HISTOPATHOLOGIC TECHNIC AND PRACTICAL HISTOCHEMISTRY

FOURTH EDITION

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NOTICE

Medicine is an ever-changing science. As new research and clinical experience broaden our knowledge, changes in treatment and drug therapy are required. The editors and the publisher of this work have made every effort to ensure that the drug dosage schedules herein are accurate and in accord with the standards accepted at the time of publication. Readers are advised, however, to check the product information sheet included in the package of each drug they plan to administer to be certain that changes have not been made in the recommended dose or in the contraindications for administration. This recommendation is of particular importance in regard to new or infrequently used drugs.

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FOREWORD

This book has been written to give a systematic treatment of recent advances in histopathologic technic. In it I have endeavored to include workable methods on unusual subjects as well as those in frequent use. No attempt has been made to attain encyclopedic scope and many methods which have appeared in previous works have been excluded as obsolete or impractical.

I have endeavored to find methods depending for constancy of results on controllable factors such as time, temperature, hydrogen ion concentration, and concentration of reagents, rather than on the skill of the individual technician. An attempt has been made to avoid methods depending on special primary fixations of tissue. Particularly I have tried to include methods which will work well after routine formalin fixation, and to choose those which may be completed with a few steps and in a short time, having in mind the demands of time on the pathologist.

I have made many modifications of older methods with these purposes in mind. My greatest regret is that in the interests of completeness I have had to include some methods with which I have had no personal experience, and others which I have tried and regard as not fully satisfactory.

I am indebted to my associates, Drs. L. L. Ashburn, K. M. Endicott, B. Highman, R. C. Dunn, and L. R. Hershberger, for some special methods, for advice, and for criticism; to Mrs. C. Jones, Miss D. Plotka, Mr. R. R. Reed, Mr. R. Faulkner, Miss A. Laskey, and Miss J. Greco for their aid in testing and devising many of the special methods included.

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 listed in "General References" have been thus used, as well as earlier editions of some of them.

R. D. Lillie

PREFACE

This edition represents a major revision. Most chapters have been greatly enlarged. Dr. Fullmer has assisted with the enormous task of bringing together in one text a broad coverage of cytologic, histopathologic, histochemical, and cytochemical methods employed in current laboratories throughout the world today. We have attempted to provide the methods in a manner that a moderately qualified technician could employ. In addition to results, the theory, mechanism of action, problems that may be encountered, and limitations of interpretations of the staining reactions are provided when known.

We are particularly indebted to Drs. W. Fishman, F. B. Johnson, and S. S. Spicer for reviewing pertinent chapters of this revision. We are indebted further to Drs. M. Hartley, Philip Pizzolato, and J. Mestecky for advice and counsel; to Dr. C. A. McCallum, Dean, School of Dentistry, University of Alabama in Birmingham, and Dr. J. F. Volker, President, University of Alabama in Birmingham, for moral and administrative support; to Mr. Clifton Link, Jr., Mrs. Patricia Donaldson, and Miss Annie Jo Narkates for technical support and Mrs. Charlotte Hughes and Mrs. Dena Darby for secretarial services.

The new material has been the special task in some chapters of one of us, in other chapters of the other, but we have both taken active part in the revisions of all, and there has been remarkably little dispute about the new material. This is probably due to our having written several joint research papers in our National Institutes of Health days. We have both been more interested in practicalities and results than in pure theory. This calls to mind a story about the old master Carl Weigert told by the late Dr. S. P. Kramer, who spent his two years in Germany under Weigert and Virchow about 1890 to 1892. Professor Weigert demonstrated one of his new staining methods to the class, and at the end of the lecture a few students came to the podium to ask questions. One young man asked, "But Professor, what is the theory of the method?" and Dr. Weigert snorted, "Ach! *Die Theorie*! You should ask my nephew Paul [Ehrlich] about that!"

This is not to state that we have not inquired diligently into the probable chemical mechanisms, not only of new histochemical procedures, but also of many traditional staining methods. These inquiries have added greatly to our understanding of stains and reactions.

Specific page references have been eliminated from this edition. Many topics come up in more than one place in the text. We suggest that the reader consult the index for complete listings of pages where information is given on any subject.

This has been a large task that neither of us could have done alone in the time it has taken, and we hope that our effort will prove useful to pathologists, histologists, and technicians alike, as well as to others who may find matters of interest in it.

The very modest list of 17 "General References" published in the first edition of this book grew to 30 in the second edition and to 46 in the third. With the appearance of more general texts in histochemistry as well as histology and enzymology, and with the necessity of entering to some extent the field of electron microscopy, this list has grown to 64 titles. The same system of referring in the text to this list simply by author's name, year, and perhaps a page reference has been followed.

R. D. Lillie
Harold M. Fullmer

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CONTENTS

1

MICROSCOPY

We 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 we 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-ml 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 6-V 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 $15\frac{3}{4}$ 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. Of late years many of the microscope manufacturers have been furnishing integrated lighting systems attached to the microscope, usually in the substage area. Description of these systems and statements as to the type of illumination furnished are to be found in the manufacturer's catalogs. Some of them include devices for polarized light and dark-field illumination and for substitution of phase-contrast optics.

As with other projection-lamp bulbs, it is often profitable to purchase bulbs of somewhat higher voltage rating than specified by the lamp manufacturer and to operate the lamp at a slightly lower voltage than the stated rating of the bulbs employed. When used this way, the bulbs last much longer, and the difference in color value of the light is scarcely appreciable.

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 we 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 almost all visual work. The latter are corrected for three colors and are preferable for photomicrography, especially in color photomicrography.

The Abbé test plate is a glass slide with a thin film of metallic leaf through which a series of parallel lines has 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 coverslip which varies progressively in thickness from about 90 μ at one end to 230 μ at the other. At the side of this coverslip 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 and 200 μ equivalent cover-glass thickness. Number 1 cover glasses average about 150 μ, Number 2 about 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 so 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 to 6× initial magnification is almost essential 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-in objective. We have found dry achromats of 45× and even 60× very useful on occasion, especially when the use of oil is inconvenient. An immersion objective of 60 or 90 to 100× 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 apochromat is in practice rather unsatisfactory because of the necessity for adjustment of the correction collar for variations in thickness of coverslips and of film of the mounting medium. An oil-immersion objective of 4 mm (40 to 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. Visual impairment in the user may be partly compensated for with 15 or 20× oculars. In selecting oculars it should be remembered that objectives do not give effective magnifications of over 1000 times their numerical aperture (NA). 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 Huygenian 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× are adequate. For the binocular instruments, only matched pairs should be used, at a constant interpupillary distance which one may determine by trial for oneself. 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 who 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 is necessary whose numerical aperture should approximate the highest numerical aperture of the objectives likely to be used.

ILLUMINATION

To obtain the best results with any suitable combination of optical components, it is necessary to relate them to one another in a definitely prescribed way. This principle is generally understood with respect to the focusing on the object of the parts above the microscope stage, and it is hardly less important with respect to the components below the stage. The necessary adjustments are (1) alignment of the optical axes of the condenser and illuminator with that of the objective and ocular, (2) focusing of the substage and illuminator condenser, and (3) regulation of the iris diaphragms of the system. If these procedures are not carried out correctly, the most elaborate equipment is no better than the plainest and is frequently worse.

Dim and uneven illumination is the most frequent consequence of faulty axial alignment. To avoid this, align the equipment as described in the following paragraphs:

Focus the microscope on a slide placed on the stage; then move the slide to obtain an empty field. Remove the ocular, and observe the back lens of the objective through a pinhole eyepiece. Close the condenser diaphragm until it begins to restrict the lighted disk seen. Move the condenser by means of its centering screws until the restriction is evenly distributed around the edge of the disk. The condenser is now centered. (The pinhole eyepiece required for this operation can be made by making a needle hole through the center of a cardboard cap fitted over the tube after the eyepiece is removed.)

Before aligning the illuminator with the microscope, the centration of the light source itself with respect to the illuminator condenser should be checked. If the illuminator is aimed horizontally at a nearby wall and the image of the lamp filament is focused upon it, the center of the filament image should be at a level with the center of the illuminator condenser. If the housing has a reflector behind the filament, the direct and reflected images of the filament should fall on top of one another, except that with filaments composed of multiple parallel coils, the two images should be displaced just enough to alternate the coils in the combined image.