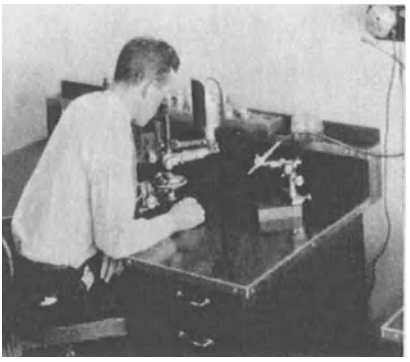


FIGURE 12.6. Dental drill and micromanipulator. The chuck of the dental drill is held in a special clamp attached to a Leitz-type micromanipulator.[2]

Another way of returning to a location in a specimen is to mark the spot with a qualitative marker (see Figure 12.7)[3] or a quantitative hardness indenter (see Figure 12.8)[2] that fits into the standard thread.[8] The Bierbaum scratch-hardness tester[9] can be used to mark a microscopic place. As shown in Figure 12.9,[2] the Bierbaum tester fits a square stage, such as that of the stereomicroscope. The test scratch can be made long enough to be easily located and followed.

12.2. MICROMANIPULATORS

Various operations require mechanical aid in controlling the X, Y, and Z directions of moving a microtool. Coarse movements can be provided by ordinary rack-and-pinion assemblies, as shown in Figure 12.6. The vertical mechanism is much like that of a simple microscopical stand on which the X—Y movements could be added with an ordinary mechanical stage. Somewhat finer adjustments are provided by micrometer threads of ordinary screw-micrometers.[3–5]

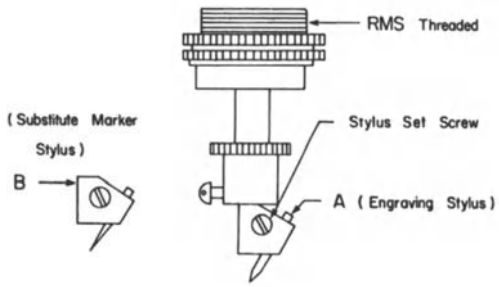


FIGURE 12.7. Sheaff microobject marker. (After illustration courtesy of Arthur H. Thomas Company.) Courtesy of Microscope Publications.

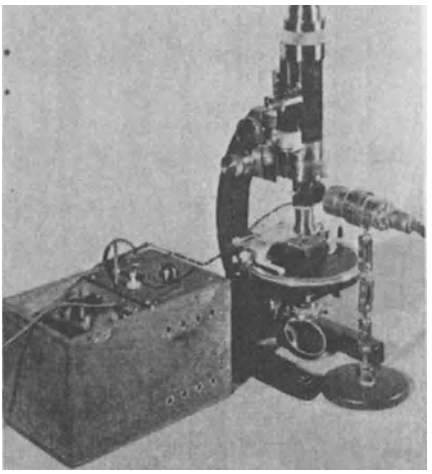


FIGURE 12.8. Vickers-type impression hardness tester (Eberbach). The Vickers-shaped diamond point is attached to a spring in a holder fitted with Society objective thread. A standardized compression of the spring, using the microscope's fine-focusing adjustment, is indicated by a signal light on the panel of the box.[2]

Useful magnifications in the range of 300–600× require finer movements in the *X* and *Y* directions. The Chambers system is based on one to four units, two of which are shown in Figure 12.10.[3] The *X* and *Y* movements in each unit are provided by modified micrometer screws with control knobs attached to flexible shafts. The shafts also absorb vibrations from the operator's hands. Clamps for holding microtools are mounted on

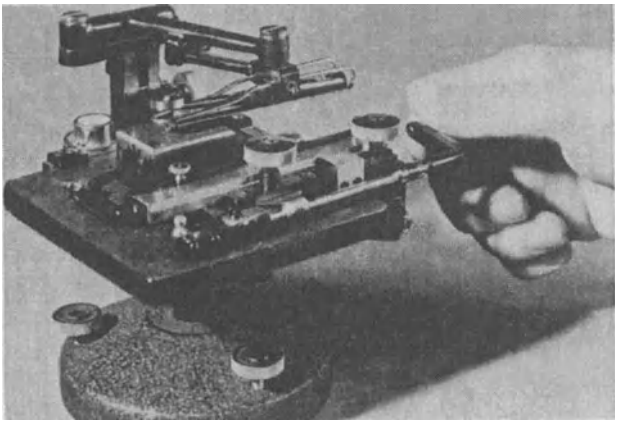


FIGURE 12.9. Bierbaum scratch-hardness tester. The tester fits a square-type microscope stage or the stand shown. The specimen is moved under the diamond point by means of a micrometer screw. The degree of hardness is indicated by the width of a scratch as measured microscopically.[2]

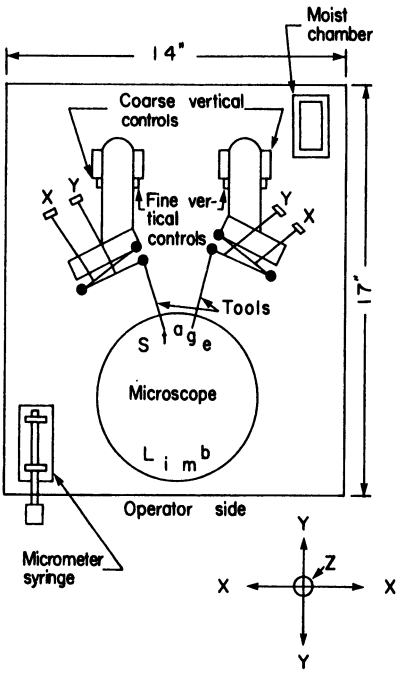


FIGURE 12.10. Typical operational layout, Chambers micromanipulators. (After photographic illustration courtesy Brinkman Instruments.)[3] Courtesy of Microscope Publications.

vertical rods for leeway in adjusting to the Z direction. Up to four units can be clustered around a microscope. Figure 12.10 shows two mounted in a moist chamber for biological purposes.

The Emerson micromanipulator[5] works on a different principle: A single vertical lever (joystick) controls an omnidirectional movement in the plane of the microscopical field. The measurement of the joystick is in the same direction as the apparent direction of motion. The extent of travel can be varied from about 0.025–3.2 mm for about 90 mm of travel by the hand. A microworm gear controls movement in a horizontal arc of 20°. The mechanical stage can be tilted about 10°. The tool holder is a tubular chuck that allows attaching tubing for gas or electrical connections through a hollow needle.[3]

12.3. HEATABLE STAGES

Heatable (hot) stages are a necessity in direct microscopical observation of thermal transitions above room temperatures. The maximum temperature to be reached is set by the materials of construction in ordinary light microscopes: about 350° C. Stages heatable to such temperatures are

adequate for studying most organic crystalline phases,[10,11] fibers,[12] many other polymeric materials, and many inorganic crystalline transitions.[7] Although most metals require higher temperatures for significant thermal studies, there are parallel physical–chemical transformations among organic systems that are very instructive in the fields of metallography[13] and ceramography. Heatable stages within the range up to 350° C can be divided into two categories: those with long working distances and those with short ones.[11] Working distance usually refers to the distance between the specimen *in* the stage and the outside lens surface of the objective with high-enough NA to obtain an interference figure.[7] In Chapter 7, we determined that the ideal NA for interference figures is 0.85 because tables of reference between measurements on interference figures and other optical properties are based on an NA of 0.85.[14] This value of 0.85 is not absolute, since there are special objectives for NA ≈ 0.85 with longer than ordinary working distance, such as those objectives intended for use with universal stages (see Chapter 7).

12.3.1. Hot Stages with Long Working Distances

The Kofler hot stage is a model[10,11,15] and originated in Austria with Reichert. The U.S. version was designed by H. Alber for the A. H. Thomas Company. It is very useful on stereomicroscopes with long working distances,[4,5] on chemical microscopes,[7,10,15] and wherever interference figures are either not needed or cannot be observed under the circumstances. As shown in Figure 12.11,[16] the Thomas–Kofler hot stage is housed in a metal cylinder *A*, fitted with metal rim *B* and glass cover *C*. The heating coil *HE* (see Figure 12.12) is electrically supplied and regulated by its variable transformer. The stage takes a half-slide (25 × 38 mm). The half-slide can be placed within fork *F* in Figures 12.11 and 12.12, so that the specimen may be moved from outside the stage. A glass baffle *D* (see Figure 12.11) can be used to ensure more uniform heating. Two thermometers are supplied (30–230° C and 60–350° C), and each is precalibrated while in its protective metal sheath *M*. There are many accessories, each with its own heat capacity, so the thermometer used with a particular assemblage of accessories should be checked with the appropriate reference melting point standards, *CR* (see Figure 12.11).[16] More precise measurement of the temperature of the specimen can be performed by placing a fine-wire thermocouple *on the specimen* just beyond the microscopical field of view. The other end of the thermocouple is kept in ice and water, bubbled with air to keep its temperature constant, in a Dewar flask.[5]

In Figure 12.12, the Thomas–Kofler stage has its own condenser *R*. As

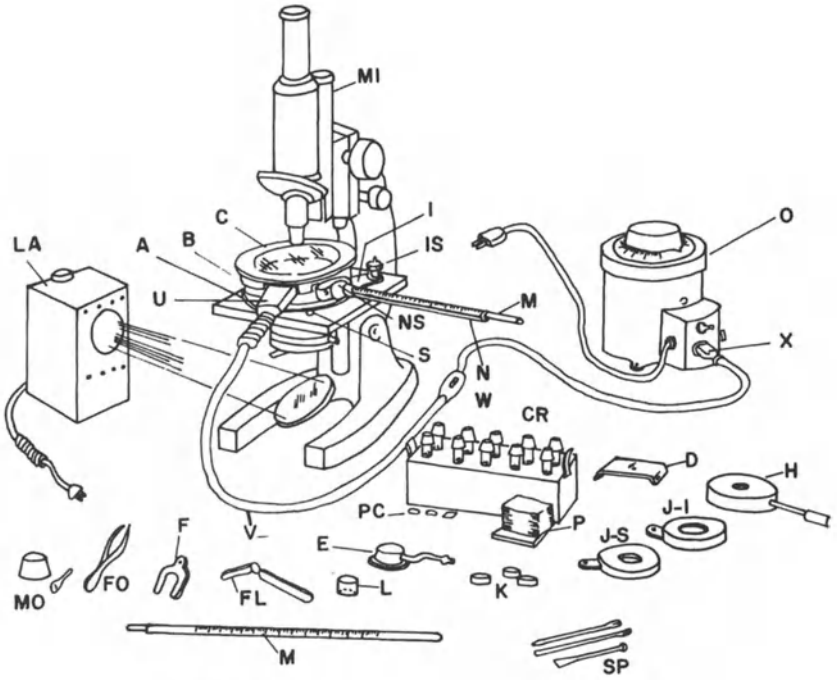


FIGURE 12.11. Accessories for the Kofler hot stage.[16]

with a stereoscopic microscope,[4,5] this lens should be removed by taking out the screws *RS*. The lens is also removed when dark-field illumination is intended with a polarizing microscope. For dark field a Leitz objective (UM-4, NA = 0.20) of long working distance is used.[17] The top lens of the regular substage condenser is removed, and an adequate central stop (see Figure 10.5) is placed in the slot under the condenser. The UM-4 objective

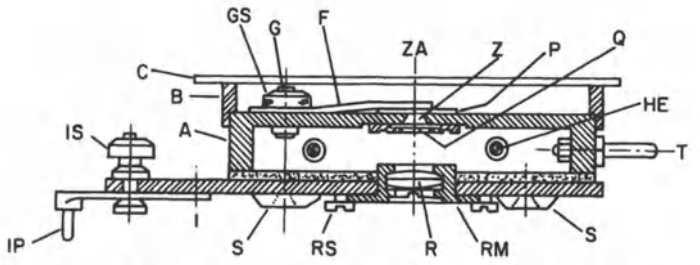


FIGURE 12.12. Cross section of Kofler microscopical hot stage.[16]

has an iris diaphragm that is closed until a dark field is obtained. With this setup of the Kofler hot stage and dark-field illumination, the growth or decline of crystalline units, such as spherulites with variation of temperature, has been reported.[17]

The Leitz Company[18] has produced a hot/cold stage for temperatures from 360 to −20° C, which allows the use of long working objectives (UM), originally designed for the universal stage. This provision for NA up to 0.6 permits observation of useful interference figures.[11]

The Mettler[19] hot stages FP2 and FP52 feature two heating coils, one above the specimen and one underneath, thus practically eliminating the vertical thermal gradient of a single coil. A second advantage of this model is the use of a small fan (*P* in Figure 12.13)[11] to circulate air inside the stage. In the older stage, FP2, air is cooled by introducing a cold gas through a fitting underneath the fan. In the newer model, FP52, cold gas is introduced directly through a tube in the housing of the stage; otherwise the two stages are alike.

In Figure 12.13, the specimen is moved by control knobs *K*. The specimen is thermally protected by a mirrored housing *O* and a current of air from fan *P*. The window *Q* is also a heat filter, which together with *O* and *P*, protects the microscope as well as the specimen. The temperature, 20–300° C, is measured by a platinum resistance thermometer in fixed position. The automatic alternative rates of heating, 0.2° C, 2° C, or 10° C per minute, can be preselected by push buttons overridden by another push button. The temperature is continuously recorded digitally to within 0.1° C at the top of the panel, while significant events (melting or other transition points) can be recorded by pressing the appropriate button. A special button arrests the temperature and maintains it as long as the button remains depressed.[11] Instructions and correction factors have been published.[20]

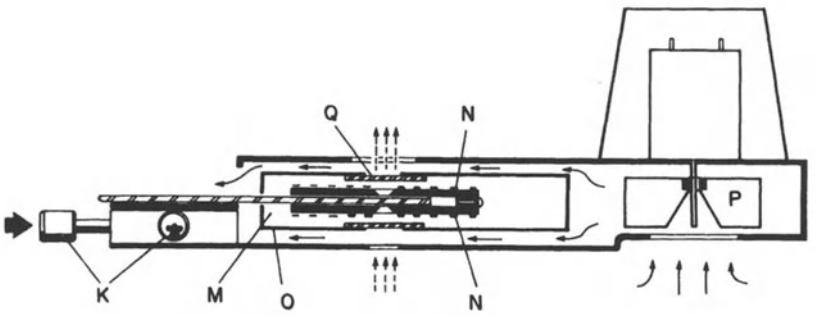


FIGURE 12.13. Mettler FP2 stage: side section (Mettler Instrument AG).[11]

There was an addition to the Mettler FP52 heatable stage: the *photoelectric sensor* employed in *thermal depolarization analysis* (TDA) (not differential thermal analysis, DTA).[11] The photoelectric sensor records changes in light transmission that accompany phase transitions of a specimen heated or cooled between crossed polars[11] (preferably synchronously rotating crossed polars). The most striking transition is melting to the point where the whole field becomes dark.[21] One eyepiece of the binocular microscope is replaced by the photoelectric sensor that signals changes in light transmission.[11] The technique has been applied to the study of thermal changes in polymers and mesomorphic systems.[21]

The mutual problem with all heatable stages having long working distances is that ordinary objectives with sufficiently high NA for observing interference figures cannot be focused close enough to the specimen. The use of long working objectives, such as those designed for universal stages,[18] has been mentioned as a solution to the problem. Another more general solution that includes using ordinary objectives employs a stage with a short working distance.

12.3.2. Hot Stages with Short Working Distances

Hot stages heated up to 150° C can be tolerated by ordinary objectives of nominal 4-mm focal length (NA = 0.85).[11] The toleration criterion requires the objectives' ability to portray interference figures not to be impaired by continual use with the hot stage. Thus the idea is to have the working distance of the stage as short as that of a 4-mm objective. Hartshorne designed two thin heatable stages: a circular one to fit onto a polarizing microscope with the conventional circular stage and a rectangular stage to fasten directly onto a microscope with the stationary rectangular stage.

The circular thin hot stage consists of a single brass disk about 10 cm in diameter and about 4 mm thick. The disk is recessed about 2 mm, leaving a rim for peripheral screws and a central ring containing the axial hole. The recess in the disk holds the nichrome heating ribbon threaded through holes in a sheet of mica. This heating unit is insulated from the disk by a whole sheet of mica. The disk's axial hole is tapered to the shape of the cone of light from the condenser (about 4 mm in upper diameter and 8 mm in lower diameter). The hot junction of the thermocouple is made by securing copper and constantan wires separately to the central boss with very small countersunk screws. A third mica sheet covers the stage, and it is fastened with small screws. There are four knurled hand nuts that attach the whole stage to the main rotatable stage of the polariz-

ing microscope. Two knurled nuts are drilled to accommodate stage clips. Heating is controlled by a variable transformer. The resulting voltage is read on a millivoltmeter with a scale calibrated with the known melting temperatures of pure crystalline standards observed to be in equilibrium with their melts at corresponding voltages.[11] The cold junction of the thermocouple is immersed in ice water bubbled with air in a vacuum flask.[5]

The circular Hartshorne hot stage has two major limitations, both arising from the thin design of its heating element in the metallic shell on the microscope's stage. One limitation is that the metallic shell raises the specimen above the substage condenser that has to furnish conoscopic illumination to obtain an interference figure. Perhaps the stop on the condenser's substage can be adjusted to raise the condenser sufficiently; if not, perhaps an auxiliary lens or different condenser can be inserted. The second limitation to the circular design is that even if the round stage is not heated over 150° C, it may be hot enough to damage the microscope's stage or polarizer or some other part. A solution may be to substitute a thermal insulator, such as an asbestos composite, for the metallic shell. (If a thermal insulator is merely stuffed under the metallic stage, the specimen is raised even farther above the condenser).[11] In any case the polarizer should be of a polarizing plastic, such as Polaroid®, rather than calcite, because the former is much less expensive to replace. If the plastic sheet must be replaced, the replacement should be a heat-resistant variety such as Polaroid Corporation makes.

If the microscopist wishes to go slightly higher than 150° C, select Hartshorne's rectangular design, because the heating element hangs under (rather than sits on) the metallic shell. Of course the rectangular model is not rotatable. The stationary aspect means that the electrical leads are also stationary and do not twist back and forth, as with the rotating stage. A point to be considered is that polarizing microscopes with synchronously rotatable polars may not be available. If such a microscope is available, the rectangular heatable stage shown in Figure 12.14 is appropriate.[11] The metallic shell of the rectangular stage is made of thin copper sheet (not more than 0.8 mm), rolled to a slight concavity upward (see Figure 12.14a, [i]), so that mounted on the microscope's stage, it will straighten out when pulled down at the corners. A thin sheet of mica (see Figure 12.14a, [ii]) has a central hole in which a circular cover glass rests on a thicker sheet of mica (see Figure 12.14a, [iii]), with an extension carrying the binding posts for the leads from the heating coil and for the thermocouple. This sheet (see Figure 12.14a, [iii]) has a central hole about 6 mm in diameter for illumination. The heating coil is made of nichrome wire or ribbon that conducts 1 or 2 A at about 36 V. The sheet also safely and carefully carries a copper–

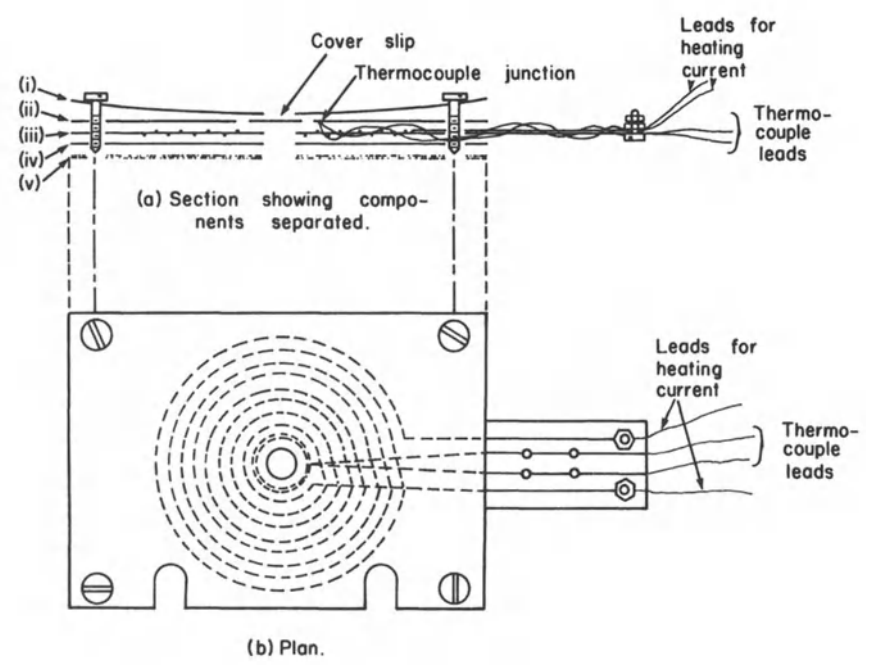


FIGURE 12.14. Simple thin hot stage. (After Hartshorne and Stuart.)[11] Courtesy of Microscope Publications.

constantan thermocouple junction as near to the center of the stage as possible. Another thin sheet of mica (see Figure 12.14a, [iv]) and one of asbestos paper (see Figure 12.14a, [v]) serve as thermal insulators. They carry holes about 6 mm in diameter for low powers and 3 mm for high powers.[11]

12.3.3. Hot-Wire Stages

A hot wire can be used to heat a microscopical specimen directly between the cover glass and slide. The resulting temperature gradient can produce two or more phases of a system in a single microscopical field. For example if we stretch a wire in a melt that freezes at a temperature within the ordinary hot-stage range (between ~300° C and room temperature) and if we remelt the solid by flowing an electric current through the wire, we obtain two or more phases on both sides of the wire, as shown in Figure 12.15.[11] Jones[22] uses no. 24-gauge platinum wire about 25 mm long, held between two clips. To keep the heating wire straight, each clip is soldered

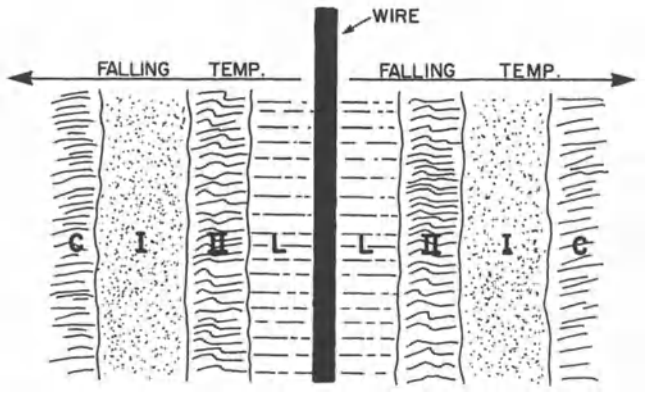


FIGURE 12.15. Digrammatic representation of a field represented by a compound having two mesophases under a hot wire. *L*, liquid; *II*, mesophase stable at the higher temperatures; *I*, mesophase stable at the lower temperatures; *C*, crystalline phase.[11] Courtesy of Microscope Publications.

to a stiff copper wire held firmly in place. The other ends of the two copper wires are soldered to flexible wires leading to a transformer. The slide and cover glass containing the specimen rest on an aluminum ring to protect the condenser from the hot slide.[11,22]

Hartshorne has elaborated on the hot-wire principle.[23] His wire stage fits onto his polarizing microscope with its rectangular stage. The polars rotate together instead of having a rotatable stage. As shown in Figure 12.16, the nichrome heating wire is stretched on a rectangular block *A* (6 mm thick) of a hard asbestos composite carrying a central hole 20 mm in

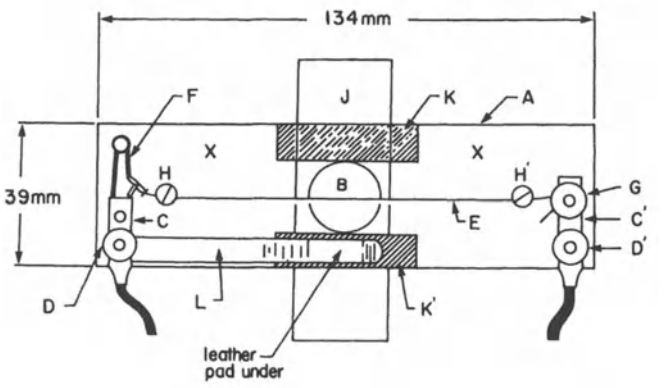


FIGURE 12.16. Hot-wire stage in plan.[23] Courtesy of Microscope Publications.

diameter. Two brass platelets *C,C'* are bolted to *A* and contain binding posts and finger nuts *D,D'* and *G*. The nichrome wire *E*, even when hot, is kept taut by a strong spring hook *F*. The posts *H* and *H'* are of such a height that the wire *E* just misses touching the cover glass over the specimen on slide *J*. Cover glasses and slides are selected within narrow limits of thickness, so that the level of wire *E* does not have to be readjusted. Clip *L* holds the slide *J* tightly against cardboard strips *K* and *K'*, which thermally insulate the slide from asbestos composite base *A*. The temperature gradient can be increased by means of a brass or copper cooling plate *M*, shown in Figure 12.17. Soldered underneath the plate *M* is a brass shim *N* the same thickness as the cover slip. The wire *E* is heated by about 6 V from a variable transformer.

Jones[22] determined melting points by means of a tiny thermocouple. Hartshorne[23] calibrated a linear eyepiece scale oriented at right angles to the heated wire. He used a compound having at least three known transition points[7,11,13] and measured the distance on the eyepiece scale between phase boundaries. (The wire's thermal gradient is not linear, and so at least three phases are needed to calibrate the eyepiece.) The standard and unknown specimen are mounted under separate strips of cover glass, as indicated in Figure 12.18. The strips are melted separately under their separated cover glasses, and any excess solidified substance is cleaned off with a razor blade. After the strips are mounted at right angles to the hot wire, they are heated and observed.[23]

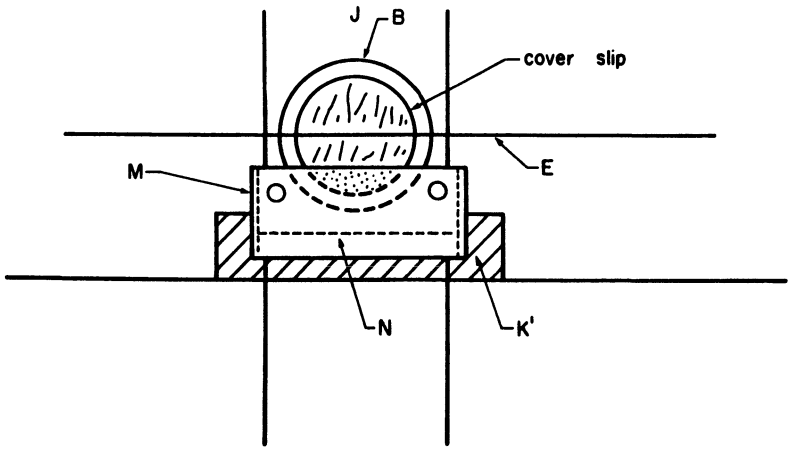


FIGURE 12.17. Cooling plate used to increase temperature gradient. Courtesy of Microscope Publications.

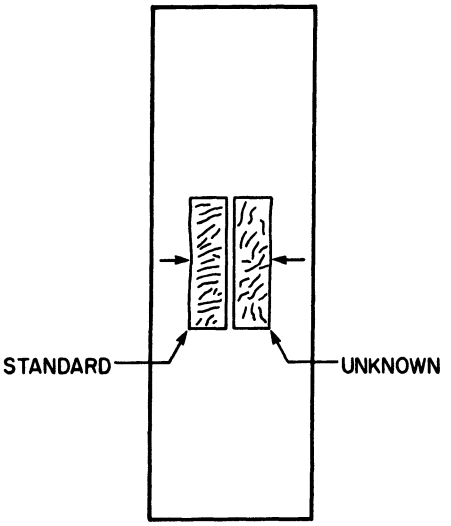


FIGURE 12.18. Comparison preparation for determining transition temperatures. Courtesy of Microscope Publications.

12.4. VERY HOT STAGES

Temperatures up to 1000° C can be obtained on a hot stage, which is a miniature water-cooled furnace.[24] Such a furnace has been used to observe heated samples of pyrotechnic systems.[25] The furnace is heated by means of nichrome wire, and temperatures are determined with a plate-type thermocouple (platinum versus 13% rhodium–platinum alloy) connected to a potentiometric recorder. The sample is placed in a 6-mm flat-bottom Inconel or platinum pan, such as is used in DTA. The pan is placed on the thermocouple plate in the furnace, whose lid has a quartz window. The lid can be rotated 360° while maintaining a gas-tight seal so that condensed reaction products can be removed from view.[25]

The sample is viewed by reflected light through a stereomicroscope fitted with a trinocular head for photomicrography. The illumination is from a 150-W quartz–iodine light through a fiber-optics cable at an effective angle. An event marker is used to record every exposure on the temperature record. Agfachrome® 50L professional color reversal film (50AS) is usually employed.[25]

12.5. COLD STAGES

Cold stages are essential for studying frozen biological tissues, chemical or physical fluids, emulsions[26] or suspensions,and for transitions. All of these can be studied by light, electron, and/or X-ray microscopy.[27]

FIGURE 12.19. Thermal stage on a light microscope. Courtesy of Sensortek.[28]

12.6. THERMAL STAGES: HOT AND COLD

Dual purpose, hot-cold stages are now available. Figure 12.19 shows a modern thermal stage on a light microscope.[28] Figure 12.20 shows a close-up of the thermal stage, and Figure 12.21 illustrates details of the thermal stage's size and construction.[28] Figure 12.22 shows the automatic thermal

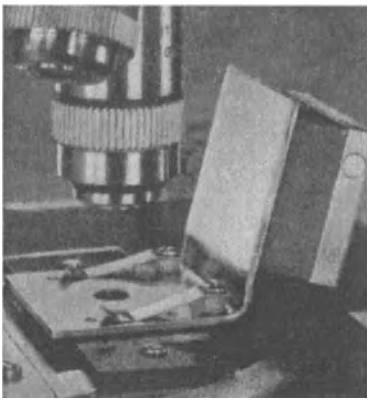
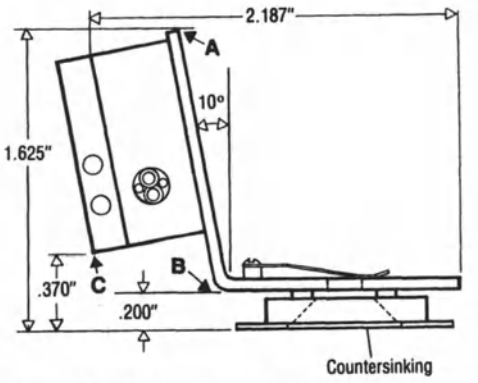


FIGURE 12.20. Close-up of thermal stage. Courtesy of Sensortek.[28]



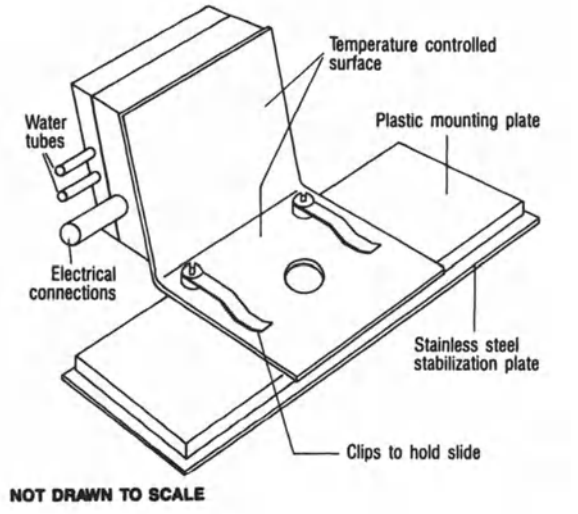


FIGURE 12.21. Detail of cold stage (not to scale). Courtesy of Sensortek.[28]

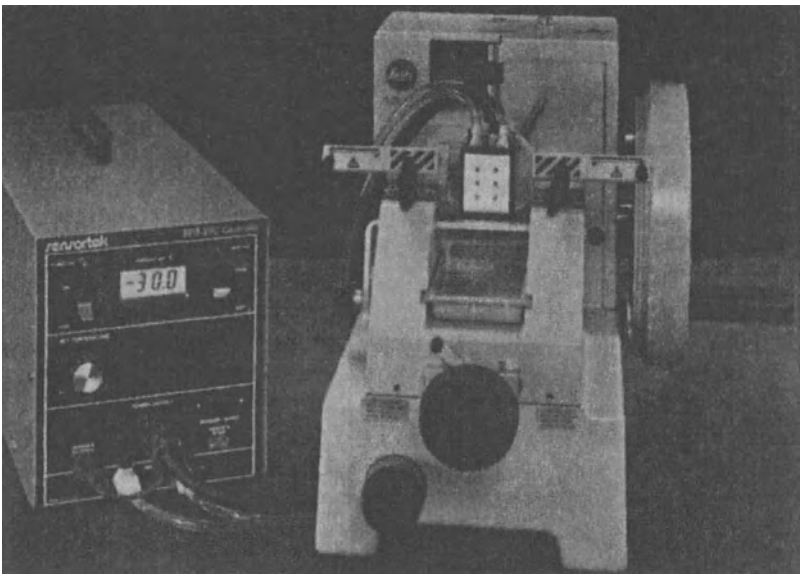


FIGURE 12.22. Automatically controlled cooling and freezing unit fitted to a microtome capable of producing precise serial sections of a frozen specimen, such as for electron microscopy. Courtesy of Sensortek.[28]

unit fitted to a microtome capable of producing single or serial sections precisely for electron microscopy, light microscopy, or any other kind.[28] Such a thermal unit usually fits or can be made to fit manual microtomes. The Sensortek units need AC electricity and a trickle of water.[28]

12.7. OTHER SPECIAL CELLS AND CUVETTES

There are many qualitative and quantitative kinds of special microscopical cells. Among the *quantitative* kinds are those of *specific volume,* such as *hemacytometer* cells, 0.10 mm deep with coordinate squares of 0.0025 mm^2 engraved on the bottom.[7]

The Nichols stage refractometer is a set of two cylindrical cells cemented to a metal slide.[3] Each cell is equipped with two glass prisms (see Figure 12.23). One set of prisms has a refractive index of 1.52, and it is for liquids having refractive indices between 1.40–1.65. The used second set has prisms of 1.72, and it is used for liquids with refractive index 1.85 and higher. In calibrating a cell it is filled with liquid of a known index, and the

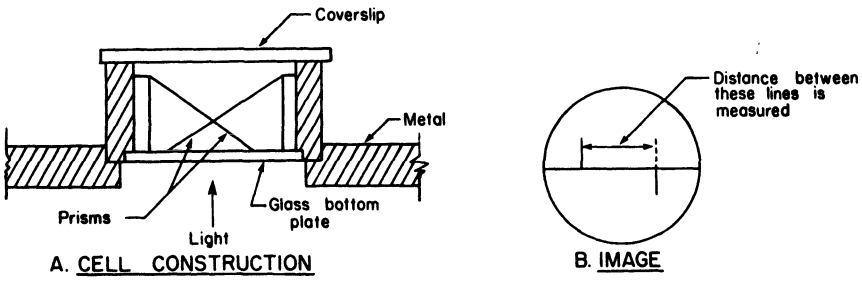


FIGURE 12.23. Nichols stage refractometer.[3] Courtesy of Microscope Publications.

distance between two lines as seen through the microscope is measured with an eyepiece micrometer. Ocular distances for other standards are all plotted against the refractive index at a constant temperature to obtain the calibration curve at that temperature.

Colorimetry can be performed to some extent under the microscope using special cells and a photoelectrometer.[7] Polarimetry on liquids can be performed in a polarizing microscope if it has a clear straight barrel that will take a long-enough tube filled to a known length to contain the liquid specimen. In this case a cap analyzer, rotatable in a ring, is used to measure the degrees of rotation of polarized light to the left or right by the fluid in the microscopical tube.[7]

There are also many kinds of *qualitative* cells, most of them supplementary to the usual stage of the microscope. Illumination by radiation in the near UV requires UV-transmitting slides and perhaps cover glasses. Far UV or infrared radiation requires quartz or fused silica slides and cover glasses.[7] Qualitative microscopical analysis for any elements in both the specimen and the ordinary glass slide requires a special slide, such as a plastic one.[29]

Orthogonal unilateral illumination, as in slit ultramicroscopy, requires a special cell for holding the fluid to be illuminated from the side.[7] A similar cell fitted with electrodes can be used in *microelectrophoresis.*[7]

Pressure cells are required in the microscopical study of some technological problems, such as those dealing with aerosol cans or polymerization under pressure. The primary problem lies in specifying and testing the glass windows.[30]

In *biology* and allied technology there are many types of microscopical cells, chambers, and cuvettes for specimens *in vivo* or *in vitro*—stationary, moving, or changing with time. Some fundamental designs are shown in Figure 12.24.[31] Some of these cells are to be used vertically, and therefore the microscope is used horizontally.[31]

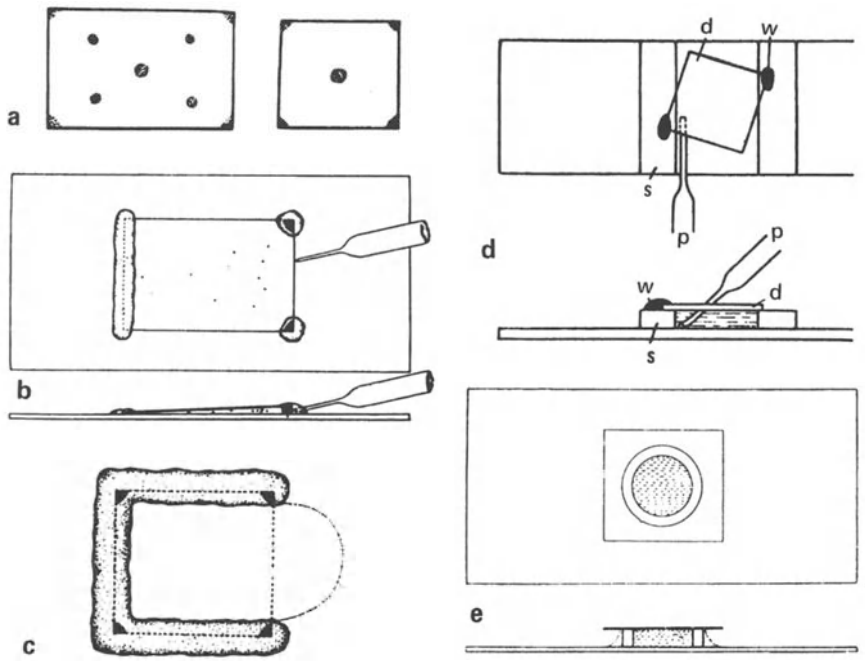


FIGURE 12.24. Typical cuvettes for microscopical specimens *in vivo* or *in vitro*.[31] (a) Support for cover glass provided by droplets of paraffin wax (after Kuhl, 1949). (b) Wedge-shaped cell viewed from above and from the side, designed to prevent damage to very tender specimens by weight of the cover glass. At the left a wall of paraffin holds the edge of the cover glass; at the right paraffin legs and pads support and hold the opposite corners of the cover glass (after Kuhl, 1949). (c) Three-sided enclosure of special advantage for living material. The reserve supply of water (drop extending at right) can be replaced as needed to prevent drying the sample and unnecessary motion of the liquid (after Kuhl, 1949). (d) Staining bridge shown in top and side views. The glass blocks are cemented to the slide, the cover glass *d* is fastened to them by clumps of wax *w*, and *p* is a pipette used to transfer staining and washing liquids (after von Wasielewski and Kün, 1914). (e) Ring-shaped cell for larger living specimens shown in top and side views. A mantel of water surrounds the ring, preventing evaporation of the experimental liquid (after Kuhl, 1949). (f) Adjustable cell for larger specimens made of glass blocks cemented to and covered by photographic film shown in top and side views (after Kuhl, 1949). (g) Combination of moist chamber and ring cell for larger living specimens or monitored sampling; made of base plate, cemented glass blocks, and large cover glass fastened at the corners with paraffin (after Kuhl, 1949). (h) *Above:* Simple cuvette. *Below:* Arrangement for constant renewal of culture medium in the cuvette. By siphon action the liquid streams from the supply vessel *V* into the cuvette *K* and is then sucked into the flask *F* (after Bode, 1954). (i) Microaquarium after Schaudinn: The cutout in the glass block is enclosed on both sides by cover glasses D_1 and D_2. The blocks *s* used for protection (after Belar, 1928).

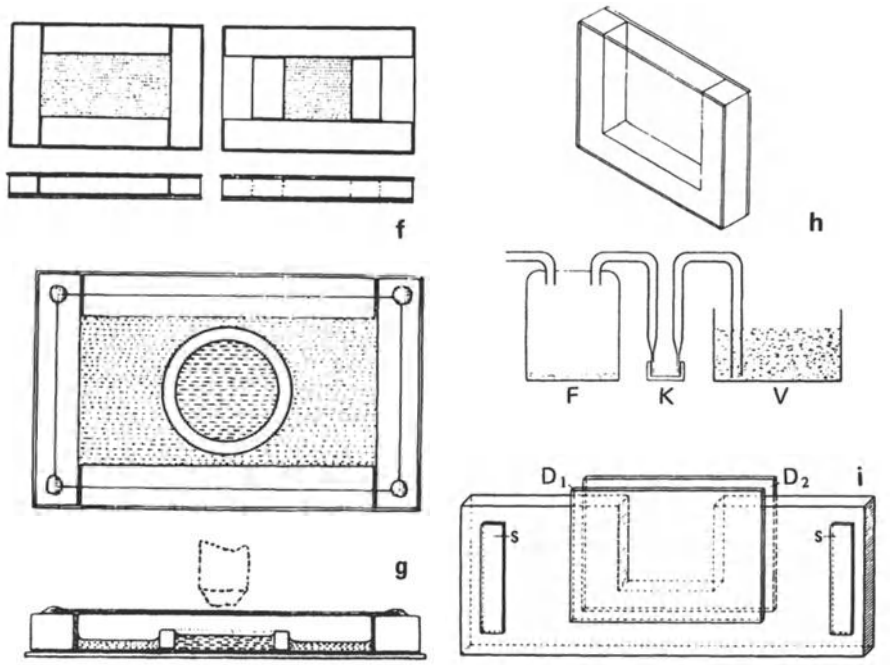


FIGURE 12.24. (*Continued*)

12.8. SUMMARY

Every microscope has a stage on which to examine the specimen. As in a theater the stage can be stationary and rectangular in shape or circular and rotatable. At the present time polarizing microscopes are usually equipped with circular stages for rotating the specimen between the polars. However there is some advantage to the old-fashioned polarizing microscope with a stationary rectangular stage and synchronously rotatable polars. The rectangular stage is relatively easy to provide with X and Y coordinate mechanical movements either at the factory or by an auxiliary attachment. The rectangular shape is also relatively easy to fit with other auxiliary stages, such as those for heating or cooling, micromanipulating, testing, or otherwise treating the specimen.

Heatable stages facilitate the study of physical–chemical changes, such as melting, crystallization, and polymorphic transformations. Commercial heatable stages are appreciably thick, presumably to provide adequate insulation, heat distribution, and chamber space, and therefore the microscopical objective must have an appropriately long working dis-

tance. Hot stages on the market that are limited to use with ordinary objectives of NA = 0.25 (10×) are adequate for most work requiring heat. However to obtain interference figures, dark-field illumination, or certain other facilities, a higher NA is required. For these purposes special objectives can be used with both long working distance and adequate NA (0.85) for obtaining interference figures. Such objectives are provided by manufacturers of universal stages, and these require placing a glass hemisphere about 1 cm in radius above the specimen.

An alternative to thick hot stage with high NA objective of long working distance is a hot stage thin enough to be used with ordinary objectives of NA up to 0.85. A thin hot stage may have to be especially built.[11] With a high-powered objective, the thin stage is not recommended for temperatures much above 150° C without further insulation and consequent compromise in purpose.

Another alternative to the thick hot stage is the hot-wire stage.[23] This is especially designed to study phase transformations, including mesophases.[11] The wire can be heated to 300° C without apparent damage to the polarizing microscope. Temperatures are measured directly with a tiny thermocouple[5] or indirectly by observing the behavior of an adjacent standard system of phases.[23]

Very hot stages for temperatures of 1000° C and above are really miniature water-cooled furnaces. They require the long working distance of an ordinary stereoscopic microscope, a simple microscope, or a telescope. Some conventional hot stages for use below 300° C can be cooled by liquid[18] or gas.[19] Cold stages specifically designed for cooling below room temperatures are commercially available.[27]

There are many kinds of special slides, chambers, and cells used in microscopy. Plastic slides are substituted when ordinary inorganic glass or special silica slides are inappropriate. Hollow slides are used to hold more liquid than will stay in place by viscosity or capillary action. To quantitatively determine particles in a known volume of liquid, such as blood, hollow slides are manufactured with precise cavities, sometimes with micrometric engravings on the bottom. The Nichols stage refractometer is a cylindric cell containing two crisscross glass wedges of known refractive index. When the cell is filled with a liquid of unknown refractive index, the measured distance between two bright refraction bands indicates its refractive index.

In biology there are many other types of microscopical cells, chambers, and cuvettes for specimens *in vivo* or *in vitro.* Specimens can be stationary, moving, or changing with time. Some of these cells are filled with liquid that will run out unless the cell is kept vertical and the microscope is used horizontally.

13

Fourier Transform Infrared Microscopy

13.1. INTRODUCTION

Infrared spectrometry and microscopy were combined in the late 1940s when all-reflective objectives became available[1,1a]; these also served as condensers. When the entire microscopical system was made to transmit the infrared spectrum, spectroscopists developed FT-IR microscopy,[2] which allowed them to analyze samples in the milligram range. Accordingly, FT-IR microscopy became highly developed in the 1980s and 1990s.[3–8] However microscopists, who routinely work with subnanogram samples,[3] lately have found applications for what they may term IR microscopy.[2]

13.2. EQUIPMENT

The FT-IR microscopes have two sources of illumination, one for the IR and the other for the visible; these are used sequentially. The IR source is normally an incandescent ceramic rod or tube. Other special items for IR microscopes are mirror lenses, called Cassegranian lenses, for focusing, and beam splitters for generating interferograms over the IR spectra range being used. A schematic for an FT-IR microscope is shown in Figure 13.1,[4] and details of a Cassegranian objective is shown in Figure 13.2.[2]

Images with reflecting Cassegranian lens cannot equal the quality of those from a proper transmitting lens, because reflecting objectives cannot be corrected for chromatic aberration. A refracting lens cannot be used, since it absorbs too much IR radiation.[2] The wavelength range generally

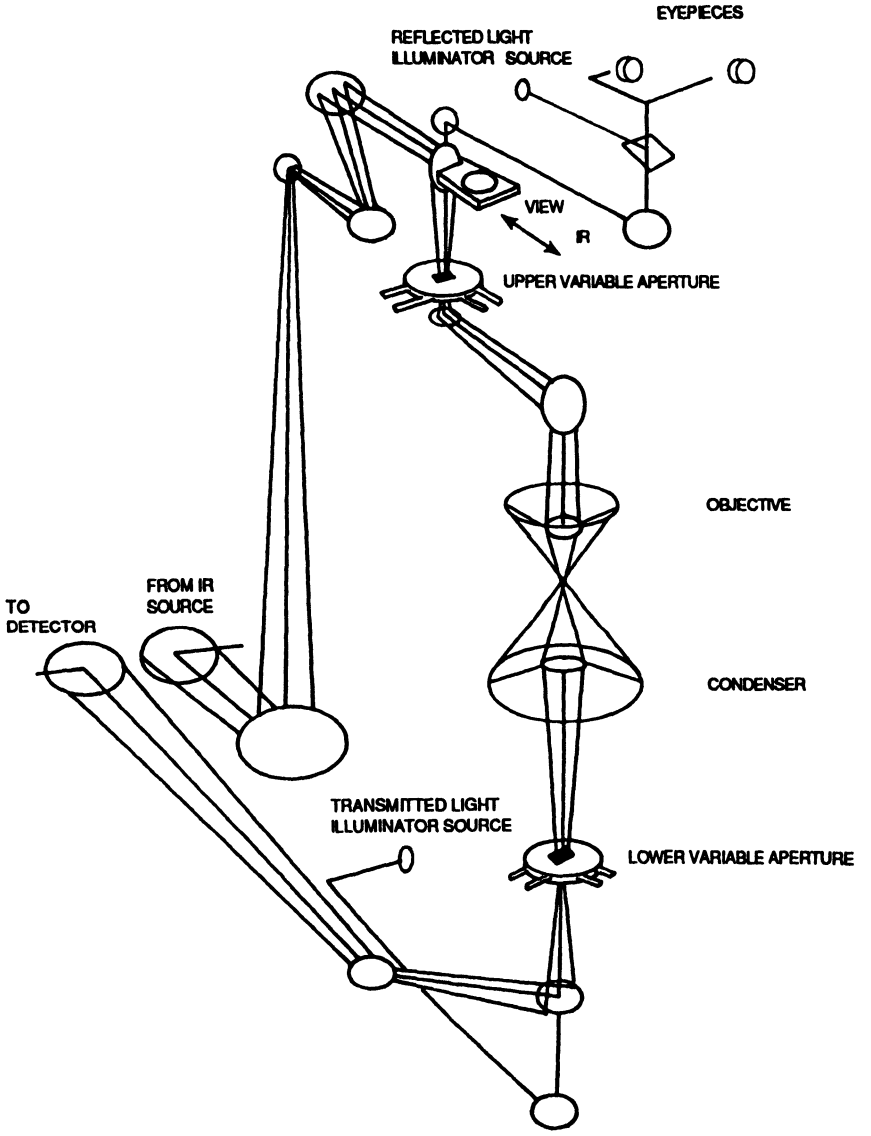


FIGURE 13.1. General schematic of an FT-IR microscope. Courtesy of Marcel Dekker.[4]

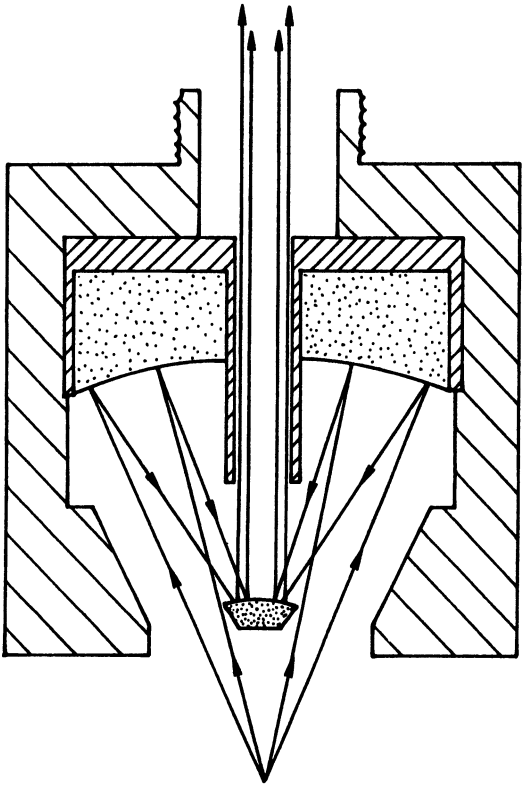


FIGURE 13.2. Ray paths through a mirror-surfaced Cassegranian objective. Courtesy of Royal Microscopical Society.[2]

used is 2.5–25 μm for the infrared to approximately 0.5 μm for the visible. As with other sophisticated tests, FT-IR microscopy can provide misleading results and therefore requires skill to be properly used.

The reader is referred to the numerous FT-IR references for information on general FT-IR principles (e.g., References 9,10). Suffice it to state here that an interferometer generates a final interference wave pattern of suitable wavelength IR illumination. A series of mirror lenses then transmits the resulting IR beam to the specimen on the stage. Specific IR frequencies are absorbed, and the emitted interferogram is modified from its original shape. Final signal modifications are functions of the absorbing chemical groups. The result is the FT-IR trace, which is normally characteristic of the viewed material.[2] A laser beam follows the same optical path as the IR beam and serves as an internal reference for the wavelength.

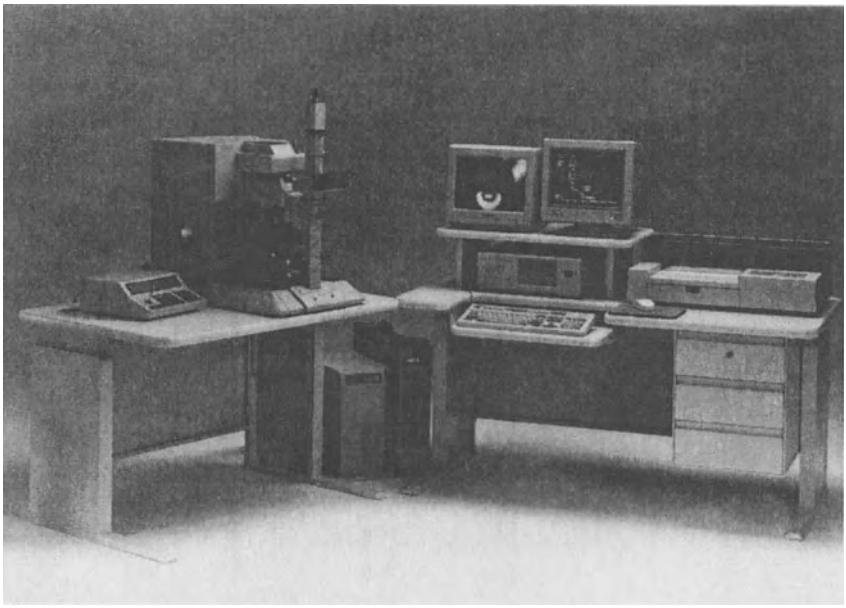


FIGURE 13.3. An FT-IR microscope (left), shown together with a FT-IR system (right). Courtesy of Spectra-Tech, division of Nicolet Instrument Corporation.[11]

13.3. FT-IR MICROSCOPES

Figure 13.3 shows an IR microscope and a FT-IR system. The FT-IR microscope can be used in either transmission or reflection modes. In reflection, if the angle of incidence from vertical is about 80°, a surface thickness in the range of a nanometer is analyzed, but if the angle of incidence is on the order of 30°, then micrometer thicknesses are assessed.[4,5] The amount of reflected radiation depends on the angle of incidence, surface roughness, refraction index, and the sample's absorption properties.[4,5]

With a computer controlled X-Y specimen stage, it is possible to scan and generate a map showing the distribution of measured components over the observed specimen. The specimen must be flat, and the scanning and general procedure are similar to that used for X-ray microanalysis with electron microscopy.

Actually the two procedures of FT-IR microscopy and X-ray microanalysis (EDX) are quite complementary. Elemental composition can be obtained from EDX, and the specific compound identification can be ob-

tained from the FT-IR microscope. Absorption information from the part of the spectrum below 1500 nm is especially useful. This region is often termed the "fingerprint" region because it contains the unique components of the spectrum.[2]

13.4. SPECIMEN ANALYSIS

Specimen preparation is generally simple: For observation by transmission the specimen should be 10 μm or less in thickness; if a specimen is too thick, some of its bands can be totally absorbed. Specimens are often squeezed to correct thickness and then sandwiched between KBr windows. Any reasonable thickness however can be used for the reflection mode. Individual particles can be used if they are mounted on a fine point or microtomed.

One of the more frequent complications is shown in Figure 13.4.[4] Spectra from an acrylic fiber sample are shown where different apertures were used for each spectrum.[4] First the specimen was embedded in polystyrene and microtomed from its original 20-μm diameter to 10-μm diameter thickness. The spectra were obtained under identical conditions except for changes in the aperture size to delineate better fiber from the embedding resin. Different chemical groups are contained in the fiber and the resin. The first spectrum with a large aperture primarily shows the spectrum for the embedding resin, and the bottom spectrum, which contains more noise, shows primarily the spectrum for the fiber. The result can be greatly influenced by experimental conditions. Figure 13.4 illustrates some of the insight needed to use the FT-IR microscope properly.

Infrared microscopy is increasingly useful with modern high-performance fibers. A specific example is using FT-IR microscopy to establish relationships between dichroic ratios, from IR microscopy, and filament modulus for different types of Kevlar fibers.[8] It is interesting that X-ray orientation measurements did not discriminate among different types of Kevlars with quite different moduli, whereas IR microscopy does. Polarized IR is needed for such an application, and a schematic of the microscope used is shown in Fig. 13.5.[7]

The FT-IR microscopy has been useful in a wide range of applications for both routine and research applications. For example, pharmaceutical, petrological, electronic, medical, biological, polymer, and forensic industries have all benefitted considerably from this new technology. Much of the work, to date, has been conducted by spectroscopists, but microscopists have recently become deeply involved, especially in forensic cases with very small samples. The FT-IR microscopy has been used to deter-

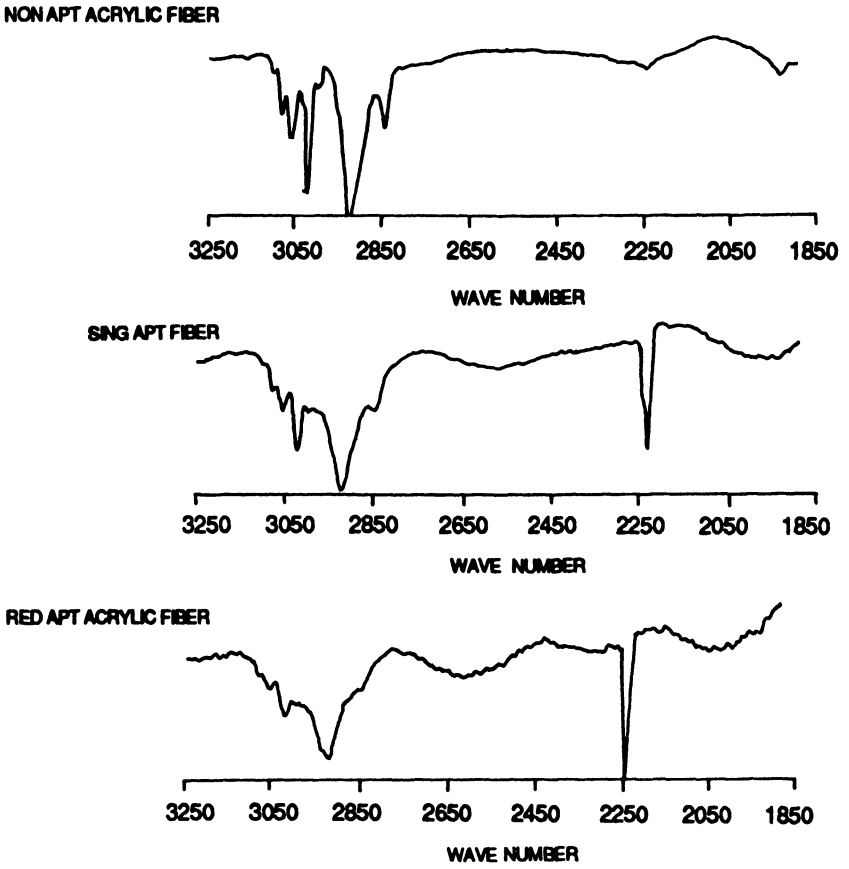


FIGURE 13.4. Signal modification from stray or spurious energy in an FT-IR microscope. All three spectra are for an acrylic fiber embedded in polystyrene. Top: Fiber has wide apertures. Middle: the fiber is defined by a single aperture. Bottom: Redundant or dual conjugate apertures define the fiber area. Courtesy of Marcel Dekker.[4]

mine contaminants and polymers in the plastics and pharmaceutical industries[12]; it can even be used to analyze surface lubricants and surface contaminants.

Different perspectives and backgrounds give these two groups somewhat different views of what to expect from FT-IR microscopy. The microscopists are more concerned with corroborating data with microscopical observations, whereas IR spectroscopists are more concerned with corroborating the image with the IR spectrum.[13,14]

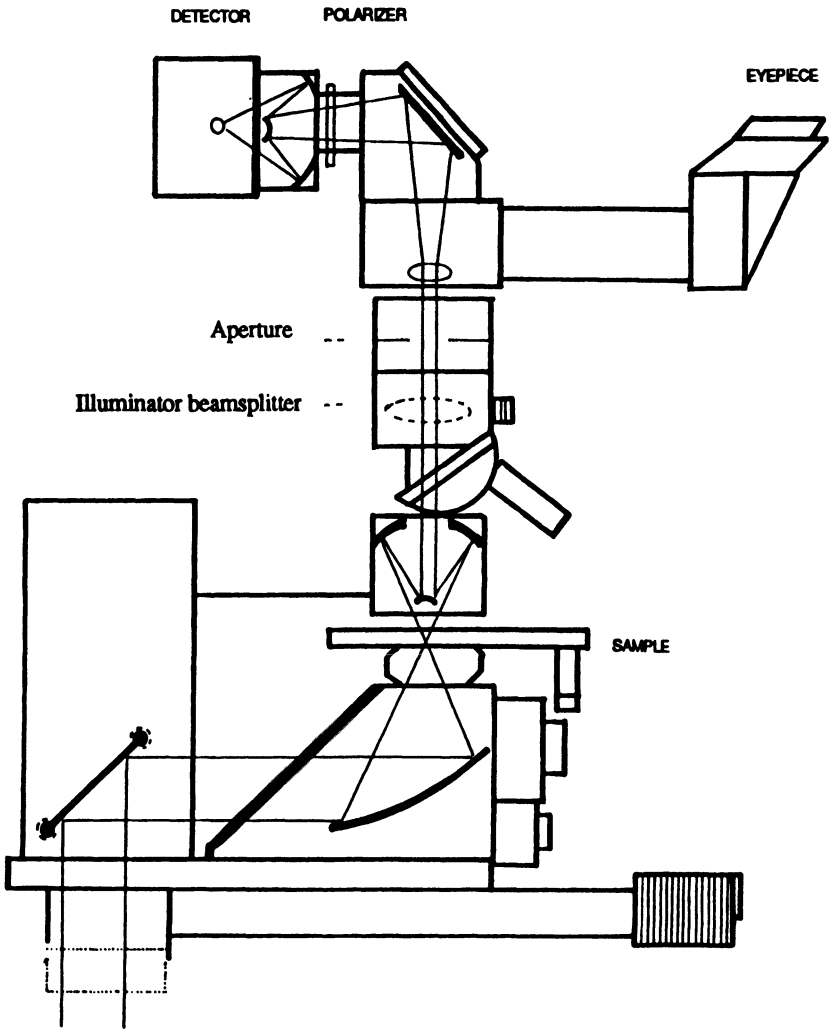


FIGURE 13.5. Paths of light in the Digilab microsampling accessory with the polarizer. Courtesy of Marcel Dekker.[7]

13.5. SUMMARY

Applications for FT-IR microscopy are as broad as those for light microscopy. The three sciences of light microscopy, analysis by EDX, and FT-IR microscopy all complement each other. Each can provide additional information for analysis. The FT-IR microscopy not only identifies ele-

ments but also the specific molecular compounds.[5] Low atomic numbers can easily be detected with FT-IR microscopy, whereas it is difficult with EDX. Skill and experience are needed with light microscopy for the proper use of FT-IR microscopy because erroneous results can be generated,[4,6] but these can be decreased with the combination of proper light microscopical applications. Resolution with FT-IR microscopy is always less than with light microscopy because of the use of uncorrected and reflecting lenses rather than corrected, transmitting lenses. This is due to the very high absorption of IR radiation in a transmitting lens and the fact that a reflecting lens cannot be corrected for chromatic aberrations over the wide range of wavelengths that are used.

14

Transmission Electron Microscopy and Electron Diffraction

14.1. ELECTRON MICROSCOPES

An electron microscope is an optical device for producing high resolution of detail in the object by a beam of electrons.*[1,1a] There are three principal kinds of electron microscopes, classified according to how detail in the specimen is revealed by electrons: transmission, scanning, and emission. In the first two types, free electrons are discharged from an electron gun to act on the atomic nuclei of the specimen, whereas in the field-emission type, the specimen itself is the source of radiation.[1] The field-emission microscope (discussed in Chapter 16), employs no lenses, whereas the TEM and the SEM (see Chapter 15) employ focusable lenses.

The focusability and extremely short wavelength of electron beams are responsible for the theoretically high resolving power of the TEM and SEM. The rapid technological development of practical resolution, contrast, and other attributes contributing to visibility account for the usefulness of these electron microscopes. Although the NA of electronic lenses is still relatively low, a compensating factor is their great field depth. Electrons react with the various atomic nuclei in the object rather than with the much larger domains in light microscopical specimens, so that electron microscopical images give unique information. Since preparing electron microscopical specimens is often different than preparing light microscopical specimens, different changes are introduced into a specimen. This must be taken into account when interpreting images.

*An electron is a subatomic particle having a negative charge of 4.8025×10^{-10} esu and a charge-to-mass ratio or specific charge of $5.2737 \pm 0.0015 = 10^{17}$ esu/g.[1]

In the TEM the image is formed by electrons passing *through* the specimen. The resultant beam contains some of the original free electrons that have not been changed in velocity or direction and some that have been changed either way or both.[2]

The TEM is both a projection microscope and a photomicroscope. Since the electron image cannot be viewed directly by the eye, the image is projected onto a fluorescent screen, and when ready it is transmitted to a photographic plate or film. As such the electron microscope can be compared optically with the light projection microscope or photomicrographic camera, as shown in Figure 14.1.[3]

A beam of free electrons emitted in a vacuum by a pointed filament *F* in Figure 14.1 can be condensed by an electromagnetic (or electrostatic[4]) lens *C* to an even smaller spot (2 or 3 μm) on the specimen *S*. Another electron lens, the objective *O*, focuses the transmitted beam to an inter-

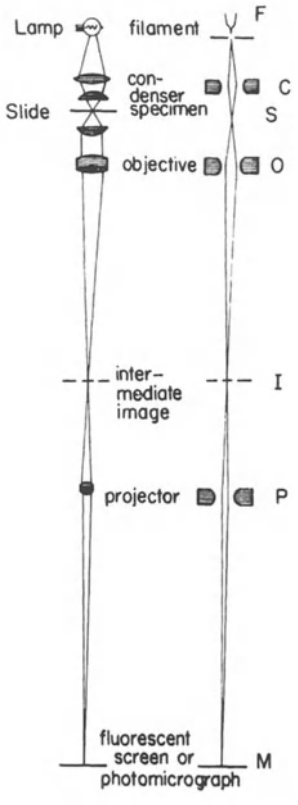


FIGURE 14.1. (left) Light-projecting microscope and (right) electron microscope compared schematically.[3]

mediate image *I*, which is enlarged by the projector lens(es) *P* to form the image on the fluorescent screen or photosensitive material *M*.

The beam of electrons is generated and accelerated in a typical electron gun,[1] shown in Figure 14.2,[2] The Wehnelt gun cylinder acts as a cathode shield, and the base has a circular aperture (1–3 mm in diameter), centered with the filament's apex. Most modern electron microscopical guns have a bias resistor applied between the cathode shield and the filament. Such guns are actually self-biased because the negative potential between shield and filament is produced by the flow of beam current through the bias resistor (rather than by a battery). As in an unbiased gun, the distance between the tip of the filament and the aperture of the gun is adjusted to yield the most intense spot as seen on an intermediate screen or the final screen at low magnification. With a biased gun the adjustment is much easier. Moreover the filament tip can be focused on a tiny single spot, resulting in much higher intensity for a given beam current. Another distinct advantage is the relative insensitivity to fluctuations in beam current.[2]

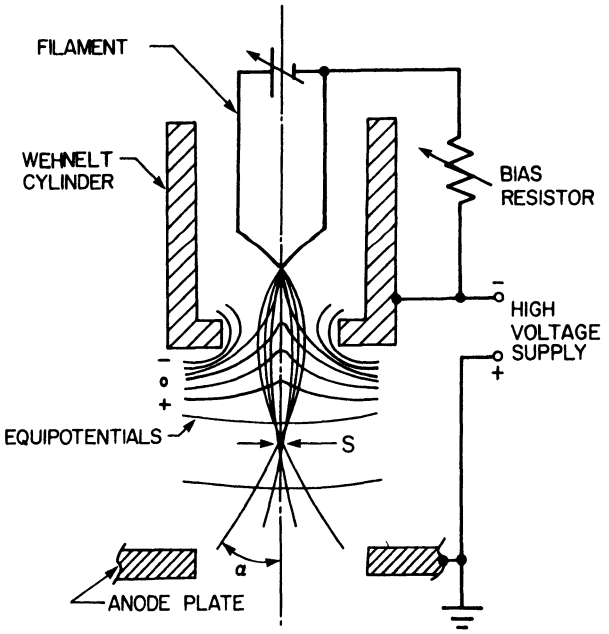


FIGURE 14.2. Self-biased electron gun showing lens-type constriction of the beam to a focal spot *S* (α = 0.50 angular aperture) (from *Practical Scanning Electron Microscopy*, J. I. Goldstein and H. Yakowitz, [eds.]). New York: Plenum, 1975. Adapted from Hall.[2]

14.2. ELECTRON LENSES

The chief lens in transmission microscopes is the *objective*. It determines the degree of resolution in the image.[2] A simplified scheme of a magnetic objective lens is illustrated in Figure 14.3.[5] Although the iron yoke Y and electrical windings W are relatively large and externally impressive, the important pole pieces P_t and P_b have very precise bores B, an aperture A, and specimen S. The specimen can be placed between the two pole pieces, it can be slightly above the top pole piece P_t. As long as the distance above P_t is greater than a certain minimum, the distance is not precise. So the specimen may be tilted through large angles. While there is less angular tolerance if the specimen is placed between pole pieces, there is great tolerance of lateral motion, a simple mechanism for inserting the specimen, and shorter attainable focal lengths; however longer focal lengths give better contrast. An aperture A is usually inserted to increase contrast by removing scattered electrons; of course the smaller the aperture, the lower the resolving power and the more difficulty in keeping the microscope clean. Stigmators S_t, devices to correct for spherical aberration (usually six or eight) are placed around the space Sp and between the two pole pieces P_t and P_b to adjust electron rays along the respective azimuths.[6]

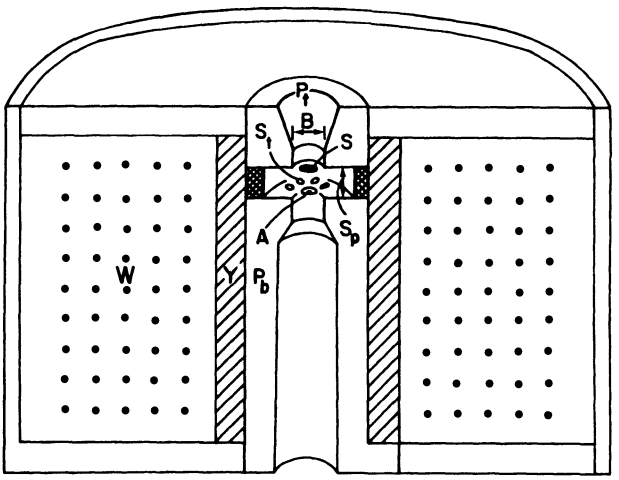


FIGURE 14.3. Simplified scheme of electromagnetic objective lens with iron yoke Y, electrical windings W, pole pieces P_t and P_b, aperture A, specimen S, space between pole pieces S_p, stigmators S_t.[5]

The *projector lens* is required to receive the small stream of electron rays and produce a relatively large image. To gain more variation in magnification, the projector lens system is usually a doublet[2] (see Figure 14.4).[5]

The *condenser* forms an image of the crossover point of between the annular rays from the aperture of the electron gun. Again a double lens adds flexibility to the illuminating system in terms of intensity versus area of the illuminated specimen[2] (see Figure 14.4).[5]

The Zeiss EM910 electron microscope, shown as Figure 14.5, features automated controls designed to produce Köhler illumination.[1,1a] This innovative system automatically selects the appropriate aperture and lens conditions to illuminate homogeneously only the imaged area of the specimen. Whereas in light microscopy Köhler illumination eliminates glare from extraneous light, in electron microscopy, Köhler illumination's main advantage has been in protecting the specimen from damage by heat.[7]

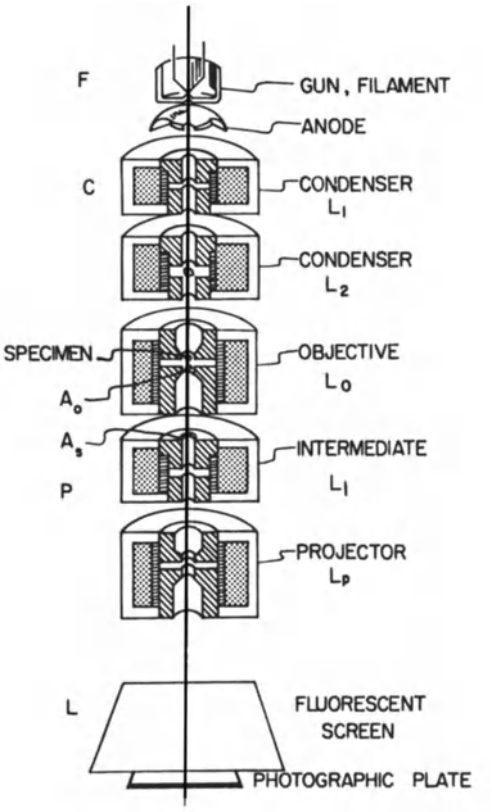


FIGURE 14.4. Transmission electron microscope with double condenser and double projector.[5]

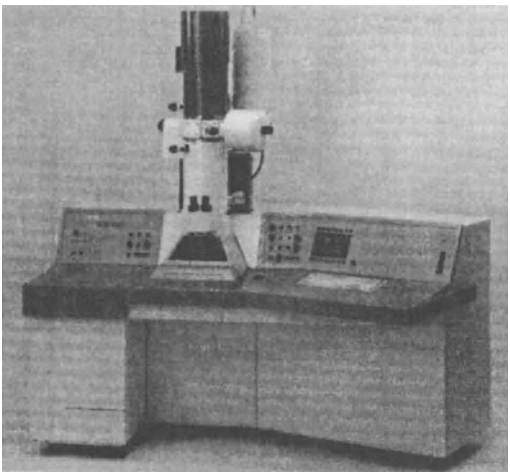


FIGURE 14.5. Zeiss electron microscope EM910 features automated controls designed to produce Köhler illumination. Courtesy of Carl Zeiss.[7]

14.3. RESOLVING POWER

As in light microscopes resolving power is fundamentally what you *pay* for: the *potential* ability to distinguish between two points, expressed as a minimum distance. Hall pointed out[2] that this reasoning is not strictly logical, since "we say that the resolving power is higher the smaller the distance." He preferred the definition used in spectroscopy: Resolving power is inversely proportional to the minimum wavelength difference between spectrum lines.

For the *approximate* minimum resolving power as distance d, we borrow from light microscopy: $d = 0.5\lambda/\sin\alpha$, where λ represents the wavelength and α equals one-half the angular aperture.[1]

Ruska[8] has estimated "attainable" resolving powers for some beam voltages (kV) and consequent wavelengths (λ). For the common voltages 50 and 100 kV and the especially high voltage of 1000 kV, his values are given in Table 14.1.

High-voltage TEMs, such as the one shown in Figure 14.6, are built to attain the limiting resolving power and test their practicability[8–10] (see Figure 14.7).[8]

Abbe had postulated that (in the light microscope) only one of the two diffracted rays of the first order is necessary (in combination with the zero order ray) to form an image of a *periodic* structure. Under this condition,

TABLE 14.1[8]

Beam Voltage (kV)	Wavelength, [λ (Å)]	Resolving Power, [d (Å)]
50	0.054	2.1
100	0.037	1.7
1000	0.009	0.7

a diffraction grating could be twice as fine as the limit resolved by axial rays. The required double angle of aperture 2α can be achieved by tilting the illuminating beam. Gold crystals have a periodic structure of atoms separated by 2.06 Å (or 0.21 nm), and they have been resolved by tilting the electron beam to 2α.[2]

14.4. RESOLUTION

Resolution is what you achieve with your microscope from day to day. Practical resolution depends on the value and constancy of the voltage as it controls the monochromatic wavelength λ, and it is inversely proportional to the angular aperture α. The objective's aperture depends not only on the size of the diaphragm as delivered, but on the size before or after cleaning, as the case may be. Resolution is reduced from 0.3 nm when the objective has a focal length of 2 mm to 3.5 nm with a local length of 14 mm.

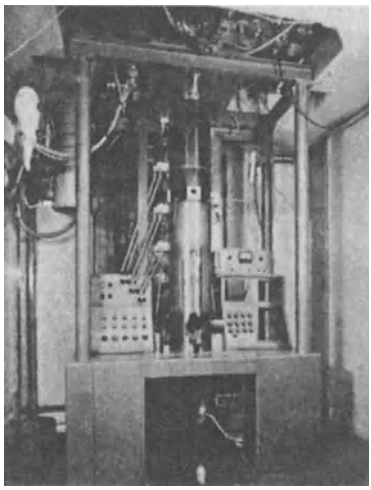


FIGURE 14.6. 750-kV electron microscope of the Electron Microscope Section in the Cavendish Laboratory, Cambridge University, Cambridge, England.

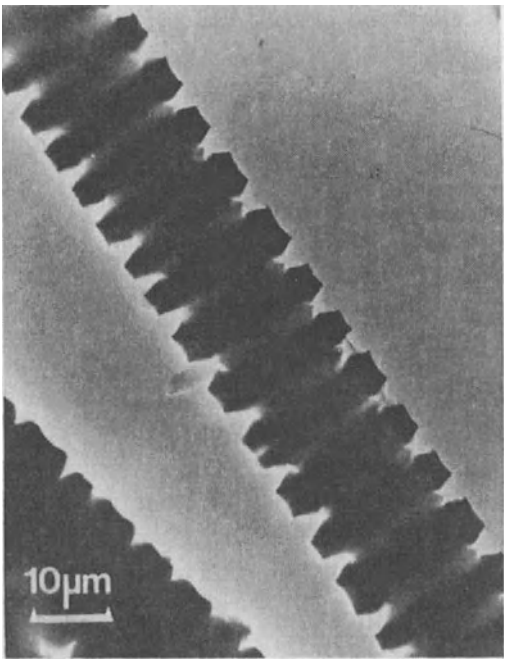


FIGURE 14.7. Commercial (drawn) polyethylene terephthalate filaments etched with 42% aqueous solution of *n*-propylamine at 30° C, showing stress corrosion and extremely fine etching. Taken at 1000 kV on a high-voltage electron microscope. The filament in the lower left is quite in focus; the central filament manifests the halo of underfocus.[10]

14.5. CONTRAST

Contrast in the electron microscopical image is a function of the nature of the specimen and the properties and adjustment of the electron optical system. Hall[2] takes up six factors: contour fringes, amorphous scattering, diffraction contrast, refraction, deflecting fields, and instrumental factors.

Contour fringes are most prominent at edges bounded by free space. They also occur at the edge of dense particles embedded completely in a matrix of lower scattering power. At true focus contour fringes are invisible, but deviation from true focus affects resolution adversely.

Amorphous scattering is a function of spherical aberration resulting in the loss of part of the incident intensity from the imaging beam. Such scattering increases contrast as if a smaller diaphragm were used on the objective. For the same reason resolution is lost as contrast is increased by amorphous scattering from spherical aberration.[2]

Diffraction contrast occurs in crystals as an interaction among diffracted and undiffracted waves, as well as multiple scattered beams. It is a complicated phenomenon manifested as fringes at the edge, extinction contours at grain boundaries, and curious patterns of dislocations.[2]

Refraction contrast occurs at boundaries between refractive material and a vacuum as a result of interference (phase delay) between the respective waves. The resulting phase contrast is manifested by over- or underfocusing.[2]

Deflecting beam electrons by a magnetic or electrostatic field is an especially important source of contrast. Boundaries between ferromagnetic domains are manifested in this manner. Phenomena in electrostatic fields in highly resistant materials are more complicated and may be erratic.[2]

The chief instrumental factors affecting contrast are the beam voltage and using an objective aperture. Decreasing the voltage increases the contrast, but the thickness of the specimen must be decreased accordingly; thus there is a very practical lower limit to the voltage. Contrast can also be increased by reducing the aperture of the objective. The limit to this procedure is set by the degree of diffraction error to be tolerated. Another limitation is that the smaller the aperture, the sooner it will be plugged by contaminant[2] (chiefly a carbonaceous product of charred organic matter). Additional contrast is generated for most biological and polymeric specimens by "shadowing"—spraying at an angle with a heavy metal.

Staining with heavy metal compounds is a major technique for creating contrast within specimens that inherently have little contrast due to their composition from mainly lighter elements.

14.6. ABERRATIONS

Aberrations in electron microscopical images can be caused by either mechanical or optical problems. Mechanical problems arise from difficulties in machining pole pieces precisely and from inhomogeneities in the iron pieces. Either or both difficulties are manifested in astigmatism, i.e., appreciable lack of symmetry around the optic axis. In modern instruments correction is effected by stigmators, whereby the azimuth and magnitude of correcting asymmetry can be applied while the microscope is operating. A mechanical method uses two compensating iron pieces. Their distance from and along the optic axis can be varied until correction is accomplished. Another method uses electrostatic electrodes equally arranged around the optic axis. The azimuth and direction of the correcting asymmetrical field are controlled by separate potentiometers. The electrostatic method has no moving parts or vacuum seals, and the appropriate compensating circuit can be switched in simultaneously with a change in the beam potential. Stigmators are used on objective and condenser lenses.[2]

Aberrations resulting from the projector lens system do not affect resolving power, but they can produce distortion in the final image, especially near the outer zones of the lens. The obvious safeguard is to avoid going to the magnification limit set by the bore of a particular pole piece. The temptation to overextend the limitations of the projection lens is not so great in the modern two-lens projector system as in the single lens. The two-lens projector permits a greater range of magnification without exchanging pole pieces.[2]

Hall lists seven *optical* aberrations from electron (as well as light) lenses[2] of which axial–spherical aberration is the most important. This is the only theoretical aberration that occurs for a point on the optical axis as well as for all other (extra axial) points in the specimen. The explanation is that from *any* point in the object, the power of the lens is greater the longer the distance at which rays pass through the lens. Electron lenses are *always convergent;* the power of outer zones of the lens is always too great compared with that of the inner zones. Since at present there are no divergent electron lenses to combine with convergent ones, correcting for axial–spherical aberration cannot be accomplished with electrons as it is with light. The tendency, therefore is to keep the angular aperture small to reduce the aberration, and this limits the resolving power.[2] Ruska[8] and Spetier[11] have attempted to overcome spherical aberration and attain the theoretical limit of the TEM.

Some varieties of spherical aberration peculiar to electron *projector lenses can be corrected.* They are manifested as either pincushion or barrel distortion. In either case the image lies on a curved surface, so that distortion occurs when projected on a flat surface. Correction is by means of a second lens that tends to neutralize the spherical aberration of the first. The pair can be either a doublet or a two-lens system.[2]

14.7. CLEANLINESS

Cleanliness in electron microscopes involves taking precautions against contamination and removing whatever contamination has nevertheless accumulated. A contaminant can be any product of bombardment by the intense beam of electrons in a high vacuum. The chief kind of contaminant is a carbonaceous product of decomposed organic matter: commonly grease used as vacuum seals or oil leaking in as vapor from the forepump. Grease is avoided by using greaseless seals such as dry, nonvolatile, elastomeric gaskets, metallic bellows, a grease-free specimen, and photographical locks. Oil is reduced by having a remote forepump with a main valve that is kept closed until the diffusion pump has warmed up.[2]

Hydrocarbon vapors from oil, etc., decompose under electronic bom-

bardment and are deposited as such or as polymers. Silicone oils may also deposit silica. If either kind of oil vapor is trapped away from the specimen, water vapor may be the chief offender by becoming ionized and etching any organic specimen. The apparent source of water is photographic negative material,[2] which therefore should be thoroughly desiccated before use. In some instances contaminants are controlled by using a trap cooled by liquid nitrogen in the microscope's column.

Of prime importance is protecting the specimen itself from contamination. Precautions should also be taken to clean scrupulously containers, instruments, reagents, substrata, replicating materials, and room atmosphere. Reagent and wash water should be freshly distilled, not stored in a metallic tank and run through pipes.

Cleanliness also means having clean lenses, open apertures, etc., just as in light microscopy.

14.8. FOCUS DEPTH

Focus depth means in practice how far the viewing screen or photographic plane can be moved along the microscope axis and still receive satisfactory focus of the image. This distance is relatively long. Hall estimates that the viewing screen and photographic plate or film can be separated successfully by several inches.[2]

14.9. FOCUS

Focus is the point at which rays originating from a point in the object converge or from which they diverge under the influence of a lens.[1] As in light microscopy, an outline of a particle is imaged most sharply when focus is exact, though contrast may then be poorest. Sometimes an over- or underfocus halo is preferred or inadvertently obtained (see Figure 14.7).

14.10. ILLUMINATION

Illumination in the TEM takes place by transmitting electrons through the specimen. The electron source is usually an incandescent filament in the shape of a hairpin. While its relatively large size provides mechanical stability and long life, its hairpin shape is surrounded by an asymmetrical electrostatic and electromagnetic field. A pointed filament is much better electron optically, but it is of course more fragile, and it has a shorter life.[2]

The condenser in the TEM serves principally to control the size and

therefore the intensity of the illuminated specimen field. Even higher intensities can be obtained electronically by means of a *double* condenser. The second condenser also allows a greater reduction in the area illuminated, thus protecting the specimen outside the field of view from injury by the illuminating beam.[2]

Illumination intensity is expressed in amperes per square centimeter, i.e., in the illuminated area of the specimen. Intensity is inversely proportional to the square of the magnification. Brightness is defined as the flux per unit area per unit solid angle, usually measured in amperes per square centimeter per steradian.[2]

Tilted illumination, as mentioned before, is a way of increasing resolving power. With the TEM it is also a method of obtaining oblique illumination or a dark-field view. Another method involves placing an eccentric diaphragm in front of the specimen. Either way a bright image is sought against a dark background, especially with crystalline substances, which manifest diffraction images.[2]

Radiation illuminating the electron microscopical specimen has a constant wavelength to the degree that the accelerating potential on the tungsten filament is kept constant.

14.11. ANISOTROPY

Anisotropy is considered in electron microscopy because the motion of electrons is somewhat analogous to the behavior of light in anisotropic materials.[2] Moiré patterns can render the structure of crystalline and other anisotropic specimens visible. For example, Holland[12] has demonstrated symmetry in a crystal of polyethylene lying on a substrate of crystalline polyethylene. The Moiré pattern is the same in two opposite quadrants but different from the pattern in the other pair of quadrants.

14.12. USEFUL MAGNIFICATION

Useful magnification in electron (as well as in light) microscopy refers to the minimum magnification required to show the desired detail in a specimen. Of course the finest detail may be beyond the practical limit of resolution by means of electrons. In such cases it may be better to define empty magnification, beyond which no new information is revealed.*[1]

*Some empty magnification may however be advantageous in making measurements.[3]

The required resolution must be in the electron micrograph before enlargement, and the enlarger must be in sharp focus on the original micrograph. Moreover photographical copying procedures generally lose resolution.

14.13. FIELD OF VIEW

Field, the portion of the object in view, is very small in transmission electron microscopy—on the order of a few square micrometers at 20,000x within the microscope.[3] At any given magnification, the field is at present limited by a low NA (~0.002); greater apertures carry unacceptable aberrations and distortions. Other limiting factors are faintness of image for viewing or photographing, specimen susceptibility to damage from more intense illumination, practical selection, and the number of fields to be viewed.

Experience in field selection may come directly through repetitive work in a specialized field, such as virology, but in broad scientific, technological, or industrialized areas, mapping out significant fields comes from experience with macroscopy and light microscopy.[3]

Relevant fields selection lies within the general science and art of sampling. The ability to discriminate a representative from a nonrepresentative field takes a great deal of experience, thought, and judgment.

14.14. ARTIFACTS

An *artifact* is a spurious image that does not correspond directly with the true microstructure of the original specimen.[1] Obviously artifacts can result in serious misinterpretations. Avoiding or reducing artifacts depends on understanding how and why they become visible. Some electron microscopical artifacts represent damaging changes in the specimen during exposure to the beam of electrons. For example films representing the specimen, substrate, or replica may shrink, tear, or curl, if they absorb energy from the electron beam. Textile or paper fibers or biological tissues may swell or evolve gas, while microorganisms may change in size or shape.[3] Observing variations in such changes with time of exposure or intensity of the electron beam should lead to preventing artifacts. Remember that no electron diffraction is observed if the crystallinity has been destroyed by impinging electrons during the preliminary viewing.

Those artifacts discussed in connection with the electron microscope

itself are introduced by contaminations. For instance specimens of carbon black particles may be observed to increase,[3] resulting from the deposition of such carbonaceous products as pump oil, gaskets, substrates, or specimens themselves.[2] Another result of contamination is due to the image drifting. If after cleaning the microscope and taking precautions against contamination from pump oil and gaskets, such artifacts recur, changes in the specimen preparation should be considered. If the specimen itself is volatile, encasing it in an evaporated film of metal or another stable element may help. At any rate changes in the specimen caused by the high vacuum in the electron microscope should always be considered and constantly studied. Some specimens may require thorough preconditioning in a vacuum comparable to the electron microscope.

14.15. DEPTH CUES

Cues to structure *depth* in an electron microscopical specimen include the built-in cue of the relatively great depth of field in a reasonable focus, about 10 μm, which is about 10 times the depth of high-aperture light microscopical objectives. The result is a more realistic three-dimensional appearance of the electron microscopical image than in high-powered light microscopy.[3]

Shadows are very important specimen cues in a depth variation, but they are obtained in a much different way than in light microscopy because electron microscopical lenses do not have a high aperture. Consequently oblique illumination cannot be obtained in the electron microscope by blocking off one side of the aperture of the condenser lens (as in light microscopy). Instead the specimen is shadowed before it enters the electron microscope; that is the specimen is prepared by obliquely depositing a material, usually a heavy metal, at an angle. The metal is evaporated from an electrically heated filament in a vacuum at great enough distance from the specimen for the metallizing atoms to approach as a beam. A vertical projection of height h casts a shadow of length $l = h/\tan\theta$, where θ is the horizontal angle of obliquity. Thus the height h can be estimated by measuring the length of the shadow cast at a known angle θ. In the positive-electron micrograph, such shadows appear light, making depth interpretations difficult for the observer accustomed to viewing dark shadows. Therefore a negative print is usually made from a transparent positive print[2] (see Figure 14.8). The print should be oriented so that the shadows fall below the object (rather than above it), since we are accustomed to illumination from above (rather than below) an object.

A third cue to depth is *stereoscopy*, made possible by the great field

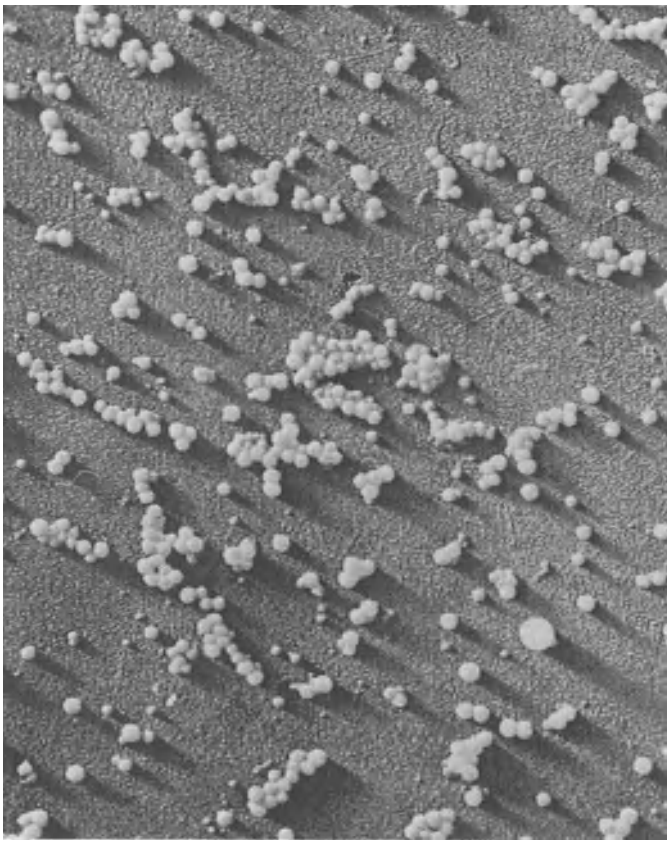


FIGURE 14.8. Polystyrene emulsion stabilized and shadowed by coating lightly with gold (at an angle of 10°). A negative print was used to make dark shadows. Electron micrography by Mrs. E. Gagnon Davis and E. J. Thomas, American Cyanamid Co.

depth in focus in the electron microscope. Two electron micrographs are taken with the specimen tilted at two different angles to the axis of the microscope. The specimens are best viewed in a stereoscope. Most commercial electron microscopes are built so that the specimen can be tilted to the appropriate angles for stereoscopy.[2]

At the present time Fourier transforms have been applied with the TEM to image periodic structures of molecules in three dimensions with a resolution of 7 Å, nearly three times the best resolution previously attainable. Fourier microscopy combines data from low-contrast electron micrographs with data from electron diffraction. Various views of the specimen

are obtained by having the specimen on a tiltable stage. From such projections an image of high resolution is constructed in three dimensions.[13]

14.16. SPECIMEN THICKNESS

Specimen thickness in transmission microscopy is very important. For optimum visibility the specimen must be thin enough to allow electrons to penetrate some parts significantly and yet thick enough for other parts to absorb electrons preferentially. Even atoms with a low atomic number scatter electrons appreciably. Therefore sections of biological tissue and other organic materials must be much thinner than the thinnest specimens (1 μm) used in light microscopy. Inorganic materials must be even thinner if they are to transmit any electrons in contrast to a silhouette. The thinner or smaller a specimen, the more likely it will be unable to support itself on the grid of the stage. Supporting films are usually no thicker than 200–500 Å (20–50 nm) to be sufficiently transparent to electrons.[14]

Particulate specimens, such as dusts, precipitates, pigments, and strengthening fillers, must be sufficiently dispersed to differentiate units from aggregates. However during preparation the size of the effective particle should not be affected if the electron micrograph is to be interpreted in terms of a problem, such as lung disease, air or water pollution, pigmentation, or particulate strengthening.[3]

14.17. FIELD DEPTH

Field depth in the TEM is sufficient to ensure that the whole field in view of an adequately thin specimen is in focus. To this extent field depth in electron microscopy is not a problem but an advantage in visibility. Of course field depth varies with the angular aperture 2α. While 2α is always small in electron microscopes as compared to light microscopes, angular aperture and therefore field depth can be varied between limits. For a resolution d of 10 Å, α is about 2.5×10^{-3} rad for beam potentials of 50–100 kV; field depth D is 4000 Å, and $D/2$ on the underfocused side is 2000 Å or 0.2 μm. For a resolution of 50 Å, $D/2$ is 1 μm.[2]

14.18. SPECIMEN STRUCTURE

Specimen *structure* also influences visibility. For example a *periodic* structure such as that of a single crystal of gold is resolved ($d = 2.04$ Å) when

illuminated *across* the periodic structure by a tilted beam at an angle equal to the angle α of the objective. This resolution is five times the usual limit of 10 Å for a nonperiodic structure. The example is simply Abbe's diffraction principle $d = \lambda/2 \sin \alpha$ when the undiffracted ray and one of the first-order diffracted rays are both included within the angular aperture of the objective. The resulting image is really a diffraction pattern of the (gold) crystal. It is a special example of how specimen structure affects visibility. Nevertheless it is a practical case, since all metals and their alloys represent periodic (crystalline) structures that can be resolved if they are illuminated by a properly tilted and directed beam of electrons.[2] Structures of other crystalline specimens also play an important role in visibility.[15]

14.19. SPECIMEN MORPHOLOGY

Morphology is the shape and size of particles, lines, areas, or volumes in a structure.[1] Seeing morphology is what microscopy is all about. Interpreting shape is relatively easy when the size is large and other attributes contributing to visibility are favorable. However some microscopists wish to see smaller and smaller particles or parts regardless of larger neighbors with the same composition and shape. Other microscopists are not particularly interested in cracking the barriers of resolution, but they have no larger relevant particles to look at. These microscopists would do well to seek or grow larger specimens. The chemists's method of digesting the finest particles and precipitating the resultant solute (in time) on the larger particles may be useful, or sublimation or annealing can be tried. Otherwise it may be worthwhile to study analogous or isomorphous species of crystals. Likewise biologists may learn relevant morphology by studying closely related but larger species. The point is that morphology, true to its Greek derivation, pertains more to shape than to size.

14.20. SPECIMEN INFORMATION

Information is a very important attribute in interpreting TEM images. If the required information does not come with the sample, the electron microscopist should ask such questions as, What is the problem? Is there macroscopical and light microscopical information relevant to the problem? How were the samples taken? Are they stable in a vacuum? Are they heat sensitive? In what are they soluble and insoluble? Are there good and bad examples present or obtainable? Old and new? If there have been changes in environment, treatment, etc., are there examples of each?

Similarly the microscopist should furnish adequate information about the electron microscopical examination with every micrograph submitted in a report. Magnifications must be accurately given and usually are, but other essential information is sometimes omitted. Which is the top side of the electron micrograph? Remember to orient the picture so that the illumination appears to be from above if you wish the viewer to interpret shadows of elevations and depressions as they would be seen macroscopically. Also remember to indicate a positive or negative print and how you made the shadows dark. You may be educating and informing your sponsor, and in the long run, the benefit rebounds to you. This means that exact details of sample preparation should be given with advantages and limitations of the method.

Procedures labeled standard should have been standardized by an official society like the American Society for Testing and Materials (ASTM).[16] The ASTM standards are the result of democratic deliberation and voting by peers representing the producer on one hand and the consumer on the other. The ASTM standards are published annually and reconsidered at least every 5 years. The ASTM procedures are reproducible by consultants and referees, and the results constitute admissible evidence in court. Microscopists should keep bound notebooks of information about procedures, interpretations, illustrations, and experiments for a filing system and maintain electron micrographs.

14.21. EXPERIMENTATION

Experimentation should be devised to test the interpretation of all images not obtained by standard or repetitive procedures. The microscopist's reputation for accurate interpretation is always at stake, and only the microscopist knows when to take time to vary procedures in sampling, preparing specimens, and checking for specimen contamination and aging. Other experiments involve gradient heating or cooling and chemical or physical reactions. Admittedly electron microscopical procedures require a lot more time than procedures using light microscopy.

14.22. SPECIMEN PREPARATION

Specimen preparation is too large a topic to be discussed in procedural detail. The reader who wishes laboratory directions is referred to monographs on the subject[16–21] and to ASTM recommended practices.[16,22] It must be emphasized here that any preparation of a microscopical spec-

imen changes it in some way and to some degree. In transmission electron microscopy the primary goal is to prepare a tiny, thin specimen that will fit into the microscope holder. In most instruments the holder is intended for a screen or grid with about 200 openings per inch. One popular screen shape is a disk about 3 mm in diameter.[2,14,17] In most cases the TEM specimen represents a very small portion of the sample (see Chapter 20).

The first problem is whether to select a representative or nonrepresentative specimen. If we are interested in contamination, impurities, forensic traces, spots, specks, or other isolated portions, a nonrepresentative specimen is in order. In the process the original sample has been changed drastically, so that an accurate record of the selective process should be made. One good way of doing this is to photograph at a low resolution an area that pinpoints the field taken at high resolution (see Figure 14.9a and b).[21]

If sampling for TEM is *representative,* there are other considerations. Even a dry powder composed of only one phase but small and large particles is difficult if not impossible to represent in a specimen of TEM size.[3] If the original sample is large enough, it may be advisable to make a quantitative size analysis macroscopically and then take random specimens of the resultant size fractions for electron microscopical illustrations. Size fractionation can be by rate of settling in a gas or liquid,[16,24] and settling in a liquid can be by gravity or centrifugation. Liquids and some gases can cause aggregation of particles, so unless such aggregates are

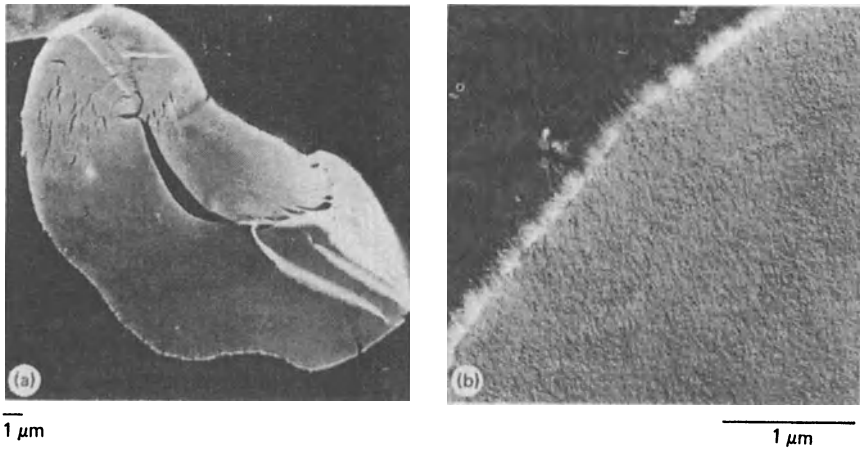


FIGURE 14.9. Transmission electron micrograph of a thin section of a cotton fiber; (a) cross section of whole dry fiber of cotton; (b) higher resolution of an area in (a). USDA micrograph by Southern Regional Center, New Orleans.

penetrated by the electron beam, they will appear in silhouette and can be mistaken for single, much larger particles. Without sufficient information it should not be assumed that dispersion in any one preparatory liquid duplicates commercial dispersion in a different medium. Many a false interpretation of *practical* particle size or shape has been made by assuming the morphology to be that of the ultimate[2] (finest) size of the particles before introduction into the commercial product.

Particles often deposited directly onto a filmy substrate, such as Formvar® polyvinyl formal resin, by settling from a dispersion in air or by dusting or rolling from a cotton swab dipped into a powdery sample. Other powders may need more shear force, such as that provided by a Tesla coil operating on a small portion of powder resting on a Formvar® film. An alternative involves an ultrasonic vibrator operating on the powder suspended in acetone so as to spray the suspension onto a carbon film. Magnetic particles, or any other dry materials that would contaminate the TEM, must be embedded in an adhesive, such as a 1% solution of collodion (cellulose nitrates) in amyl acetate. Dispersion is by means of a spatula or muller on a glass plate[17] or a tiny mortar and pestle. Enough solvent is added to leave a sufficiently thin film after evaporation. If aggregation occurs during this treatment, it may be better to use a vehicle and a thinner that represent natural or industrial conditions. Particles are usually shadowed by evaporating a heavy metal at a known angle[17] (see Figure 14.8).

Surfaces are generally prepared for the TEM by replication. Carbon replicas are preferred because they are thermally and electrostatically conducting, and they can be made thinner than plastic replicas. A typical method of making a carbon-positive replica of a solid surface is shown in Figure 14.10. First a 4% solution of collodion in amyl acetate is applied, dried, stripped off, and discarded to clean the surface. Then several layers are stripped off and shadowed by evaporating a heavy metal on the replicating side. Next carbon is applied as a positive replica from a carbon arc in a vacuum.[17]

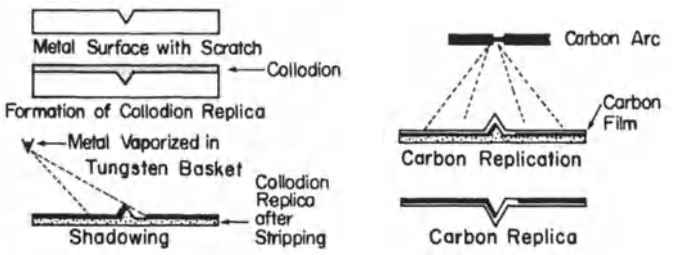


FIGURE 14.10. Steps in making a carbon-positive replica.

Surfaces sensitive to a plastic solvent can be replicated dry by using polystyrene disks made by flattening pellets at 165° C. Powders, such as dry yeast, are especially amenable specimens; they are simply sprinkled on the disks, covered with a weighted glass slide, and placed in the oven at 165° C for about 5 minutes. After cooling the specimen is removed, and carbon is projected from a carbon arc in a vacuum as usual. Since carbon replicas are very delicate, special techniques have been developed to handle them.[17]

Microtomy is discussed in Chapter 20.

Freeze fracturing biological and other aqueous specimens is accomplished by freezing the specimen in Freon® 12 refrigerant and producing a fracture surface with a precooled razor blade. The specimen is transferred to a special accessory for the Ladd vacuum evaporator, where at the precise moment the specimen is shadowed with platinum and carbon, then replicated with carbon. The replica is protected with a drop of 1% collodion in amyl acetate, and a slice of the specimen and replica is cut off with the razor blade. The specimen is dissolved in Chlorox® sodium hypochlorite solution, and the collodion is dissolved by fumes of amyl acetate, leaving the carbon replica for electron microscopical examination.[17]

14.23. ELECTRON MICROGRAPHY

Accelerating voltages of 50–100 kV produce electrons, each one of which is capable of passing through as many as 100 photosensitive grains and losing enough energy to make at least some of the grains developable. The number of grains developed depends on the developer and development time. The information in a ray of electrons is the signal. There is also noise (without information); However the *signal-to-noise ratio* for electrons is 100–1000 times greater than that for light! The photographic record also has a signal-to-noise ratio; the signal is the optical density, and the noise is the developed granularity.[26] If there is no granularity[1] developed, the signal-to-noise ratio in the electron micrograph is the same as in the electron beam. Such a film is on Kodak® high-resolution plates,[25] which are practically without graininess[1] but far too slow for any except the most stable instruments and specimens.[26]

Among glass photographic *plates*, the projector slide grades are the most practical.[25] As far as electrons are concerned, there is little difference between the two grades medium and contrast. One great advantage to both grades is that useful exposure time to electrons (speed) can be changed by adjusting conditions during development. Normally Kodak® developer D-19® solution is recommended for 3 minutes at 68° F (20° C).

However development time can be varied from as little as 1 minute to as much as 8 minutes.[26] Photographic plates have the greatest dimensional stability, and they are readily prepumped before use.[27] But the disadvantages of heavy, bulky, and breakable glass plates are obvious when it comes to storage, both before exposure and after processing.

Fortunately Kodalith® LR film, Estar® polyester base available in cut sheets overcomes the problems of glass plates. Moreover the polyester base pumps down almost as fast as glass and much faster than acetate bases. Polyester also has less curl and more dimensional stability than acetate. Kodalith® LR film, when developed in D-19® for 2 minutes at 68° F (20° C), has the same electron reaction speed as medium projector slide plates with 50-kV electron acceleration and two-thirds the electron reaction speed with 100 kV. Because of gradual fogging however, increased development is impractical, and a Kodak® safelight filter 1A (instead of 1) is required during all handling prior to fixation. Kodalith® LR Estar® film is available in all sheet sizes.

For TEMs equipped for 35- or 70-mm roll film, the fine-grain variety has been the most popular. A drawback for some users is the fact that acetate base requires four times as long to pump down as glass plates. The electron reaction speed of this film is slower than that of projector slide plates, but this is acceptable because the bellows length is shorter and therefore the electron beam intensity is higher. The signal-to-noise ratio is low enough to be just barely detectable by the eye; this characteristic may or may not be important. Kodalith® LR Estar® film was available in rolls 35 or 70 mm wide.[26]

Besides potential granularity in a photographic emulsion, the spread function limits the acceptable extent of enlargement in TE micrographs. The spread function measures the area over which an electron's energy is expended in its deviated path through the gelatin (not the photosensitive grains) of the photographic emulsion. Two narrow electron beams separated by less than the radius of spread (scatter) will not record separate information. For accelerating voltages of 50–100 kV, the spread function is about 5–10 μm, giving a maximum enlargement of 20×. Less than 20×, developed granularity limits the photographic resolution of information with all materials except Kodak® high-resolution plates, which have practically no granularity.[26]

After exposure to electrons and specific development of the negative, stopping, fixing, washing, drying, and printing are similar to processes used after exposure to light;[27] the reader is referred to current literature.[28]

There is however a drawback in the photographical systems of TEMS that do not accommodate the fast self-developing, direct-positive camera backs, such as Polaroid®. These cameras must be used outside the vacuum

system of the TEM. Focusing on the outside of the fluorescent screen is unsatisfactory because of the granular nature of the screen. Instead a grainless single crystal of europium-activated calcium fluoride is employed. The activated crystal, assembled with a mirror, is placed in one of the two portholes provided for accessories in most TEMs. As indicated in Figure 14.11, a special light microscope *M*, consisting of an objective camera lens (f = 1.4) and an ocular, is placed in the other porthole. Connected to the microscope *M* is a Polaroid® camera[30] *C*, supported on a stand *S*. The fluorescent crystal's assembly *F* includes a vacuum-tight sleeve that permits focusing on the fluorescent image by viewing through a hand magnifier held in the plane of the camera back. The sleeve *F* also allows the whole assembly to be drawn out of the way when not in use. Figure 14.12 is an electron micrograph of a diffraction grating taken as a test for resolution and calibration.[29]

With the TEM it is important to select an area to be micrographed before the specimen changes, burns up, or contaminates the microscope. This is especially difficult at first if the image is weak or low in contrast. While some TEMs are equipped with image intensifiers, others are not. For the unequipped microscope there is an accessory that intensifies both brightness and contrast of the image, and it fits on the microscope in place of the binocular viewer. The accessory focuses the original image on a photocathode and reproduces it on a phosphor associated with an anode. The net gain in luminance is about 4×10^4.[29]

The accessory image intensifier can be connected to a cathode ray (video) tube. Such a system has been used to examine electronmicroscopic particles from celestial space. Some of these particles decompose readily in

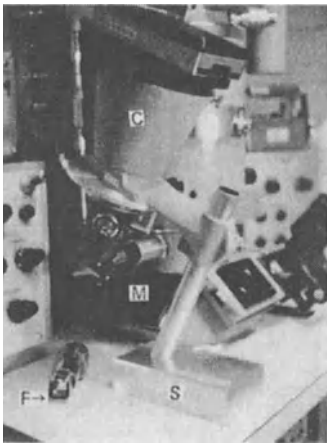


FIGURE 14.11. Polaroid® camera system attached to a TEM. A fluorescent crystal mirror assembly (*F*) is on table in foreground.[29]

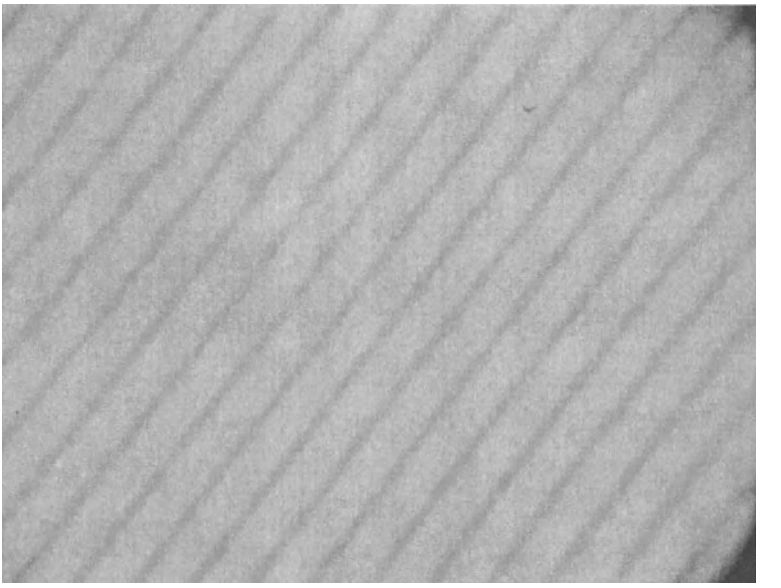


FIGURE 14.12. Micrograph of 1134 line/mm grating replica at 5000× made with a Polaroid® camera system of a TEM.[29]

the electron beam and therefore are examined at extremely low-beam intensities with the image intensifier. The video aspect of the system is an apt introduction to the STEM (see Chapter 15).

Additional useful magnification can be obtained with photomicrography and subsequent enlarging. Figure 14.13 shows a lattice image with a stacking fault. Most of the useful magnification occurred in the microscope, but additional useful magnification was achieved in photographic printing.[31]

14.24. ELECTRON DIFFRACTION

Electron diffraction is routinely conducted with conventional transmission electron microscopy to obtain additional information, such as crystalline structure, crystalline habit, and molecular orientation. Related insight is provided by X-ray diffraction (XRD), but the required sample sizes are quite different. Electron diffraction (ED) from an area 1 μm × 1 μm is relatively easy to obtain with a suitable sample for transmission electron

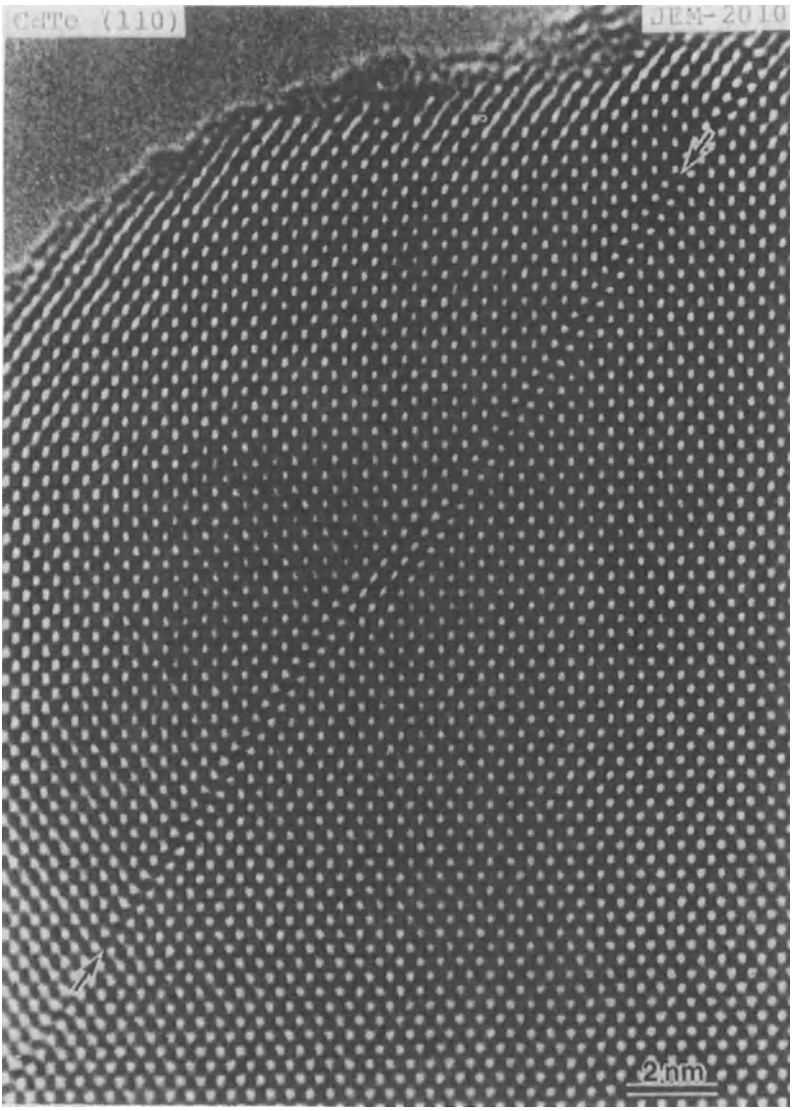


FIGURE 14.13. A high-resolution TEM photomicrograph of Cd Te, a semiconductor compound with a face-centered cubic structure, observed in the (110) direction. The lattice image clearly shows a stacking fault on the (111) plane. Shifts in the lattice plane at the fault are indicated by the arrows. Courtesy of JEOL, USA, Peabody, MA.[31]

microscopy, whereas a sample size of more than twenty times this is necessary to provide XRD.[32] Thus with small apertures or masks in the electron beam, many subareas can be analyzed even though they are too small to provide diffraction information by XRD. In addition considerably more information is provided in the ED pattern than in the XRD pattern because of differences in the utilized wavelengths, ca. 0.002 Å for ED versus ca. 2 Å for XRD. This difference is due to differences in wavelengths, a topic beyond the scope of this book but involving a larger Ewald sphere for ED and the sphere's intersection with the crystal's reciprocal lattice points. This concept is often explained in texts on ED[32] and XRD.[33]

While crystalline lattices are discussed in Chapter 7, it is pertinent to illustrate the crystallographical advantage of the high resolution of the TEM. Figure 14.13 is a TEM micrograph of CdTe, a semiconducting compound with a face-centered cubic structure, observed in a certain direction (specified as 110). The lattice image shows a stacking fault on the 111 plane, indicated by the arrows.[31]

A simple cubic unit cell is represented in Figure 14.14. Spacings between such planes are given by Bragg's law from X-ray diffraction, i.e., $\lambda = 2d \sin \theta$, where λ = wavelength of electrons, d_{nkl} = spacing between the periodically spaced planes in the unit cell and crystals that diffract the waves, and θ = angle between the incident electrons and the specific set of atomic planes. Constructive interference occurs only when many parallel planes are at an angle such that Bragg's law is satisfied; otherwise scattered waves intersect destructively and are not seen.

Small differences do exist between ED and XRD. Because the wavelengths of electrons are typically one thousand or more less than those of X-rays, Bragg's law is fulfilled by crystallographic planes almost parallel to the original, undeviated electron beam. Also because thin crystals are used

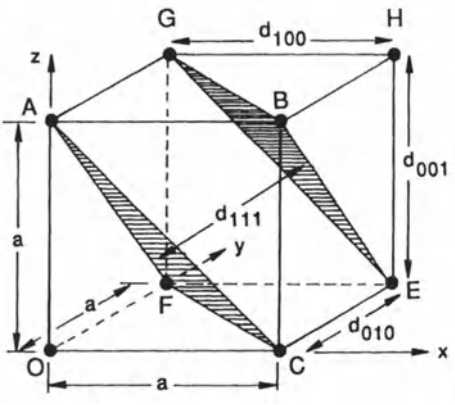


FIGURE 14.14. Cubic-unit cell showing the d_{100}, d_{010}, d_{001}, and d_{111} lattice plane spacings. After Beeston, Horne, and Markam.[32]

with ED, typically 10–50 nm thick, limited constructive interference, or incomplete destructive interference, does occur when crystal alignment deviates slightly from the Bragg condition.[34]

As indicated in Figure 14.15, when an image is formed in the image plane of the objective lens, a diffraction pattern (VW) is formed in the objective's back focal plane. It also indicates that all rays from the same point in the specimen are focused at the same corresponding point in the image plane. Rays from point *A* are focused at *B*, and rays from *C* are focused at *D*. Figure 14.15 also shows that all rays parallel from the specimen are focused at the same points in the back focal plane where constructive and destructive interferences occur.[32] It is also understood in Figure 14.15 that rays scattered or diffracted in the same direction are patterned in the back focal plane of the objective. Rays from a point in the specimen are collectively focused at the same point in the image plane, and rays scattered in the same directions from the specimen are collected at the same points in the back focal plane.[32] On being combined in the back focal plane, the rays undergo constructive (additive) or destructive (subtractive) interferences, and small electron diffraction patterns can be formed. When an image is focused in the image plane, interference of diffracted waves can occur in the back focal plane. This concept is similar to the concept of light interference patterns discussed in Chapter 5.

Figure 14.16 illustrates electron scattering from stacked crystalline lattice planes. For scattering angles other than those indicated by the Bragg law, waves associated with the scattered electrons interact destructively,

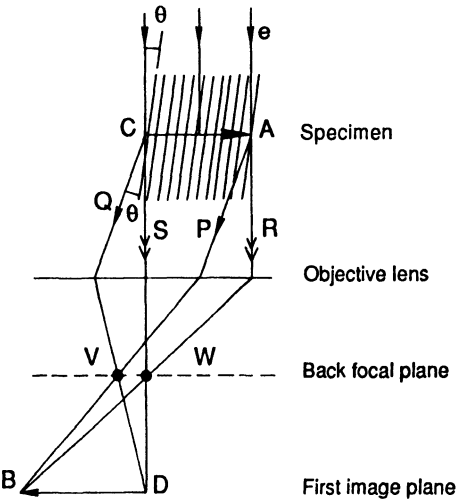


FIGURE 14.15. Schematic diagram of electron rays passing through a periodic structure, such as a crystal, to form a characteristic electron diffraction pattern in the back focal plane of the electron lens. After Beeston, Horne, and Markam.[32]

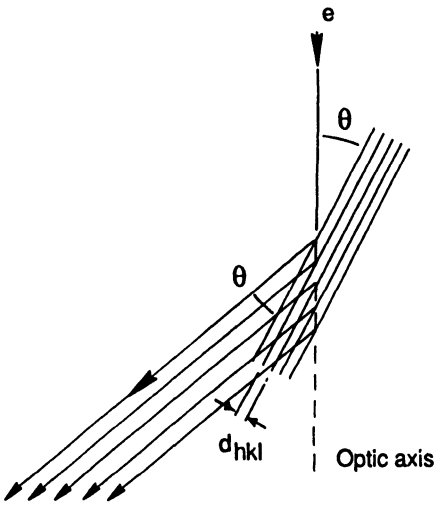


FIGURE 14.16. Schematic diagram illustrating the origin of an electron diffraction pattern from parallel crystal lattice planes of spacing d_{hkl}, lying at an angle θ to the incident electron beam. After Beeston, Horne, and Markam.[32]

and only a small amount of diffuse scattering is observed if the specimen is noncrystalline. If the specimen is crystalline, the diffraction pattern is composed of discrete spots if the crystals have a preferred orientation; "preferred" means that the crystals are not randomly oriented. In contrast if the crystals are randomly oriented, they diffract at all angles, and the observed diffraction pattern is composed of complete rings with inter-ring distances determined by inter-lattice planar spacing; this is due to an inverse relationship.

A very high magnification photomicrograph of a crystalline lattice with atomic resolution is shown in Figure 14.17. Superimposed on this lattice image is its ED pattern. Such an ED pattern could be made at much lower bright-field magnification, where the atomic structure could not be resolved. However through the ED pattern considerable information could be generated on such parameters as interatomic spacings, type of unit cell, approximate crystal size, and atomic orientation in the matrix.

Generally two projector lenses function together to magnify the initial image formed by the objective lens. For viewing the ED pattern however, the first projector lens is weakened because less current flows through its lens coil to decrease its focal length; therefore it becomes focused on the back focal plane of the objective rather than on the first image plane. The image is given additional magnification by a succeeding lens, or several lenses, and finally the image is focused on the viewing screen.[32] Of course when the general image is viewed, the strength of the lenses after the objective lens must be changed. Thus the resulting image is of the first

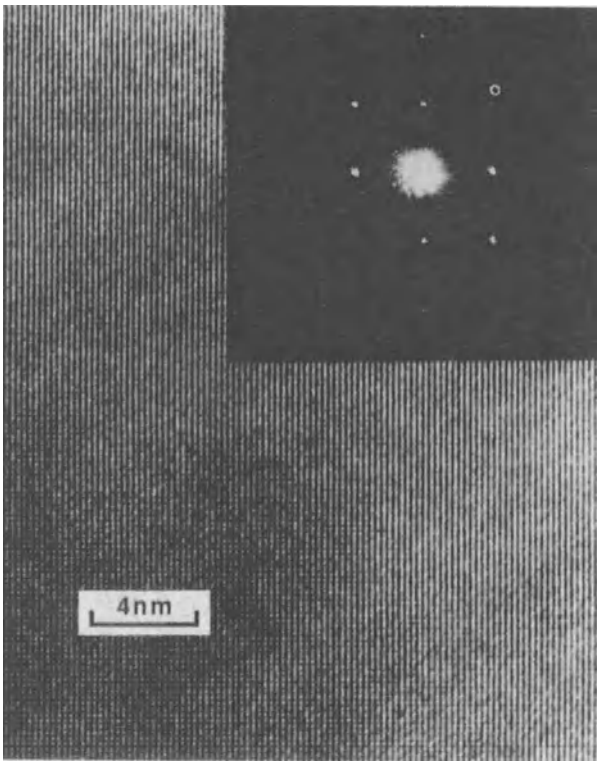


FIGURE 14.17. Transmission electron micrograph with crystalline lattice resolution of a gold single crystal. Magnification on the original print was 4,000,000. Superimposed on the lattice image is an electron diffraction pattern from the same microcrystal.[31] Courtesy of JEOL, USA, Peabody, MA.

image plane rather than the back focal plane as it was in the diffraction case.

Specimens normally interact destructively with electron radiation, and such interaction of electrons is generally destructive to the specimen, especially many crystalline, organic specimens. The radiation dose is so concentrated that it is often compared to what occurs in nuclear explosions.[35] Specimen heating is usually not a severe problem because of the specimen's large surface-to-volume ratio, although it can create artifacts with temperature-sensitive specimens. This point emphasizes why it is so important to minimize as much as possible the specimen's exposure to beam current by using low-intensity currents. The potential increase in the specimen's lifetime is also a major advantage of the scanning transmission

electron microscope (STEM) over the conventional transmission electron microscope (CTEM). Often images, especially those of organic materials, degrade detectably while being viewed or even before first viewing if the microscopist does not work quickly. This is often a main reason for using cold stages to decrease the rate of specimen degradation.

In summary considerable information about a specimen suitable for transmission electron microscopy, can often be fairly easily obtained with ED.

14.25. SUMMARY

Electron microscopy began with the realization that a beam of electrons behaves, like visible light, both as a train of waves and a stream of particles. Since the de Broglie wavelength of electrons decreases with their kinetic energies, fast-moving electrons have very short wavelengths associated with them and so are capable of very high resolution if that wavelength can be used in an appropriately designed instrument.

The TEM is one such instrument. It is arranged much like an ordinary microscope designed for examining translucent specimens by the transmission of visible light except that magnets are used instead of light-bending lenses to deflect and focus the beam of electrons. The electrons originate in an electron gun that usually has a hot filament (sometimes a cold-cathode emitter) as the source and an arrangement for defining and accelerating a narrow beam of electrons. The accelerating voltage can be anywhere from 30,000–1,000,000 V or more, but it is usually in the range of 50,000–100,000 V. The accelerated beam is then focused on a tiny area of the specimen by a pair of concentric toroidal electromagnets that act as condensing coils. The sample is held on a screen of extremely fine wires, so the beam passes through one of the holes in the screen and on through the objective and projection electromagnets. Since different parts of the specimen absorb electrons differentially, as the projected beam of electrons falls on a fluorescent screen, it shows bright areas where the sample has absorbed least and darker areas where the sample has absorbed more of the electrons. By removing the fluorescent screen and allowing the projected beam to fall on a photosensitive plate or film, electron micrographical negatives can be taken. Direct positive quick-developing systems, such as Polaroid require an external adapter.

Since the electron beam would be scattered by collision with air molecules, the interior of the electron microscope has to be pumped down to an extremely low pressure by efficient diffusion pumps and forepump. It follows that any specimen that dries out or loses volatile components in a

vacuum is altered, and this limits the applicability of the method and tempers the interpretation of the image. Furthermore volatile carbonaceous material is decomposed by the hot cathode and the stream of energetic electrons, which deposits decomposition products in the fine bores of the coils and fouls the microscope. Sample decomposition by the energetic beam produces the same undesirable result. Volatile components of lubricants, sealing compounds, and pump oil must be eliminated. Some contamination of the microscope is unavoidable though, and periodic, thorough cleaning of critical parts is necessary.

The resolving power of a TEM is theoretically proportional to the wavelength of the electrons and inversely proportional to the angular aperture. The theoretical limit is never attained in practice, but a resolution of about 4 Å at 50 kV and 3 Å at 100 kV is attainable. Tilting the sample improves the resolution. In general greater resolution is obtained on an electron photomicrograph than on the visual fluorescent screen in the microscope, because the photographic emulsion has much finer grains.

Contrast in the TEM image depends on many factors, but operationally contrast can be increased by reducing the beam voltage. This allows greater differential absorption by the sample, but of course it reduces resolution. Similarly reducing the aperture of the objective (by means of a diaphragm) increases contrast at the expense of resolution. Greater contrast in the photomicrograph can be obtained by using high-contrast plates and developer and/or stains.

Some aberrations of the magnetic lenses can be corrected by adjustable electrostatic deflectors within the TEM. Some progress is being made to reduce spherical aberration to approach theoretical resolution.

Focus depth presents no problem in the TEM, which is adequate for all purposes. Deliberate under- or overfocus increases contrast but reduces sharpness.

The useful magnification of the TEM depends on its resolving power. At a resolution of 10 Å, a magnification of 50,000 is routine. The field of view under such conditions is very small, only a few square millimicrons. Hence the operator must usually choose between several kinds of field, based on experience, and justify the choice. Recognizing spurious images as the result of damage to the specimen by the electron beam or fouling of the microscope is also a matter of experience.

Preparing the specimen is an especially important part of operating the TEM. The sample must be thin enough to transmit electrons yet thick enough to show differential absorption. Variations in depth can be enhanced greatly by shadowing the sample—depositing a heavy metal at an acute angle by evaporating the metal from a hot filament off to one side in a vacuum chamber. Further details of structure can be revealed by tilting

the sample in two or more directions. Experiments can be conducted on the sample material to select a range of particle size, to determine the effect of drying or heating, or to gain greater dispersion of solids, but of course any alteration of the sample material deemed necessary as a result of such experiments must be justified in the report. Many suggestions for sampling material, preparing a specimen, and interpreting the image are given, but for details see references.

Electron diffraction conducted can easily be on a specimen suitable for transmission electron microscopy. Properly obtained results from ED can tell whether the specimen is crystalline or not; and if it is, the ED can tell the crystalline habit and whether or not the crystals have a preferred orientation.

15

Scanning Electron Microscopy and Compositional Analysis

15.1. INTRODUCTION

In an SEM the image is formed in a cathode ray tube synchronized with an electron probe as it scans the surface of an object.[1] The resulting signals are secondary electrons, backscattered electrons, characteristic X rays, Auger electrons, and photons of various energies.

In a typical SEM an electron gun and multiple condenser lenses produce an electron beam whose rays are deflected at various angles off the optic axis by the first set of electromagnetic scan coils. The second set of scan coils deflects the beam back across the optic axis. Both sets of scan coils are in the bore of the final lens. Figures 15.1 and 15.2[2] help show the components; Figure 15.1 also indicates that all the rays pass through the final aperture of the final lens. From this final crossover, the rays, one at a time, strike the specimen at various points. The scan coils and the cathode ray tubes are powered by the same scan generator, so that each scanned point on the specimen is unique as reproduced on the displaying or the recording cathode ray tube and video amplifiers. To these amplifiers, one or more of the resultant signals are fed: high-energy backscattered electrons; low-energy secondary and/or backscattered electrons, X rays, and cathode-luminescent radiation in the UV, visible, and infrared regions. All results can be monitored separately or simultaneously by means of the appropriate detectors.[3–5] Many types of signals exist, as illustrated in Figure 15.2. Consequently SEMs are complicated and expensive instruments; see Figure 15.3.

Interpreting scanning electron micrographs is different from interpreting images formed directly by bending light or electron rays from object to image. The SEM indirectly constructs a pattern or map that can be

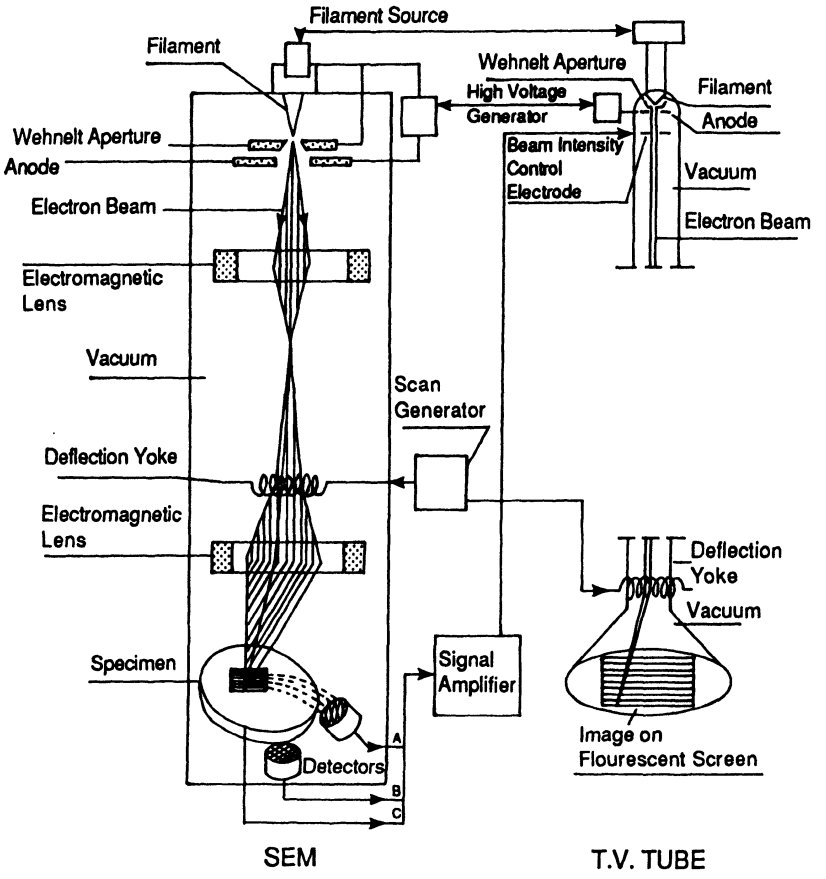


FIGURE 15.1. Schematic of an SEM and a television. Many of the general principles are the same for both instruments. Courtesy of Philips Electronic Instruments Company.

interpreted as an image of the object. Interpretation of the SEM image is facilitated by the many other attributes contributing to visibility, particularly resolution, contrast, focus depth, morphology (topography), apparent illumination, and its three-dimensional aspect.

One of the most striking aspects of the SEM image by secondary and backscattered signals is its resemblance to images of depressions and elevations illuminated from above by an oblique beam of light. For example the ant and chip shown in Figure 15.4 look as though they were illuminated by an oblique beam of light from above. We have been familiar with this type of illumination in macroscopy since childhood. It is also the type of illumination most frequently used with simple magnifiers and

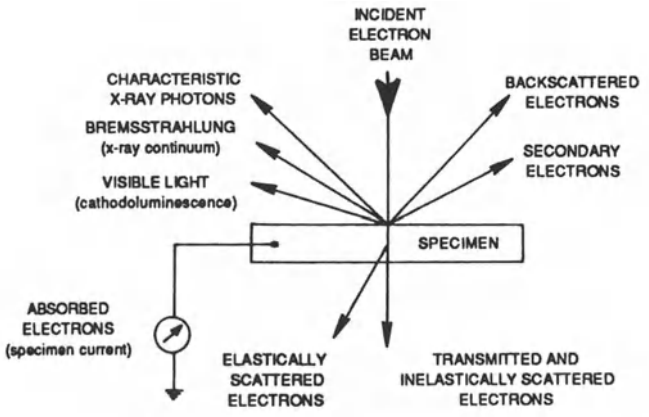


FIGURE 15.2. Signal types generated by electron–specimen interactions.[2]

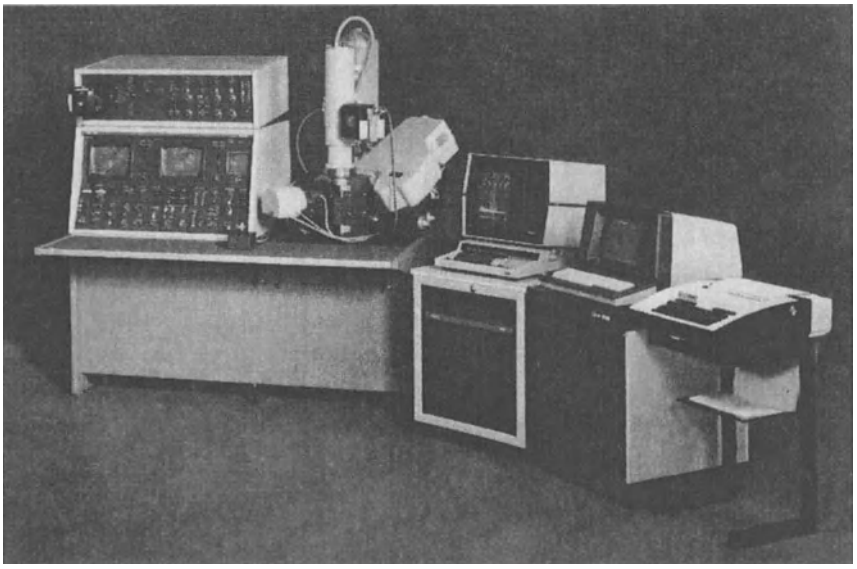


FIGURE 15.3. Typical SEM; the left-hand display screen shows the specimen, and the right-hand display screen shows elemental analysis. Courtesy of Philips Electronic Instruments Company.

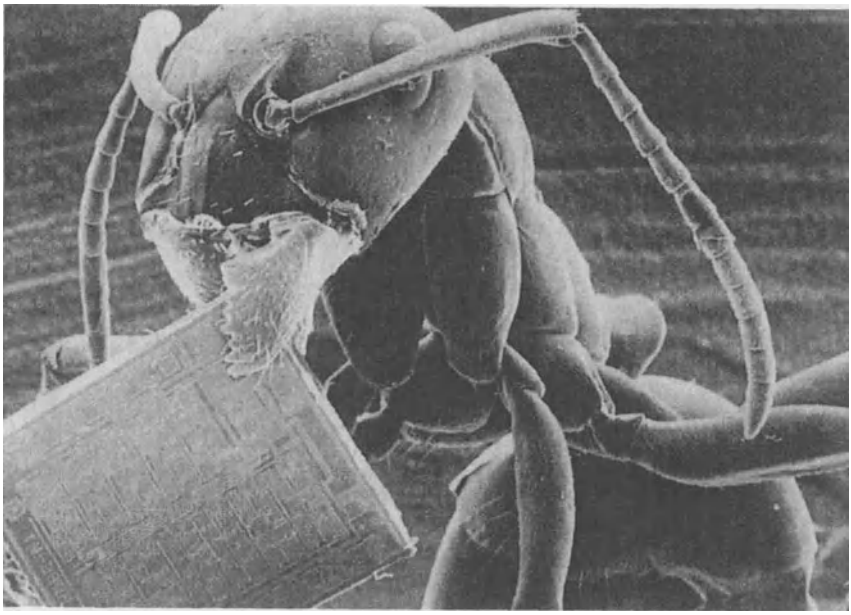


FIGURE 15.4. Scanning electron micrograph, not a photo montage, of a chip in the mandibles of an ant. Original magnification was 30×, and the chip is approximately 2 × 2 mm. This award-winning micrograph was made in the Application Laboratory for EM, Philips Einhoven, and is reproduced by courtesy of Philips Einhoven, The Netherlands.

stereoscopic compound microscopes; consequently structure and morphology are easily inferred.

Even when magnification is boosted to 2000× so that resolution and focus depth are beyond that of the light microscope, a scanning electron micrograph is easy to interpret because it looks as though it were illuminated by a unidirectional beam of light from above the specimen[6] (see Figure 15.5). Another aid to interpretation is the perspective these scanning electron micrographs manifest.

15.2. MAGNIFICATION AND RESOLVING POWER

Magnifications from around 10–200,000× or more are possible with SEMs. Normally lower magnifications solve the problem and the microscopist should always ascertain that resolution is adequate to support the magnification. Magnification calibrations are often established by observing diffraction gratings of known spacing. Since no actual image is formed

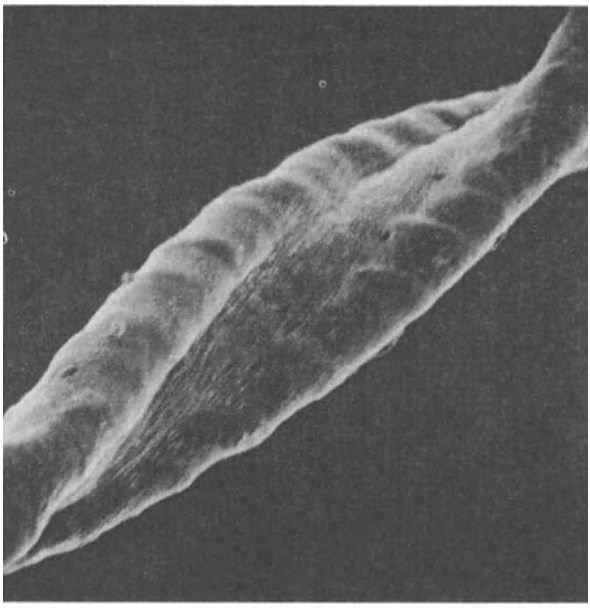


FIGURE 15.5. Cotton fiber coated with a thin layer of gold and palladium by evaporation in a vacuum and photomicrographed by SEM.[6] The photo vividly shows thickening of flat fiber, fibrils, and two spiral structures. USDA micrograph by Southern Regional Center, New Orleans.

in the microscope's column, magnification is the ratio of the area scanned to the display area. Since the size of the display is generally fixed at between 10–20 cm, magnification is increased by decreasing the area scanned, as depicted in Figure 15.6. Magnification is generally calculated as the ratio of the length of the display divided by the corresponding length of the scanned area.

The *resolving power* of the SEM depends primarily on the effective beam diameter of the probe. The secondary collection efficiency is assumed to be unity and the specimen capable of producing the 25% contrast necessary to satisfy Raleigh's criterion (see Chapter 2); a theoretical limit of resolution at 90 Å (9 nm) was calculated earlier for the ideal specimen.[3]

15.3. RESOLUTION

The practical *resolution* of the SEM is limited to 200 Å (20 nm) for most specimens in optimum position relative to the detector for secondary

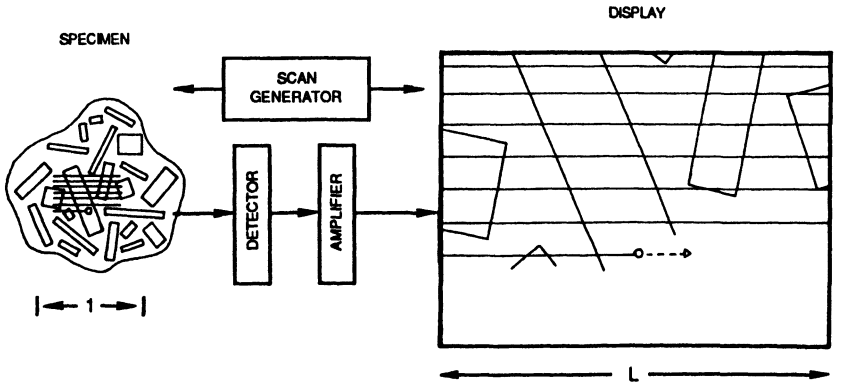


FIGURE 15.6. Image formation and magnification in the SEM. Courtesy of *The Mineralogical Record.*[2]

electrons. Some pairs of points on the surface of the specimen may not be in the optimum position for detecting secondary electrons, and therefore they are not resolved even though the limiting pairs in optimum position are resolved.[3]

Nonconducting specimens are coated with a metal to reduce damage by the thermal effects of the electron beam and to eliminate electric charges on the surface.[3] Thin as such coatings are (100–300 Å [10–30 nm]), they do cover up the finest topographical features, thereby reducing the practical resolution of the very finest detail.

15.4. CONTRAST

Contrast in scanning electron microscopy is defined as the ratio of the change in signal between any two points on the specimen and the average signal. Differences in *atomic number,* i.e., nuclear charge, of the atoms composing the specimen, are important in the SEM just as in the TEM. In the SEM however, we are concerned chiefly with backscattered electrons rather than transmitted electrons. The higher the *atomic number,* the greater the backscattering and the less the transmission of electrons. For a given phase, such as that of an alloy, ore, rock, ceramic, or cement, the effective backscatter coefficient is the weighted average of the backscatter coefficients of the pure elements. In smooth plane surfaces we are dealing entirely with atomic contrast[3] (see Figure 15.7).

In the case of rough surfaces, we are dealing with *topographic contrast,*

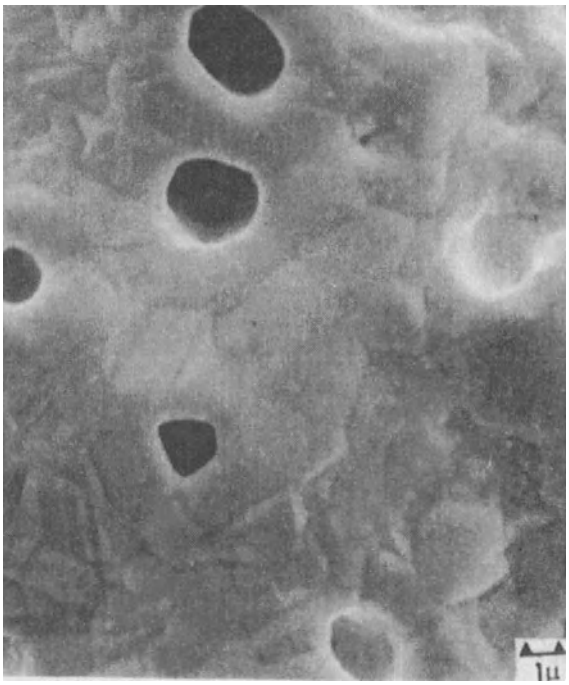


FIGURE 15.7. Pure gold wire after annealing to a smooth surface (plus voids, *black*) at 950° C (1742° F) for 2 hr.[13] Courtesy of A. C. Reimschuessel.

whether backscattered or secondary electrons are detected. When a plane specimen is tilted away from the normal incidence of the beam, electron *backscattering* increases, gradually approaching unity at grazing incidence. Therefore a faceted surface (see Figure 15.8) with its facets at various angles to the beam emits signals of varying strengths. The peak in backscattering by each facet lies in a plane containing the normal to the surface and the direction of the incident beam[3] (a sort of oriented luster). The Everhart–Thornley (E–T) detector is highly directional and picks up only trajectory electrons traveling toward the detector. Thus the resultant image contains a high proportion of black (shadowed) facets. If the specimen's current signal (SCS) is employed to form the image, trajectory effects are lost, and topographic contrast is due solely to the relative number of electrons from each facet.[3]

Secondary electrons can escape from the surface of most materials at depths of less than about 10 nm (100 Å). At such shallow depths there is minimal elastic scattering; electrons travel in a beam parallel to the direc-

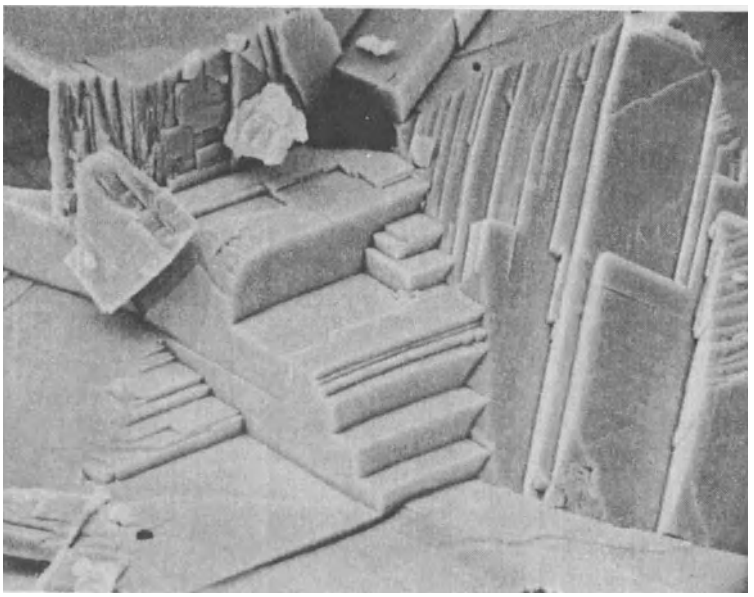


FIGURE 15.8. Lead sulfide, galena (isometric), taken by F. G. Rowe with field-emission electron gun (see Figure 15.11).

tion of incidence. A greater number of electrons escape from surfaces at a higher tilt.[3]

Contrast in the SEM can be varied in other ways[3]:

Electron channeling contrast, related to the crystallographic nature of such a specimen.

Magnetic contrast related to crystalline structure.

Magnetic contrast related to noncrystalline specimens.

Voltage contrast from beam-induced current contrast.

Electron-beam-induced current contrast, a phenomenon of electron hole pairs in semiconductor devices.

Cathode luminescences from the emission of long-wavelength electromagnetic radiation of light. A very important advantage of cathodoluminescence is that it introduces natural *color* contrast into the SEM, so that photomacrographs or photomicrographs can then be taken in color.[3]

Major influences on contrast are the incidence angle of impinging electrons onto the specimen and the collecting angle from the specimen to the positively charged collector. *Synthetic* color can be introduced into a series of correlated black and white (e.g., X-ray) images of the very same area (e.g., an alloy) by arbitrarily assigning a different color to a different image.[3]

15.5. ABERRATIONS

Spherical aberration is caused in the SEM when electrons move more strongly in trajectories away from the optical axis being focused than those near the axis (see Figure 15.9 top). Consequently the image of point P is a disk or circle of confusion. Aberration can be reduced by diaphragming with a lower intensity of the probe, or the object can be placed nearer the lens; however a decrease in working distance and a loss of freedom from the condenser's magnetic influence result.[7]

Chromatic aberration is caused by a variation in the energy from E_0 to $E_0 + \Delta E$ (see Figure 15.9 center). Of course a variation in the magnetic field also produces a disk of confusion in the scanning probe, but sources of trouble can be reduced by stabilizing the respective power supplies. Nevertheless there remains a fundamental variation ΔE in the energy representing the spread of energies leaving the cathode (2–3 eV for a tungsten or lanthanum boride source; 0.2–0.5 for field-emission cathodes).[3,7]

Since there is wave motion in the electron beam and a diaphragm of radius R, *diffraction* causes its own disk of confusion dr in the scanning probe of electrons (see Figure 15.9 bottom). The radius of the first diffraction minimum dr subtends an angle θ at the lens.[7]

Astigmatism results when a circular object does not yield a circular image. Astigmatism enters the electron probe lens through machining deviations, inhomogeneities within the iron pieces, asymmetry in the windings, and an uneven accumulation of contaminants. Astigmatism is usually corrected by a stigmator with two controls: one for the *magnitude* of asymmetry and the other for the *direction* of asymmetry. The two controls are alternately adjusted and refocused at medium magnification, 5,000–10,000×.[3]

15.6. CLEANLINESS

Cleanliness in the SEM is as important as in the TEM and for the same reasons: to keep the apertures open and avoid accumulating carbon and

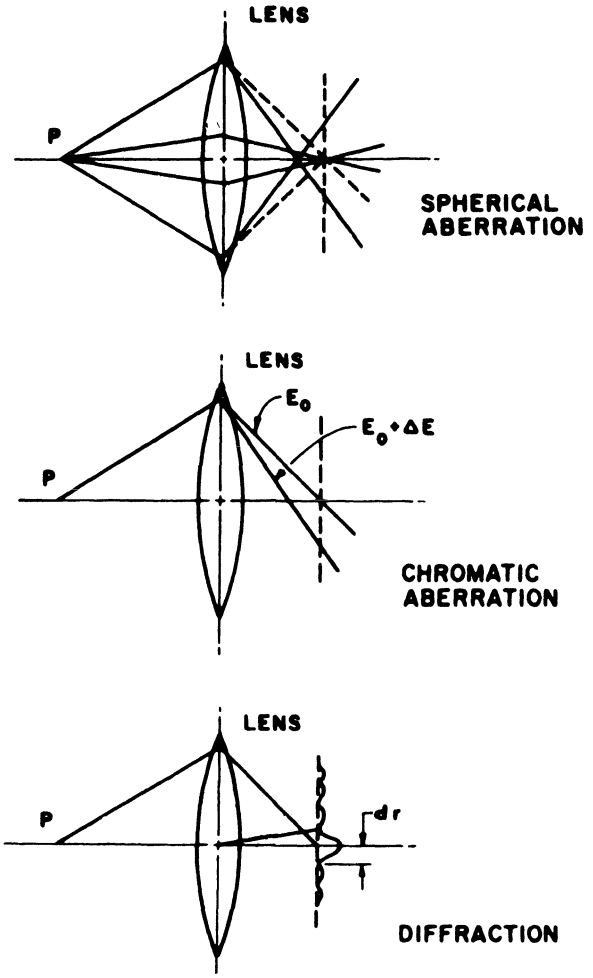


FIGURE 15.9. Schematic drawing showing spherical and chromatic aberrations as well as diffraction at a lens aperture.[2] Adapted from Hall[7] and Oatley.[8]

any other matter that can deviate the electron beams. Again the chief offender is carbon or carbonaceous matter from sealing grease, pump oil, or the specimen itself. Precautions, too, are the same: greaseless seals, cold oil traps, and prompt removal of carbonaceous specimens. In Auger electron analysis,[8,9] an oil-free, ion pump is required.

In X-ray analysis contamination by hydrocarbons may lead to polymerization rather than decomposition. X rays of long wavelength, such as from beryllium, boron, or carbon, may be highly absorbed, leading to a high degree of polymer contamination and an increasing carbon $K\alpha$ count

as a function of impingement time. One preventive method is to direct a stream of gas at low pressure onto the specimen. Air is then introduced to burn the carbon deposit. Another method is to provide a nearby cold "finger" on which the organic molecules preferentially collect. A combination of the two devices may be worthwhile[3] (see Section 15.8).

15.7. FOCUS DEPTH

Focus depth of the SEM is the greatest among microscopes, because the final condenser lens has such a very small aperture to demagnify the image of the electron source sufficiently to make it an adequate probe. Focus depth is a great advantage for keeping in focus all parts of a rough topography, but a compromise must be made between field depth and resolution (see Figure 15.5). Since the specimen is tilted toward the electron collector, there is a substantial difference in the path length from one side of the specimen to the other (see Figure 15.1). Most manufacturers have introduced a second lens to correct the focus as the beam moves across the specimen (dynamic focusing).[3,9,10]

15.8. FOCUSING

Focusing the SEM is much like focusing a television set. The operator should first set the contrast and brightness controls to suit personal preference. It is generally easier to focus on a screen that is not very bright. If the contrast is not suitable, consider using either the black level or the gamma control. Increasing the black level makes a flat surface appear less flat. Increasing the gamma control is useful in looking at the bottom of holes or in obtaining a picture when the specimen is changing. Both the black level and gamma controls produce artificial pictures, so their use should always be recorded.[10]

For magnifications over 2000×, the image should be tested for astigmatism with the fine-focusing control by focusing. If the picture seems to be smeared at an angle to the scanning axes on one side of focus and at the opposite angle on the other side of focus, there is astigmatism. Use the stigmator, going back and forth through focus until correction is optimum.[10]

Focusing and astigmatic adjustments should be checked at a higher magnification than the one to be recorded. If the beam voltage, aperture, or lens currents are changed, the image should be refocused and corrections for astigmatism should be made again.

15.9. ILLUMINATION

Illumination in the SEM picture should appear to go toward the top of the specimen, because the observer is accustomed to such illumination and therefore finds it the easiest to interpret in microscopy. Therefore shadows are below elevations and in the upper part of depressions. For single pictures illumination appears to come from the top, but with stereopairs the illumination usually appears to come from the side of the integrated image. Effective illumination from the top of the stereopair can be obtained by tilting the specimen around an axis parallel to the line between specimen and detector. Since this kind of tilt is not usually provided in SEM stages, a goniometer stage can be used to rotate the specimen on an axis normal to the specimen–detector line.[3]

The source of illumination in the SEM must be an electron gun of great intensity because the specimen's responsive signals are relatively weak. The thermionic gun made of tungsten filament (the same as for TEM; see Figure 14.2) is reasonably satisfactory at moderate magnifications, but for higher magnifications or shorter scanning times, more intensity is needed. The lanthanum hexaboride gun[3] (see Figure 15.10) of Broers is 10 times as bright as the tungsten filament and has a longer life

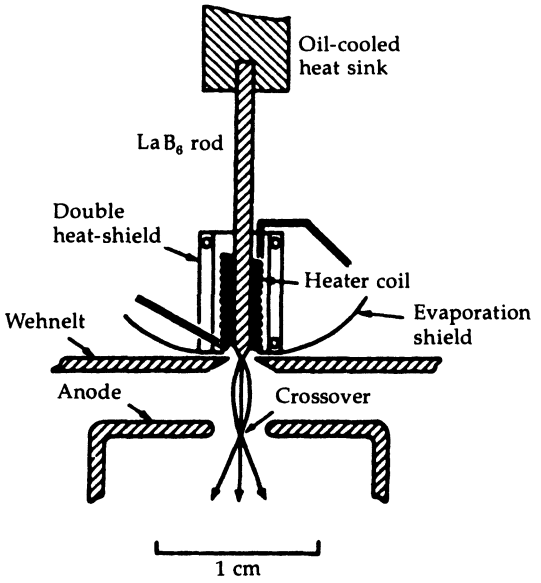


FIGURE 15.10. Electron gun with LaB_6 cathode[3] (from Broers).

at a cost of additional complication and expense.[8] The field-emission gun[3] (see Figure 15.11) is capable of very high intensity and small spot (less than 0.2 μm).[8] Its major problems are the high vacuum required and relative instability.[3]

15.10. RADIATION

The radiation used in the SEM is one or more of eight different types of signal, as indicated in Table 15.1.[9] So far we have discussed emission and reflection from a surface of *secondary* and *backscattered* electrons as information signals in the SEM. However some electrons are *transmitted* to give information about the thickness and composition of foils and films. The degree of electron *absorption* can thereby be determined to give complementary information about the specimen.

In certain specimens high-energy electrons create pairs of electron holes. If the pairs combine, energy is manifested as radiation of long wavelengths. Such *cathodoluminescence* can include visible light of various wavelengths (colors). Cathodoluminescence is usually weak and requires an efficient light-collecting system, including elliptical mirrors and fiber optics.[3] Results are both qualitative (pictorial) and quantitative (photometric), and these are feasible from both industrial and biological specimens. Figure 15.12 illustrates the more common signals and the location of the specimen volume from which they originate.

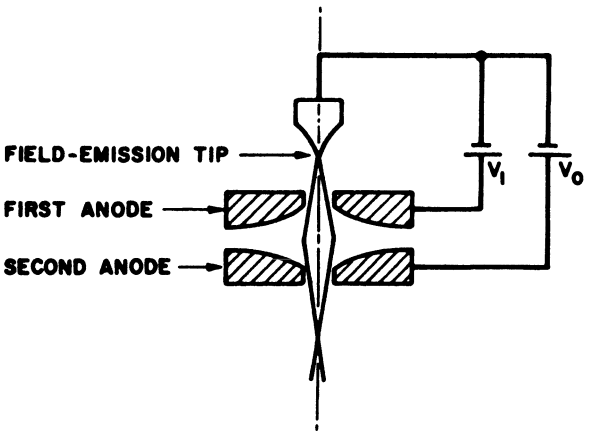


FIGURE 15.11. Electron gun with field emission.[3] Adapted from Crewe *et al.*

TABLE 15.1
Types of Radiation Signaling Information in SEM[9]

Radiation	Signal	Location	Information
Emission	Secondary electrons	Within 5 μm of surface	Topography of surface
Reflection	Backscattered electrons	Within 1 or 2 μm of surface	Nature of specimen
Transmission	Transmitted electrons	Thin foils and films	Thickness and composition
Absorption	Specimen current	Through specimen	Complementary to preceding information
Beam induction	Current in external circuit	Within specimen	Semiconductors
Cathodoluminescence	Photons of selected wavelength	Light	Various phases in a specimen
X rays	Selected wavelengths of X rays	Kinds of atoms	Spectrochemical analysis
Auger	Auger electrons, selected wavelengths	Auger electrons	Chemical elements present

X rays are photons of electromagnetic radiation. Therefore they have a wavelength λ related to their energy E through the equation $\lambda = hc/eE$, where h is Plank's constant, c is the velocity of light, and e is the electron's charge.[3] In microanalysis X-ray spectrometry can employ either a wavelength-dispersive spectrometer (WDS) or an energy-dispersive spectrometer (EDS). Since the SEM of the EDS is easier to use, it is more common than the WDS type, and the current discussion is limited to the EDS.

When a electron beam of sufficient energy impinges on a solid, X ray

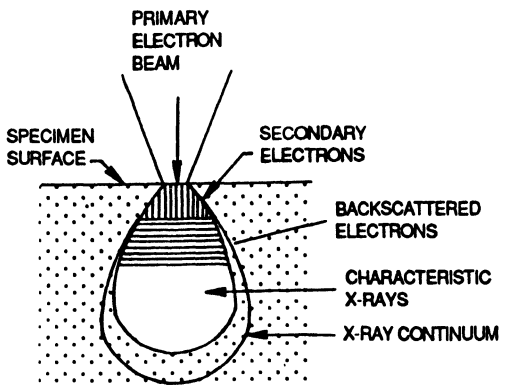


FIGURE 15.12. The volume within a specimen from which various types of signals originate. Courtesy of The *Mineralogical Record*.[2]

photons may be emitted by core scattering, manifested as a *continuous* background (*bremsstrahlung*) of X rays and inner-shell ionization, which yields the *characteristic spectrum* of X rays.[3]

The intensity of the continuum is a function of atomic number and accelerating voltage. As the voltage increases the continuum manifests shorter wavelengths and increased intensity. The extent of continuum radiation also increases with increasing atomic number, because heavier elements have more nuclear scattering and less energy loss from interactions among electrons. Continuum radiation forms the background x-radiation[1] in the electron microprobe SEM. In most instances it is desirable to keep the continuum (background x-radiation) to a minimum.[3]

Characteristic x-radiation results from the interaction of incident electrons with electrons belonging to the inner shells of atoms in the specimen. If an incident electron has sufficient energy, it may dislodge an electron from an inner shell (e.g., *K*, *L*, and *M*[1] [see Figure 15.13],[3] leaving the atom in an excited (ionized) state. The atom returns to its original state by transitioning an outer electron into the vacancy in the inner shell. The atom loses specific energy by emitting a photon of x-radiation (see Figure 15.14). The discrete energy level is described by a principal quantum number of the atom: one energy level for $n = 1$ (*K* shell), three energy levels for $n = 2$ (*L* shell), five energy levels for $n = 3$ (*M* shell), etc. The emitted X-ray photon has a discrete energy level equal to the difference in energy between the initial and final states of the atom. That is *wavelengths of the characteristic X-ray photons* from a specific chemical element are critically

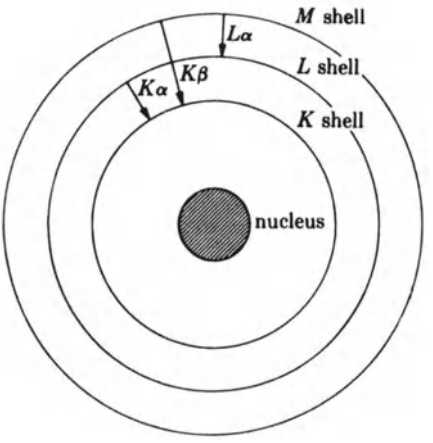


FIGURE 15.13. Electronic transitions in an atom.[3] Courtesy of J. I. Goldstein *et al.*

related to a discrete energy level E in keV.* Determinative tables are available.[3]

Figure 15.14 indicates that following ejection of an orbital electron (top), electron relaxation (center) may cause an *Auger* electron to be ejected (bottom left) *instead* of an X-ray photon (bottom right). In either case the energy is characteristic of the emitting element. The Auger yield is very high for light elements, while the X-ray yield is low, so Auger analysis may prove to be more useful than X-ray analysis for light elements.[3]

The qualitative and quantitative use of X rays excited by a microprobe of electrons has been offered separately by manufacturers of the EPMA. However the EPMA and SEM can be one and the same instrument,[3] and they are treated as such in this chapter.

If a solid-stage *X-ray* detector (*SD* in Figure 15.15) is placed *close* to the electron probe of an SEM or EPMA, X rays pass through a thin beryllium window into an evacuated chamber containing a cooled reverse-bias lithium-drifted silicon crystal. X ray absorption produces a charge pulse that is converted into a voltage pulse by a sensitive preamplifier. After more amplification a multichannel analyzer sorts the voltage pulses and displays them on a cathode ray tube, x-y recorder, or computer.[3]

A schematic depicting principal components of an EDS is shown in Figure 15.15. A primary electron beam is scanned across the specimen in synchronism with the cathode ray tube's scanning electron beam. As with normal image scanning, the area of the specimen being scanned can be controlled, and hence the resulting magnification is variable in a controlled manner. Beam intensity in the display cathode ray tube is modulated by detecting a characteristic X ray, and this causes a single dot of light to appear on the cathode ray tube.[3,4] In this manner as the electron beam is scanned across the specimen, a set of characteristic dots can be generated. This dot pattern forms a map of the elemental distribution superimposed and displayed on the more common image of the specimen. Thus we can visually associate the elemental distribution with the specimen morphology.

*Electronic transition involves a change from one discrete energy level to another, and the change in energy ΔE (by Planck's law) is equal to $h\nu$, where ν is the frequency associated with the transition. In terms of wavelength λ, since

$$\lambda\nu = c \qquad \text{(the velocity of light)}$$

then

$$\nu = c/\lambda$$

and

$$\Delta E = hc/2$$

where h and c are constants and ΔE can be expressed in kiloelectron volts (keV) instead of ergs (1 keV = 1.602×10^{-9} erg).

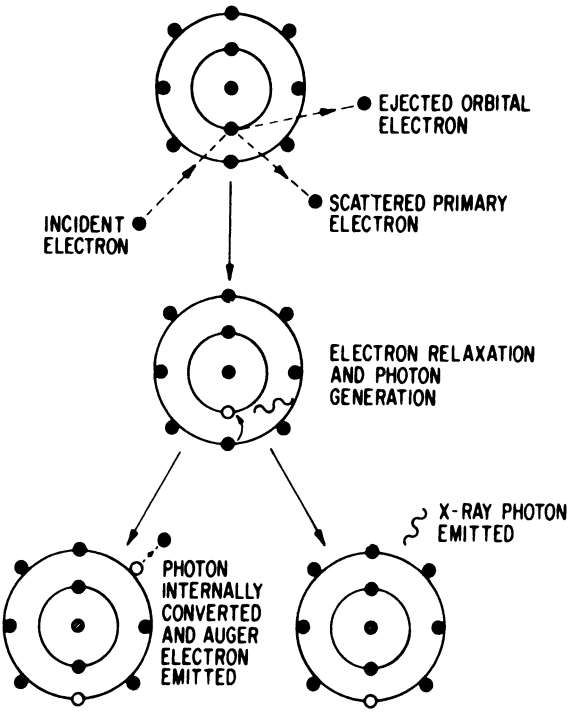


FIGURE 15.14. Electron excitation manifesting characteristic X rays or Auger electrons[3] Courtesy J. I. Goldstein *et al.*

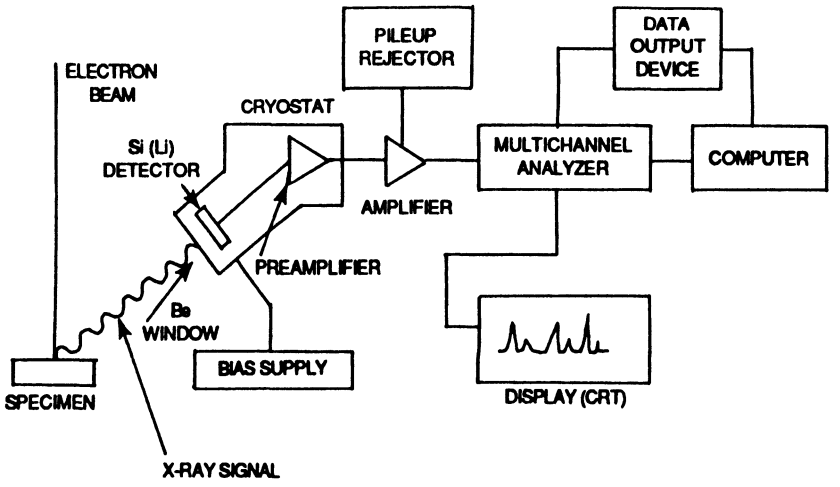


FIGURE 15.15. Principal components of an EDS.[3]

Detecting light elements (Be, B, C, N, O, and F) is difficult because of the low-energy X rays they generate. Because of their low energy, these signals are absorbed by the beryllium window generally used as the detector cover shown in Figure 15.15. It is possible to detect the low-energy signals, but this is beyond our discussion, so the reader is referred to specialized texts.[3,4]

Superposing the detected distribution of identified elements on the image of the specimen provides considerable structural information. Not only can an elemental content be obtained, but the microdistribution of elements, as shown in Figure 15.16, is also provided. Considerable caution must be taken in interpreting relative dot concentrations as ratios of elemental composition because the relative intensity of dots depends on such factors as specimen surface smoothness and different excitation energies (which are related to atomic numbers).[4]

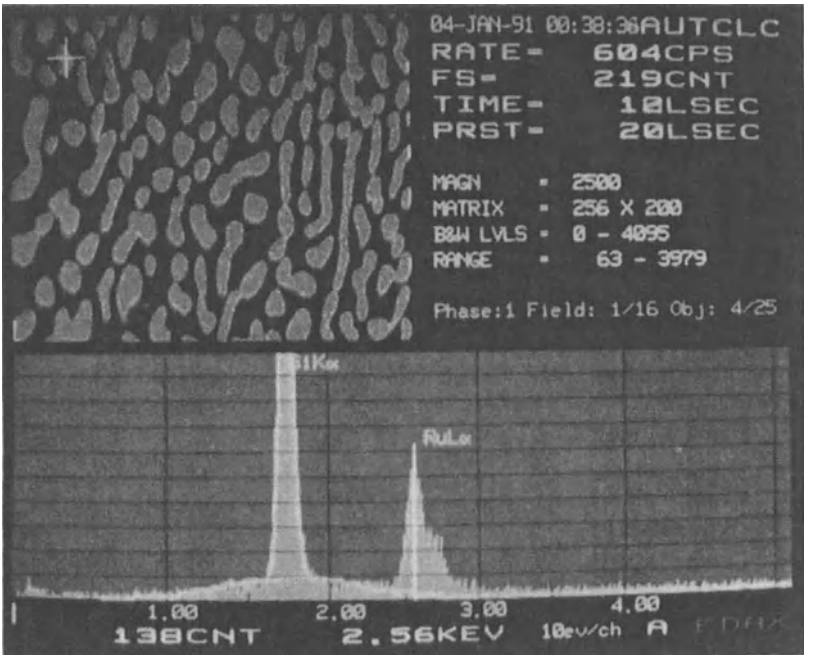


FIGURE 15.16. Elemental analysis obtained at the cross in the upper part of a microscopical reference photomicrograph obtained at 2500x. The elemental distribution displays peaks for SiKα and RuKα, which with other information proves that ruthenium osmium silicide composed this region. This analysis was performed by an EDAX elemental detector and analyzer. Courtesy of EDAX International, Inc.

Conventional maps with discrete dots representing detected characteristic X rays (called continuous rastering) have been produced for many years. In this case the emitted X-ray beam signal is displayed in synchronism with continuous scanning of the electron beam in the display cathode ray tube. Even though the scanning is termed continuous, dot images are not continuously recorded, since the detector system produces a pulse up to tens of microseconds long for each processed X-ray signal.[4] Because of this required time per signal, the detector system can be overloaded, so that generated signals may not be collected nor displayed.

Discrete rastering is now more common than continuous rastering. Actual initial generation of the detected X-ray signals is similar in both cases however. With the discrete technique, each point at which the scanning beam stops and information is generated is called a pixel.[4] Generally 128–512 points are detected along the scanned line, and the same number is often used in the display. The point-by-point scanned area is stored in the computer memory as a two-dimensional array of pixels.

A typical analysis of the components in an alloy is shown in Figure 15.16. By computer processing the collected signal, much more can be done with the collected signals. For example different signals can be displayed with different colors to enhance visibility, or component signals can be artificially added or removed to assist in understanding the problem.

An excellent survey of compositional imaging and X-ray spectrometry has been published.[5] Activities during the past 35 years are surveyed. The first 25 years concentrated on operating with dot maps for compositional imaging. Each dot represented a detected X ray from the specimen. However in recent years there has been a dramatic contribution from the computer-controlled electron beam and computer-controlled manipulated compositional images. This has permitted routine mathematical smoothing across the discrete dots and the display of a continuous colored region or area to represent each detected element. Recent inexpensive digital storage components with multimegabit capacity have greatly expanded existing procedures.[5]

15.11 USEFUL MAGNIFICATION

One aspect of the SEM's *useful magnification* is different from the TEM: Micrographs taken on the SEM cannot be enlarged very much because of raster lines.[12] The limit of directly useful magnification is about 20,000×, since higher magnifications tend to be fuzzy.[11]

Useful magnification can be determined on manufactured linear scales of a variety of spacings. A composite photomicrograph should be

made of a particular scale in two positions of rotation 90° apart; a better set of standards uses cross-ruled diffraction gratings. All standards should be photomicrographed at the working distance used to record the specimen image.

15.12. FIELD OF VIEW

The *field of view* of the SEM is comparable to that of the light microscope, and it is large compared to that of the TEM[10] (see Figures 15.4,[60] 15.17, and 15.18[13]).

15.13. NOISE

Noise should not be greater than one-fifth the signal change caused by the specimen if the eye is to distinguish the true signal change in the

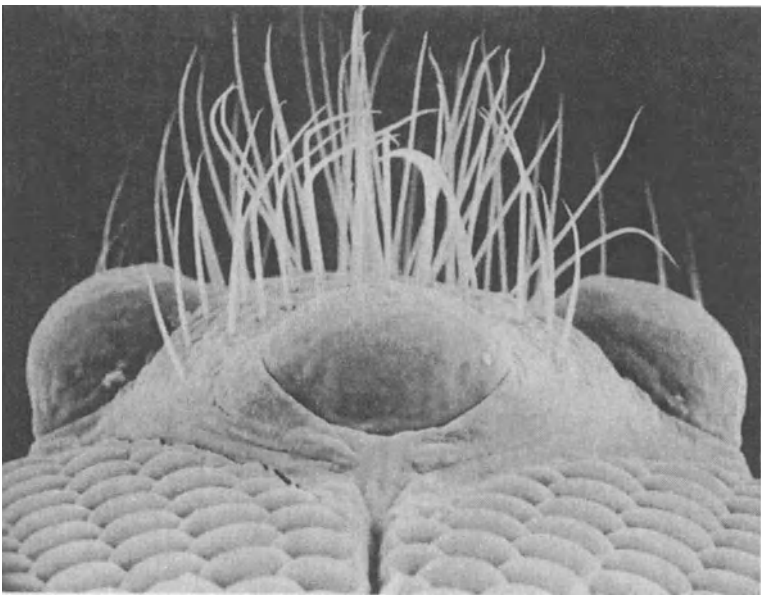


FIGURE 15.17. Eyes and back of head of sweat bee. Taken by F. G. Rowe with a Coates and Welter SEM, Model 106A with field-emission electron gun.

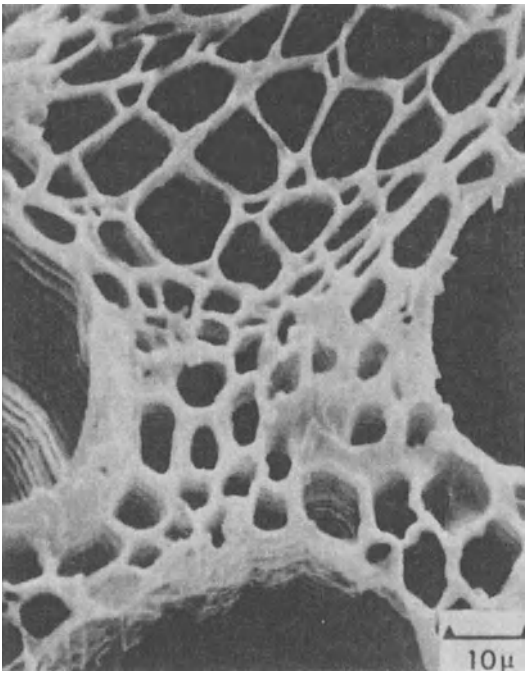


FIGURE 15.18. Vascular bundle in *Scirpus lacustris,* a reed.[13] Courtesy of A. C. Reimschuessel.

scanning-electron image. Noise can be introduced at all stages in the signal path from the specimen to the cathode ray tube (see Figure 15.1). In the transmission electron scintillator–photomultiplier system, the number of informative electrons or photons is smallest at the intersection between beam and specimen, where secondary electrons are collected. In an efficient scintillator the signal is increased preferentially but without further information in the image. Noise manifests itself as graininess ("snow") in the image. Noise results from too little current for the scanning beam, which should be at least twice the threshold (minimum acceptable) current.[3]

15.14. DEPTH CUES

The *depth cues* in the SEM image are very impressive and effective. With single SEMs the main cues are the distinct shadow and the apparent

perspective. In the SEM image the microscopic specimen appears as though you were looking at macroscopic elevations and depressions of wet sand (see Figure 1.4). In both cases depressions have their shadows at the lower half of the visual image. The illustrator must assume responsibility for correctly orienting photocopies of photographs in reports and publications. Shadows should fall in the natural azimuth, and perspective should also be natural. Parallel lines should appear to converge; larger parts should be in front of smaller ones. Note these aspects in Figures 15.5, 15.8, and 15.17.

15.15. WORKING DISTANCE

The long *working distance* of the SEM is the distance between the surface of the specimen and the front surface of the objective lens,[1] which is of special advantage in arranging movement of the specimen. Working distance from the bottom pole piece of the objective lens to the specimen's surface is around 5–25 mm,[3] so that the specimen and its low-energy secondary electrons are outside the magnetic field of the lens. The working distance also makes it possible to have a large space to hold the specimen on a special stage for heating, cooling, bending, ion etching, or electromigration.[12]

15.16. FIELD DEPTH

The great *field depth*[1,4] is one of the most important advantages of the SEM (see Figure 15.4). It is hundreds of times greater than in the light microscope of the same magnification,[8] and it is capable of showing an inclination with its highest and lowest points in simultaneous focus.

15.17. STRUCTURE

Structures are composed of units of increasing complexity: atoms, molecules, domains, crystalline grains, and biological cells.[1] Periodic structures are particularly interesting at this point. Figures 15.19 and 15.20 for example show the *three*-dimensional periodicity of certain diatoms.[13] Figures 15.21–15.24 show the linear periodicity, respectively, of yellow, pink, orange, and black scales taken from colored butterfly wings.[13] The same figures also show interlinear structure that may or may not contribute to the structural color.[14]

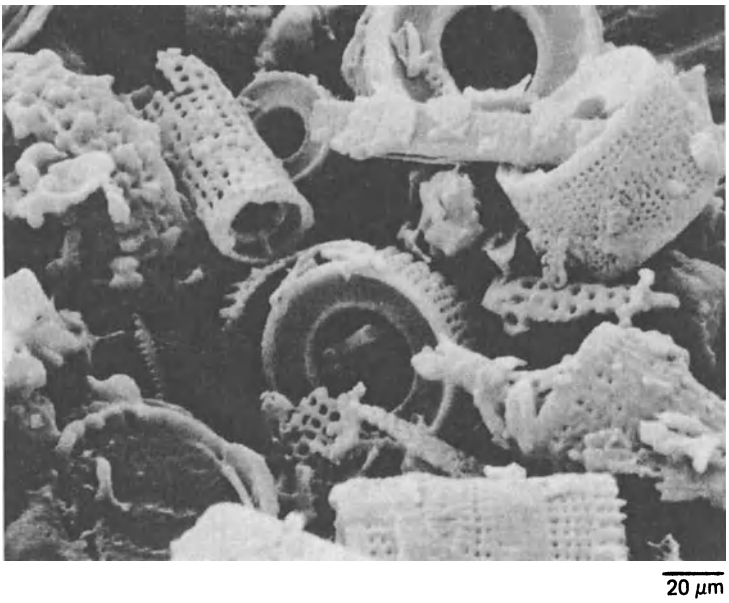


FIGURE 15.19. Filter aid: diatomaceous earth. Taken by F. G. Rowe with a Coates and Welter SEM, Model 106A with field-emission electron gun (see Figure 15.11).

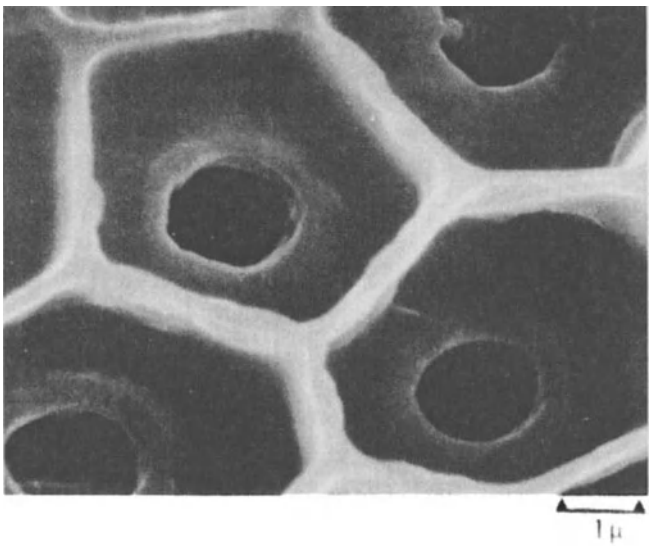


FIGURE 15.20. A single fragment of shell in diatomaceous earth.[13] Courtesy of A. C. Reimschuessel.

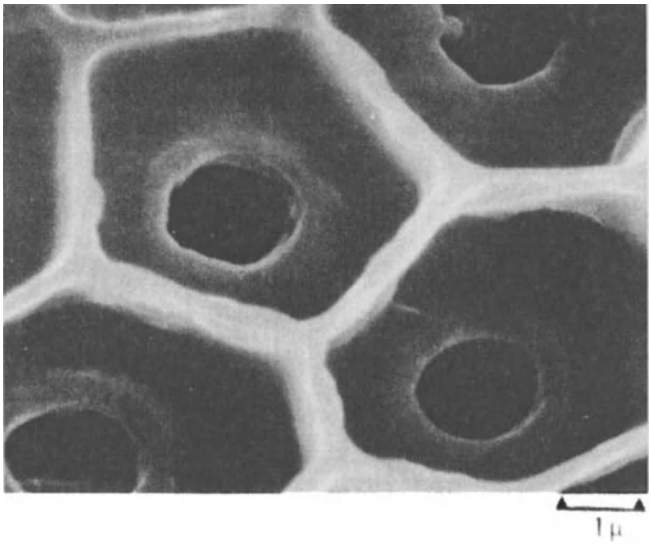


FIGURE 15.21. Yellow scale of the wing of the butterfly *Colias eurythyme*. Periodicity is 3–4 μm.[13] Courtesy of A. C. Reimschuessel.

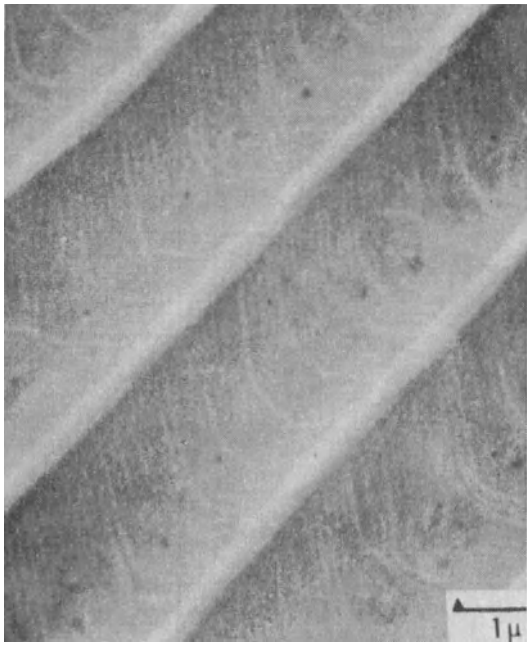


FIGURE 15.22. Pink scale of wing of the butterfly *Colias eurythyme*. Periodicity is 2–4 μm.[13] Courtesy of A. C. Reimschuessel.

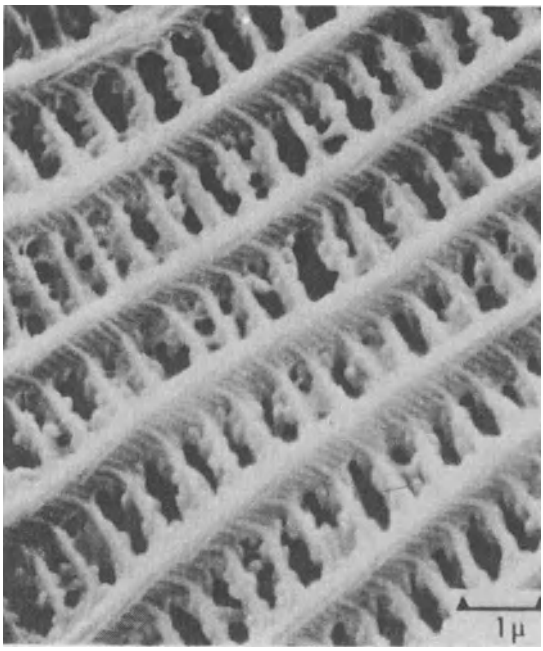


FIGURE 15.23. Orange scale of wing of the butterfly *Colias eurythyme.* Periodicity between ribs is 1.5 μm; between cross ribs, 0.5–0.7 μm.[13] Courtesy of A. C. Reimschuessel.

15.18. MORPHOLOGY

Morphology, as has been stated,[1] involves the size and shape of a structure. In Figures 15.21–15.24, the distance between lines should be compared to the wavelength (color) of light from butterfly wings.

15.19. INFORMATION

To continue our search for *information* about the color of scales from butterfly wings, we must know macroscopically whether the color is yellow, pink, orange, or black. Is a pigment present to contribute to the total color by reflected light? What is the color by transmitted light with the specimen mounted in a permeating liquid of similar refractive index, as compared to the color in a liquid of very different refractive index?[14] With such information, scanning electron micrographs, such as those in Figures 15.21–15.24 have much more meaning than would otherwise be possible.

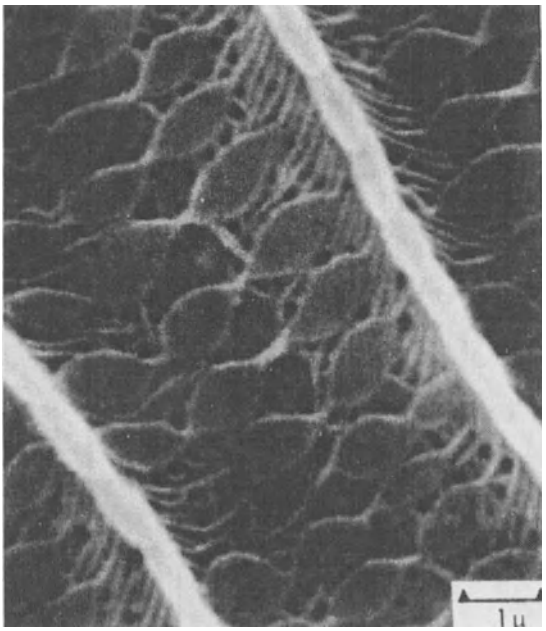


FIGURE 15.24. Black scale of wing of the butterfly *Colias eurythyme.* Periodicity is 3–6 μm.[13] Courtesy of A. C. Reimschuessel.

15.20. DYNAMIC EXPERIMENTATION

Dynamic experimentation is a specialty of the SEM, which has great field depth in focus and a large working space. Substantial specimens can be moved with considerable freedom with or without accessories.

SEM *stages* strong enough to stretch metals, alloys, and semiconductors have been devised,[3] and micromanipulators have been built to operate on specimens in the SEM. Closely related mechanisms are made for observing and measuring scratch and indentation hardnesses.[12]

Special stages have also been developed to change the temperature, magnetic field, electric field, liquid medium, etc.[3] Heating, cooling, mechanical manipulation, etching, and electromigration can all be performed on the specimen within the SEM. For example explosive chemicals have been studied as they slowly decompose at carefully controlled temperatures below 200° C. Nickel particles have been thermally etched at 1000° C or 1300° C. Change in topography of metal surfaces has been studied at various temperatures within the SEM.[12]

Ion etching has been performed by an argon ion beam at 5 keV

striking the specimen at right angles. Some of the materials studied *in situ* by ion etching are dental tissues, iron oxide films, aluminum on oxidized silicon,[12] and a wide range of inorganic and organic substances.[10] Electromigration is a process of moving metal atoms along a conductor in which a current is flowing. Aluminum has been studied in this way.[12]

With beam diameter greater than about 1000 Å, the current can be made strong enough to employ television rates for scanning. Then dynamic events can be recorded on either cinefilm or video tape. With narrower beams the SEM is limited to imaging repetitive events that can be examined stroboscopically.[12]

15.21. SPECIMEN BEHAVIOR

Specimen *behavior* can be observed postmortem by exposing the specimen to conditions outside the TEM's chamber, stopping the reaction, and putting the spent specimen into the TEM. Such procedures include weathering, wear, fracture (see Figure 15.25), corrosion, erosion, annealing (see Figures 15.20 and 15.26), and certain manufacturing processes.

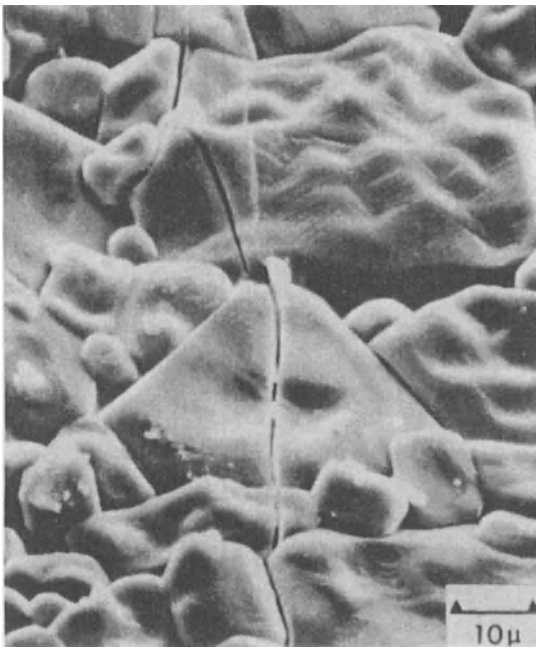


FIGURE 15.25. Intragranular fracture in alumina.[13] Courtesy of A. C. Reimschuessel.

FIGURE 15.26. Pure gold wire before annealing. Compare with Figure 15.7 sample after annealing.[13] Courtesy of A. C. Reimschuessel.

15.22. SPECIMEN PREPARATION

Preparing a specimen for examination in the SEM by secondary electrons can range from almost nothing to do to a complex procedure. No preparation is needed if the surface is of interest and is conductive as received. If the interesting surface is an insulator, it can become charged and ruin the image. If the insulating layer is thin enough for some electrons to penetrate an underlying conductor, a satisfactory image can be obtained from the secondary electrons. If not, the usual procedure is to coat the surface by vaporizing with a thin layer (~100 Å) of metal such as gold or gold plus palladium. A different approach involves viewing the insulating surface through a hole in a foil of aluminum.

Surfaces of wet specimens are preserved by freeze-drying. For example, fibers of wet paper fresh from the machine are not collapsed during freeze-drying, whereas they are by ordinary drying. Of course, in finished paper, fibers are not only flattened by commercial drying but even more so by the calendering (glazing) process. The freeze-dried surface does not

represent the surface of paper, but it helps us understand changes in fibers during paper making.[3]

In many instances a suitable surface of the material has to be made for the SEM. Biological material can be cut once with a razor blade or microtome knife (see Figures 15.18 and 15.27), and watery tissue can be frozen and fractured. Ice crystal size can be controlled by adding glycerine or gelatin or both, or the water can be displaced by alcohol or acetone, the sample quenched in liquid nitrogen, and then fractured.[12] Treatment with hydrophobic liquids, such as chloroform, ether, amyl acetate, or epoxy resin (unpolymerized), may change the appearance of the material so much that the image may not be interpretable. More complicated procedures, such as chemical digestion, may make interpretation easier.

15.23. PHOTOMICROGRAPHY

Photomicrography is simpler with the SEM than with the TEM because the image is exposed to the photosensitive material outside rather than

FIGURE 15.27. Section of *Scripus lacustris*, a reed, to show distribution of stomata.[13] Courtesy of A. C. Reimschuessel.

inside a vacuum. With the SEM, Polaroid® or other fast-developing photosensitive material may be used directly in the commercial camera back, so that photomicrographs can be made very quickly.

On the other hand photomicrographic exposure is longer with the SEM than with the TEM, because SEM signals are integrated slowly into the image [less noise (fewer dots) appears during slow integration than during fast exposure]. An exposure of 40 seconds is usual; 100 or 200 seconds may be required to clear up the noise. Yet a mere 20-seconds exposure records a transient image and prevents damage to the specimen by the electron beam. The number of lines per frame should be adjusted to 1000 or up to 2000 with higher resolution. More lines per frame may overlap and blur the picture, while fewer lines may be so far apart that they show in the final photomicrograph.[10] In any event a canning electron micrograph, with its raster structure cannot be enlarged to the same extent as a transmission electron micrograph, with its very fine grain structure.

Many SEMs have cameras that automatically select exposure times according to overall brightness and contrast in the image. If the operator is interested in optimum brightness and contrast for a small part of the image, it is possible to override the automatic mechanism with a manual control. With instruments not having automatic cameras, the operator has to make a decision by studying a line across an interesting part of the image in darkness. The scan line should not be left very bright on the record screen in darkness because the screen is easily burned, and it is expensive to replace. Using Polaroid® positive film matched to the ASA rating of the negative film speeds up the trials with fewer errors.[10] Scan rotation units make it easier to arrange the image in a position that lends itself more readily to interpretation.

15.24. SUMMARY

The SEM differs from the TEM in several important respects:

1. The SEM deals with various signals *scattered* from the *surface* of the specimen.
2. These signals are received by *cathode ray tubes* which are *synchronized with the scanner* probing the surface of the specimen.
3. The picture (pattern) on a cathode ray tube, like that on a television tube, is composed of scanner (raster) *lines* that limit the degree of useful total magnification but permit photomicrography with a camera placed outside any vacuum. The Polaroid® type of quick photography is the most popular.

4. The photomultiplier receives the secondary and/or backscattered electrons at an *average* angle to the surface of the specimen and at *various* angles to various facets of grains. The resultant image manifests modulated *shadows* and a perspective, which add three-dimensional aspects and render the image *easily interpreted.*

5. A faceted or rough surface manifests *topographic contrast* because the detector is highly directional. Only certain facets are so oriented that most of their backscattered electrons are aimed exactly at the detector, while the remaining facets contribute fewer electrons thereby providing more contrast between facets.

6. Crystalline specimens also contribute contrast by electron channeling and magnetic or semiconducting effects.

7. In addition there are noncrystalline specimens contributing contrast by other effects of the initial electron beam: magnetic, voltage (from beam-induced current), and luminescence.

Like the TEM, the SEM has *great depth* of field in focus because the wavelength is so short and the angular aperture is so small. Resolution by means of the SEM is somewhat less than that of the TEM, but it is of course greater than the resolution of the light microscope.

The most important comparison however emphasizes the *differences in the kinds and extent of information* provided by the three different types of microscopes.

8. The qualitative and quantitative use of X-rays excited by a microprobe of electrons is offered separately by some manufacturers as EDS, WDS, or EPMA. The EDS is the most common of these instruments.

16

Emission Microscopies

16.1. INTRODUCTION

Emission microscopes without lenses can achieve high resolution by using a very small source or detector in combination with scanning of either the source, detector, or specimen.[1] Chapter 16 discusses field-emission or point-projection microscopes, scanning tunneling and atomic force microscopes, and scanning near-field light microscopes.

16.2. FIELD-EMISSION MICROSCOPES

Field-emission (point-projection) microscopes are usually operated with the projection point cold. In the electron-emission microscope the specimen can be heated (see Figure 16.1).[2,2a] In other hot-emission microscopes, the specimen can be broad and glow with visible or infrared radiation (becoming the source of illumination). If however the surface of a broad hot specimen is irradiated with UV light, photoelectrons can be extracted at high potential and imaged on a fluorescent screen.[3,4] Two kinds of field-emission microscopes[2] were invented by E. W. Müller (see Figure 16.2). The older device, a specialized cathode ray tube (see Figure 16.1),[2] uses field-emitted *electrons* from the *negatively* charged tip of a very sharp needle into a vacuum by point projecting the image onto a positively charged, fluorescent screen.[2] The newer device (1950) emits ions from an anode.[2a]

The electron field-emission microscope* is unique in its ability to

*The field electron microscope is sometimes loosely called a shadow microscope. It is not to be confused with another, very different shadow microscope, much like the TEM except that it produces an image by direct (point) projection instead of by a projection lens.[3]

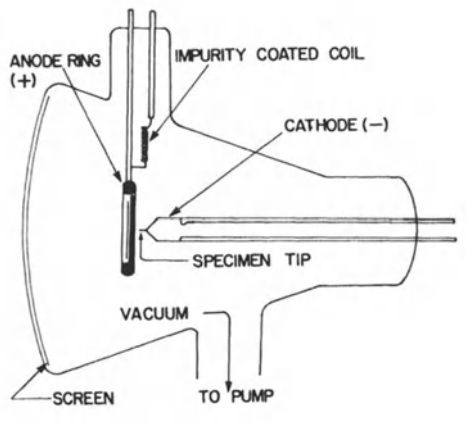


FIGURE 16.1. A schematic drawing of the electron field-emission microscope, showing the remarkable simplicity of the apparatus. The coil coated with an impurity can be heated to evaporate its atoms onto the point for the purpose of making emission studies.[2] Courtesy of E. W. Müller.

detect if not resolve single atoms or small groups of atoms on the surface of the needle tip. Thus the purity of the metal is established. If the coil shown in Figure 16.1 is coated with a potential additive, such as barium, and heated, such atoms migrate to the metallic tip of the cathode where they are visible as bright spots in motion. The device is useful in studying electron emission as a function of surface structure. Organic compounds can be substituted for the inorganic additive and evaporated onto a clean metal point. Phthalocyanine shows a pattern curiously like the structural formula of its molecule.[2] Caution must be taken however when interpret-

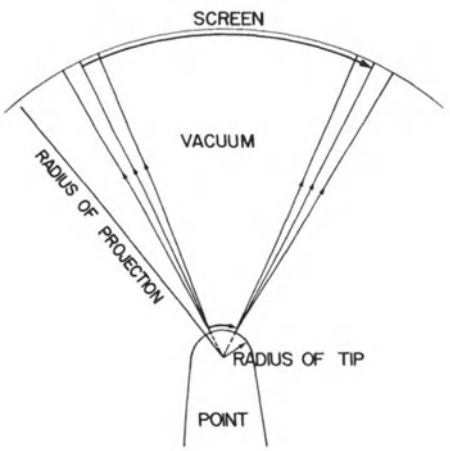


FIGURE 16.2. Principle of both kinds of point-projection or field-emission microscopes.[2]

ing such patterns, since a large number of molecules, regardless of their shape or symmetry, produce similar patterns.[5,6]

Müller's newer device (1950) is the field-ion microscope (FIM).[2,7] It operates with the metallic needle tip *positively* charged in the high vacuum. A gas, such as helium, is introduced and ionized at the tip of the cooled specimen. The tip is needled down from fine metal wire (e.g., tungsten) by etching to give it a radius of only 5–100 nm. The etching roughens the tip of the metal so that its atoms stand out in a terrace of ledges (see Figure 16.3).[4] A potential of several kilovolts is applied to the tip; these are positive with respect to the negatively charged fluorescing screen. Figure 16.4 explains the formation of an image (or pattern) on the fluorescent screen.[2] Incident atoms of the gas (e.g., helium) are attracted to the rough, sharp tip of the specimen. Some of them hop along the tip, losing electrons to the specimen cooled by a cryogenic bath. The resulting gas ions are projected onto the fluorescent screen, where they release light in a typical pattern (micrograph).

Figure 16.5[4] shows a diagram of the typical apparatus. Field-ion microscopy is concerned with the study of the atom itself, not only in metallurgy, but also in fundamental physics and chemistry.[8–11]

Professor Müller added the *atom probe* to the FIM to identify atoms chemically. Selected atoms are fed into an ion detector. Data are collected slowly by a skilled operator and require careful interpretation.[4,10]

Figure 1.6 shows a field-ion micrograph of a platinum crystal that was taken in the 1960s[2] at the Pennsylvania State University by the late Professor E. W. Müller. The micrograph shows the distribution of Pt atoms in a Pt crystal. The micrograph was provided by his successor, Professor T. T. Tsong.[10] In 1933 Professor Tsong published an up-to-date account of field-ion microcopy.[2a] This technique "now permits the study of mechanisms and energetics of atomic processes on solid surfaces and the single-atom and atomic-layer chemical analyses of surfaces."[2a]

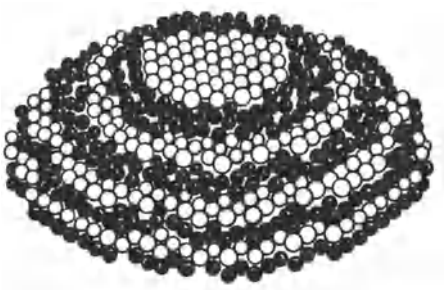


FIGURE 16.3. Illustration of a ball model that approximates the end of a field-ion emitter. The surface is not smooth; protruding atoms are imaged in the FIM.[4]

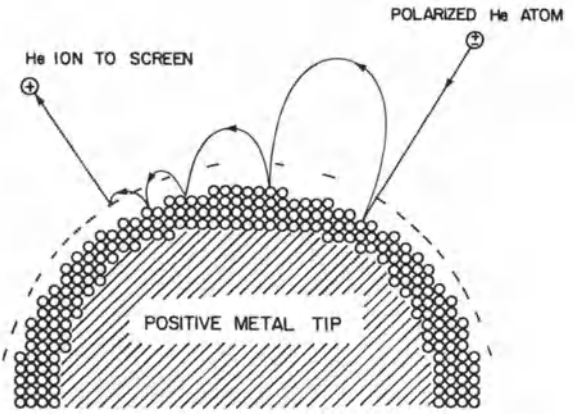


FIGURE 16.4. A polarized helium atom is attracted to the metal tip, slowed down in a number of hops, and ionized in the ionization zone (0.2-Å thick) over a protruding atom. The helium ion is accelerated toward the screen.[2]

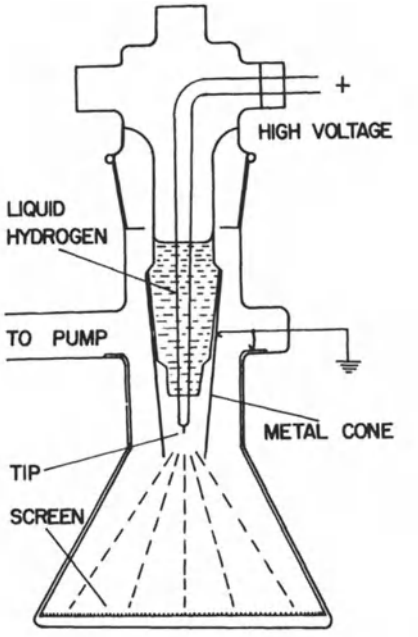


FIGURE 16.5. Schematic drawing of a FIM.[2]

16.3 ATTRIBUTES CONTRIBUTING TO VISIBILITY BY FIELD-EMISSION MICROSCOPY

16.3.1. Thought, Memory, and Imagination

The mental processes of thought, memory, and imagination are especially important when interpreting images by field-emission microscopy. In the absence of lenses, illuminating devices, and automatic controls, a field-emission microscope is less of an apparatus and more of a direct aid to the eye and brain. Together the mental processes furnish, Müller wrote,

> the skill, experience, patience, and ingenuity which the microscopist can muster for the preparation of the specimen, the making of the observation, and, finally, the interpretation of the image. It seems as if the evasive atoms still hide from the curious eye of the casual sightseer, and reveal themselves rewardingly only to the serious researcher.[2]

16.3.2. Resolving Power

The FIM is capable of visually separating individual atoms.[2] Each atom is depicted as a bright dot on a black background, like stars in the night sky. The theoretical resolution is about 1.5 Å, since the wavelength of ions (protons) is so much less than that of electrons and the temperature of liquid hydrogen or nitrogen is used to reduce thermal motion.[3]

16.3.3. Resolution

The practical *resolution* of the modern FIM is as small as a triangular spacing of 2.3 Å. Resolution amounts to separating bright spots or halos with certain elements—helium ions and liquid nitrogen. Neon, nitrogen, oxygen, and argon can be used instead of helium, but the images are inferior. Elemental specimens that give best resolution and those that give satisfactory images are discussed in Section 16.2.15.[2,10]

It should be emphasized that resolution with the FIM involves separating atoms as halos (bright spots or tiny stars). There is no resolution within the atom. With the field-emission electron microscope, while resolution of phthalocyanine (see Figure 16.3) is 12 Å on a side,[3] each square is divided into two or four parts, giving a resolution of at least 6 Å.

16.3.4. Contrast

There is usually plenty of black and white *contrast* between bright spots. In 1960 Müller used color contrast to detect quick changes in two successive images by printing one on red film and the other on green. When the two are superimposed, deletions from the first pattern are red, additions are green, and unchanged portions are yellow.[5,10]

The frequent problem of low intensity in field-ion images is due to contrast. Müller found some success by post accelerating ion beams through a fine, high-transmission wire mesh, but disturbing double images may occur. Converting the ion image into an electron image by using secondary-electron emission from a fine grid is somewhat more promising. External electronic image intensifiers based on photoelectric emission have proved to be more successful.[2]

16.3.5. Aberrations

Aberrations in field-emission microscopy originate at either the tip emitter or the fluorescing screen (since there are no lenses). The tiny tip is the chief source of aberrations. Most of them arise from the mechanical stress exerted by the electric field on the conductive surface, about 1 ton/nm,[3] which may exceed the yield strength of the particular metal by a factor of 50. The shear component of the stress, due to nonsphericity of the tip, may cause dislocations to move until the tip fractures. One correction involves reducing the tip's radius to less than 200 Å, so that there is insufficient room for defects to grow.[2]

Another kind of correction involves reducing the field strength to shape the tip without introducing severe lattice imperfections. Hydrogen promotes field evaporation and therefore shaping of the tip; it can reduce the required field strength by 5–20% for most metals used as tips.

16.3.6. Cleanliness

Cleanliness is of supreme importance in field-ion microscopy because some of its main applications involve the purity of metals—detecting and locating impurities and studying solids, liquids, or gases adsorbed on the surface. Therefore the metal specimen used for the emitting tip should be as pure as possible, as should be gases for ionization or adsorption and any other substance introduced into the microscope. All these ingredients must also be scrupulously free of dust, dirt, oxidation, and any other contamina-

tion. Metal for the specimen tip should be kept free of mechanical changes that would alter crystallographic conditions, such as dislocations, slippage, twinning, orientation, etc.

16.3.7. Focus Depth

Focus depth does not apply to the microscope itself, since it is without lenses, but it does apply to the photographic lenses in the cameras used to record either snapshots or moving pictures. Especially with the FIM images are of such low intensity that the lens should have a high aperture (low *f*-number). This means short focus depth, which in turn means that the fluorescent screen should be as flat as possible.[6] Another reason for a flat screen in either kind of emission microscope is that images are often changing or migrant, and this means short exposures, wide-open lens, and consequently short focus depth.

Atoms are generally in *focus*, but occasionally an atom jumps out of focus and appears brighter on the fluorescent screen.[10]

16.3.8. Illumination

Illumination in emission microscopy is due to the point projection of electrons or ions from the specimen-emitter onto the fluorescent screen. Intensity of image in the FIM depends on the specimen, its tip, the ionizable gas, etc. When these factors cannot be improved upon sufficiently for visual examination or for photography, Müller has used an external image intensifier (see Figure 16.6).[2]

16.3.9. Radiation

Radiation in the field-emission electron microscope forms a cone of electrons, as in an ordinary cathode ray tube. In the FIM, a stream of polarized atoms of a gas, such as helium, is attracted to the cold specimen at a high positive potential. Here gas atoms are slowed down in a number of hops and ionized and accelerated toward the screen.[2,14]

Most of the metals examined to date are isometric and thereby isotropic. Some however such as rhenium, are in the hexagonal system and therefore *anisotropic.*

Orientation of the metal grains does, however, contribute to the field-emission image, so that grain boundaries, twins, dislocations, etc., are recognized.[2] In the field-electron microscope, the orientation of some

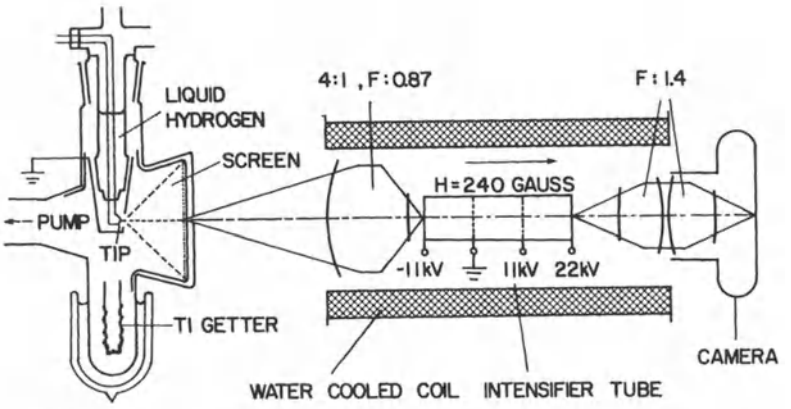


FIGURE 16.6. Schematic drawing of a FIM with external image intensifier.[2]

molecules, such as phthalocyanine, apparently makes a difference in the image, but the explanation is not clear.[5]

16.3.10. Magnification

Magnification is quite incidental in field-emission microscopes. It depends primarily on the radius of curvature of the tip. But the variation in local magnification due to bumps on the tip are not directly related to the radius of the bump because of a strong compression factor. Magnification of course also depends on the projection distance.

16.3.11. Field of View

The *field of view* in emission microscopes is small because the tip has a volume of only about 10^{-21} m^3. Larger fields require higher potentials than the usual 30 kV. Larger volumes also result in higher stresses on the specimen with greater potential damage.

16.3.12. Artifacts

The chief *artifacts* in field-ion microscopy come from disturbances to the specimen by extremely high mechanical stresses induced by very high

electrical fields. Such stresses are great enough to cause fracture. Stress both nucleates and rearranges dislocations, and it can induce deformation twinning; it usually leads to relatively high artifactual vacancies. As long as such artifacts are understood, they can be turned into an advantage in performing experiments *in situ*.

In the FIM the *third dimension* is limited to one or two layers of atoms, a true surface. Yet in a homogeneous specimen, we can infer much about the third dimension from the surficial arrangement of atoms and from previous knowledge of the space lattice of the respective metal (see Figure 16.3). Field-ion microscopy is already accepted in studying three-dimensional metallography.[13]

16.3.13. Working Distance

The *working distance* in both kinds of field-emission microscopes is fixed by dimensions within the sealed chamber. Nevertheless the working distance is ample for any practicable experiment, since no lenses are involved.

16.3.14. Field Depth

The *field depth* in emission microscopes is completely adequate because there are no lenses (and no focusing), and the object space consists of only one or two rows of atoms.

16.3.15. Structure

The atomic and crystalline *structures* of the metal are very important attributes contributing to visibility in the FIM. The chemical nature of the ionizable gas is also important. By the ionization of helium, for example, the following metals at the temperature of liquid hydrogen yield satisfactory field-ion images: tungsten, rhenium, iridium, platinum, molybdenum, tantalum, niobium, and rhodium; marginal are zirconium, vanadium, palladium, titanium, nickel, and iron. With neon gas gold, iron, nickel, copper, and zinc can also be imaged at or below their evaporation rate, as can metals in the first of the two preceding lists. Various changes in crystalline structures can be seen at the atomic level, such as dislocations, vacancies, impurity atoms, twinning, slip bands, and fatigue cracks.

16.3.16. Morphology

Morphology of the field-emission microscopical specimen at present refers chiefly to sizes and shapes of particles of a second phase distributed in an alloy. Quantitative particle-size distribution is difficult to obtain due to the complex shape of the specimen. Calculations are best performed on a computer.[4]

16.3.17. Information

Crystallographic *information* about the specimen amenable to field-ion microscopy is published and generally available.[9–11]

16.3.18. Experimentation

Various *experiments* can be performed inside the FIM. By preferred field evaporation of weakly bound, substitutional impurity atoms or by corrosion through field-induced chemical reactions with strongly adsorbed oxygen, carbon monoxide, or nitrogen, surface vacancies can be made to appear. Slip bands can be produced *in situ* by pulling at the tip by applying a reduced electric field at elevated temperature. The growth of fatigue cracks has been seen by superimposing an alternating voltage component onto the tip voltage to cycle the stress. The difference between annealed and field-evaporated specimen tips can be observed. The reversed action—rearranging atoms when annealing a field-roughened tip—can be witnessed, and at the same time activation energies can be measured *in situ*.[2] These are a few examples of experimentation in the FIM.

Experiments can also be performed by field-emission electron microscopy. Emission is critically influenced by monatomic layers, and single atoms or small groups of atoms can be detected as scintillating bright spots on the fluorescing screen. Migration of the atoms is readily observed as the surface structure is varied. Organic molecules can be introduced by evaporation from the heater coil within the microscope. The scintillating spots have structure, but interpretation awaits further experimentation.[3,5]

16.3.19. Behavior

Behavior can be observed in field-emission microscopes, since the environment is changed relatively easily in such a small, sealed space. Oxy-

gen, carbon monoxide, nitrogen, and other atmospheric gases can be introduced at will; likewise various other molecules can be introduced if they can be volatilized satisfactorily within the microscopical chamber by means of the high vacuum and localized heat. In this way corrosion and stress corrosion can be studied *in situ*. Specimen behavior in organic atmospheres may also be studied.[3]

16.3.20. Specimen Preparation

Specimen preparation is an important factor imaging by radial projection. The first crude steps are chemical etching and electropolishing a fine wire of the specimen metal, such as platinum. The etched specimen tip is spot welded to the tungsten leads sealed in glass (see Figure 16.5). The microscope is evacuated, and evaporation occurs at the tip when thermal activation permits atoms to leave the surface. However when a sufficiently high electric field is applied, some surface atoms may leave as ions by shedding an electron or two apiece. Field evaporation takes place preferentially at the sharp tip, which had been roughened by the etching. The tip becomes an oblate microhemispheroid, characterized by a uniform field strength.[2]

The problem of preparing *photographs* of field-emission images lies in recording a fleeting or moving subject at a low level of light intensity. A fast photosensitive emulsion is required. Fortunately there is plenty of contrast: white spots on a black background. Visual monitoring can be done in a darkened room. The external image intensifier shown in Figure 16.6 helps in severe cases, especially in taking moving pictures.[2,10]

Even with fast film, fast lenses are required in field-ion microscopy. Hydrogen-ion still pictures can be taken at $f = 1.4$, but with helium ions $f = 1.0$ is required. For 16-mm motion pictures $f = 1.0$ is also required.[5,10]

Müller reports the technique of taking two successive exposures and superimposing them to make a positive picture of very fast changes; printing the first picture on red film and the second on green is another technique.[2,10]

16.4. SCANNING-TUNNELING AND ATOMIC FORCE MICROSCOPIES

The scanning-tunneling microscope is among the latest inventions, having been developed in the 1980s. It has subatomic resolution; unfortunately the STM does not operate with nonconductive specimens,

whereas the subsequently developed AFM can be properly operated with insulating specimens.

Although developed almost independently of the earlier FIM, the two instruments are related, but the STM has many advantages over the FIM. The STM has greater resolution than the FIM and generally scans over the surface in a raster pattern to assess a much larger field of view than is obtained with an FIM. Scanning is similar to that with a SEM, but here scanning is a physical movement of the probe, whereas it is electronic in an SEM.

The AFM is similar to the STM but its probe tip or objective actually contacts the specimen's surface, whereas that of the STM does not. Resolution is greater on the STM than on the AFM.

For both types of microscopes, no free particles are collected. The tunneling microscope detects voltage changes that vary exponentially with the probe's distance from the surface. With the quantum tunneling concept, there is no sharp discontinuity between surfaces, just an exponentially decreasing probability of the two sets of electron clouds overlapping. The AFM detects surface contours through either the tunneling principle, capacitive techniques, or interferometry.

Tunneling current provides a very sensitive atomic-level sensor or probe of the surface profile—in other words it is a microscope. Since vertical position of the objective must quickly respond to changes in atomic elevation, computer controls are necessary.

16.4.1. Scanning-Tunneling Microscopy

The STM produces an image by monitoring electrons that tunnel between the two electron clouds bridging the narrow gap separating the conductive specimen surface from the probe. The probe is approximately triangular in shape with the sensing end ideally terminating in a single atom[12,17,18] (see Figures 16.7 and 16.8). Wave functions in both the microscope's probe and that of the specimen overlap, so that with a small applied voltage between the probe tip and the specimen, electrons tunnel between the two surfaces. Since the tunneling current is extremely sensitive to distance, a rapid-response, computer-controlled feedback mechanism maintains a constant distance between surface and probe. In this manner the probe measures the surface contours while scanning, and its motion is interpreted by a computer and displayed on a monitor.[15,16]

Scanning over the field of view yields a three-dimensional image of the surface. For proper operation the two surfaces are less than 10 Å

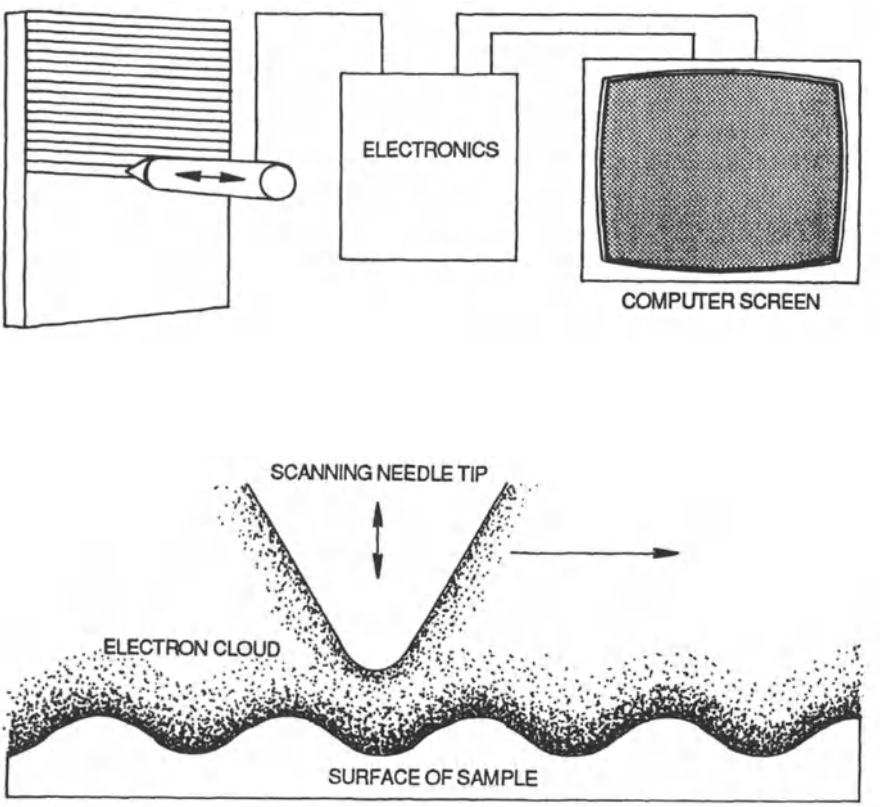


FIGURE 16.7. Electron tunneling, (bottom); scanning an electron tunneling tip and a screen display of the surface morphology are shown (top). Electrons tunnel across very small separations, and because the tunneling current depends exponentially on the degree of the separation, the tunneling current provides a very sensitive microscope of the surface's morphology. From Gerg Binnig and Heinrich Rohrer, "The Scanning Tunneling Microscope," copyright © Scientific American (Aug. 1985); all rights reserved.[17]

apart,[17,18] and the applied voltage is in the range of 0.1–1V.[12,17] The interaction strength of the two overlapping sets of electron clouds decreases exponentially with distance, which makes the instrument extremely sensitive to minute surface irregularities. A change in the separation distance of approximately one atomic diameter changes transmitted voltage by as much as 1000 times.[17]

Natural atomic and subatomic scale irregularities isolate the tunneling current, and these are the source of the extreme resolution.[12] Vertical and

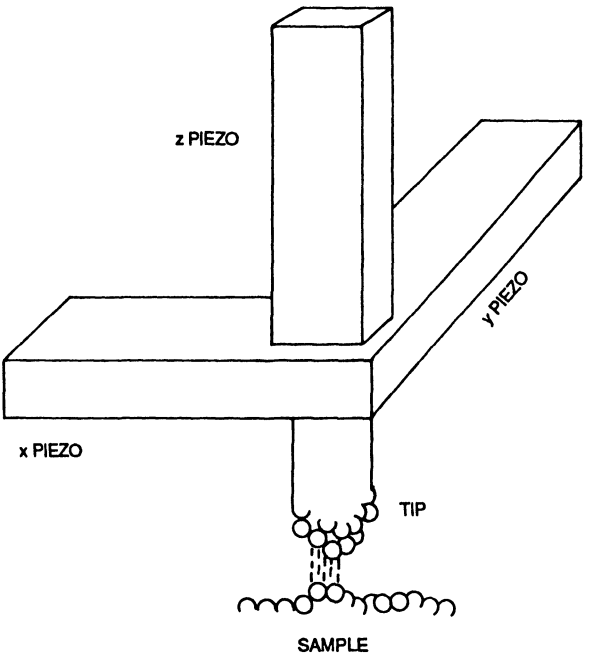


FIGURE 16.8. Schematic diagram of vacuum tunneling showing the tip and piezoelectric controls for the *x*, *y*, and *z* directions.[12] Tips are generally less than 10 Å in diameter. The piezoelectric control marked *z* controls the working distance between the specimen and tip. *X* and *y* piezoelectric controls scan the tip in a raster pattern over the field of view. During scanning the tunneling current is monitored and creates the displayed image.

horizontal resolutions are approximately 1 Å (0.1 nm).[19,20] With the very tip of the probe generally one atom in size, the sensitivity or resolution is subatomic. In fact a new tip is checked by simply measuring the resolution: If resolution is not atomic or subatomic the tip is normally replaced with another fairly inexpensive one. The tip is often of tungsten or platinum-iridium.[20] Since sensitivities yield magnifications of 10–100 million; however isolation from any vibrational noise and precise translational drives are critical.[12,17]

Both the STM and the AFM can scan a field of view as large as 130 nm. Because scanning is a contact technique, it does not resolve steep walls well.[20] The AFM has provided high-resolution images on nonconducting materials, such as polymers, and biological materials, such as amino acids and blood cells. Both air and liquids have been used as the media around the specimen.[27] Excellent recent contributions on this subject were re-

viewed in *Ultramicroscopy*, which has a series of relevant symposium articles.[26–28]

Figure 16.9 shows the most critical parts, a larger view of the instrument AFM's appears in Figure 16.10. A nanoscope AFM is shown in Figure 16.11. The upper part of the instrument is a reflected light microscope, and the "head" of the AFM is in the protective housing at the front focal plane of the light microscope. With the light microscope initial alignment to within 1 μm can be accomplished. Figure 16.12 shows the atomic resolution of NaCl atoms.

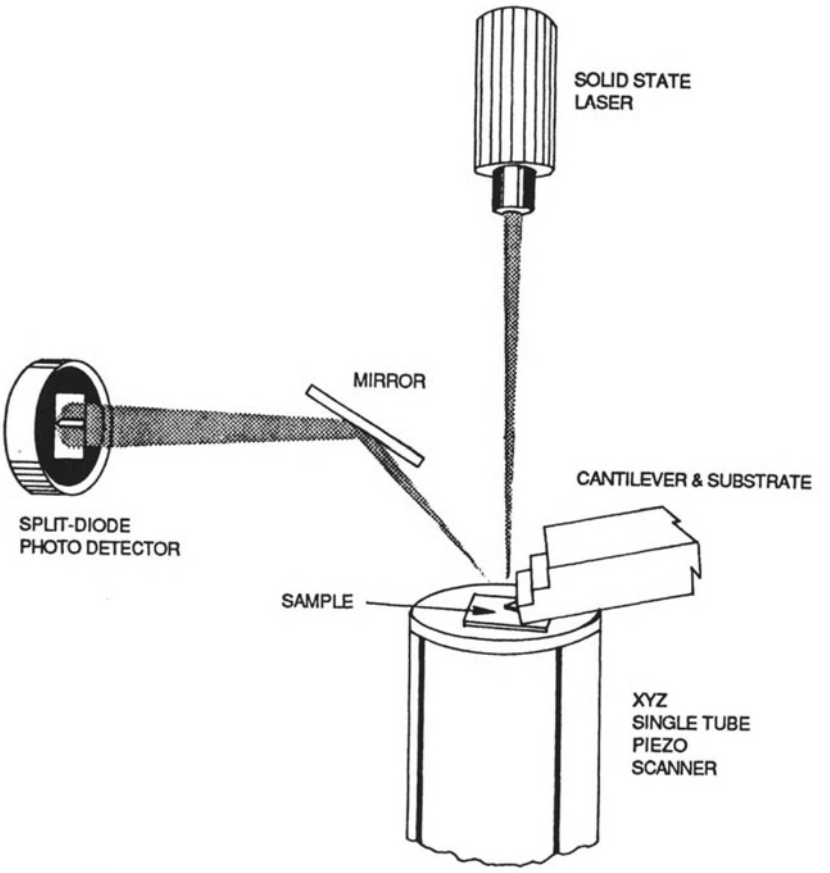


FIGURE 16.9. Schematic of an atomic force microscope showing the optical lever for detecting cantilever deflection. Reprinted from *American Laboratory* **23**, 4(1991), 40. Copyright 1991 by International Scientific Communications.[25]

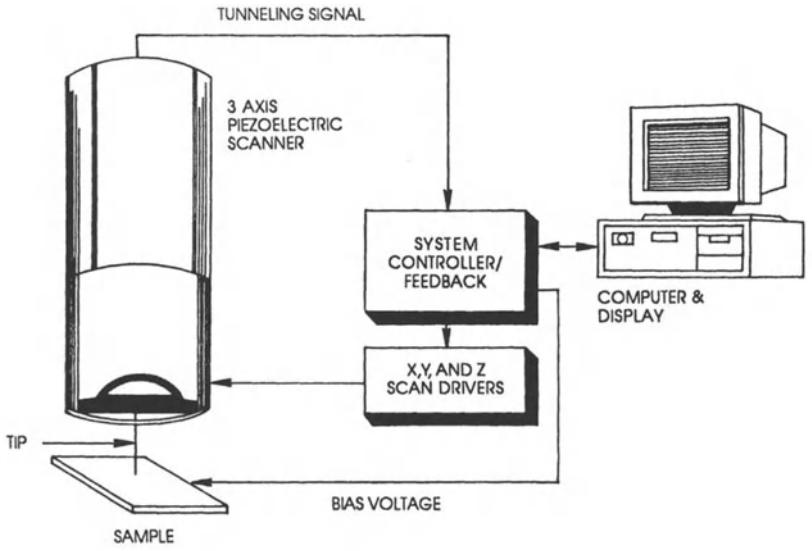


FIGURE 16.10. Diagram of a scanning-tunneling microscope. Reprinted from *American Laboratory* **23,** 4(1991), 36. Copyright 1991 by International Scientific Communications.[25]

16.4.2. Scanning Near-Field Light (Optical) Microscopy

The SNLM is still in the experimental stage, but it has already extended the resolving power of light microscopy by more than an order of magnitude over the resolution limit developed from classical theories (i.e., from 20 nm to 0.2 nm)[29] (see Figure 2.1). Although sometimes called an optical microscope, light is the preferred term, since the medium is light and optical also pertains to other media.[5,5a] Development of the SNLM was stimulated by the earlier appearance of the STM, and they both use the same close proximity between the probe objective and the specimen's surface.

Scanning near-field light microscopes, invented at Cornell University by Professor Michael Isaacson and his associates, do not have lenses. The resolving power of lenses is limited, not only by the wavelength(s) of the emanation, but also by aberrations imposed on an image formed relatively far from the object, that is, in the *far* field. Professor Isaacson uses a simple analogy: a garden hose with a nozzle of a certain diameter supplied with water at a certain pressure. If we observe the aperture of the nozzle at close hand, we will be sprayed by water in a spot whose diameter is close to the diameter of the hole in the nozzle. But if we stand, say, 3 feet from the

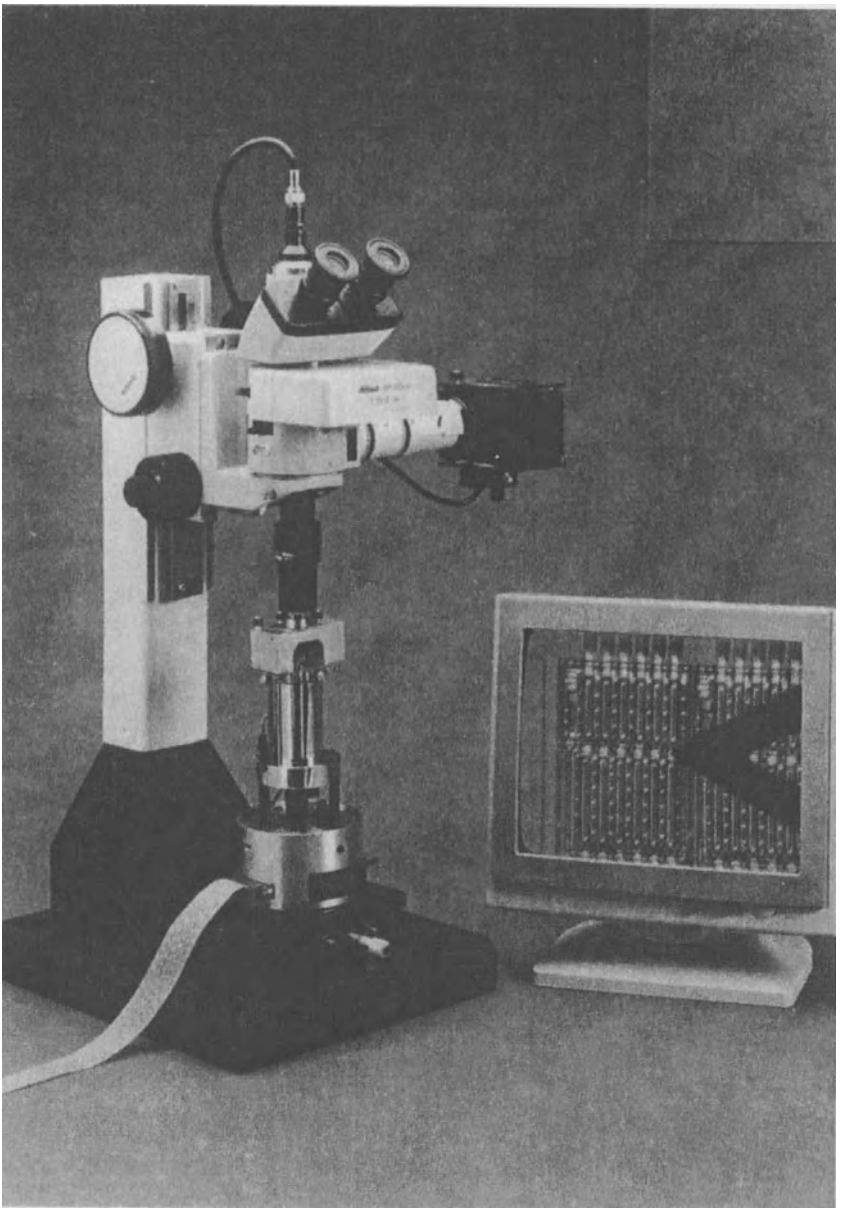


FIGURE 16.11 Nanoscope AFM and Nikon light microscope with video display. As shown on the video screen, one can position the probe tip while simultaneously viewing the sample magnified by light from above. The tip can be initially positioned before commencing the AFM scanning to within 1 μm. Courtesy of Nanoscope, Digital Instruments, Santa Barbara, CA.

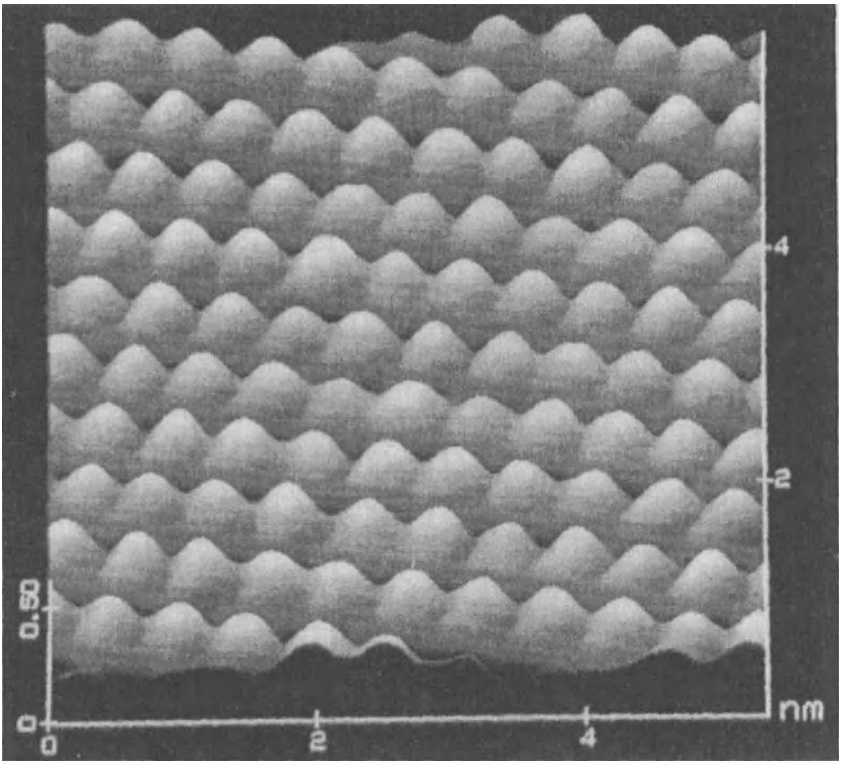


FIGURE 16.12. Resolution of atoms on the surface of NaCl to show the ability of the AFM to resolve atoms on the surface of nonconductors. Courtesy of Nanoscope, Digital Instruments, Santa Barbara, CA.

nozzle, we will be sprayed all over with water.[33] Therefore instead of focusing light with a lens, Isaacson passes light through a *very* small hole and a sample so close to the hole that the light beam has no chance to spread out. Using yellow green light (λ = 550 nm), Isaacson's team obtains a resolution of about 40 nm. Thus they are not resolving atoms (as with an STM), but they are using light. The limit is not so much the wavelength of the light as the fact that the amount is small enough to be undetectable.[33]

Aaron Lewis at Hebrew University in Jerusalem and Raoul Koppelman at the University of Michigan plug the 50-nm hole with a tiny crystal of anthracene because it fluoresces in UV light, yielding excitons that pass easily through the tip and turn into visible light (fluoresce). The result is a much brighter image, say, of a biological cell, from a near-field light microscope.[33]

Since the resolution of SNLM is similar to that of a SEM, what are its advantages? Potential advantages include its different type of illuminating, lack of need for a vacuum environment, potentially considerably lower price, and use of well-developed light contrast mechanisms over electron microscopy.[30] In the future this technique may be used to develop a near-field infrared microscope for chemical analysis of submicron specimens.[31-34]

At first the SNLM seems to contradict the basic laws of optics; however these laws exist for the far field, while the SNLM functions only in the near field. Figure 16.13 shows a schematic of the SNLM.

The far field is well explained by the classical work of Abbe and others, but theoretical explanations for the behavior of light in the near field have not yet been completely developed, although the general understanding does exist. Near-field behavior depends on evanescent or transient waves. These waves cannot propagate in free space, but they provide the mechanism for light to transmit through an aperture smaller than the wavelength.[31] Light emanating from an aperture of submicron diameter forms an illuminated spot of subwavelength diameter and this spot of light is scanned over the specimen to generate the image.[31] During light transmission through the submicron-sized aperture the light's wavelength is collimated to the aperture size, and the transient light through the aperture is mainly independent of the initial wavelength—this evanescent light extends only a distance approximately equal to the aperture's ra-

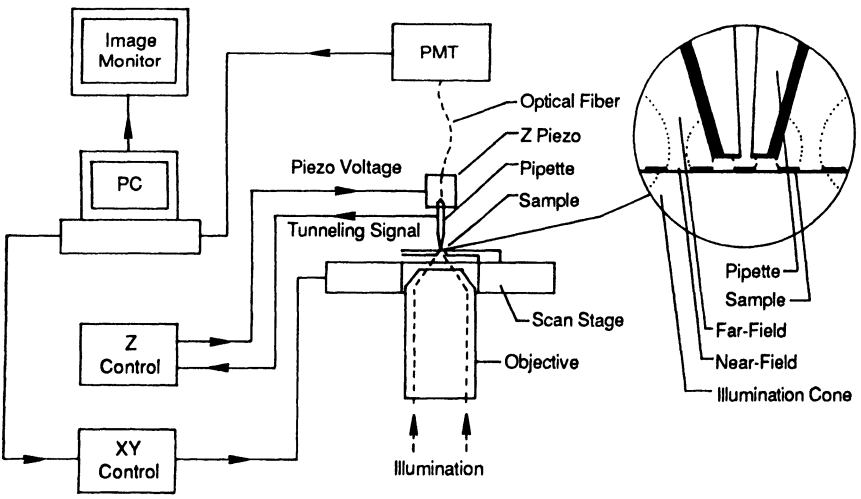


FIGURE 16.13. Schematic of the near-field scanning light microscope developed at Cornell University.[30]

dius,[32] at greater distances its behavior follows classical laws. For a 500-Å diameter aperture, the near field extends only about 200 Å.[32] Tunneling current from protrusions on the aperture has been used in conjunction with the light probe to control the objective's or probe's working distance from the sample.[30]

At first forming the submicron-sized apertures seemed a major obstacle, but this was overcome by drawing hollow glass capillary tubes to the breaking point.[32] Metal is then evaporated onto the drawn capillary, making it opaque, and the hole is left in the end of the capillary.

Work has been done with the aperture functioning as the illumination source, and some recent developments use it as the collecting objective.[30] The illumination system is scanned across the specimen similar to the scanning in an STM or SEM. A major problem with continually decreasing the diameter of the aperture is the amount of light emitted through the minute aperture. The emitted light can be as little as one ten-thousandth or less of the illuminating light, and techniques are being developed for increasing the illumination.[33] Betzig and Trautman provide a comprehensive review of the SNLM.[34]

16.5. SUMMARY

Emission microscopy is one of the most rapidly evolving developments, and it potentially has major importance. It permits atomic resolution and detection of the chemical composition at the atomic scale. The latest development in this class of microscopes uses light and the investigators have achieved a resolution which is a small fraction of the light's wavelength.

In a *field-emission microscope,* the specimen is the source of radiation by virtue of a strong electrical field and a very high vacuum. The specimen source is cold (at or below room temperature) unlike thermal emission. Lacking lenses, cold field-emission microscopes achieve magnification by straight projection of electrons or ions rays; therefore the point of the thin-wire specimen is made extremely sharp (5–100 nm radius). With a projection distance of 10 cm, magnification (over one million times) is useful to the extent of resolving atoms or small groups of atoms, depending on whether ions or electrons are emitted.

The *electron* field-emission microscope is much like an ordinary cathode ray tube except that the specimen, which is the cathode, is pointed at a resolving power of 2 nm. In this state the field-electron microscope is useful for studying the emission of electrons as a function of surface structure.

The *ion* field-emission microscope employs the extremely pointed specimen as the *anode*, which is kept very cold in a liquified gas. Helium atoms are introduced and find their way to the cold tip, where they are slowed down in a series of lower and shorter hops on the roughly etched tip of the anode. Some of the slow helium atoms lose an electron to a protruding metal atom. The resultant helium ions are shot onto the luminescent screen, producing a dim pattern that can be photographed on fast film during long exposure or intensified photoelectronically. The resultant pattern (image) is of refractory metal atoms organized or disorganized on a surface or succession of surfaces. Grain boundaries, slip planes, dislocations interstices, and vacancies are also made visible. Interpretations lead to surficial binding energies and work functions; surface chemical reactions of interest include corrosion and catalysis.

The STMs, AFMs, and SNLMs are among the latest and potentially most important technological developments. No free particles are collected with either microscope. The detector is either within tunneling distance of the surface or actually contacting the surface.

Scanning-tunneling microscopy is related to earlier field-ion microscopy, but it has many advantages: Scanning-tunneling microscopy has greater resolving power than field-ion microscopy and generally scans over a larger field of view. The AFM is similar to the STM, but its probe tip actually contacts the specimen's surface, whereas the STM collects information by sensing electronic tunneling signals from the specimen's surface. The AFM's resolution is less than that of the STM, but it can examine the surface of an insulator, whereas the STM cannot.

The SNLM is the latest in this evolving series. Its resolution is subwavelength through use of a condenser objective of much smaller inside diameter than the initial wavelength. Like the STM and AFM, the scanning near-field light microscope is a near-field instrument and therefore its fundamental resolution and capabilities do not follow the classical law developed for the far field. Understanding is still at an early and evolving stage. In a strict sense an SEM is an emission microscope, but since it is so well established independently of the others, it is discussed in Chapter 15.

The scanning probe microscopes discussed in Chapter 16 and others are reviewed in considerable detail in an article, "The Scanning Probe Microscope,"[35] which should be studied for additional detail. Unique capabilities of these microscopes make them an exciting topic, and they hold considerable potential for the future.

17

X-Ray Microscopy

17.1. X RAYS

X rays are electromagnetic radiation of the same nature as visible light but with only one-thousandth of the wavelength.[1] X rays behave like light toward photosensitive materials. For example when a dentist takes a radiograph she/he places an unexposed photosensitive film next to the teeth and turns on the X rays. Cavities show up dark on the negative film because they transmit X rays so well. Sound teeth show up less dark because they are denser than air or flesh. Ceramic and plastic fillings are still darker if they are denser than teeth and bone. Silver and gold show up very dark when exposed to X rays because they are very dense (such have high atomic numbers).

The dentist's radiograph is approximately life-size (1×). Dioptric magnification by transmitting X rays is not feasible because lenses have not been developed for it. X rays at glancing incidence have been reflected from concave mirrors or curved surfaces of crystals, but so far focusing is not sharp enough to construct an X-ray microscope this way.

The dentist's radiographs and other radiographs may be usefully enlarged to 10×, but not much more. An X-ray microscope based on the principle shown in Figure 17.1 has no more magnification than the simple light microscope or equivalent that enlarges the contact negative. The enlarger cannot remove the contact negative's blur, which is caused by the crossover of peripheral X rays from such a large source.[2,3] Still the ordinary source of X rays may be useful in low-power microradiographic studies of the distribution of heavy elements among lighter ones in an essentially inorganic specimen. In an organic system contrast in the preferential absorption of X rays is increased if a selective stain can be found bearing a heavy element in its composition. In any case it helps in simple

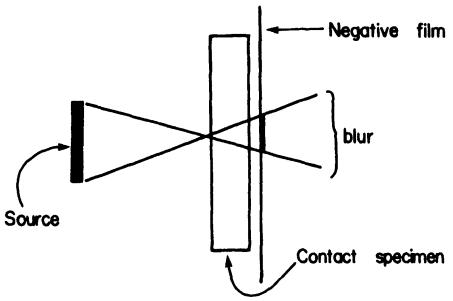


FIGURE 17.1. Ordinary source of X rays for microradiography blurs the contact image.[2]

microradiography to have the specimen as thin as commensurate with selective absorption among the specimen's constituents.

The boom to moderately high-power (100 or 200×) microradiography came with the development of a *point source for X rays.* As shown in Figure 17.2, the point source provides a very narrow bundle of almost parallel X rays to impinge on the specimen. As the source point becomes smaller and smaller, the crossover angle of the peripheral X rays through the specimen is reduced accordingly. Therefore the contact image is made sharper, and the permissible thickness of the specimen may be a little greater.

17.2. CONDENSER LENSES

One way of producing a concentrated point of X rays is to use the objective lens of an electron microscope to focus electrons on a target

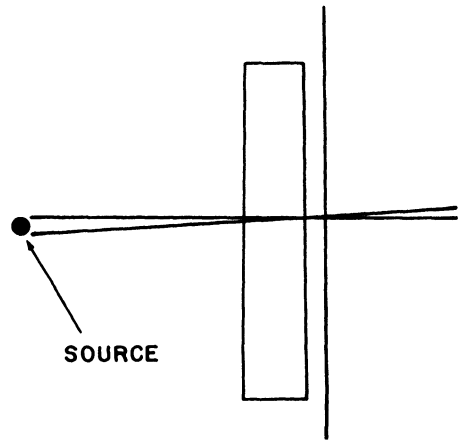


FIGURE 17.2. Point source of x rays for microradiography improves the contact image.[2]

situated near the specimen and photosensitive film. Figure 17.3[4] shows a homemade adapter for the point projection of X rays from a target through the specimen to the film[4] Subsequently electromagnetic lenses were built into commercial point-projection X-ray microscopes.[2,3,5] Figure 17.4 shows a diagram of a double electromagnetic X-ray microscope.[3] Such a microscope is ideal for studying inorganic chemical constituents, especially those with high atomic number. Nevertheless the specimen should be thin. If it is thick, the catalytic pellets for absorbing automotive leaded gasoline vapors, the pellets are abraded until they are only 25 μm thick. Figure 17.5[6] shows a negative microradiograph of thinned unused experimental pellets, taken on a Philips instrument.[5] The photosensitive film was Kodak fine grain, processed through a fine-grain developer. The finished negative was enlarged 20×. Other pellets that had been exposed to fumes from leaded gasoline were treated and x-radiographed in the same way. The commensurate negative enlargement is shown in Figure 17.6. While peripheral rings show the distribution of lead atoms in the exposed pellets.[6]

Point-projection radiography is also very useful in studying organic systems.[3,7-9] Botty and his colleagues studied plastic foams by employing soft (low-voltage) X rays of low-penetrating power, in which low-mass materials (e.g., air and polymers) are differentiated. In Figure 17.7, an enlarged negative microradiograph of an organic artificial foam, *air* bubbles photograph black, and the polymeric walls photograph white and grey.[7] The point of X rays is so sharp and the specimen is so thin that a useful magnification of 100× was achieved.

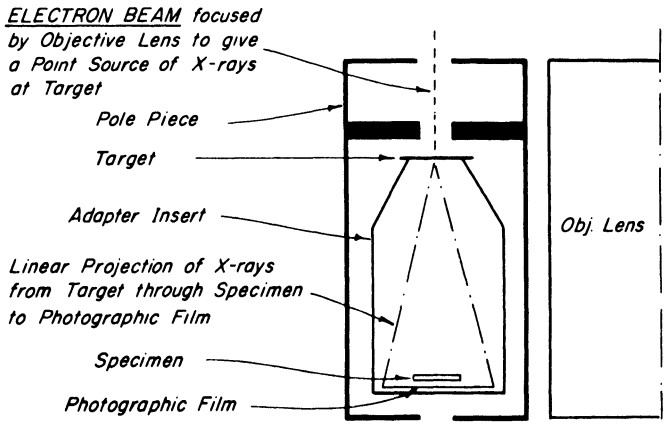


FIGURE 17.3. Schematic drawing of adapter showing target, specimen, pole piece, and paths of electron and X-ray beams.[4]

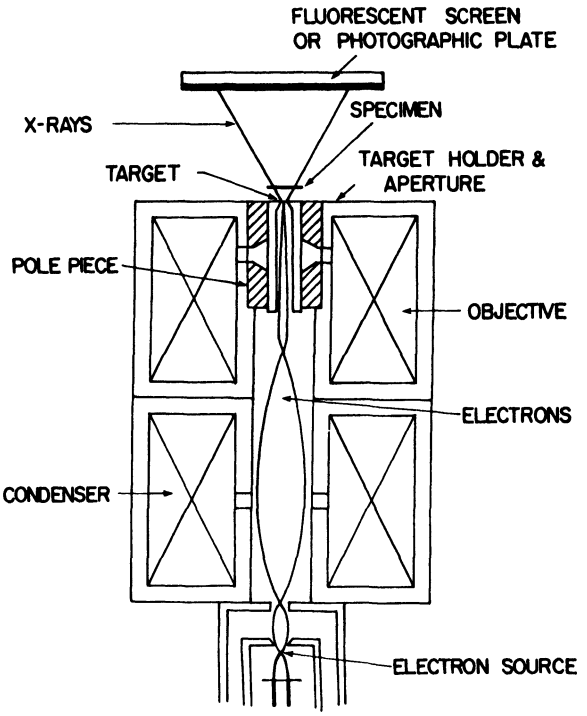


FIGURE 17.4. Diagram of the point projection X-ray microscope.[3]

17.3. RECENT DEVELOPMENTS

X-ray microscopy, with either synchrotron radiation[10] or laser-produced plasmas as x-ray sources,[11] has recently begun to realize its promise in biology, engineering, radiobiology, geology, and materials science.

Both illumination sources and detectors have been improved in recent years.[12] Brightness for example has been increased millions of times over that of conventional X-ray tubes by means of synchrotron radiation, X-ray lasers, and X-ray-emitting plasmas. Detectors have been improved through both new electronic developments and such recording techniques as microscopic resist materials.[12]

Synchrotron radiation is being used as the source of soft X-ray photons of 0.1–1 keV energy with 1–10 nm wavelength. There are three major potential advantages of soft X-ray microscopy over light and electron microscopies[13]:

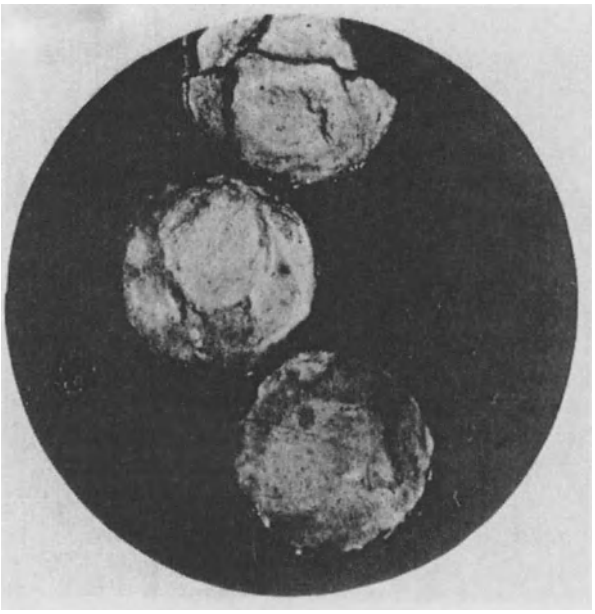


FIGURE 17.5. Microradiograph of experimental auto exhaust catalyst pellets before exposure to leaded gasoline vapors. Thin ground sections (approximately 25 μm thick).[6] Philips CMR Instrument.[5] Pellet diameter: 1.5 mm.

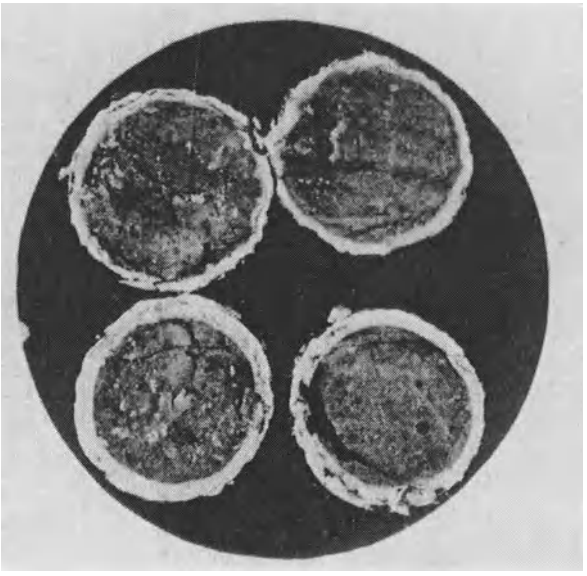


FIGURE 17.6. Microradiograph of experimental auto exhaust catalyst pellets after exposure to leaded gasoline vapors. Ground thin sections. The white peripheral rings show the distribution of Pb condensate.[6] Philips CMR Instrument.[5] Pellet diameter: 1.5 mm.

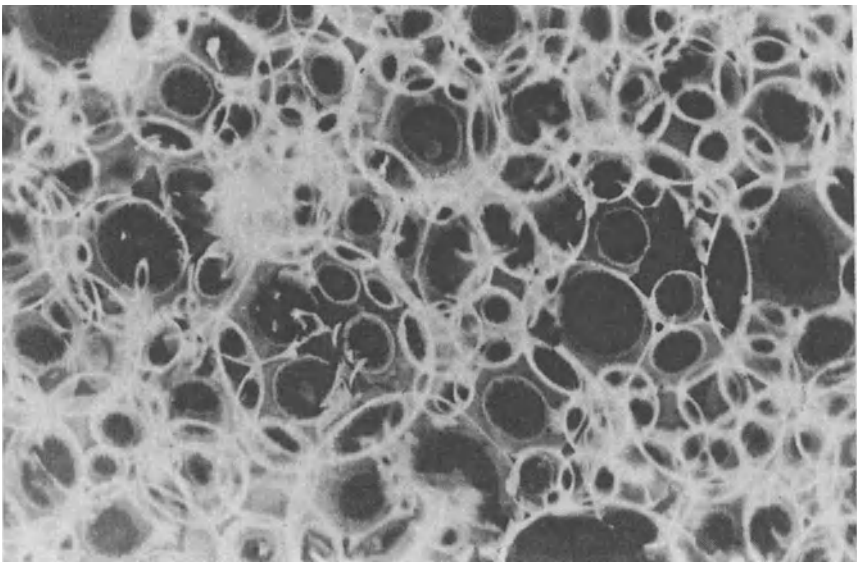


FIGURE 17.7. Contact microradiograph of a thin section of thermosetting polymeric foam showing holes, or thin-walled air bubbles (black spots), at the periphery of the thin cell windows.[7] Exposure conditions: 2 kV, 3.5 mm. Philips[5] CMR Unit: standard specimen holder; Kodak film.

X-ray microscopy has a potential resolution 25–50 times greater than light since their wavelengths are much shorter than those of light.

In the X-ray spectrum from 2.3–4.4 nm wavelengths, there exists a large difference, an edge, in the absorption of carbon containing molecules and water.

Due to their high penetrating power relative to electrons, the internal structure of relatively thick (10–20 μm) specimens can be examined in transmission.

Scanning microscopes with such sources have been constructed at centers throughout the world. An example is the National Synchrotron Light Source at Brookhaven National Laboratory in Upton, New York.[12] Images can be recorded digitally, so that quantitative information on specimen absorptivity as a function of position can be quickly obtained.

With synchrotron X-ray sources it is possible to choose different wavelengths of radiation to use the atomic absorption coefficients of structural materials selectively.[14] From such information elemental maps can be

drawn.[15] Resolution is currently better than 600 Å, and current microfabrication techniques are increasing the resolution.[12,16] In addition to synchrotron radiation sources, considerable activity in laser development is occurring in applications of X-ray lithography, and X-ray microscopy is also benefiting.[11,17] A schematic of this technique is shown in Figure 17.8.[17]

The basic technique, which is similar to contact printing and in fact termed contact microradiography,[17] involves placing a specimen near the recording medium, which is then exposed to the X-ray source. High resolution is possible; X-ray photoresists are used[1] to record the image. Much of the necessary technology evolved from fabricating integrated circuits using microlithography. While earlier techniques to miniaturize circuits involved electron beams, X rays have been used more recently. For this type of microscopy, a specimen is placed in intimate contact with the resist surface. A typical high-resolution resist is poly(methyl methacrylate) (PMMA). After exposure to X rays and subsequent development, the resulting relative heights in the contact image are functions of the specimen's X-ray absorption; Figure 17.9 illustrates the contact-imaging process.[17] After preparing the contact image, it is magnified in either an SEM or a TEM. Damage can occur to the photoresist image in the electron beam used for viewing so techniques are being developed to form metal replicas of the resist image.[11]

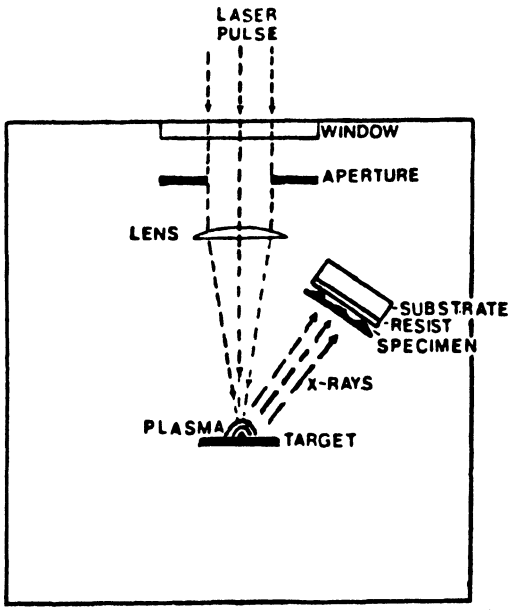


FIGURE 17.8. Schematic of a plasma generated by a laser pulse. Drawing shows a possible configuration of the source and specimen (from p. 288 in *Examining the Submicron World*, Plenum, 1986).[17]

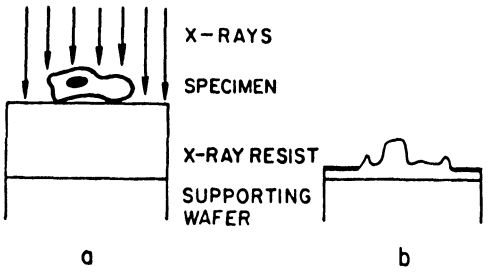


FIGURE 17.9. Schematic of contact imaging. (a) Contact imaging of specimen into resist where incident X-ray beam is modified by the absorption of the specimen. (b) The resist after development and metallization. The resist profile is then magnified and read out by electron microscopy (from p. 282 in *Examining the Submicron World*, Plenum, 1986).[17]

Imaging X-ray microscopy and scanning X-ray microscopy are more direct techniques than soft X-ray contact microscopy.[12] These microscopes depend on one or more Fresnel zone plates. A circular Fresnel lens is defined by ASTM as "a sheet of transparent material into which concentric groves have been formed in such a pattern that light will be focused as with a lens."[1] A Fresnel zone plate is similar to a Fresnel lens, and it consists of alternating transparent and opaque rings whose spacing diminishes with distance from the center.[12] Waves are diffracted as they pass through the transparent rings. By controlling the spacing between the rings, or the wavelength, we can control the distance from the plate to the common focal point. Recently zone plates have been constructed with grating spacings as fine as 30 nm. Fresnel zone plates can serve as condenser and objective X-ray lenses, and these function much as lenses in a transmitted light microscope.[12]

Scanning X-ray microscopes are becoming more common. A zone plate is used to sequentially focus an X-ray beam onto a small, localized area of the specimen. As the beam is scanned, picture elements (pixels) are generated much as in an SEM. The size of the illuminated spot determines the resolution. Scanning minimizes radiation damage, and since a sufficiently coherent soft X-ray beam is needed, scanning X-ray microscopy can be accomplished with only a major synchrotron source.[12]

17.4. SUMMARY

X rays hold great microscopical promise for several reasons:

X rays for X-ray microscopy are generated by conventional X-ray sources and now also by synchrotrons, X-ray lasers, and X-ray emitting plasmas.

Common wavelengths are short enough (0.1–4 nm) to give high-resolving power.

X rays absorption spreads among the chemical elements as the cube of the atomic number.

Since X rays travel well in air, making a vacuum unnecessary, wet or living specimens can be examined *directly* without desiccation.

Specimens are so transparent and X rays have such penetrating power that thicknesses of 20–50 μm are permissible for shorter wavelengths.

If parts of a specimen differ (or are treated to differ) in atomic number, there is plenty of contrast. Also with soft X rays of 1–10 nm wavelength, even materials with lower atomic number provide sufficient contrast without any treatment. However with these longer X rays, specimens must be sufficiently thin.

With synchrotron radiation, different wavelengths can be selected to use absorption coefficients. In this way elemental maps are obtained. Resolution better than 600 Å has been achieved, and higher resolution is anticipated. This resolution is possible by recording on photoresists with subsequent magnification by scanning or transmission electron microscopy.

Since X-ray micrographs are pictures of absorption, they may give better contrast than light micrographs of inorganic parts or particles of metals, ores, minerals, rocks, ceramics, silicones, pigments, and fillers. Moreover elements with a high atomic number can be added as selective stains to parts of organic systems[8,9] for automatic analysis.

The full potential of X-ray holographic microscopy has not been realized. Submicron resolution has been achieved but considerable difficulties exist because of the low sensitivity of the recording media and difficulties in recording contrast readout reconstruction. Research is currently underway to develop X-ray microscopical holography as a practical technique.[18]

18

Acoustic Microscopy

18.1. ULTRASOUND WAVES FROM MICROSPECIMENS

Acoustic microscopes employ electromagnetic signals and transform them into *acoustic* waves by means of a piezoelectric transducer. The resultant acoustic waves are the same propagating elastic type as water waves in the ocean and sound waves in air.[1–8] Being of very high or ultrahigh frequency, 100–2000 megacycles/sec or megahertz (MHz), microscopical acoustic waves are ultrasonic (they cannot be heard). As in macroscopical use of ultrasound,[7] the primary significance is that such acoustic waves are reflected or deflected by the specimen's variations in *density* or *stiffness* rather than by differential refraction, absorption, or reflection (as with light or electrons). Consequently a whole new microscopical area of structural information is open for correlation with properties or behavior and composition or treatment. Much of the contrast with acoustic microscopes is due to variation in the material's elastic constants. With light microscopy however much of the contrast is due to variation in dielectric constants. Therefore acoustic microscopy complements light microscopy.

In acoustic microscopy the specimen is contained in a cell filled with a liquid, often water. Incident acoustic waves are deviated by the specimen and are collected and converted into electronic signals that are translated into an image on a cathode ray tube, in video fashion. Figure 18.1[9] shows what can be resolved in acoustic microscopy.

18.2. ACOUSTIC MICROSCOPES: SLAMs VERSUS SAMs

There are two versions of the scanning acoustic microscope, which are classified according to how scanning is performed. In the Sonomicroscope® scanning laser acoustic microscope (SLAM), scanning is done with

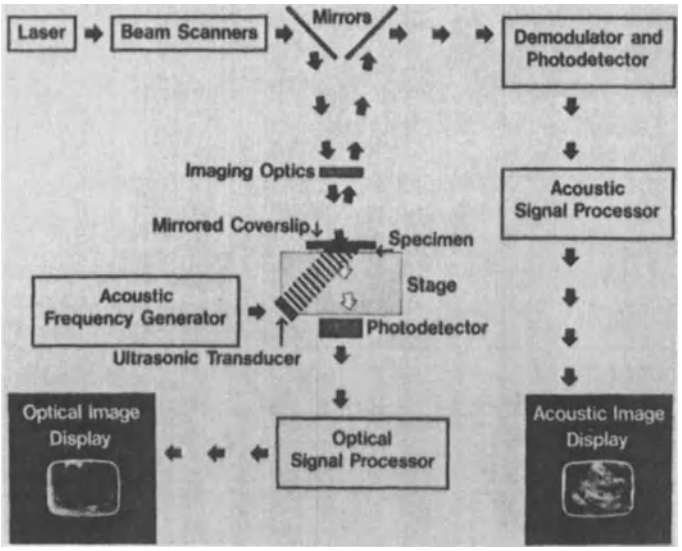


FIGURE 18.1. Simplified block diagram of the Sonomicroscope® SLAM.[9]

a laser. As Figure 18.1 shows, the specimen is mounted in shallow liquid and held on a stationary stage that is activated acoustically at 100 or 500 MHz. The specimen is covered with a mirrored semitransparent cover slip. The resultant differential disturbances of the mirrored slip are detected by the scanning laser (*flying spot*) and converted into electrical signals by a photodetector. The (weak) signals are amplified and decoded to produce an acoustic image display (lower right, Figure 18.1). An optical image can simultaneously be produced with the small amount of laser light transmitted differentially and displayed (lower left, Figure 18.1) or differentially reflected from the specimen and displayed (not specified in Figure 18.1). Hence a special feature of SLAM is that the laser light image simultaneously be compared with the acoustic image.[9]

An optional feature of the Sonomicroscope® involves feeding acoustic and optical signals into separate channels of a color television monitor. Thus the separate identities of the acoustic and optical images are maintained in contrasting colors. This feature is important in interpreting the novel acoustic image in terms of the conventional optical image. Interference patterns can be produced by means of an electronic phase reference. The result is a graphic display of changes in density and elasticity within the specimen.

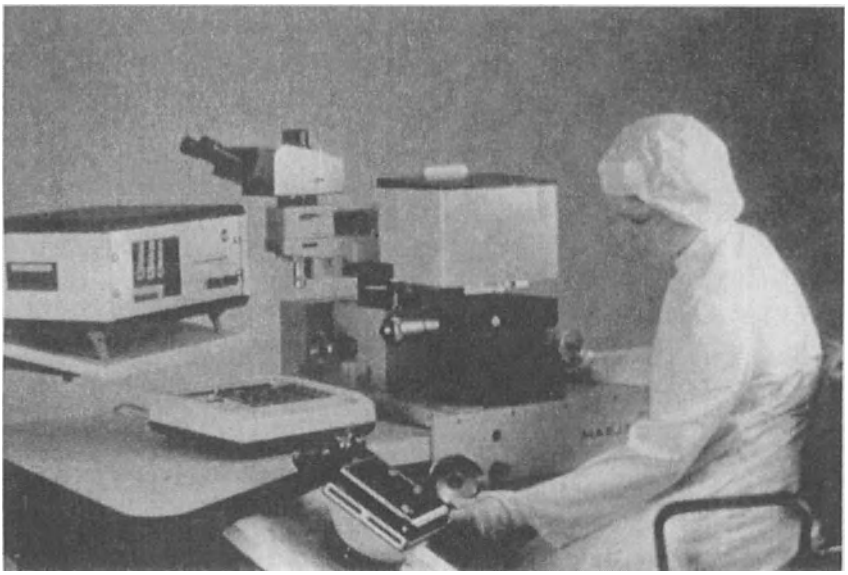


FIGURE 18.2. Photograph of the SLAM by Sonomicroscope®, Inc., for producing acoustic micrographs of specimens.[9]

In the SAM, scanning is performed *mechanically* by moving the specimen, as shown in Figure 18.3.

Figure 18.4 (left) shows an electromagnetic input signal being *transformed* into an *acoustic* beam by a piezoelectric film of zinc oxide on the input sapphire crystal. The acoustic beam travels through the liquid (water) in the cell that also contains the object, supported by Mylar® polyester film. The surfaces of the input sapphire crystal and the output sapphire, which are in contact with the spherical part of the specimen's cell, are concave. Thus the input acoustic beam is focused (condensed) on the specimen, which scatters the output acoustic beam into the output (objective) lens (liquid + sapphire) system. The output acoustic beam is converted into the output electromagnetic signal by the respective piezoelectric film transducer (see Figure 18.4, right). The acoustic cell in the middle constricts the acoustic beam to less than one wavelength (approximately 2 μm) in diameter. The object is scanned in a raster pattern. The acoustic output modulates the intensity of the output electrical signal and displays it on a cathode ray tube (see Figure 18.3). The resultant image corresponds either to amplitude modulation or phase interference. In Figure 18.5a the amplitude (intensity) mode is shown in an acoustic micrograph of a fixed preparation

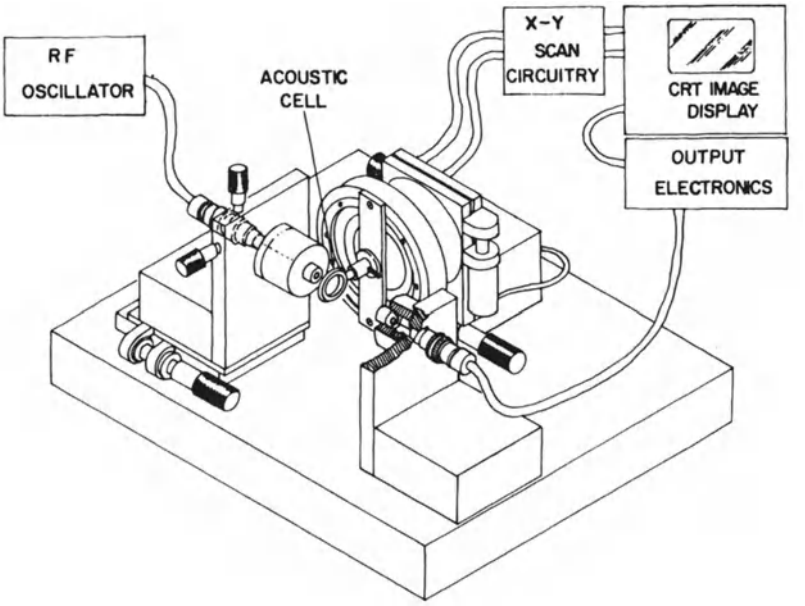


FIGURE 18.3. View of the SAM showing scanning drives and associated electronic equipment.[10,11]

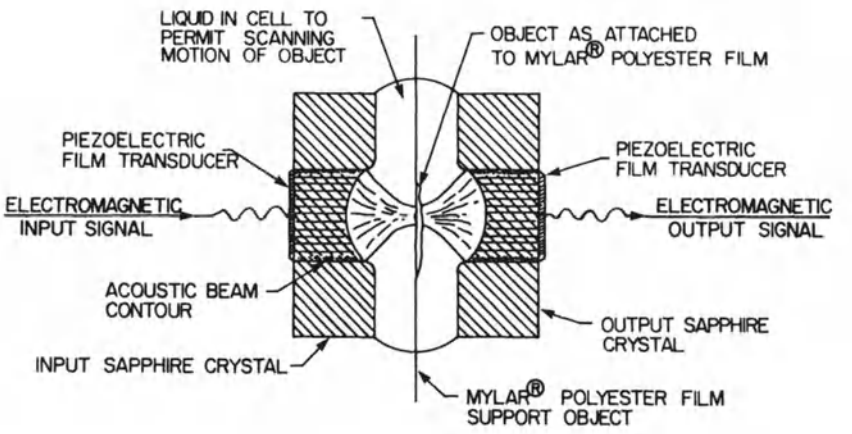


FIGURE 18.4. Schematic representation of the acoustic cell as used in the SAM.[1,10]

of red blood cells. The change in amplitude of the acoustic beam gives information about acoustic absorption and impedance. Figure 18.5b (the phase model) shows a point-by-point display of changes in the velocity of sound when the section is uniformly thick.[1,10–14]

Difficulties exist in routinely aligning separate transducers for transmitting and collecting signals in acoustic microscopes. With reflection rather than transmission, only one transducer is needed. Techniques have also been developed for generating *V(z)* curves to assess changes in intensity of the acoustic reflection as a function of sample depth. Such plots, which provide information about acoustic velocities and elastic properties, are unique for each material.[6]

Results of a study of composites show that the internal angles of individual fibers can be determined in an opaque-to-light matrix by observing the interference fringes that appear on the specimen's surface.[6]

Another useful result is that interference of surface acoustic waves, i.e., Raleigh waves, detect the existence of microcracks near the surface, that are far smaller than the image resolution of the microscope. These cracks may be a nanometer in diameter, and their interference results are easily seen.[6,13] A potential limitation of the microscope is that at a resolution of ca. 1 μm with a frequency of ca. 1 GHz (1000 MHz), signal attenuation limits microscopical information to a depth of only about 1–100 μm.[6]

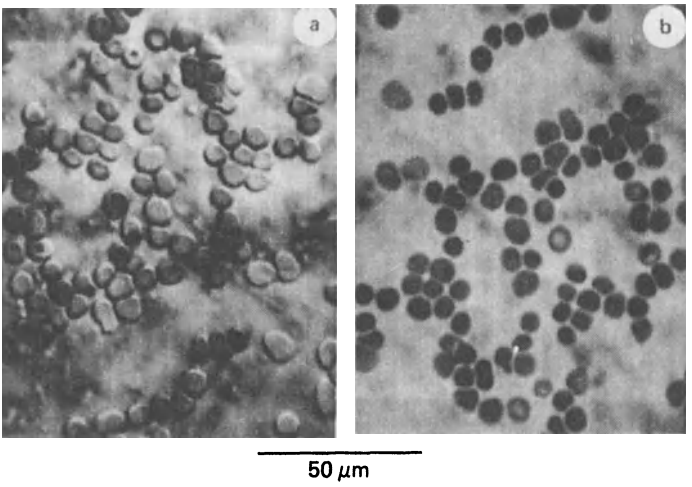


FIGURE 18.5. Scanning acoustic micrographs (a) amplitude modulation and (b) phase interference of red blood cells fixed with methanol. 1000 MHz. May 28, 1976. Courtesy of C. F. Quate.

The SAM is being applied to such medical problems as malignance in blood cells, mammillary glands, lymph glands, and lung tissue.[4] Problems involving even greater constituent differences in density and stiffness could include teeth, bones, and various types of kidney stones. Foreign microscopic particles involved in lung diseases include siliceous minerals, asbestoses, other fibers, soil minerals, and air pollutants. The physiologically detrimental compounds of lead and mercury also seem to be acoustically determinative.

In nonbiological fields acoustical studies are being done on solid-state materials, such as a thin aluminized pattern deposited on silicon. Another example is a circuit of silicon on sapphire, in which microscopic flaws were discovered solely by acoustic microscopy. In cold-worked aluminum acoustic micrographs revealed not only the surfacial slip lines but also structural changes below the surface. Other variations in stiffness and density may be revealed among constituents in alloys, welds, ores, slags, ceramics, cements, pigmented materials, and reinforced plastics, among others. Thus the SAM provides considerable quantitative information that is otherwise unobtainable on composites.

Conceivably the acoustic frequency in the SLAM could be changed to improve the resolving power. Even at 500 MHz, resolving power of the SLAM depends on the distance between the object plane of interest and the cover slip, since the SLAM operates like a general x-microradiography.

18.2.1. Theoretical Resolving Power of Acoustic Microscopes

The theoretical *resolving power* of the acoustic microscope of course depends on the acoustical wavelength in a particular medium. Table 18.1 shows how the wavelength at 100 MHz varies with the medium according to its density and stiffness. The resolving power of the Sonomicroscope® 100 (MHz) type of SLAM is stated to be between 0.7–1.3 wavelengths. In the Sonomicroscope® 500, the input frequency is 500 MHz, with a correspondingly higher theoretical resolving power.[9]

At present a still higher frequency in the SAM (2000 MHz) ensures a correspondingly higher theoretical resolving power. In water the wavelength is 1.5 μm, and the theoretical resolving power is possibly half of that. The net optical resolvability is less than the audio but comparable. Both audio and optical resolvabilities are less than the resolving power of the conventional light microscope, but again they are comparable.[1] Liquid helium has been employed instead of water to reduce acoustic velocity to as little as one-eighth and yield a resolution near 100 nm (1000 Å).[1,4,5]

TABLE 18.1
Typical Wavelengths at 100 MHz in μmm[9]

Material	Compressional Waves	Shear Waves
Air	4	
Water	15	
Soft tissue	14–17	
Hard tissue	30–60	
Plastic—acrylic	27	11
Metals		
Aluminum	63	31
Brass	43	20
Iron	59	32
Titanium	60	31
Glass	56	34

18.2.2 Practical Resolution of Acoustic Microscopes

In addition to the wavelength, practical *resolution* from an acoustic microscope depends primarily on the effective diameter of the detector. In the lensless SLAM, the laser beam diameter can be adjusted to the limit of the optical wavelength. In the laserless SAM the diameter of the acoustic beam at the waist of the cell (see Figure 18.4) is slated to be less than one wavelength in water, i.e., <1.5 μm.[1] The resulting resolution limit is about 2 μm. Water is used as a mounting liquid not only because it is compatible with biological and other specimen types, but also because it supports a sufficiently low acoustic velocity to effect good practical resolution at a given input frequency. At the same time water has high enough absorption to prevent stray multiple acoustic reflections from degrading the image (provided that input and output electrical connections are well shielded from one another).[10]

For maximum practical resolution, all relevant adjustments must be at their best. When used as a transmission microscope, the two lenses (condenser and objective) in the SAM, must be carefully aligned in all three directions, since the beam's diameter is so small (≈ 1 μm). This difficulty is removed by using the instrument in the back reflection mode.

In both types of acoustic microscopes, practical resolution also depends on maintaining the frequency of incident ultrasound. In the SLAM the laser beam scanner must be sharply controlled, and in the laserless SAM the mechanical scanning mechanism must function within certain specifications.

18.2.3. Contrast in Acoustic Images

Contrast is generally adequate in acoustic images because the variations in elastic parameters are greater than those in optical parameters. Furthermore, in either the SLAM or the SAM, electronic amplification of contrast is readily available within the bounds of the signal-to-noise ratio.[1] Both types of acoustic microscopes provide an interference mode of operation by comparing either the transmitted or the reflected signal with a constant portion of the initial electrical oscillation. The resulting phase-interference micrograph can then be compared with the amplitude (intensity) micrograph to a study contrasts. Using color contrast between acoustic and laser micrographs has already been mentioned as an optional feature of the SLAM.[9]

18.2.4. Aplanatic Lenses in SAMs

Aberrations in the spherical lens of the cell (see Figure 18.4) are negligible at this stage in development of the mechanical SAM. In fact the lens is inherently *aplanatic*, because the concave spherical face of the sapphire contacts water instead of air. The acoustic velocities of water and sapphire are in the ratio of 7.45:1, so that acoustic rays are sharply refracted at an angle closely parallel to the radii. The focal length of the lens is only 15% greater than the radius[12] (a very low f number and a very high NA).

18.2.5. Cleanliness in Acoustic Microscopes

Cleanliness is a factor in both types of acoustic microscopes because the specimen is immersed in the liquid medium (e.g., water). Solutes bleeding from the specimen into the liquid medium could change the velocity of sound significantly, and local concentrations of solute would introduce aberrations. Such considerations pertain largely to biological specimens that may contain a natural serum, an isotonic solution, or a fixing reagent unless it were cleaned of such matter. Of course when light is introduced into either type of acoustic microscope, any turbidity becomes an optical problem.

18.2.6. Focus Depth in SAMs

The *field in focus depth* (penetration depth) in the SAM is probably on the order of 1–100 μm. However with a thick section (6–8 μm) section,

although more information is obtained about differential densities and stiffness. But with opaque or relatively dense materials, such as metals, inorganic glass, kidney stones, or bone, the image comes chiefly from the surface and is therefore in sharp focus.[12] The SLAM accommodates samples of up to several millimeters thick. The acoustic penetration is generally inversely proportional to the acoustic frequency of the specimen (see Table 18.1). Evidently the field depth in focus is greater in the SLAM (at 100 or 500 MHz) than in the SAM (at 1000 MHz).

18.2.7. Focusing SAMs

The SAM is focused with a micrometer screw that holds the specimen in the cell (see Figure 18.3). When the object plane coincides with the narrowest cross section of the acoustic beam, the sharpest focus is achieved.[10]

18.2.8. Acoustic Radiation

The acoustic *radiation* in the Sonomicroscope® 100 is at a frequency of 100 MHz (or, as an option, 500 MHz).[4] As shown in Table 18.1, compressional (and in some materials, shear) waves of characteristic wavelength are set up in the mounting medium and the specimen. Coincidentally a flying spot of laser-coherent light scans the microfield, as shown in Figure 18.1. The flying spot detects perturbations of the mirrored slip covering the vibrating specimen. A photo detector converts the signaling laser beam into electric signals that are amplified and decoded into the amplitude-(intensity-) modulated acoustic micrograph. At the same time some of the incident light of the laser beam is reflected and/or transmitted by the specimen. Either the reflected or transmitted light beam can be photodetected and displayed by a separate video system. The resultant optical micrograph can be compared with or superimposed on the SLAM acoustic micrograph.[9]

The *radiation* currently in the SAM is up to 2000 MHz with water immersion. The *angle of incidence* for the acoustic lens can have a total aperture of 120° or more.[5] The acoustic cell (see Figure 18.4) can be tilted 90° to place the acoustic axis in a vertical position. Thus the entire cell can be immersed in a medium suitable for maintaining the cell's environment in a viable condition. Then living cultures can be viewed over an extended period of time.[7] The 90° tilt places the specimen in a horizontal position. With the condenser (input lens) on top, the acoustic microscope resembles an inverted light microscope, with some of the same advantages.

The *incident* angle in the Sonomicroscope® is normally 10° for aqueous media. Stages with other incident (insonification) angles are optional.[9] In the SAM the incidence is axial (0°).

18.2.9. Magnification

The *magnification* quoted[15] for the Sonomicroscope® 100 is 100×; for the 500 it is quoted as 5000×. At a practical resolution of 20 μm, a magnification of 10 or 20× would be sufficient to meet the minimum requirement (200 μm of the eye). At a resolution of 4 μm for the 500, 50 or 100× would be useful. How much more magnification is useful rather than empty (see Chapter 2) depends on use (such as in micrometry). The quoted[15] magnification of the SAM is 500–1000×. At a practical resolution of 2 μm, 100 or 200× is sufficient for the eye. How much more magnification is useful depends on other uses besides simple qualitative visual examination. Obviously if the theoretical resolution of 0.5 μm is achieved, 500–1000× is useful magnification.

18.2.10. Field of View

The *field of view* with the Sonomicroscope® is 1–12 mm^2,[1,15] and 30 images are produced each second on the television monitor, making it possible to observe dynamic activity. The field of view with the SAM is much smaller, 0.25 mm on a side. This area amounts to 5×10^4 elements of information and takes about 1 sec to scan.[10,15] Larger areas are composited (see Figure 18.6).[12]

18.2.11. Stray Acoustic Radiation

Stray acoustic radiation produced in the SAM by multiple reflections is attenuated enough to avoid degradation of the image (glare) if care is taken to shield the electrical *in* and *out* connections from each other.

18.2.12. Three-Dimensional Aspect of SLAMs

The *three-dimensional aspect* of the SLAM is heightened by superimposing acoustical and optical micrographs.[16,17] The Sonomicroscope® accommodates any sample, transparent or reflecting, as thick as several millimeters.

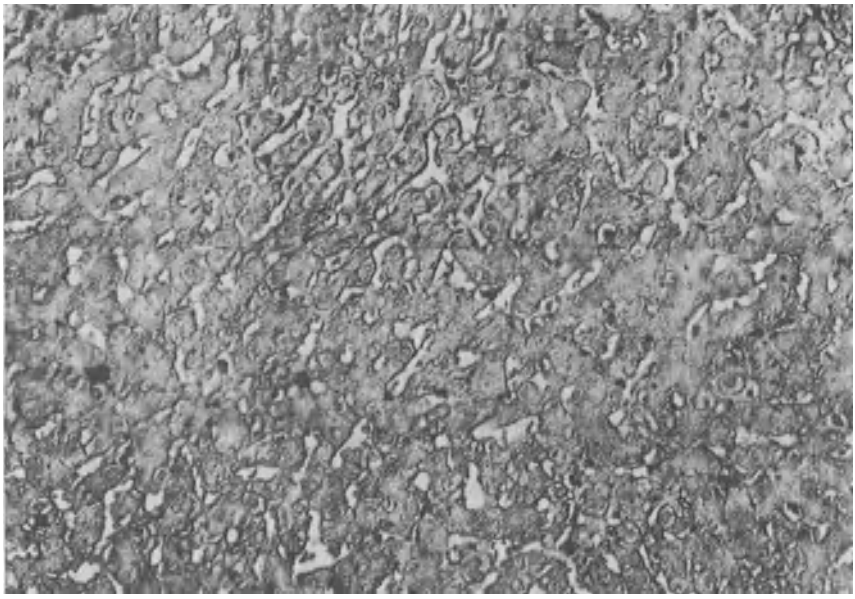


FIGURE 18.6. Human liver tissue as invaded by hepatitis. Acoustic frequency of 900 MHz in the SAM.[12] (Sample prepared and analyzed in the laboratory of Dr. J. Edward Berk.)

18.2.13. Specimen Thickness

The *depth* of thick biological sections for use in transmission with the SAM is 6–8 μm and often cut from frozen fresh samples. The depth of thin sections, generally cut from mounted fixed specimens, is about 1 μm.[6] Reflective materials, such as metals, their compounds, coal, cements, and ceramics, are studied at their surfaces. Specimen thickness is therefore a dimension of convenience. An example of limited field depth is shown in Figure 18.7. This can be a very useful feature of acoustic microscopy.

18.2.14. Working Distance

Working distance above a specimen in the SLAM is apparently ample for experimenting with the specimen *in situ*. Specimens can be placed directly on the stage or a glass slide; however they are covered with a semireflective cover slip. Interchangeable stages are available for the Sonomicroscope® for polarized shear-wave insonification, alternate

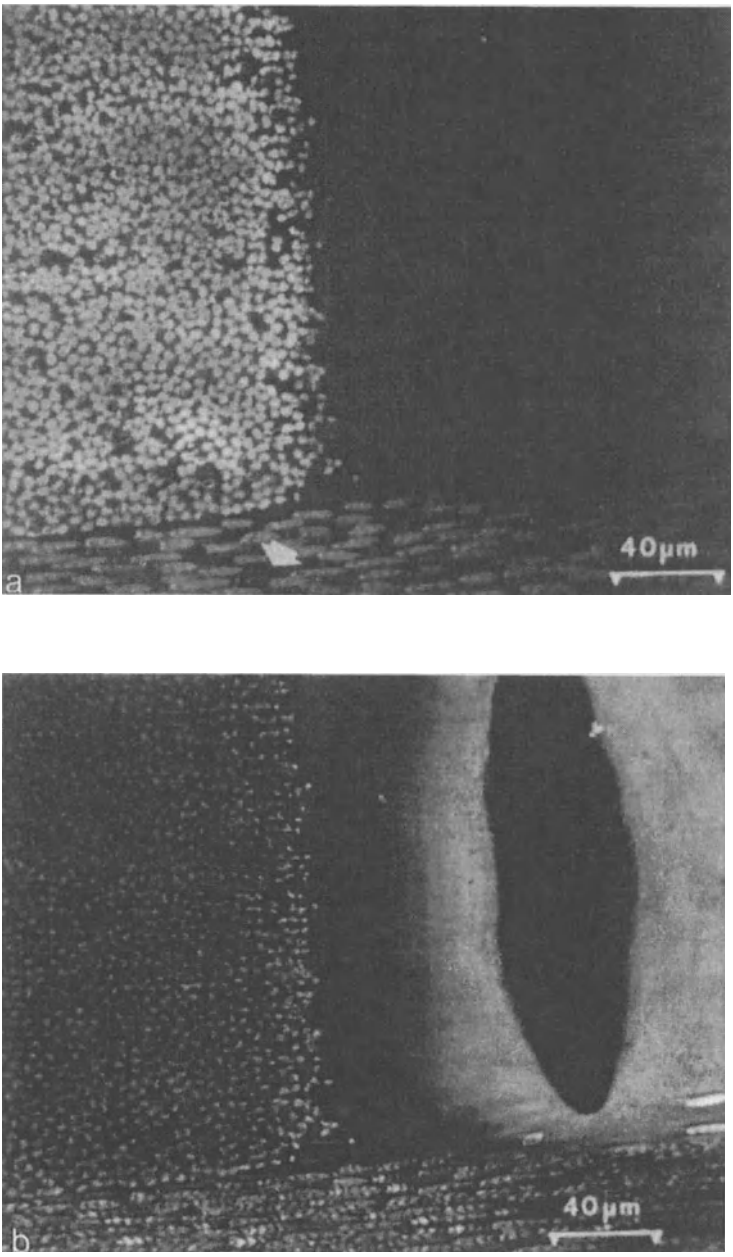


FIGURE 18.7. Two views of a woven composite. (a) Graphite fibers are in the focal plane; (b) the image is focused 10 μm below the field in view (a). (b) A void is clearly distinguished by good contrast with the resin. Original composite specimen courtesy of Mansour Mohamed, NCSU. Photomicrograph courtesy of D. Downs, X. Cui, A. El-Shiekh, P. Tucker and J. Russ, NCSU.[6]

insonification angles, and other special work.[3] In the SAM the specimen is mounted on a Mylar® polyester membrane stretched across a thin metal ring (see Figure 18.3). The ring is connected to the movement of a loudspeaker cone. The ring assembly is enclosed in the cell, which is filled with a liquid (see Figure 18.4). Consequently there is no practical working distance beyond the specimen. Working distance with the SAM in the back reflection mode is similar to that in a light microscope.

18.2.15. Specimen Structure

Structure contributes fundamentally to acoustic microscopy, since differentiating features of the image represent microvariations in density, stiffness, viscosity, or viscoelastic properties. Table 18.1 shows certain types of matter that differ greatly in acoustic properties. Even in microscopic amounts such different materials as air, water, soft tissue, hard tissue, plastic, elastomer, metal, or inorganic glass are sharply contrasted against one another in their composite acoustical image. Indeed brass is a composite in itself, and various possible constituents, such as α and β solid solutions of copper and zinc, are probably distinguishable, since the former are based on the crystallographic space lattice of copper and the latter on that of zinc. Iron can be present as α, γ, or other crystallographic structure. Acoustic absorption probably differs not only with the iron's structure but also with the amount of carbon in solid solution in steels. Moreover alloy steels vary in crystal structure and physical properties with the kind and amount of alloying element, such as vanadium, chromium, or nickel. There is perhaps no more dramatic example of the effect of structure on elastic properties than that of carbon crystallized as either diamond or graphite. Other industrial examples of crystalline allotropy are the white pigment TiO_2 as rutile and/or anatase, and $CaCO_3$ as calcite, aragonite, or vaterite.

18.2.16. Anisotropy

Anisotropy is a related structural phenomenon that can be acoustically important. Variations in elasticity according to propagation direction of ultrasound waves may be due to anisotropy in particular molecules—crystals, glasses, fibers, films, or other solids or semisolids.

Structure change has been investigated in stretched and unstretched elastin or collagen fibers.[1] The same kind of experiments would be interesting with elastomeric fibers and strained polymers.

18.2.17. Specimen Morphology

The study of *morphology* in both the SAM and the SLAM is limited by their respective resolving powers. Hence in pioneering research at this stage of development, it may be well to choose systems or problems involving relatively large microscopic morphology.

18.2.18. Information about Acoustical Images

Relevant *information* is inherent in the SLAM, since laser light images are readily available and superimposable on acoustical images.[9,18] With the SAM ordinary light images are independently formed.[1,10,11] In either case information accumulated during the long history of light microscopy (see Chapter 1) is available for interpreting acoustical images. Likewise macroscopical information about elastic properties and material strength should be considered when interpreting acoustical images.

18.2.19. Experimentation

Among all the dynamic *experiments* that can be performed with acoustic microscopes, the most relative are studies of the hearing mechanism in humans, land and aquatic animals, insects, and other creatures. Aquatic studies are especially relevant because of the use of water as the mounting medium. Indeed biological material in general can conveniently be studied *in vivo* or *in vitro*. Inanimate aqueous or hydrophilic material is likewise amenable to acoustical experimentation; examples are wet-strengthened paper, wet fibers, tow, yarn, cloth, wood, or sponge. It is conceivable that such specimens can be dynamically stressed *in situ*. Acoustic studies of the static and dynamic stiffening of fibers (dyed as well as undyed), at various temperatures seem to be logical experiments. Similar studies with other plastic materials, especially those containing pigments, carbon black, pulverized minerals, or man-made fibers, are needed by industry. Hydrophobic materials can be studied in a nonaqueous liquid suitable in the SAM cell. Metallic fibers, powders, foils, wires,[19] and fabrics can be studied acoustically with regard to cold working, annealing, plating, welding, alloying, and compositing. Copper-plated paper laminates, widely used as microcircuits in the electronic industries, present many problems that might be solved uniquely with the SAM. Solid-state materials and devices have already been examined.[11,20] Acoustic microscopy is providing unique information about a wide variety of subjects, including biological, electrical microcircuits, and composites.

18.2.20. Specimen Behavior

Behavior of disease on tissue has been studied in the SAM by post mortem examination and from samples of live objects. Comparing the elasticity and density of diseased tissue with that of healthy tissue is a unique capability of acoustic microscopes. Also unique is the opportunity to compare changes in elasticity and density (by audio scanning) with changes in optical parameters (by coherent or noncoherent[1,10,11] light). In the SLAM the two kinds of images can be compared by separate displays or a single superimposition with differentiating colors, as when locating the advancing front of a disease.[9]

While the study of elasticity and density in most biomedical specimens is new,[21] the study of such properties in industrial materials, even in microscopical specimens, is an old part of physically testing stress–strain relationships. In the acoustic microscope however, we have a new and distinctive feature that allows us to view directly the changes in elasticity. For example it is well known that natural rubber ages poorly, particularly in oxygen or ozone; with an acoustic microscope actual aging can be viewed. Changes in elasticity can be studied microscopically in other materials, such as plastics, metals, alloys, ceramics, and inorganic glasses.[20]

18.2.21. Specimen Preparation

Preparing a polished thick section of a specimen, such as metal, ore, rock, plastic, bone, or teeth, for SLAM or SAM is similar to the preparation for examination by reflected light (see Chapter 4). The only special requirement is that the so-called thick section be only a few millimeters thick.

For thin (1-μm) microtomed sections, the less preliminary preparation, such as dehydration and fixation, the better until it is learned what effect the treatment has on the specimen's elastic properties. Microtomy of a frozen specimen was, however, found to be more difficult than fixing, mounting in, and releasing from poly(methyl methacrylate).[12]

18.2.22. Photomicrography

Photomicrography is provided by the full-range of camera backs and automatic fast-developing photographic film. With the advantage of speed, simplicity, and convenience of the Polaroid® system come the restrictions on choice of photosensitive materials, variations in photoprocessing, multiple photoprinting, and transparencies for projection.

Photomicrography by means of the video image[9–12] brings limitations to resolution caused by raster lines. A video tape recorder[9] can relieve some of that limitation, at least during information storage, and at the same time permit dynamic events to be recorded, such as cardiovascular contraction and the fracture process in steel.

18.3. SUMMARY

There are two kinds of acoustic (ultrasound) microscopes. The SLAM employs a scanning laser beam microphone.[9] The other acoustic microscope, the SAM, employs mechanical movements.[10,11]

The SLAM is lensless; it is an acoustic shadowgraph projection microscope whose resolving power is limited by the wavelength of the ultrasound and diameter of the laser flying spot. With an input frequency of 100 MHz, the limiting resolution of the SLAM is about 20 μm; with an input of 500 MHz, the limiting resolution is about 4 μm.[9] In transmission, the SAM possesses two small, identical planoconcave lenses: One acts as a condenser of the acoustic beam, and the other acts as the objective. Both lenses are sapphire crystal, immersed in a liquid usually water (see Figure 18.1). Although each lens is a single component, it is practically aplanatic, because the ratio of acoustic velocity in sapphire to that in water is so high (7.45:1). As a result the acoustic rays are very sharply focused. Since the focal length of each water-immersion lens is only 15% longer than the radius, the NA is very high. The velocity of sound in water is so low that the wavelength (λ) at a frequency of 1000 MHz is only 1.5 μm. This corresponds to a limiting resolving power in the SAM of 0.5 μm.[12] Because of the difficulty in aligning both the transmitting and receiving lenses at higher resolution, most recent SAMs have used back reflection with a pulsed signal. In addition to alignment problems, this is also because acoustic waves need a solid or liquid for conduction, and they have an attenuation proportional to the square of the frequency in most fluids.[5]

After characteristic interaction with the specimen in the SAM, acoustic energy is converted by a piezoelectric transducer into an electromagnetic signal that is imaged on a television screen. In the SLAM the output acoustical beam modulates the laser beam as it scans the field. The modulated laser beam is received by a photodetector that yields two signals: One depends on the optical features of the specimen; the other is proportional to the *acoustic* amplitude within the specimen. Each type of signal is television monitored separately, but the two images may be superimposed, even in contrasting color. With the laserless SAM, ordinary light is used separately to make an optical image for comparison with the acous-

TABLE 18.2
Guide to Various Applications for the Three Most Common Techniques of Acoustic Imaging[9]

	C-scan		SLAM			SAM	
Frequency	1 MHz ...	10 MHz ...	30 MHz ...	100 MHz ...	200 MHz ...	500 MHz ...	1 GHz
Wavelength	1.5 mm			15 mm			1.5 mm
Medical ultrasound							
Conventional ultrasonic nondestructive testing							
Coarse-grain metals: defect detection							
Cracks in plastic encapsulated integrated circuit (IC) devices							
Composite materials							
Cracks in ceramic IC packages							
Spot welds							
Heat sealing food pouches							
Ceramic chip capacitors: delaminations and cracks							
Hermetic seal reliability							
Polymer-foil package lamination							
IC die attach							
Thick-film adhesion and porosity							
Laser spot welds							
Fine ceramics: defect detection							
Fine-grain metals: defect detection							
Seam welds on tin cans							
Lead bonds on hybrid circuits							
Ceramic substrate porosity and cracks							
Cracks in silicon wafers							
Thin-film adhesion							
Grain structure determination							
Fine-line inspection on silicon							

Source: Courtesy Sonoscan[9]

tical image of the same field. In both acoustic microscopes the optical image is helpful in interpreting the acoustical image, especially during experimental applications.

Acoustic interference microscopy can be incorporated into either instrument by combining signals from attenuated acoustic rays with a reference signal. The resultant interferogram consists of light and dark bands. By analyzing the position of the bands, quantitative data on density and elastic properties are derived on a microscale.

Applications of acoustic microscopy in the micron range stem from experience in the macroscopical (millimeter) range with the familiar pulse-echo devices used in medical diagnoses and in material flaw detection. The micron range of resolution is also that of light microscopy. Thus the exploration of *elastic* or *dense* microstructure by ultrasound waves is where light microscopy was four centuries ago. It is now hoped that acoustic microscopy will progress to areas where electron microscopy began.

Table 18.2 lists various applications for the three most common techniques of acoustic imaging (also see the frontispiece).

19

Image Collection, Analysis, and Reconstruction by Computer

19.1. INTRODUCTION

Automated image processing has facilitated new insight into complex data; for example it is no longer necessary to view a three-dimensional specimen as a series of two-dimensional slices. A three-dimensional image can be reconstructed, and this new image can be electronically rotated or subsections of it studied at high resolution, although a more common application is automated statistical characterization of a complex two-dimensional image. Parameters can be measured and aspect statistics calculated on a large enough population to make such numerical characterization impossible by manual means. We must recall the concept of empty magnification versus resolution when we hear that it is possible to magnify up to one thousand or more by video microscopy with a low-resolution objective. Classical principles still apply to these new techniques.[1]

As with most tools hardware and software can be fairly simple or extremely complex. A typical system includes a microscope, video system, display monitor and (or) a recorder, and for a few systems integration into a multisite or even multicountry network. The specific microscope selected determines requirements for other components by determining or greatly influencing available light levels, resolution, contrast, image size, and type of necessary information storage.

The human eye and brain plus conventional photomicrography do an excellent job of acquiring and processing two-dimensional information, but these are deficient when processing and reassembling three-dimensional information or four-dimensional information that has time as a

variable. Such three-dimensional and four-dimensional processing was much more difficult and expensive until the advent of relatively inexpensive, automated image acquisition equipment, such as frame grabbers, and image-processing equipment, such as personal computers (PCs). This hardware has been accompanied by numerous packaged software programs from many suppliers. Of course increasing the complexity of image manipulation may exceed the abilities of a PC and thus require an integrated image analysis system from one of the larger microscopical dealers or even connection to a massive computer analysis network. However simpler, less-expensive systems satisfy most needs.

All involved subtopics are undergoing such rapid changes that it is difficult to keep current. Inoué[2] lists many journals with useful information, including *Journal of Microscopy*, *IEEE, Transactions*, and *Microscopia Acta*; others that should be included are *Computer-Assisted Microscopy* and *Bioimaging*, and a complete list would include major journals for almost every discipline. Also recommended are relevant trade journals. Inoué also provides a useful glossary of technical terms to assist the user with complex terminology acronyms.[2]

Numerous small companies now provide packaged systems, and such companies often have considerable expertise or occasionally very limited expertise. If expert advice is needed with the purchased items, it is wise to check on the professional reputation and support ability of the potential supplier, since software development is a complicated, evolving field currently lacking clearly documented guidelines.

Proper use of automated image processing always depends on correctly using the microscope, which has been the main thrust of this text.

19.2. MICROSCOPES

Automated image collection can be done through any type of macroscope or microscope. Automated stages are special features that translate in three directions, *X*, *Y*, and *Z*, in synchronization with data collection. Such automated translation is normally done discretely rather than continuously, and sufficient time must be allowed at each increment or step to allow accumulation of desired data from the field of view. Image equipment can also be purchased to refocus automatically the microscope as the stage is translated. Such systems can free the microscopists of many complex or tedious procedures, and the tasks can be automated; however automated stage systems are expensive, and it may be necessary to employ many people to approximate their operations manually.

19.3. VIDEO SYSTEMS

The standard digitizing board or frame grabber provides a 525-line image, with each line composed of 525 pixels (discrete picture elements) and 30 displays or frames per second. Many of the newer systems provide 640 pixels per scan line and 480 scan lines per display.[3,4] The standard-format camera for generating the pixels in black and white is known as the RS-170; other systems exist outside the United States, and even within the United States, for color.[1] Cameras can achieve higher resolution, even beyond 2000 lines; a tube camera, such as the vidicon camera, is the standard, earlier model. Similar, newer cameras are the nuvicon and plumbicon, which have greater light sensitivity;[3,4] however vidicon cameras do have spectral sensitivity closer to that of the human eye.

More recent solid-state devices that function as cameras are the charge-coupled device (CCD) and the charge injection device (CID); the CCD is the more common of the two. The basic concept for both devices involves using separate devices or elements for each picture point or pixel to be measured and measuring all points simultaneously. For low-light intensities, such as that used with fluorescent microscopy, solid-state cameras can be obtained to integrate the intensity for as long as several minutes. Such cameras are often cooled to control the amount of noise.[3]

The CCD and CID cameras are simpler and more rugged than tube-type cameras;[3] however these former lack the resolution of tube-type cameras because of the need for separate sensing cells for each picture point to be shown. The CCD cameras are recently being marketed with up to 2048 × 2048 pixels with 12 bits per pixel of resolution, so they are rapidly evolving.[3a]

Digitization by digitizing boards or frame grabbers converts the camera's analogue *(A)* signal to a digital *(D)* signal for the computer; the digitizing boards and frame grabbers are known as *A/D* converters.

Resolution with frame grabbers can be improved by collecting a series of scans of the same image and averaging across this series.[4] A special *C*-mount adapter is normally required to connect a video camera to the microscope, just as a similar adapter is needed to connect a light-photographic camera.

Since the components are in series, there is no advantage in using a microscopical resolution higher than that imposed by the camera. Actually there may be disadvantages, such as unnecessary loss of size and field depth.[5] As a general rule 300 dots/inch appear continuous at a normal viewing distance, and Webb and Dorey note that a person with 20/20 vision can distinguish dots 1 arc-second apart, which is equivalent to a

200-μm separation at a 1-m viewing distance.[5] Lack of an adequate true three-dimensional display of images is a limitation with videoimaging, especially when micromanipulating specimens as with a stereomicroscope.

19.4. COMPUTERS AND SOFTWARE

All current larger PCs can be used for image processing. As with other microscopical equipment, it pays to first decide what tasks are to be done and learn the general concepts before purchasing equipment, since software selection is a complicated task.

It is generally advisable to see prospective software demonstrated on your specific tasks before making a purchase. A vendor's ability to provide continuing technical support should also be considered.*

Software exists for both image-acquisition software and image-processing software, and it can be obtained to conduct almost any desired measurement or data manipulation. Major light and electron microscopical companies also have software in depth, and it should be considered if such a wide range of programs is necessary for your tasks.

Digitized images occupy considerable storage space. An image of 512 × 512 pixels and 1 byte per pixel requires one-fourth of a megabyte (Mb) of storage.[7] Storage media can either be archival as with magnetic tape, which has a lower cost but longer access time; more expensive Winchester hard disk with fast access time; or more reasonably priced optical disks, which can store up to 650 Mb of data.[7] Programs and systems have recently been developed to operate with workstations that would previously have required main frame computers because of the complexity of the operations.[8]

19.5. DISPLAY AND NETWORKS

If you use a single unit without windowing capabilities, you can alternate between the video image displayed on the computer screen and the computer menus. With a double-monitoring system, you can dedicate one monitor to display results from the frame grabber and use the other for computer menus.[7] Multiple monitors can be used to compare results from different processes.

*More than 40 suppliers of image analysis products have recently been listed.[6]

Most monitors have a resolution of 512 pixels per line and 480 lines per display,[6] but special monitors have higher resolution. Polaroid for example has a higher resolution system where the original image is recorded photomicrographically onto film and then scanned into a computer using a digital scanner with approximately four times the resolution of standard CCD cameras.[9]

Palatini *et al.* review their use of a PC to capture images and then a Digital Equipment Corporation VAX computer to store, retrieve, and distribute images on site and internationally.[10] Such an image system is illustrated in Figure 19.1. It is not necessary to illustrate the video camera's image in the final report; the electronic camera and computer results can be processed for the desired data and a conventional photomicrograph recorded as an illustration, since high-resolution video printers are at the stage "where instant film was 15 years ago."[1]

A review of terminology for computer networking has been published;[10a] critical concepts, such as modem (an acronym for MOdulator/DEModulator) and baud rate, are defined and recommendations are made for purchasing total systems. A modem converts the digital computer signal into an analog signal that can be sent by telephone transmission; baud rate refers to the data transmission speed of the modem.[10a]

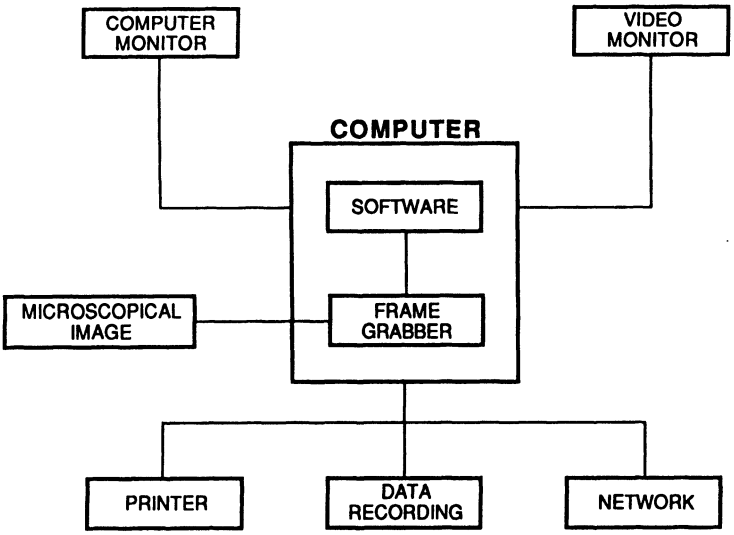


FIGURE 19.1. A typical image analysis system.

19.6. CONTRAST, LOOK-UP TABLES, AND COLOR

Contrast and color, i.e., gain and offset, of the video image can be artificially influenced if desired; of course a fairly representative color of the original can also be produced.

For artificial color each pixel is associated with a specific gray that is coded from zero for the darkest gray (or black) to 255 for the brightest white.[12] A similar manipulation exists for color images with a much wider possible range of values. We are able to distinguish only about 30 different gray levels, but we can distinguish hundreds of thousands of colors. Shades of gray can be converted into artificial colors, and such pseudo color can improve contrast. On the other hand if improperly used such artificial color contrast may lead a viewer to concentrate on image details and neglect the information.[4]

Gray or brightness values of discrete pixels are converted into color by using look-up tables (LUTs) to find the gray value of a specific pixel, then converted it into a number from 0–255. Numbers in this range are divided into three groups: blue, green, and red, and the shade of each is related to the grayness of the original signal.[12] In this manner artificial colors are assigned to different shades of gray from a monochromatic camera. True color images are considerably more expensive to collect[4] because three different collection sensors must be used: one each for blue, green, and red. After the color image is collected it is also considerably more expensive to store these three images, and the software is also much more complicated for analyzing the three color combinations.

19.7. SUMMARY

Automated image analysis is practical and beneficial. While the human eye and brain do an excellent job of acquiring and processing simpler two-dimensional images with conventional photomicrography, reassembling three-dimensional information or four-dimensional information, which varies as a function of time, proves to be more difficult. Resources are generally not available to collect and process large amounts of routine microscopical data, which is relatively simple with an image analysis system.

With most microscopical systems a video camera is needed with the microscope to supply the analog signal to a digitizing board or frame grabber, which converts the analog signal to a digital signal for the computer. With SEMs for example, separate initial digitizing boards are not

needed, since an image is not initially generated since there is a direct feed of the electrical signal to the computer.

Numerous packaged programs exist to process collected data. Either two-dimensional or three-dimensional images can be reconstructed. Two-dimensional digitized information units are called pixels, and three-dimensional digitized information units are called voxels. Series of images as a function of time can also be obtained if needed. Most monitors display 512 pixels per line and 480 lines per display, but higher resolution can be obtained at considerably increased cost. Since all components are in series, the final resolution is no better than the poorest link, and this means that resolution of the total system must be increased to increase the final resolution. Collected information can be processed on the computer and then stored or distributed on a network.

Details on a total system involving many programs and different features are published in *Microscopy: The Key Research Tool*,[11] which should be consulted for information on three-dimensional reconstruction and computer graphics.[11] These software programs are written in generic C language and run on a variety of machines using the UNIX operating system.

The *MSA Bulletin*, published semiannually, contains a section, "In the Computer Corner." The MSA has a growing list of computer programs, reference spectra, and tables of physical constants that can be obtained through an electronic network, BITNET. Communication can also be made by electronic mail. These services are free or available at nominal cost.*

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20

Specimen Preparation

20.1. INTRODUCTION

Specimen preparation depends to a great extent on what information is already known and what is sought about *composition, treatment, properties, behavior, structure,* and *morphology* (see Figure 20.1).[1] We usually know the composition and condition but seek the structure and morphology to predict properties or behavior; of course we could know the composition and condition and test for properties and behavior to determine structure and morphology—and so on.

Often while testing for properties, microscopical information is obtained simultaneously. For example Figure 20.2[2] shows the fracture surface of a test bar of thermoreacted resin that exhibited a certain statistical strength according to ASTM standards.[3] Coincidentally the close-up view discloses the *granular texture* of the fracture surface imparted by the wood flour filler.[2,4]

Figure 20.3 shows typical areas in two sheets of rubber, one (left) varnished and the other (right) unvarnished. Both surfaces were weathered simultaneously by a standard procedure.[3] The unvarnished surface (right) became cracked in a typical granular pattern while the varnished rubber surface seemed to be unchanged (by comparison at the time with a piece of original sample).[2]

Figure 20.4 shows a *cross section* of a sheet of artificial rubber after testing in acid and simply cutting across it with a wet razor blade.[2]

Sometimes a macrotest can be turned into a microtest to allow for some microscopy. For example the paper industry's most important resin (the one that makes writing paper, not blotting paper) is *rosin,* a natural mixture of complex unimers derived from the black liquor by-product of pulping coniferous wood. However recovered rosin is so pure that it sometimes crystallizes out of solution before it can be applied to the paper

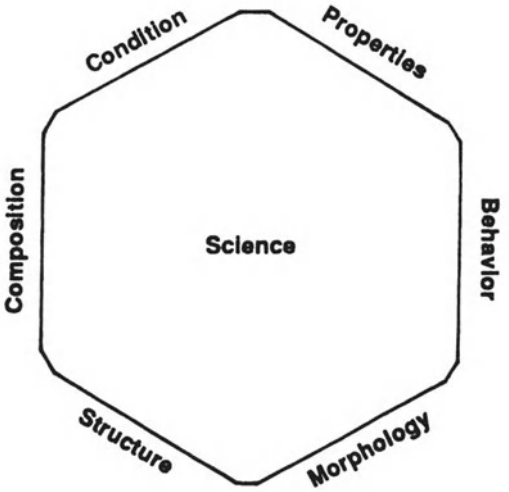

FIGURE 20.1. Facets of the scientific area: *composition* in physical–chemical terms; *condition* as used in mechanical tests; *properties* for practical utility; *behavior* in use, weather, or test; *morphology* in terms of size and shape; *structure* in terms of construction units.

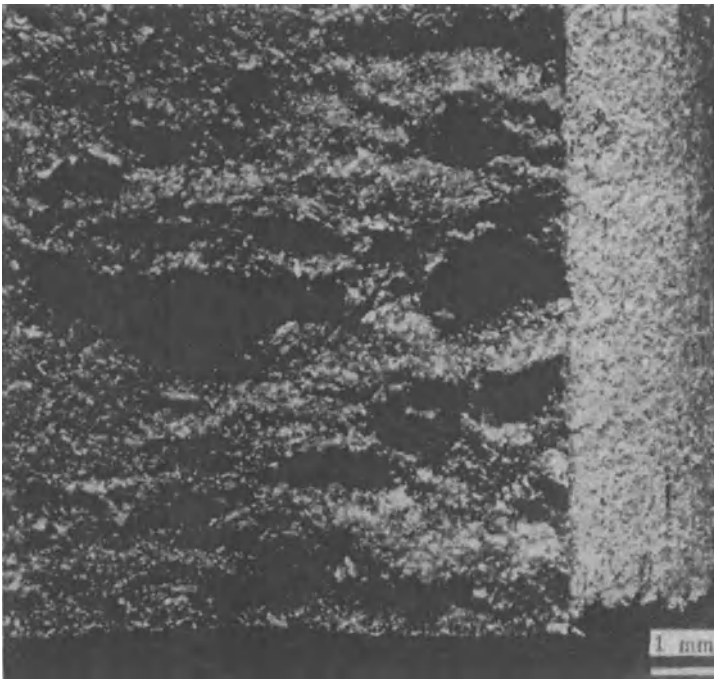

FIGURE 20.2. A fractured test bar of thermoreacted resin by oblique reflected light; left: Fracture surface; right: Milled slot that directed the fracture during an impact test.[2] Courtesy of McCrone Research Institute.

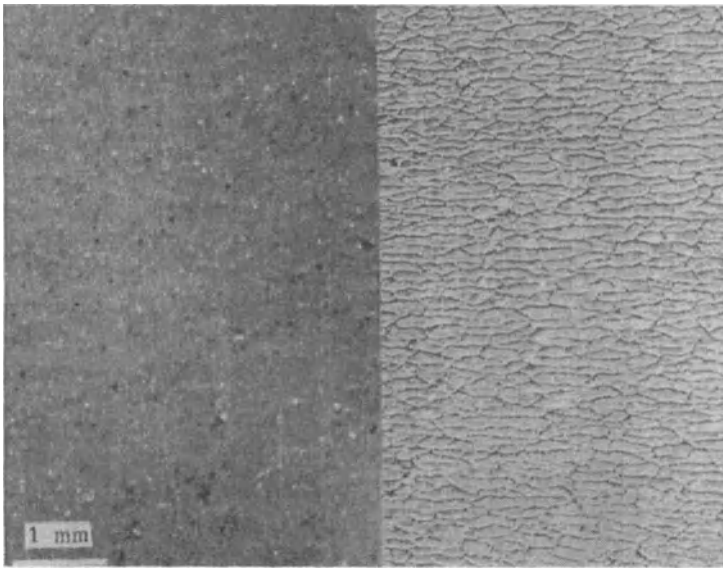

FIGURE 20.3. Sheets of rubber after weathering; by reflected light; left: varnished; right: unvarnished.[2] Courtesy of McCrone Research Institute.

pulp. Inhibitors are added, so there are standard macrotests[4] for crystallizability. The Kirk flask[5] (see Figure 20.5) was devised to be large enough to give reproducible test results yet small enough and with side sufficiently flat to reveal crystals between crossed polars with a stereoscopic microscope (see Figure 20.6).[2]

By looking into a stereoscopic microscope with one eye and putting a camera over the other eyepiece, an automated microdynamometer was developed[6] (see Figure 20.7) and improved[7] (see Figure 20.8). Figure 20.9 shows the dimensions of a standard microspecimen adopted for the orig-

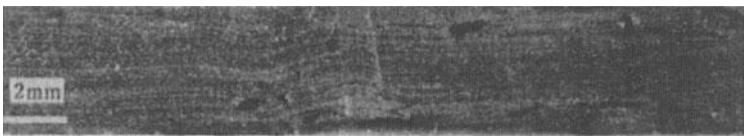

FIGURE 20.4. Cross section of an artificial rubber sheet; oblique, reflected light. Specimen shows cracks and voids after testing in acid. Preparation: As cut with wet razor blade.[2] Courtesy of McCrone Research Institute.

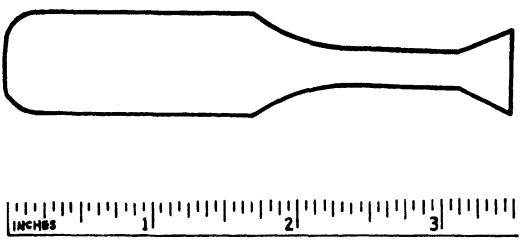

FIGURE 20.5. The Kirk Flask #1000 for incubating rosin or rosin size and subsequent microscopical examination; devised by A. F. Kirkpatrick and furnished by R. C. Ewald.[5] This cell is thick enough to permit reproducible growth of crystals from the viscous medium. It is easily filled yet easily sealed by drawing the neck closed in a flame. The sides are flat enough for viewing under a stereomicroscope. Courtesy of McCrone Research Institute.

inal automated microdynamometer.[2,6] Figure 20.10 shows a typical microspecimen stretched to breaking in the microdynamometer.[2,6] Figure 20.11 shows a load time curve for Lexan® polycarbonate, plotted by a microdynamometer, with frames from a cinamatographic record corresponding to the times shown by selected points on the graph.[6] In this particular case the testing device was brought to the microscope instead of vice versa.

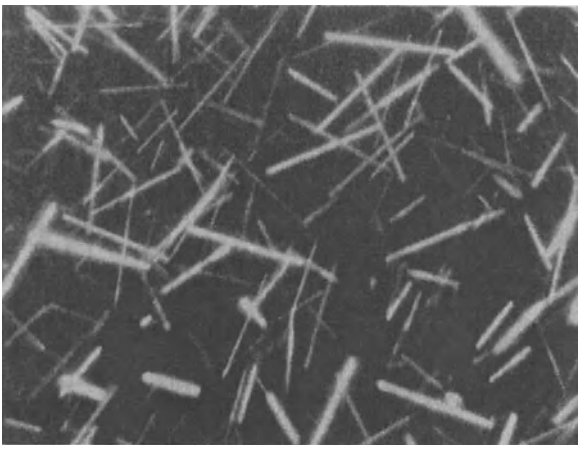

FIGURE 20.6. A specimen of rosin size after a crystallizability test in a Kirk flask (see Figure 20.5). The average crystal length is 19 mm, yet the crystals are well within the focus depth of the stereomicroscope. The crystals, shown here between crossed polars, are highly anisotropic, as indicated by those that happen to be in positions of brightness.[2] Courtesy of McCrone Research Institute.

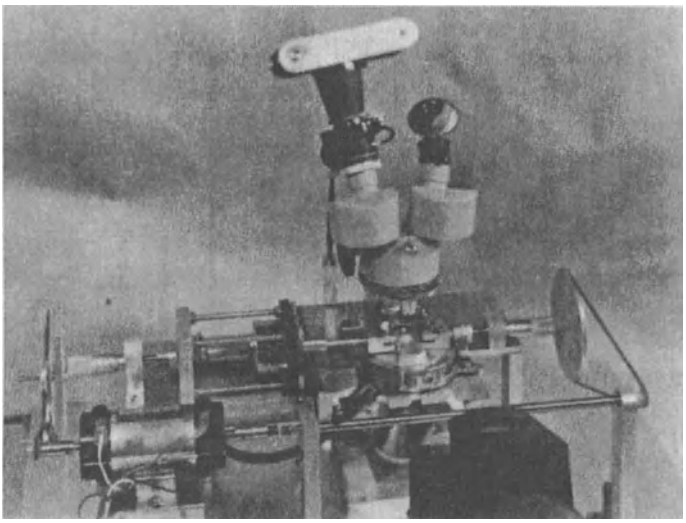

FIGURE 20.7. Prototype of an automated microdynamometer developed at the Research Laboratories of American Cyanamid Company in Stamford, CT.[6] Courtesy of McCrone Research Institute.

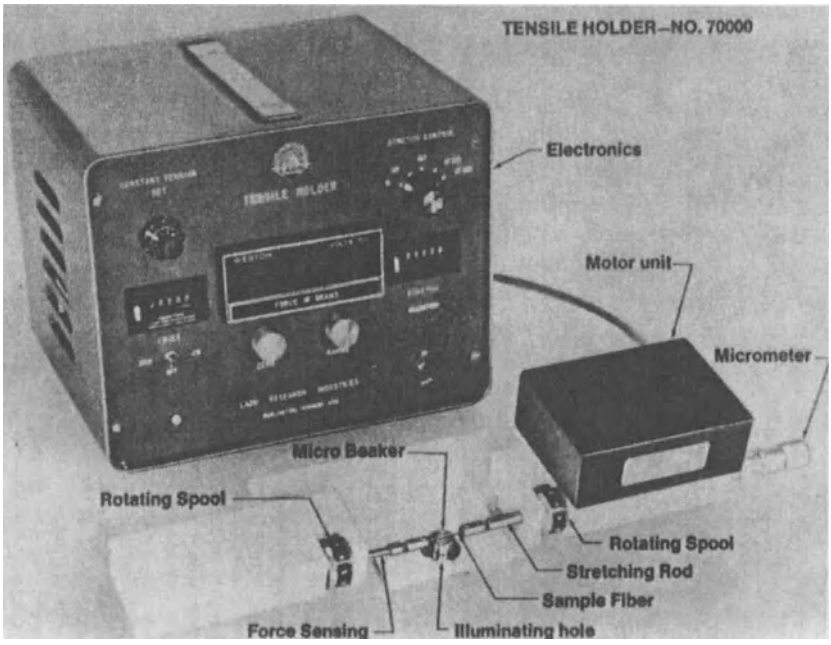

FIGURE 20.8. The Ladd Tensile Holder. Courtesy of Ladd Research Industries.[7]

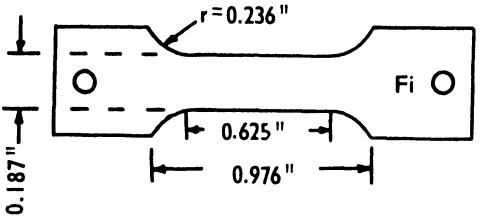

FIGURE 20.9. Diagram and dimensions of a standard[3] microspecimen of transparent plastic foil (film) for the microdynamometer.[6] Courtesy of McCrone Research Institute.

20.2. PREPARING FIBER, FUR, AND HAIR

Natural and man-made fibers are important units in textiles, paper, cordage, forensics, etc. Such fibers are fit for the *polarizing light microscope* because of their relevant microscopic size, characteristic anisotropy toward light (see Chapter 6), and ease of preparation.[2,4] Indeed for longitudinal views, fibers need no more preparation than immersion in a colorless, medicinal grade of mineral oil (n = 1.48).[4a]

Cross-sectional views can be prepared with a "microbread" slicer,[8] an assemblage of two single-edge razor blades firmly clamped together.[2] Perhaps even shorter lengths can be obtained with a dual-edge razor. Another quick method of cross-sectioning fibers is to stuff them into one of the 36 ready-made holes in a piece of black vinyl board the size of a

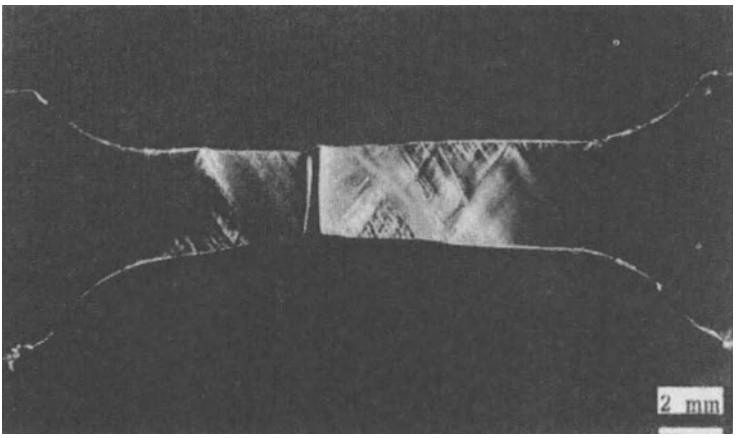

FIGURE 20.10. Microspecimen of Lexan® polycarbonate stretched and broken *between crossed polars* in the microdynamometer.[6] Courtesy of McCrone Research Institute.

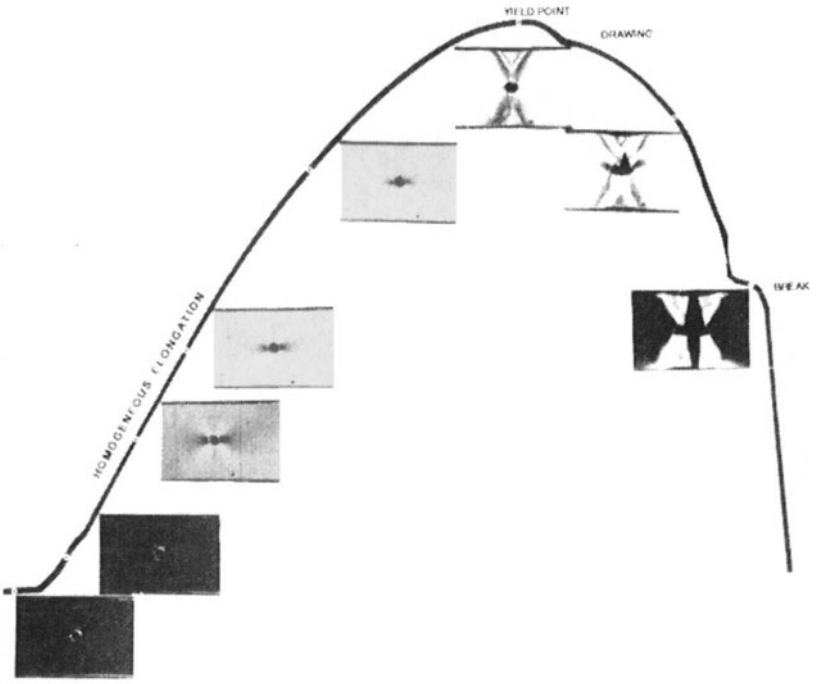

FIGURE 20.11. Automated resinography of a plastic film. Load-time curve for Lexan® polycarbonate plotted automatically on the microdynamometer with frames from a cinematographic record corresponding to times shown by selected points on the graph.[6] Courtesy of McCrone Research Institute.

microscopical slide.[9] Since only one hole is used per sample, a slide can hold as many as 36 different specimens, easily filed for future reference. Stuffing involves the following steps:

Push a flexible needle threader through a hole until the wire loop opens up. The open loop is filled with specimen fibers *plus a strong black thread.* Pull the black thread into a loop. When the loop becomes small, it is filled with specimen fibers.

Pull the black thread so that the bundle of specimen fibers bends, doubles, and stuffs the hole firmly.

Cut away excess fibers with scissors.

Shave with a sharp (fresh) single-edge razor blade.

In the old-fashioned method of cross-sectioning fibers, (1) a loop of thread is inserted into a hole in a cork stopper with a sewing needle, (2) fibers are inserted into the loop and pulled into the cork, and (3) the fibers are sliced. A newer method involves stuffing fibers or yarn into a piece of plastic tubing and making thin slices of the composite[12a]; this is an especially good technique when using an SEM.

For light microscopical examination, the perforated slide is placed on an ordinary glass slide on the microscope's stage and illuminated from below. Some textured yarns and all dull (pigmented) yarns require a mounting liquid (such as mineral oil) to decrease the scattering of light. Compared with cork the perforated plate produces a more uniform and reproducible section, which is simultaneously mounted and preserved. Both methods are suitable for classroom use (see Figure 20.12). A drawback to purchasing perforated plate is the minimum number per order of 1000.[9] Perhaps one or more of our microscopical supply houses would be willing to accept smaller orders.[10,11,11a,12]

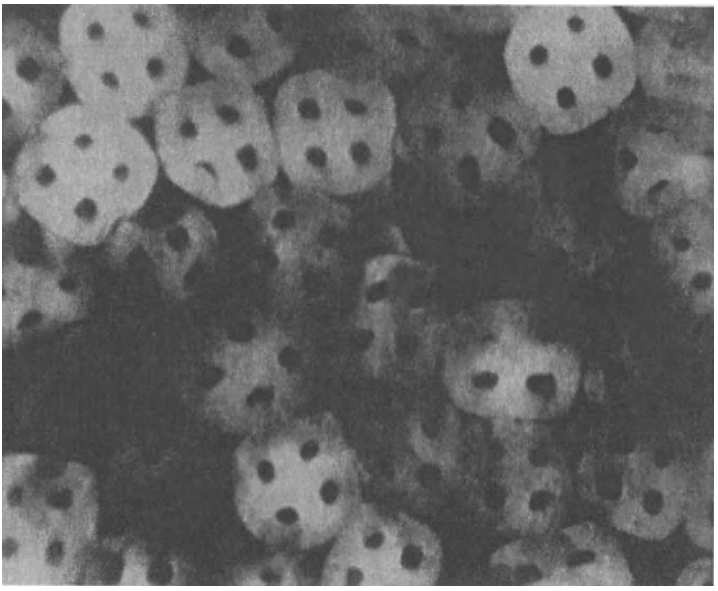

FIGURE 20.12. Cross sections of Antron® II nylon fibers mounted in a hole in a black vinyl card, then cut on both sides with a razor blade and photomicrographed in class.[2] Courtesy of McCrone Research Institute.

20.3 MICROTOMY

Microtomy is the technique of cutting sections suitable for examination in the *light* microscope by transmitted illumination.[13] A microtome consists of a holder for the specimen, a feeding mechanism for regulating the thickness of the cut, and a guide for the knife.[14a] Microtomes range in size from the small Hardy type[15] for fibers to heavy bench types for three-dimensional specimens, including those extensively prepared by fixing, polymerizing, impregnating, etc. Figure 20.13 illustrates the use of a microtome[16–18] for fibers, including the use of collodion[12] to attach the section of a fiber to a microscopical slide. Figure 20.14, which is related to Figure 20.1, shows the correlation of a composition and *condition* with structure and morphology to learn about properties and behavior through cross-sectioning (see Figure 20.13) and employing light microscopy.

If the material to be sectioned is porous, friable, or has poor tenacity,

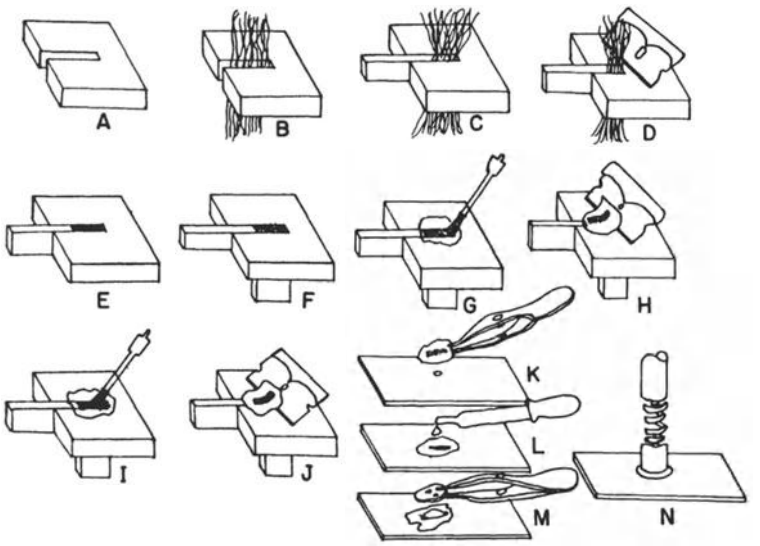

FIGURE 20.13. Sketches showing the use of a small microtome for fibers.[2] (a) The slotted top chromium-plated for protection against scratching by razor blade, forceps, etc.; (b) filling the slot; (c) plunger pushing fibers together; (d) trimming bundle with razor blade; (e) appearance after trimming; (f) further compaction with plunger; (g) embedding ends (or edges) prior to sectioning with a lacquer, such as MICO-LAC solution; (h) preliminary cutting with unused blade; (i) coating with collodion or other lacquer; (j) slicing; (k) removing slice; (l) lubricating with water; (m) removing slice; (n) firming slice. Courtesy of McCrone Research Institute.

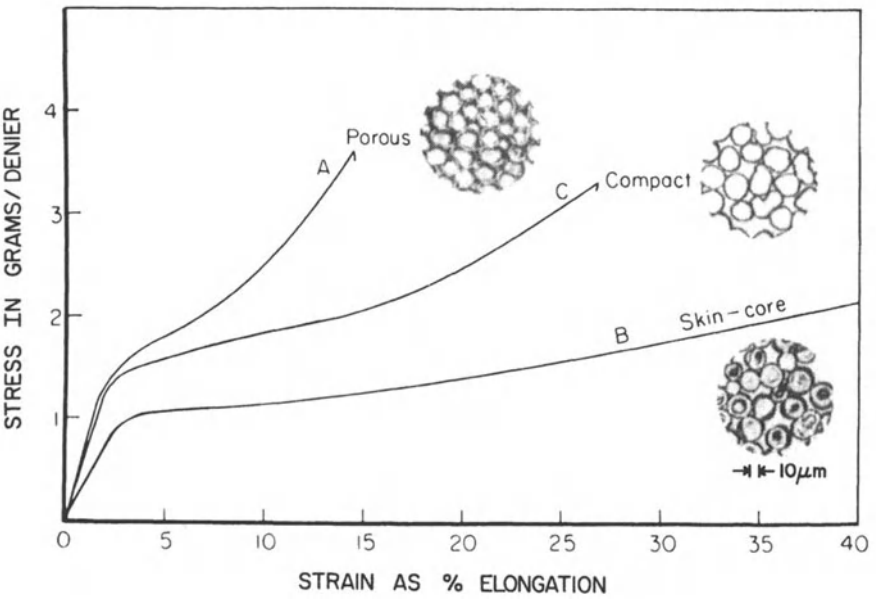

FIGURE 20.14. Resinographic study of experimental acrylic fibers cut successively from the same wet tow and given various treatments before drying. Corresponding structures and stress–strain properties are depicted.[1]

some sort of embedding must be used. Dry, porous materials can be simply and quickly infiltrated by molten paraffin[12] or harder vegetable waxes, provided they are not affected by the medium at a temperature of 60–70 °C (140–158 °F). Paper, leather, and other porous materials can be supported this way during microtomy. Wax however is not strong or tough enough to hold textile fibers, and these must generally be embedded in plastic resin, as described for ultramicrotomy. Moist or wet materials, such as plant and animal tissues,[19] shrink or are otherwise distorted during heating. Such materials generally behave well during quick freezing, and evaporating ether or carbon dioxide in an attachment to the microtome keeps the specimen frozen while it is being sectioned.

20.4. ULTRAMICROTOMY

Ultramicrotomy is the technique of cutting sections thin enough for examination in *electron* microscopes.[13,14] In the 1940s and 1950s, specimens

had to be *ultrathin*, less than 100 nm (0.1 μm) thick at the available electron accelerating voltages. With the development of higher accelerating voltages, and especially the construction of STEMs, thicker specimens (0.1–2.5 μm and even thicker) are acceptable (see Figure 14.9); nevertheless ultrathin specimens cut on an ultramicrotome continue to be required. Because now-a-days the specimen is moved past the cutting edge (sharp glass or diamond), the bulk material must be firm or it becomes distorted while being cut. From the 1970s to the present, biological and aqueous nonbiological specimens are dehydrated and embedded in a resin. The results are not always consistent, apparently because of variations in the embedding medium, process, ageing, temperature, humidity, etc. Because most biological specimens and many nonbiological materials contain water or can be wetted, they can be frozen and ultramicrotomed at low temperatures; elastomers and other soft materials can be stiffened and then ultramicrotomed at low temperatures.[13]

Reid and Beesley[13] describe ultramicrotomes on the market as of 1991, and list sources of ultramicrotomes, microtomes, cryoattachments, steel, glass, diamond, or sapphire knives, equipment for embedding, block trimming, and other items. Hayat[20] gives directions in transmission electron microscopy as of 1986, for preparing fixatives, tissue blocks, sectioning, staining, support films, and specific biological preparations.[20,21a]

20.5. REPLICATION

To answer the question, "why one side of a specimen of commercial nylon film slides easily when folded against itself but encounters great resistance when folded the other way," each of the two sides was positively replicated by evaporating silica and then electron micrographed.[1] Figure 20.15 shows that the rough side is due to projecting crystalline grains of nylon, whereas the other side is smooth. Figure 20.15 also shows that the molten nylon was cast against a smooth solid to form a smooth surface, but on the other side of the film, spherulites were free to grow in air, creating a relatively rough surface.

Another type of replica employs the extraction technique. There are three kinds: (1) direct carbon replica, (2) carbon replica shadowed with platinum, and (3) two-stage plastic–carbon replica.[21] The purpose is not only to extract particles from their matrix but also to maintain them in their relative positions. Accordingly particles must be small enough to be supported by a carbon film; instructions are provided by Goodhew,[21] who also gives advice on mounting and storing specimens and a list of materials and suppliers as of 1984.

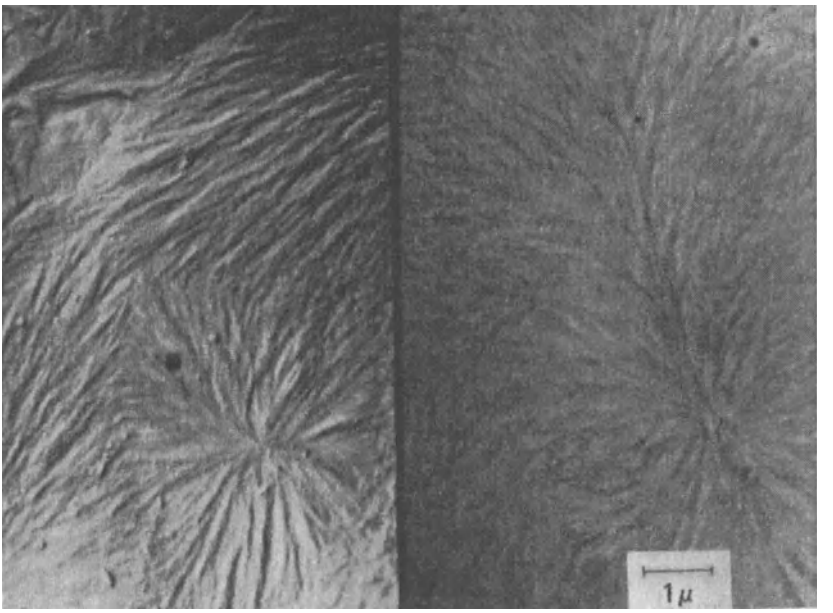

FIGURE 20.15. Commercial film of nylon showing differences in texture between the two sides. Electron micrographs of positive silica replica; no shadowing metal. Right: smoother side cast against a smooth solid; left: rougher side cast in air.[1]

20.6. FRACTURE SURFACES

Many materials are too hard to be satisfactorily cut with a microtome or ultramicrotome. Such materials may be brittle, or they can be embrittled sufficiently to be prepared for microscopical examination by fracturing.[1,2,14] For revealing macromolecular domains, freeze-fracturing has been successful.[22] The Botty[23] fracture technique involves wrapping a specimen, such as test bar or sheet, in aluminum foil, then cooling it to about −60 °C (−76 °F) in contact with solid CO_2 for 2 hours. When the aluminum foil is removed, the test bar or sheet is fractured in a cooled tensile tester. Aluminum foil is used as a wrapper to contain fragments and prevent contamination. The fracture surface is replicated (negatively) with a 5% solution of gelatin in water at a temperature of about 55 °C (131 °F). This layer is strengthened with a layer of 10% gelatin solution to facilitate stripping off the negative replica from the fracture surface. A positive replica is then made of silica by vacuum deposition to a thickness of about 150 Å[23] (see Figure 20.16).[22]

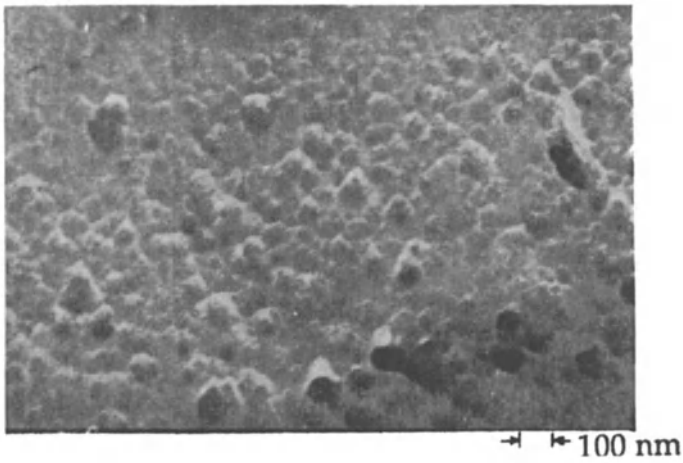

FIGURE 20.16. Unshadowed SiO_2 replica of fractured melamine-formaldehyde molded resin.[22] Macromolecular domains 70–80 nm in diameter are evidently composed of smaller domains 15–20 nm in diameter..

Another way of producing a fracture surface in a test bar of consolidated polymeric plastic involves bending the bar, which is held by screw clamps at each end, over a central fulcrum as indicated in Figure 20.17. The bar flies apart, usually with a loud noise, if a drop of ethyl alcohol is placed on the part of the bar over the fulcrum.[24] The fracture surface is best observed by an SEM.

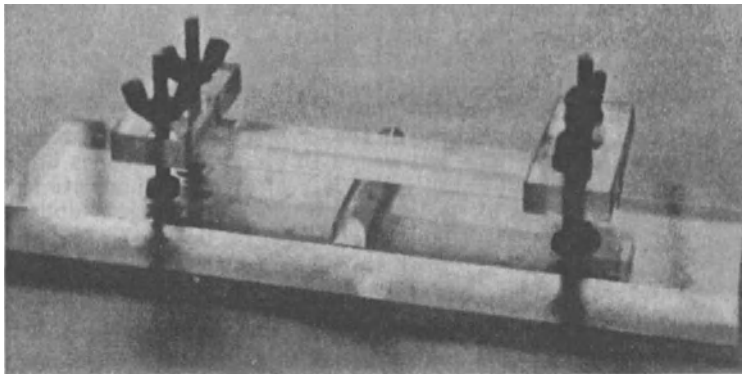

FIGURE 20.17. Sample apparatus for wet stress crazing of test bars. After turning the thumbscrews to the desired extent of stress, a drop of wetting liquid (e.g., ethyl alcohol) is placed on the specimen (e.g., PMMA) over the fulcrum.[2]

20.7. THIN SECTIONS OF HARD MATERIALS

Such materials as bone, horn, sea shells, and some minerals are too hard to be microtomed, but they are soft enough to be hack-sawed by hand.[25] Moreover hacksaw dust from a clean blade, is adequate for a preliminary microscopical examination by transmitted light.[25] However for a microscopical examination of rocks, minerals, and the like, a petrographic thin section is required.[25] This involves cutting off a specimen with an abrasive wheel, such as a metallic disk rimmed with diamond dust.

The usual sequence of operations for preparing the petrographic type of specimen is illustrated in Figure 20.18. The standard thickness of petrographic sections is 0.03 mm. Since the most common mineral in a rock is quartz, which has a very recognizable first-order polarization color at a thickness of 0.03 mm, the professional technician grinds rock sections to the color for quartz, as indicated on the Michel–Lévy scale of birefringence (see Figure 5.8). Of course with other types of specimens, a thickness micrometer may (or may not) be used. Since preparing petrographic (thin) sections is a specialized technique, the general microscopist may wish to send occasional requirements to a professional technician.[26]

20.8. TRANSPARENT PARTICULATE SPECIMENS

Transparent powders, granules, foils, papers, yarns, and fabrics must be consolidated before they can be thin-sectioned by either microtomy or grinding. Most of these specimens are either thermosensitive, porous, or otherwise not amenable to briquetting by means of a pressure-sensitive resin, such as phenol-formaldehyde. If the specimen is not thermoplastic, it can be embedded in paraffin[12] or a mixture of camphor (62%) and (napthalene), which freezes at 32 °C (89.6 °F),[14] for microtomy; a preformed capsule is convenient for this purpose.[2,12] If the specimen is porous like some yarns, fabric, paper, or sponge, warming at an allowable temperature with or without a vacuum is desirable.[2]

20.9. MONOMERIC EMBEDDING MEDIA

Butyl methyl methacrylate embedding medium has regained favor with some electron microscopists because it can be cured with UV radiation at room temperature or even refrigerated.[28] Another reason for its return to favor is the availability of azo-1-cyanocyclohexane as a catalyst

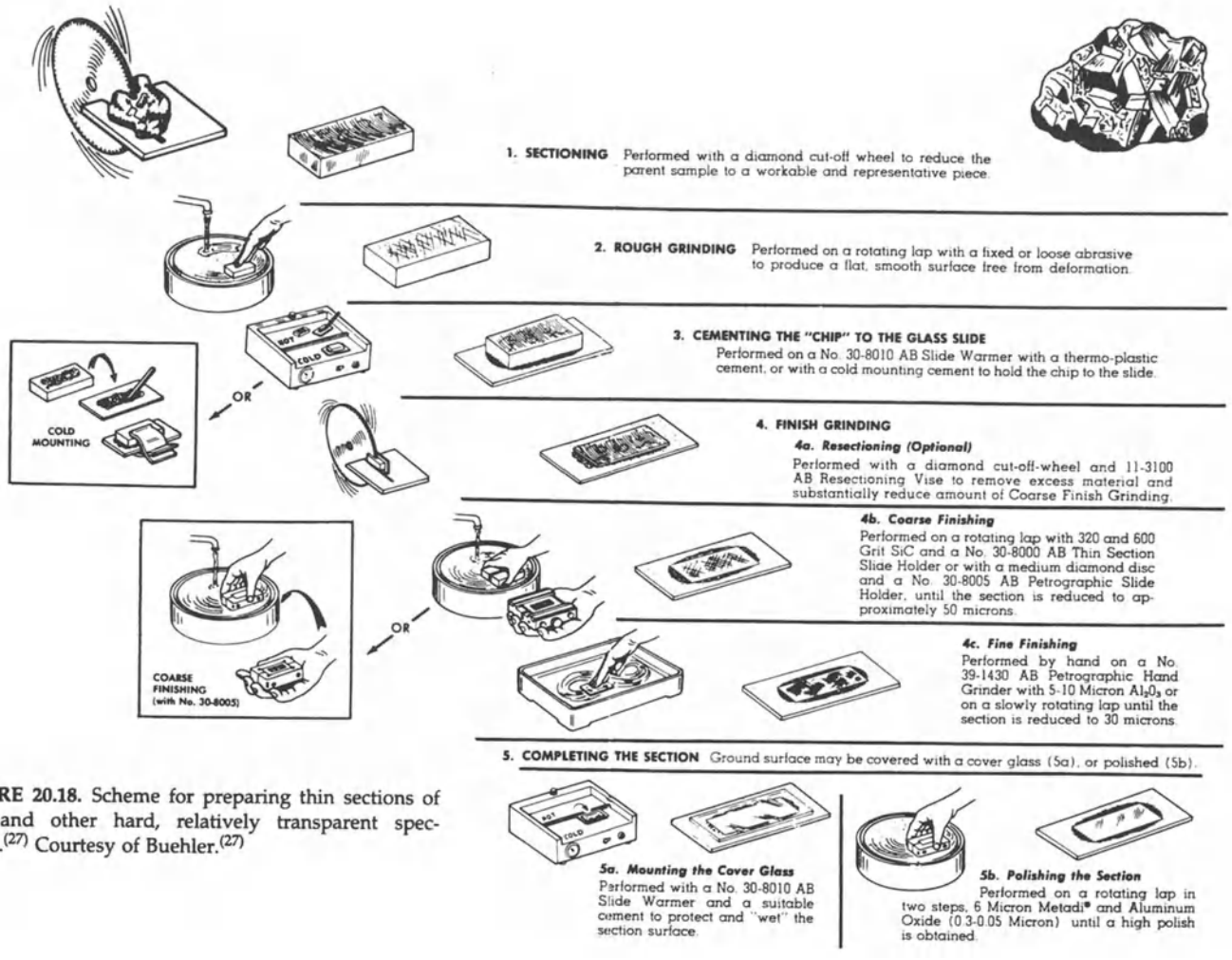

FIGURE 20.18. Scheme for preparing thin sections of rock and other hard, relatively transparent specimens.[27] Courtesy of Buehler.[27]

to replace the potentially explosive benzoyl peroxide. Poly(butyl methyl methacrylate) may also appeal to the light microscopist, who should follow directions for keeping quantities of the catalyzed monomer down to capsule size (or thereabouts). Methacrylate polymerization is a strongly exothermic reaction that is catalyzed by heat, light, and active oxygen. Since the monomer must not come in contact with the skin, it is necessary to wear gloves and goggles and to use an *active* exhaust hood.[2]

Epoxy-anhydride embedding media have certain advantages over the methacrylate media in resinography: Since resultant (cured) epoxy resins are thermoset, they can be sawed, abraded, polished, or heat-treated without getting sticky. At the same time they can be formulated to be soft enough to be microtomed into thin sections[29] or to be hard enough to be abrasively sawed and polished into thin sections.[30] A favorite mixture is Dow Epoxy Resin (DER) 332 (harder) and DER 732 (softer) with diethylene triamine as a curing agent; a good combination to start with is 45% by weight of DER 332, 45% by weight of DER 732, and 10% of diethylene triamine.[31] Specimens should be cured at 60° C (140° F) for 35 minutes and they must be dry. The mixed resin should be stored in a desiccator or on top of the curing oven; the amine, which is more hygroscopic than the mixed resin, must be stored in a desiccator. The separate mixed resin has a long shelf life. Use a disposable paper cup and a wooden stick[2] to combine the mixed resin with the amine hardener.

Flexible capsules of silicone potting agent[32] are cast in a negative mold, as shown in Figure 20.19. This negative mold is fashioned after Sanders's[33]; but none of the pieces has to be turned on a lathe; instead they can all be sawed from a 0.25-in. sheet of poly(methyl methacrylate). The sawed edges must be straight and smooth.

To cement the pieces together, clamp them, then use a medicine dropper to lubricate the joints with a good, volatile solvent, such as methyl ethyl ketone. If air spaces return, fill them again with solvent. Keep the pieces clamped together overnight, then inspect them again. If there are no air spaces at the joints, you can remove the clamp. If there are air spaces, fill them again, this time using a syrup of PMMA and solvent. When the negative mold is ready, fill it to the top with the silicone potting agent, which has been mixed according to directions. After curing, remove the flexible mold, then cut *shallow* slots with a razor blade at the apex and the center of each capsule to hold a sample of yarn. Note: Do not make the slots until you need them because they can easily be cracked as you flex the mold to remove the capsules.

Composite yarn made on the integrated spinning system[29] is a typical example of the preceding method of preparation by cutting a *sledge microtome.* This type of yarn combines the strength of a bundle of continuous

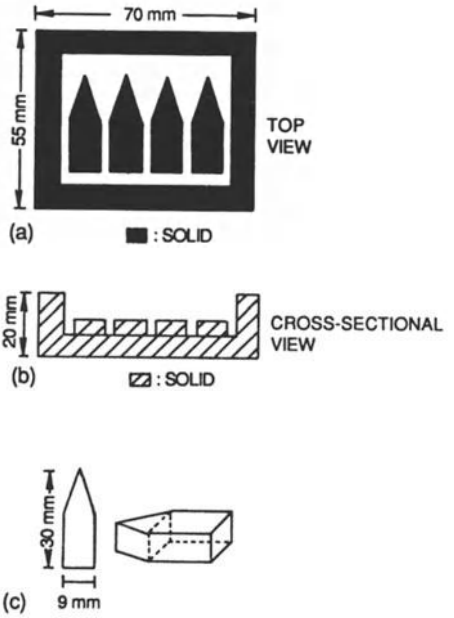

FIGURE 20.19. Flexible silicone negative mode (a and b) to produce (c) positive casting(s) of specimens for either microtomy or grinding/polishing.[2] (a) Flexible, negative silicone mold, top view; (b) side view of flexible, negative silicone mold; (c) positive, hollow, unit cell to receive specimen.

filaments with the appearance and thermal insulation of cotton, wool, or man-made staple yarn. The staple is stuck onto the continuous filaments of polyester or nylon or continuous ribbon of polyolefin. The assembly is then twisted to integrate the staple fibers. To perform resinography without disturbing the yarn's cross section, a short length of a composite yarn is encapsulated in 50:50 DER 332:732 catalyzed with 10% diethyl amine. The yarn is cured at 60 °C (140 °F) for 35 minutes, then thin sections are cut on the sledge microtome.[29]

In an earlier investigation,[30] two-component Bobtex integrated composite-spun yarn was embedded in epoxy resin that became hard enough to slice on a cut-off wheel and to prepare as though it were a piece of rock. The resin was Epon 828 (88%) cured with metaphenylene diamine (12%). First specimens were placed in a small cardboard box and heated to 60 °C for a half hour to expel moisture. Then the specimen was transferred to the bell jar shown in Figure 20.20. The vacuum pump was used for a half hour to expel air from the specimens. Then Epon 828 metaphenylene diamine mixture was allowed slowly to fill the specimen box almost completely. Evacuation of air and volatiles was continued for 8 hours at 25 °C to jell the resin; curing was at 53 °C (127.4 °F) for 2 hours and at 149 °C (300.2 °F) for 2 hours. The block of hard, dark resin was trimmed

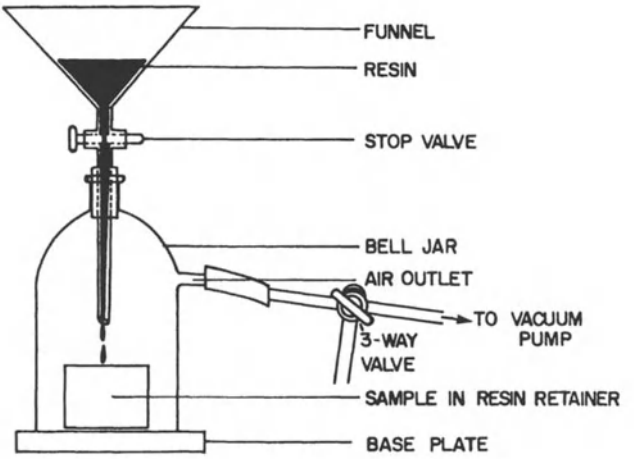

FIGURE 20.20. The vacuum system by which entrapped air is removed from the sample.[2]

with a diamond-studded cut-off wheel[33] (see Figure 20.20), and enough of the block was cut away to expose the specimen. Then one side of a thin slice of the specimen was fastened with water-soluble adhesive (gum arabic solution) to a solid, nonporous block as a handle. The opposite (exposed) side of the specimen was smoothed with successively finer abrasive papers. The abraded surface was washed with water and dried with a current of air. This surface was polished on successively finer diamond-impregnated laps.[34] When free of microscopic scratches and pits, the handle was dissolved in water. The polished surface was washed and dried, then permanently cemented to a microscopical glass slide with Epon 828 catalyzed with metaphenylene diamine (see Figure 20.21). After curing the adhesive resin, the other side of the thin section was abraded

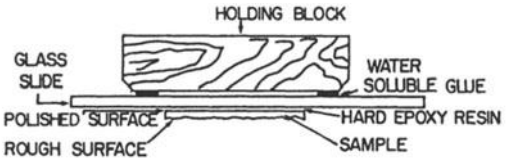

FIGURE 20.21. Diagram of specimen assemblage with the rough surface still to be polished. The polished surface is permanently fastened to a microscopical glass slide with hard epoxy resin. The other side of the glass slide is held on the holding block with water-soluble glue.[2]

and polished like the first. Care was taken not to go right through the wafer when polishing.

Epoxy resins make good permanent specimen mounts and coverslip seals. Since they are fairly permanent however, this may create difficulties in later studying the sample in various refractive index liquids. Small, convenient, dual packages of epoxy fluid and hardener are marketed as patching and mending materials. They all probably make good seals; as mounting media, they should be tested to assure that they are sufficiently transparent when cured.

All epoxy materials but particularly amine hardeners are harmful to the skin, especially the eyes, so it is important not to rub the eyes while working with catalyzed uncured epoxy resin. If you get epoxy material on your hands, wash it off immediately and wear thin gloves during preparation. Some of the amines, especially crystalline metaphenylene tetramine as melted for action, are dark-colored and can stain clothing so it is important to wear an apron and keep hands clean.

20.10. PREPARING FOR REFLECTED ILLUMINATION

Thermoset resins, ores, coal, and other opaque materials are prepared for examination by *reflected* light, electrons, X rays, etc. If transparent constituents are closely associated, they, too, are prepared to be examined by reflected emanation. If the specimen is solid and it can be cut to a handy size, it is already fit to be abraded, polished, etched, stained, etc.; otherwise the specimen must be consolidated into a convenient size and shape. If the specimen is in parts, these are compressed, impregnated, composited, or otherwise consolidated into a convenient size and shape.[1,2,14,25,28]

20.11. PREPARING METALS AND OTHER OPAQUE MATERIALS

Metallographic specimens preparation is well documented.[3,3a,14,25,35–37] Similar methods have been adapted for other opaque materials, such as ores, coal, filled thermoset resins (see Chapter 4), tires,[1] composites,[1] ceramics, plasma coatings on metals, and cemented tungsten carbide.[38] Ceramics, plasma coatings, cemented tungsten carbide, and other hard materials require diamond dust for cutting, abrading, and polishing.[39] Figure 20.22 shows a diamond loaded cut-off wheel on a low-speed saw fitted with a sample-holding arm fitted with an electronic load-sensing circuit to prevent injury to the operator (or the machine).[39] For softer

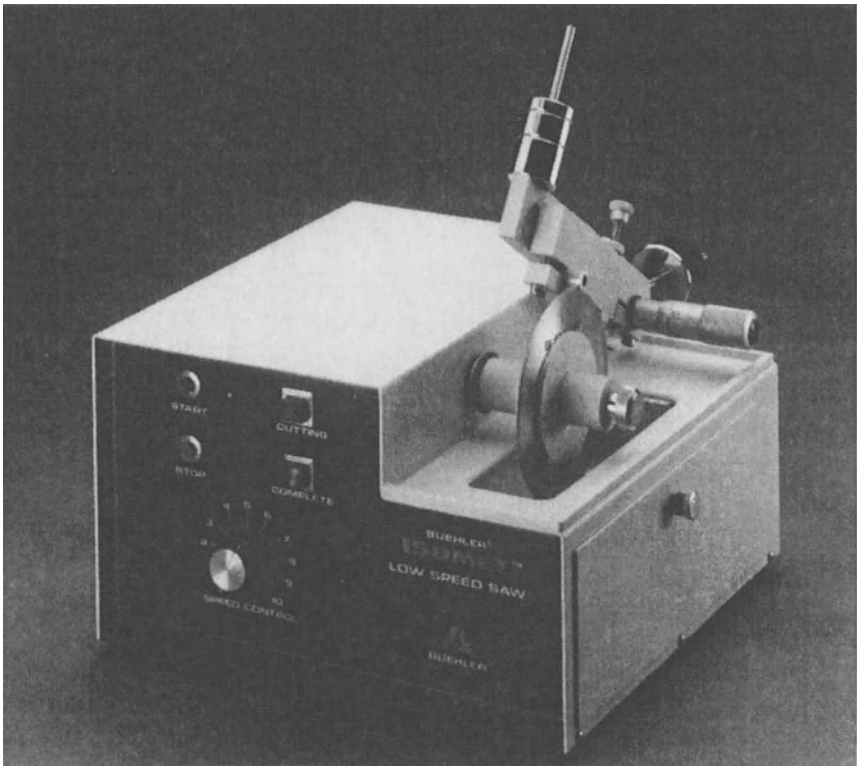

FIGURE 20.22. Buehler Isomet® low-speed diamond saw.[26a] Courtesy of Buehler.[26a]

specimens silicon carbide and/or aluminum oxide abrasive and polishing materials are recommended.[33]

Figure 20.23 is of a polished cross section of a commutator brush for an electric generator. The brush is a composite of particulate copper and particulate graphite consolidated in thermoreacted melamine-formaldehyde resin. The specimen had been prepared by mounting the article in thermoreactive black bakelite BM120 and using the compression mold with rectangular cavities shown in Figure 20.24. Compared to round briquets, rectangular briquets are easier to hold, orient, and return to their previous locations. Once the front side has been designated (by inscription) and a particular location has been recorded, it can be relocated by means of graduated coordinates on a rectangular mechanical stage, such as the one shown in Figure 20.25. Moreover this briquet fits in the mech-

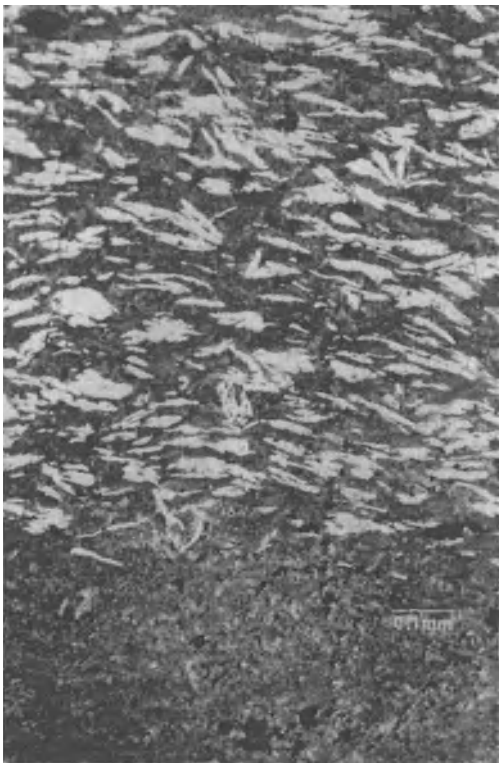

FIGURE 20.23. Polished cross section of a composite commutator brush for an electric generator. White = copper (for conductivity), light gray = graphite (for lubrication), dark gray = melamine-formaldehyde resin (consolidation), and black = air (pores).[1]

anical holder of the particular automatic polishing machine (see Figure 20.26).[25]

Briquetting with a thermosetting resin permits surfacing and polishing a wide variety of coexisting phases, for example soft and hard constituents, nonreflecting and reflecting ones, and particulate as well as solid materials.

Figure 20.27 shows the cross section of an adhesive bond between two sheets of aluminum alloy as polished for examination. Before coating each sheet with phenolic varnish (two dark layers), each aluminum sheet had been anodized (dark dots) to make the phenolic resin adhere better. The central layer (medium gray) is an elastomer to give some more resilience to the joint. The results of tensile tests on this composite were good, but costs of so complicated a sandwich structure were much higher than the cost of using a composite, one-step "spread" (see Figure 20.28). The continuous phase is a phenolic varnish, and the particulate phase is an elas-

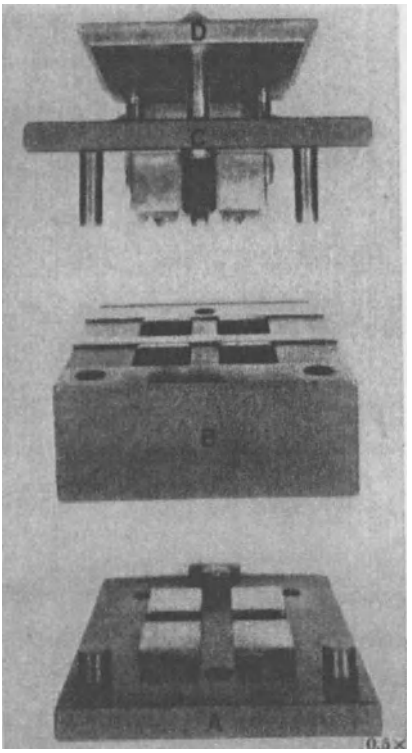

FIGURE 20.24. Four-cavity specimen briquet mold (Eagle Tool Co.): (a) bottom plate; (b) chase; (c) top plate; (d) stripping plate.

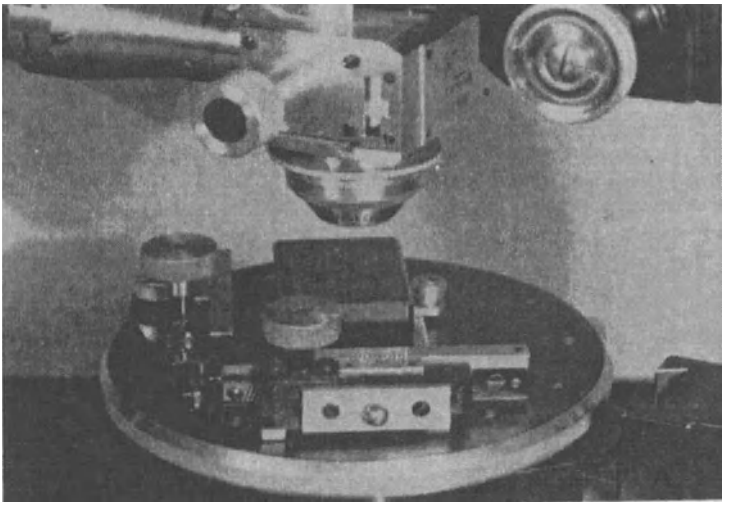

FIGURE 20.25. Rectangular briquet fitted in a mechanical stage (Leitz) on a rotating stage (Leitz).[25]

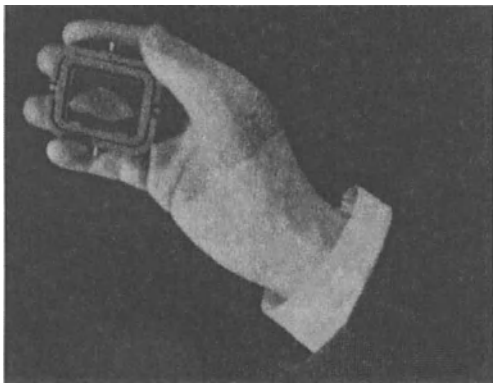

FIGURE 20.26. Mechanical holder (Mico Instrument Co.) for a rectangular briquet.[25]

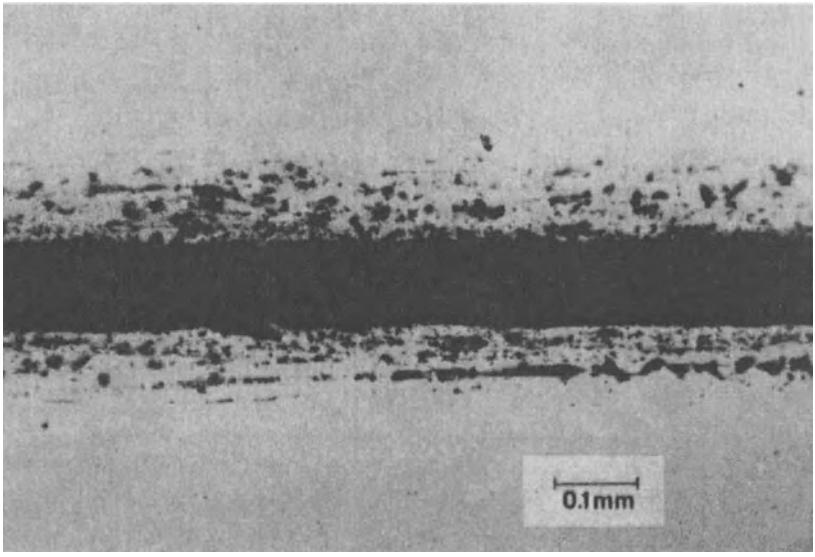

FIGURE 20.27. Resinous bond for aluminum alloy. The white areas, top and bottom, are aluminum alloy; the central, medium gray layer is elastomer; the two dark gray layers are varnish primer.[25]

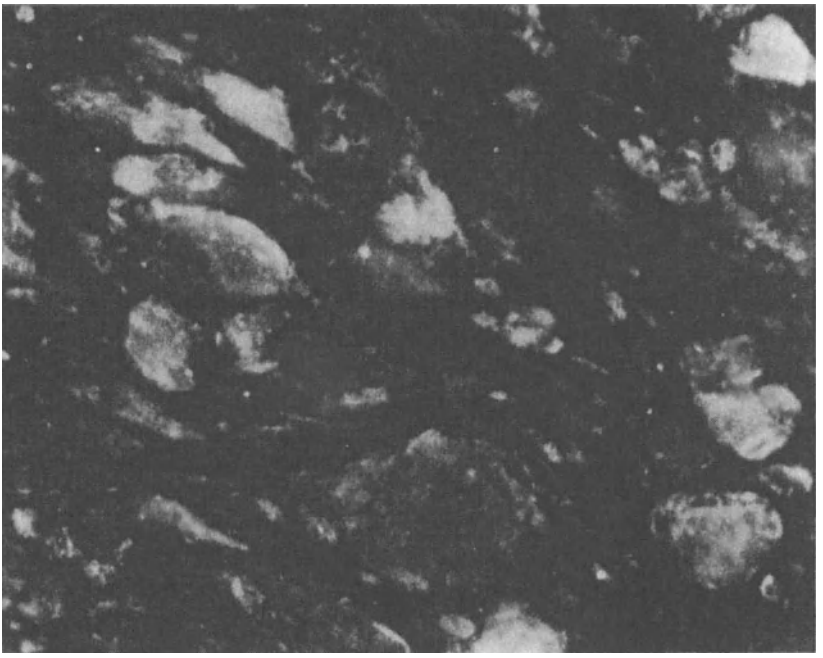

FIGURE 20.28. Elastic mixture or spread for one-step bonding of aluminum sheets. Continuous phase is phenolic varnish; particulate phase is an elastomer.[25]

tomer. An advantage of the particulate system is its three-dimensional application and properties.[25]

20.12. SUMMARY

The preparation of a material for microscopical examination depends upon what is to be examined and by what kind of microscope. In most scientific, technical, and practical endeavors, light microscopy and electron microscopy are the most important kinds. Since methods of preparation are generally related, the two types are discussed together. A list of topics follows:

Microscopical examination of the specific specimen surface that has failed in service or a specific test

Microscopical examination of fibers, fur, or hairs by methods that require little preparation

Microtomy for light microscopy

Ultramicrotomy for electron microscopy

Surface replication as is or as prepared

Fracture surface as is or as prepared

Thin sections of hard materials, such as materials, rocks, and ores, in the manner of *petrography*

Transparent particulate specimens, such as powders, granules, fibers, foils, papers, yarns, and fabrics embedded in paraffin or a mixture of camphor and naphthalene

Monomeric embedding media that polymerizes to a solid

Thermoset resins, ores, coal, etc., prepared for examination by reflected light

Metal preparation (metallography).

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