Histopathologic Technic and Practical Histochemistry

By

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" ὅταν δὲ ἔλθῃ ἐκεῖνος, το πνεῦμα τῆς ἀληθείας,
ὁδηγήσει ὑμᾶς εἰς την ἀλήθειαν πᾶσαν." Ιω. xvi–13
δίδασκε ἡμᾶς, κύριε, γνῶναι ταύτην ἀλήθειαν.

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Foreword

Since the previous edition was written, there has been a great deal of active investigation of histochemical procedures. I have endeavored to bring selected variants of the newer methods into this book, and to emend them so that the methods may be followed without personal instruction by one who has had previous experience, or a good deal of experimentation to find optimal times, temperatures, pH levels and reagent concentrations. This has entailed in many instances a considerable amount of experimentation in arriving at workable conditions.

I am indebted to Dr. J. H. Peers, Dr. G. Laqueur, Dr. G. Brecher, Dr. B. Highman, Dr. R. W. Mowry, Dr. J. D. Longley, Dr. E. R. Fisher and others of the staff for their suggestions and cooperation in evolving these variants, and to Miss A. Laskey and Mrs. J. Greco Henson, and Mrs. H. Burtner for active help in performance and evaluation of procedures.

I also acknowledge my indebtedness to my predecessors and colleagues from whose works I have borrowed freely. Such of these borrowings as have been taken directly from their original publications are usually so cited in the text, but many have been taken, often in modified form, from other laboratory manuals. These texts are usually cited simply by the author's name, except that in the case of Ehrlich's "Encyklopädie" I have often cited the contributor's name. This last text I have often preferred as a source of those older methods which are still used in unmodified form. The following texts have been thus used, as well as earlier editions of some of them.

General References

Amer. Assn., Textile Chemists & Colorists: Yearbooks, 1939–1949, Howes, N.Y.

Conn, H. J.: "Biological Stains," 4th and 5th ed., Geneva, N.Y., Biotech., 1940, 1946.

CONN., H. J., and DARROW, M. A.: "Staining Procedures," Geneva, N.Y., Biotech., 1943–1945, 1947–1949.

COWDRY, E. V.: "Microscopic Technique in Biology and Medicine," Baltimore, Williams & Wilkins Co., 1943, 1948.

EHRLICH, P.: "Encyklopädie der mikroskopischen Technik," Berlin & Wien, Urban & Schwarzenberg, 1903.

FEIGL, F.: "Qualitative Analysis by Spot Tests," 3rd ed., Elsevier Publishing Co., New York & Amsterdam, 1946.

FEIGL, F.: "Chemistry of Specific, Selective and Sensitive Reactions," Acad. Press Inc., Publ. New York, 1949.

GLICK, D.: "Techniques of Histo- and Cytochemistry," New York, Interscience Publishers, Inc., 1949.

GOMORI, G.: "Histochemical Staining Methods" in Methods in Medical Research, M. B. Visscher, Ed., Chicago, Year Book Publishers Inc. 1951.

GOMORI, G.: "Microscopic Histochemistry, Principles and Practice," Univ. Chicago Press, Chicago, 1952.

GRAY, P. E.: "The Microtomist's Formulary and Guide," New York, The Blakiston Company, Inc., 1954.

HICKINBOTTOM, W. J.: "Reactions of Organic Compounds," 2nd ed., Longmans, Green & Co., London, New York & Toronto, 1948.

HUECK, W.: "Die pathologische Pigmentierung" in Krehl-Marchands: Handb. d. allg. Path., 1921.

JONES, RUTH MCCLUNG: "McClung's Handbook of Microscopical Technique," New York, Paul B. Hoeber, Inc., 1950.

KARRER, P.: "Organic Chemistry," 4th ed., Elsevier Publishing Co., New York, Amsterdam, London & Brussels, 1950.

KRAJIAN, A. A. and GRADWOHL, R. B. H.: "Histopathological Technic," 2nd ed., C. V. Mosby Co., St. Louis, 1952.

KRAUS, GERLACH, and SCHWEINBURG: "Lyssa bei Mensch und Tier," Berlin & Wien, Urban & Schwarzenberg, 1926.

LANGE, N. A.: "Handbook of Chemistry," Sandusky, Ohio, Handbook Publishers, Inc., 1944, 1949.

LANGERON, M.: "Précis de microscopie," 5th ed., Paris, Masson, 1934, 1949.

LEE, A. B.: "The Microtomist's Vade-Mecum," 11th ed., edited by J. B. Gatenby & H. W. Beams, New York, The Blakiston Company, Inc., 1950.

LILLIE, R. D.: "Histopathologic Technic," Philadelphia, The Blakiston Company, 1948.

LISON, L.: "Histochemie et Cytochemie Animale," 2nd ed., Paris, Gauthier-Villars, 1953.

MALLORY, F. B.: "Pathological Technic," Philadelphia, W. B. Saunders Co., 1938.

MCCLUNG, C. E.: "Handbook of Microscopical Technique," New York, Paul B. Hoeber, Inc., 1929.

MCLEAN, R. C. and COOK, W. R. I.: "Plant Science Formulae," London, MacMillan & Co., 1941.

PEARSE, A. G. E.: "Histochemistry, Theoretical and Applied," Boston, Little Brown & Co., 1953.

RAMON Y CAJAL, S.: "Histology," translated by M. Fernán-Núnez, Baltimore, Wm. Wood, 1933.

ROMEIS, B.: "Taschenbuch der mikroskopischen Technik," 13th ed., Munchen & Berlin, R. Oldenbourg, 1932.

ROMEIS, B.: "Mikroskopische Technik," München, Liebnitz Verl. 1948.

ROULET, F.: "Methoden der pathologischen Histologie." Wien, Springer, Verl. 1948.

ROWE, F.: "Colour Index," 1st ed. & suppl. Soc. Dyers & Colourists, Bradford, Yorkshire, 1924 and 1928

SCHMORL, G.: "Die pathologisch-histologischen Untersuchung methoden," 15th ed., Leipzig, Vogel, 1928.

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Contents

Chapter 1

Microscopy

I do not propose to enter into any theoretical discussion of the optics concerned in the use of the compound microscope. Rather, the purpose of this chapter is to bring in certain practical points in the use of the microscope in which I have found it necessary to instruct technicians and physicians in training in pathology.

Light

The advice in older manuals about the necessity for north windows for microscopic work, the avoidance of direct sunlight, and the preferability of a white cloud as a source of illumination is still applicable for the monocular microscope. However, daylight seldom gives adequate lighting for binocular microscopes or for more than low powers; hence some form of artificial lighting generally is necessary for microscopic work. Such lighting has the further advantage of not being subject to variations in the weather.

A tungsten-filament electric lamp gives satisfactory illumination for most purposes. The slightly yellowish color of the light can be corrected by insertion of a thin blue glass disk into the microscope substage, by the use of blue glass daylight bulbs, or by the interposition of a water filter containing a weak solution of copper sulfate to which sufficient ammonia water has been added to change its color from green to blue. In form this water filter may be a cell with flat parallel sides such as the microscope-lamp manufacturers often supply, or it may be spherical, such as a 500 cc. Florence flask. The latter serves also as a converging lens.

Filament images, which give rise to uneven illumination of the microscopic field, are avoidable by the use of ground-glass disks placed in the microscope substage, by the use of frosted or milky glass bulbs, or, in more elaborate lamps, by the use of a homogeneous light source large enough to fill the field completely, such as the six-volt ribbon filament lamp.

In all lamps which do not possess a focusing or condensing device to produce parallel or converging light rays, it must be borne in mind that the intensity of the illumination is inversely proportional to the square of the distance of the light source from the object. The same law applies when a ground-glass disk is inserted in the path of a parallel or converging light beam, since this ground-glass surface acts as though it were the light source, and the available illumination diminishes with the distance from the disk to the object.

With the larger microscope lamps, which employ lens systems to focus the light accurately on the condenser of the microscope, it is advisable to mount both lamp and microscope in permanent positions on a baseboard, so that once proper optical alignment is established, it need not be disturbed. In this case it is desirable to have a cloth bag or some form of rigid cover to place over the microscope when it is not in use, in order to protect it from dust. The old-fashioned bell jar functioned well in this respect, but it was heavy and breakable. A cylinder of cellophane or similar transparent plastic, of sufficient diameter and height to cover the microscope readily and with a handle on top, makes a very satisfactory lightweight, transparent substitute which is not readily broken.

The baseboard on which the microscope and lamp are mounted may be made of sufficient thickness to support the microscope at a level such that the eyepieces are at the most convenient height for the individual observer. Among seven workers in one laboratory the most convenient height of the eyepieces above a table 76 cm. (30 in.) high varied from 33 to 40 cm. (13 to 15¾ in.). A swivel chair with adjustable height may also be used to bring the user's eyes to the approximate level of the eyepieces. Larger lamps with large light sources and focused beams are needed for critical work at high magnifications, for dark-field work, and, principally, for photomicrography.

The Microscope

For the average worker, the use and care of the microscope are adequately described in the booklets furnished by the manufacturers. Only a few practical points will be discussed here. As Schmorl aptly states, the microscope should be obtained from a reputable manufacturer, and from personal experience I would recommend that when possible the manufacturer's plant be in the same country as the user. Necessary repairs and adjustments are greatly expedited if it is not necessary to send instruments or lenses out of one's own country.

When practical, it is preferable to have a binocular microscope with inclined ocular tubes, so that wet mounts may be studied without standing up over the instrument. The binocular instrument furthermore lessens the fatigue of prolonged use, as compared with the monocular. By training both eyes to observe, it also guards against incapacity during temporary losses of the use of one eye.

Either achromatic or apochromatic objectives may be selected. The former are corrected for two colors only, and are considerably cheaper. They give quite satisfactory service for ordinary clinical laboratory work. The latter are corrected for three colors and are preferable for research work and for photomicrography. If color photomicrography is to be done, they are essential.

The Abbé test plate is a glass slide with a thin film of metallic leaf through which a series of parallel lines have been scored so as to leave clear lines bounded by narrow, opaque, metallic bands with jagged edges. This film is covered by a long, narrow cover slip which varies progressively in thickness from about 90 μ at one end to 230 μ at the other. At the side of this cover slip are graduations indicating the approximate cover-glass thickness at any point.

This test plate is used for testing objectives for chromatic aberration, for spherical aberration, for sharpness of definition, and for flatness of field. A complete substage with a device for oblique illumination is needed. Low-power objectives should be tested between 150 μ and 200 μ equivalent cover-glass thickness. No. 1 cover glasses average about 0.15 mm. (= 150 μ), No. 2 about 0.21 mm. (= 210 μ) in thickness. Test 4 mm. apochromats with correction collars at at least two points, with corresponding adjustment of the correction collar. Immersion objectives should be tested immersed in their proper immersion fluids.

With oblique illumination, achromatic objectives give relatively broad fringes of complementary colors on the edges of the metallic strips. With apochromats, these fringes are narrower—often almost inappreciable.

When sharply focused with the condenser centered and properly focused, a good objective should continue to give sharply defined points on the edges of the metallic bands when the illumination is decentered across the direction of the bands. Similar performance should be obtained in the central and peripheral portions of the field.

Relative flatness of field can be judged by the amount of focusing necessary to give sharp definition respectively in the center and at the periphery of the field. It should be borne in mind that lenses with the greatest resolving power in the center of the field ordinarily do not give as flat a field as some others inferior in resolving power. This property of flatness of field is more important with lower powers and for photographic purposes.

Resolving power may be tested on various test slides. For instance, the diatom *Pleurosigma angulatum* at 250 × should show three distinct striation systems. One runs perpendicular to the median rib; the other two cross obliquely at an angle of about 58°. At higher magnification the striae appear as material between rounded globules which is dark at high and low focus, bright at normal focus. The wing scales of *Epinephele janira* ♀ show longitudinal striation at 40 ×. Between these striae a fine cross striation is seen at 150 ×. At 800 to 1000 × the longitudinal striae are doubly contoured and

contain round granules. (The material for this paragraph was derived from Romeis. For a fuller account consult Langeron.)

The objectives to be selected for a microscope naturally vary widely with the purpose to which each is to be put. For general pathology the following seem the most desirable. An achromat of about 3–6 × initial magnification is useful for general views of sections. Achromats or apochromats of 10 and 20 × (16 and 8 mm.) are needed for more detailed study. A 31 × (5.5 mm.) achromat has proved quite useful in practice. It is similar in performance to the English ¼ inch objective. An immersion objective of 60 or 90 × initial magnification (3 or 2 mm.) is required for very high magnification. These last are available in three grades: achromatic, fluorite, and apochromatic, in ascending grades of performance and cost. The second will serve almost all purposes; the last are somewhat better for photography and maximum resolution. The 4 mm. (45 ×) achromat is a useful lens for moderately high magnification when it is desired to avoid the use of immersion oil. However, the apochromat of the same designation is in practice rather unsatisfactory because of the necessity for adjustment of the correction collar for variations in thickness of cover slips and of film of the mounting medium. An oil immersion objective of 4 mm. (40–45 ×) has been found very useful for differential cell counts of leukocytes in thin blood films, because of the larger field afforded. This magnification is still adequate for identification of ordinary blood leukocytes, but for marrow films a 2 or 3 mm. (90 × or 60 ×) objective is required.

Among eyepieces the 7.5 ×, 10 ×, and 12.5 × seem the most useful. In selecting oculars it should be remembered that objectives do not give effective magnifications of over 1000 times their numerical aperture. Hence a 60 × objective with NA 1.40 can be used with 15 × oculars, giving about 900 diameters final magnification; but a 90 ×, NA 1.30 objective will accept only a 12.5 × eyepiece, giving 1125 diameters; or perhaps better, a 10 × eyepiece, yielding 900 diameters. Attempts to obtain higher magnifications by use of higher oculars result in blurring of detail.

For apochromatic objectives, compensating eyepieces should be used. For achromats, the Huyghenian type is satisfactory. For fluorite objectives, eyepieces should be either compensating or of an intermediate grade designated as hyperplane or planoscopic. These last can also be used with achromatic objectives and even with apochromats, though they are not recommended for the latter.

Generally two eyepieces separated by 5 × in magnification are adequate. For the binocular instruments, only matched pairs should be used, at a constant interpupillary distance which one may determine by trial for himself. Both should be brought to focus on some individual detail in a microscopic field by means of the focusing collar on one of the ocular tubes.

In regard to the question of parallel or converging ocular tubes, both

have their defenders and either seems to be satisfactory to the individual observer when he has become accustomed to it. Changing from one to the other is difficult. Note that the parallel design has been adopted by three of the four manufacturers whose microscopes have been commonly used in the United States.

Condensers are commonly used in the substage of microscopes to bring to bear on the object a sufficient amount of light at an adequate angular aperture to illuminate the field adequately. For work with ordinary transmitted light, the usual Abbé condenser serves for routine work with achromatic objectives. For apochromatic and fluorite objectives, an aplanatic or achromatic condenser with a wider aperture is necessary; and its numerical aperture should approximate the highest numerical aperture of the objectives likely to be used.

In order to utilize fully the wide-aperture objectives, it is necessary to fill the aperture with light. If the beam of light entering the objective from the condenser is of too narrow an angle to fill the aperture of the objective, the full resolving power of the objective is not attained. The essential condition for attainment of full resolving power is the even filling of the whole objective aperture with light. For this purpose either *critical illumination* or *Köhler illumination* is used.

For the attainment of critical illumination it is required in theory that all of the light waves reaching the object-point and forming the image at any one instant should also emanate at the same instant from a single point in the light source. This condition is fulfilled when the light source is focused on the object, as is the case when a beam of parallel rays from a very distant source or from a collimating lens on a lamp strikes the front or lower surface of the microscope condenser. For this type of illumination, the light source must be uniform over its surface to obtain even lighting. If the primary source does not cover the field, an enlarged real image can be used.

In the Köhler illumination the specimen is evenly lighted by forming an image of the light source in the lower focal plane of the substage condenser, and hence also in the back focal plane of the objective; and by forming the image of the lamp condenser in the plane of the object. In practice the image of the light source is focused on the substage iris diaphragm by the use of one or two convex lenses (or a concave mirror) in the light path between the light source and the microscope condenser. This light-source image should be just large enough to fill the aperture of the condenser. Since the microscope condenser focuses the image of the lamp condenser in the plane of the object, a diaphragm set next to the lamp condenser should be regulated to exclude all light not being used.

With the Köhler illumination, the size of the illuminated field and its aperture of illumination are easily controlled. At high magnifications, however, only a small aperture can be used at the lamp source. (From *Photomicrography*, 14th ed., Rochester, N. Y., Eastman Kodak Co.)

Schwind (*Blood* 5:597, 1950) gives more detailed directions for the use of Köhler illumination with immersion systems, which I summarize here:

1. Use the flat side of the substage mirror and check the centering of the substage condenser (p. *infra*).
2. Nearly close the substage diaphragm, and with the focusing device on the lamp condenser, focus the image of the lamp filament on the substage diaphragm, with the aid of a small mirror.
3. Place the slide on the stage, making immersion contact between the under surface of the slide and the top element of the condenser.
4. Focus on the object with a 16 mm. (10 ×) objective. Nearly close the field diaphragm of the lamp. Then focus the substage condenser so that the object and the image of the edge of the lamp-field diaphragm are simultaneously in focus.
5. Place a drop of oil on the cover glass, change to an immersion objective, and focus on the object. Refocus the condenser to bring the outline of the lamp-field diaphragm into focus.
6. Open the lamp-field diaphragm until its edge just disappears from the field.
7. Remove the ocular from the microscope and adjust the substage diaphragm so that its edge is just visible in the rear lens of the objective, and the rear lens is completely filled with light. If the condenser is not immersed, a dark ring is seen at the periphery. Replace the ocular.

To center the condenser, close the substage diaphragm and focus with the coarse adjustment and the substage focusing adjustment until the circle of the nearly closed diaphragm is in sharp focus. Then adjust the size of the opening of the substage diaphragm to a circle of approximately the same apparent size as the field of the microscope. Then, using the centering screws of the condenser, bring the circle of the field and that of the substage diaphragm into approximate coincidence.

One should make a practice of refocusing the condenser to give the most satisfactory illumination on each change of objectives. Generally, the highest powers require the highest focal position of the condenser to give adequately bright illumination, while low powers require a low condenser position in order to fill the field and illuminate it evenly. For very low powers, such as the 32 mm. objective, a lower-powered condenser is desirable, or one from which an upper element may be readily removed so that a larger field is evenly lighted.

Special condensers are required for dark-field and for fluorescence microscopy.

Generally the microscope can be used without eyeglasses except when the wearer's optical defect includes a considerable degree of astigmatism or is extreme in grade. So-called ***high point*** oculars are essential for persons who

find it necessary to wear their spectacles while working at the microscope. These oculars are now readily available. Or individual caps carrying small lenses of the worker's eyeglass prescription may be procured from the microscope manufacturers and placed over the microscope eyepieces.

Objectives, eyepieces, and condensers should be cleaned by breathing on the glass and wiping with the lens paper made for that purpose. Immersion oil should be cleaned off daily at least, or when the use of that objective is finished for the time being. To do this it is well to dampen a spot on a piece of lens paper with a drop of xylene and wipe first with this damp spot, then with the remaining dry lens paper in a single movement.

For immersion objectives it is preferable to use one of the nondrying oils now made for the purpose. These are available in high and low viscosities and may be blended according to the particular need of the user. Low viscosities are better when rapid motion of the slide is to be used with short working distances, and when fresh wet preparations are being studied under a cover glass. The most practical oil for routine use may be an equal-volume mixture of the two grades. On one occasion it was found necessary to make immersion oil of a very low viscosity by mixing approximately four volumes of light mineral oil with one volume of alpha-bromonaphthalene. If it is necessary to make such mixtures, they should be checked for index of refraction with a refractometer if possible. The index of refraction should be between n_D 1.515 and 1.520.

If the refractometer is not available, the proper index of refraction may be approximated by immersing a white glass slide (or a glass rod of the proper refractive index) in the mineral oil and then gradually adding alpha-bromonaphthalene until the glass can no longer be seen through the oil.

If one persists in the use of thickened cedar oil for immersion, the utmost care should be taken to have it cleaned off the immersion lenses at least daily. Dry balsam or cedar oil is probably best removed by carefully chipping off the outer portion with a knife, avoiding contact with either metal or glass, and then removing the remainder with lens paper or a soft cloth moistened with benzene or xylene. Alcohol should be avoided, since it softens the cement in which the front lenses of many older immersion objectives were mounted.

Immersion oil is conveniently removed from cover glasses covering fresh resinous or glycerol-gelatin or similar mounts by first inverting the slide on a blotter and pressing down lightly. This absorbs the bulk of the oil. The small residue is readily removed by gently wiping on a blotter wet with xylene, or by dragging a piece of lens paper moistened with xylene across the soiled area, steadying the cover glass with a finger on one corner if necessary.

Blood films may be cleaned of cedar oil by dipping repeatedly in a jar of xylene, or by dropping xylene slowly on the slanted slide just above the oil drop until it runs off the edge of the film. The nondrying mineral oils may be removed in the same manner; but it is not necessary to remove them from

blood films, since they apparently do not cause fading of Romanovsky stains, as did cedar oil. In fact, it appears that this type of oil may even act to better preserve Giemsa and Wright stains.

To use the immersion objective, one finds the desired object with a lower-power dry lens and brings it to the center of the field. The finding lens should be centered on the same point as the immersion lens. If it is not, one brings the object to such a point in the lower-power field as experience indicates will bring it within the smaller field of the immersion objective. The objective is racked up. A drop of oil is then placed on the illuminated area of the slide; the condenser is racked up to near top focus; the immersion objective is swung into position and racked down until it touches the oil drop, and then raised slightly but not enough to break immersion contact. Observing now through the eyepieces, one cautiously focuses down, using the coarse adjustment and preferably both hands (if controls are available on both sides) until the object begins to come into view.

During this focusing, one sometimes notes tiny air bubbles moving in the field. These indicate that the lens is still above focus. The coarse adjustment is recommended because it is possible to tell by feel if contact is made with the cover glass; with the fine adjustment, this contact is not perceived. Violent eddy currents in the optical field indicate that the cover glass is being pressed down by the objective. One should then inspect the preparation for undue thickness of the film of the mounting medium or for the possible presence of two cover glasses stuck together. Failure to attain immersion focus may be due to placing a slide on the microscope stage with the cover-glass surface down. Many prefer to use the fine adjustment for this focusing-down step. It is safer, though slower, since most fine adjustments exert only the pressure of a small spring when contact is made with a cover glass.

When the utmost resolution is required with lenses of a numerical aperture above 1.0, and for dark-field work, a drop of oil is also placed between condenser and slide. Usually this procedure is unnecessary and (if much movement of the slide is required) both troublesome and messy.

If after immersion contact has been made and focus attained a grayish haze appears moving in from one side to obscure the image, a tiny air bubble in the immersion fluid is probably responsible. The trouble is remedied by swinging the objective out laterally to break the immersion contact, and then back in again without changing the focus.

Oblique Illumination

Oblique illumination is used to make more prominent the lineal details transverse to the plane of oblique lighting. It is obtained by excluding light from all but one side of the under surface of the condenser. A device for this purpose is included in research model microscopes, and a similar device may be improvised for student microscopes by sliding a piece of cardboard gradually across the under surface of the condenser from the desired side. Utiliza-