McGRAW-HILL BOOK COMPANY

Handbook of Basic Microtechnique

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SECOND EDITION

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Preface

This little book is intended, as was the first edition, to supplement Gray's "Microtomist's Formulary and Guide," a work too large for practical use in elementary classes. It seemed both to the author and his publishers, when these two works were first projected, that the field of microscopical technique presented an unusual dilemma. One horn was the necessity of providing an authoritative and exhaustive account of the main techniques developed since the invention of the art; the "Microtomist's Formulary and Guide," published in 1954, covers every branch of biological microtechnique and contains some forty-five hundred literature references. The other horn was the necessity of providing college teachers with a practical, inexpensive text that would cover the main requirements of undergraduate students in the fields of bacteriology, botany, zoology, premedicine, and medical technology. This horn proved much the sharper and this second edition of the "Handbook of Basic Microtechnique" differs considerably from the first edition of 1952. The author cannot too strongly express his gratitude to the several hundred teachers who have written to him suggesting improvements, and regrets only that it is impossible here to acknowledge by name his debt to each of them.

The first major change has been the introduction of a new first section, of three chapters, on the microscope and elementary photomicrography.

The former first section on the processes and materials of slide making has now become the second section and has been both altered and expanded. The alterations have chiefly involved the substitution of methods or formulas (e.g., Delafield's hematoxylin) preferred by most teachers for methods or formulas (e.g., Carazzi's hematoxylin) preferred by the author. The expansion of this section has been through the insertion of a brief synoptic chapter on types of microscope slides and the introduction of new techniques and methods in general demand. In this respect it has not been possible to satisfy every request without violating the stated purpose of this book. There is scarcely a technique in the 175 double-column pages of Part I, or a formula in the 500 closely set pages of Part II, of the "Microtomist's Formulary and Guide" that some-

one would not wish to see in the "Handbook of Basic Microtechnique." The author is deeply sympathetic to the idea that celloidin embedding, or the silver impregnation of nervous tissues, should be part of an elementary course in microtechnique. But he shares these views with a small minority of teachers to whom he would suggest that reference copies of the larger work be made available to their classes. He would also make the same suggestion to those who have requested that lists of supplementary reading material be appended to the various parts of this little book. Not only is the big book copiously furnished with references but it contains a list of the 300 journals and 120 texts used in its compilation. In addition to this the author, in collaboration with his wife, has separately published a comprehensive, classified, and annotated bibliography ("Annotated Bibliography of Works in Latin Alphabet Languages on Biological Microtechnique" by Freda Gray and Peter Gray. William C. Brown Co., Dubuque, Iowa, 1956), which contains also a history of the field.

The third part of this edition, "Specific Examples of Slide Making," has been very greatly improved by the addition of photographic illustrations. The author has, however, gone beyond the suggestion of many teachers that pictures of good slides be included. He has been at pains to select examples of very bad slides and to publish photographs of these with an explanation of how they differ from the good ones. This section also contains three new specific examples, only one of which has been transferred, in response to numerous requests by teachers of premedical students and medical technicians, from the big book.

Although the author's main debt is due to the correspondents who have suggested the changes described, he has other acknowledgments to make. His colleagues in the Department of Biological Sciences at the University of Pittsburgh have offered many suggestions in the specific fields in which they are expert. In particular, Drs. Ralph Buchsbaum, Eliot Spiess, and Ian Sussex, though they cannot be held responsible for any remaining defects, have provided wise counsel in the revision of invertebrate, genetic, and botanical methods. Finally, the author's special thanks are due to his secretary, Mrs. Leah Porter, for her unfailing and continuous help.

Acknowledgment is made with thanks to the Fisher Scientific Company for Figs. 71 through 79, 92, 130, and 131, and to the American Optical Company for Figs. 104, 105, and 127.

Peter Gray

Contents

PART ONE

The Microscope

CHAPTER 1

Principles of Microscopy

The word microscope means "little seer" or "seer of little things." The first simple microscopes were glass globes filled with water, and Pliny has left a record of their use in the first century. No one, however, can examine the carved gem stones of antiquity without realizing that microscopes were in use at least five hundred years earlier. There is no theoretical limit to the magnifying power of such simple lenses, and Loewenhoek was able to make them so well that he discovered bacteria. He never saw what we now call a microscope.

LENSES AND IMAGES

There are many practical disadvantages to simple lenses of high magnifying power. The distortions they produce, which will be discussed later, are not the chief of these. The greatest objection is that focal length decreases as magnifying power increases so that Leeuwenhoek had almost to push his eye into his lenses to see anything. This difficulty was overcome, about the year 1600, by using the newly invented telescope to examine from a reasonable distance the image made by a simple lens. This is exactly what we do today when we use what is now called a microscope, shown diagrammatically in Fig. 1.

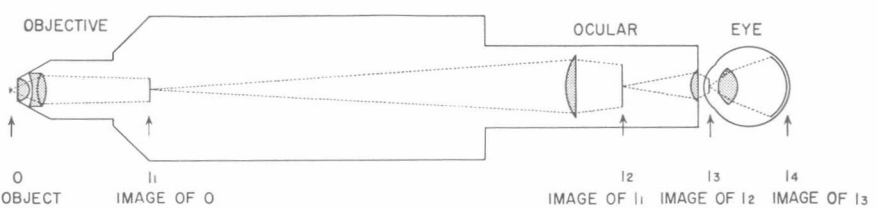


Fig. 1. Diagram to show production of image by a compound microscope. A magnified image at I_1, thrown by the objective, is examined through a telescopic device known as the ocular, from which an image is formed on the retina by the lens of the eye.

The objective at the front end of the barrel is a powerful "magnifying glass" that produces an enlarged image of the object O at I_1. The extent to which this image is magnified is called the "primary magnification," and nowadays this figure is usually engraved on the barrel of the objective. The lowest power on laboratory microscopes is usually $\times 3.5$ or $\times 4$. The highest is either $\times 45$ or, if there is an oil immersion, $\times 90$.

This magnified image I_1 is examined through a telescope, known as the ocular or eyepiece, at the top end of the barrel. The first lens of the ocular produces a magnified image of I_1 at I_2, and the second lens of the ocular produces a small image—usually about a millimeter in diameter—of I_2 at I_3. This is a real image that may be demonstrated by holding a piece of translucent paper just above the eyepiece and lowering it until the small disc-like image is sharply defined. This image is called "Ramsden's disc."

Ramsden's disc is rather small and it requires the human eye to transform it into the illusion of a magnified image of the object. This is done by advancing the eye until Ramsden's disc (I_3) is just inside the cornea. Under these circumstances the lens of the eye casts an image of Ramsden's disc over the whole surface of the retina at I_4. The image, in fact, "fills the eye." The extent of the apparent magnified image can be obtained by extending the dotted lines running from the lens to the retina as far as the plane of the object O. The production of a real magnified image on a photographic plate will be discussed later.

The distance between the outer surface of the top lens of the ocular and Ramsden's disc (I_3) is known as the "eye relief" of the ocular. In most oculars it is so stupidly short that wearers of spectacles have to remove them in order to get Ramsden's disc into the cornea and thus fill their eye with the image. A few manufacturers offer a limited range of oculars with "long eye relief" and it is ardently to be hoped that these will in time become universal.

Aberrations of Lenses. The distortions of shape and color in the images made by simple lenses tormented the early makers of microscopes. These distortions are due to two simple facts illustrated in Fig. 2, a section of a glass prism with a beam of light going through it. The beam, entering from the left, is of so-called "white" light. That is, it is a pencil of rays of mixed wavelengths which, when combined on the retina, cause the sensation that we have learned to call white. When a ray of light passes from a medium of one optical density to a medium of another—as from air to glass, or glass to water, or water to air—it is bent or "refracted." The extent to which it is bent depends first on the angle at which it enters the new medium and second on the difference in optical density between the two media. This difference is usually expressed as an index of refraction, which is the relative optical density of the

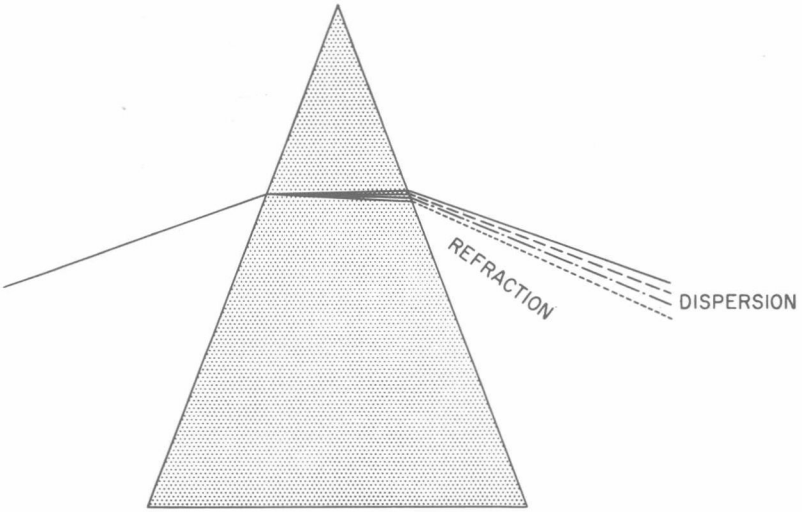


Fig. 2. Dispersion and refraction by a glass prism. Refraction is a measure of the amount a beam of light is bent. Dispersion is a measure of the amount by which rays of different colors are bent.

medium in relation to air. Glass, for example, has about one and one-half times the optical density of air, so that its index of refraction, i in most books, is about 1.5. The value of i for water is about 1.3 and for a diamond about 2.4.

All these figures have been given as "about" so much because an accurate figure can only be given for a single wavelength, that is, a single color, of light. Each wavelength is bent a different amount so that a pencil of white light passing through a prism, or lens, is dispersed as well as bent. This is also shown in Fig. 2. The difference between the index of refraction for red light, which is bent least, and violet light, which is bent most, is known as the "dispersion." In glass, and most naturally occurring transparent substances, a high index of refraction and a high dispersion go together. For example, a diamond has a value for i of 2.41 for red light and 2.47 for violet light, giving a dispersion of 0.06. These facts, as will be apparent in a moment, make life very difficult for lens designers.

Now let us examine the formation of an image by a lens. Figure 3 shows a simple lens forming an image of an object. This object is an arrow seen in front view at the far left of the picture, and in side view just to the left of the lens. Rays diverging from the lower part of the object to the lower part of the lens strike the lens at a relatively small angle and are therefore bent relatively little. It follows that they go a relatively long way before converging again to form the bottom part of the image. Rays

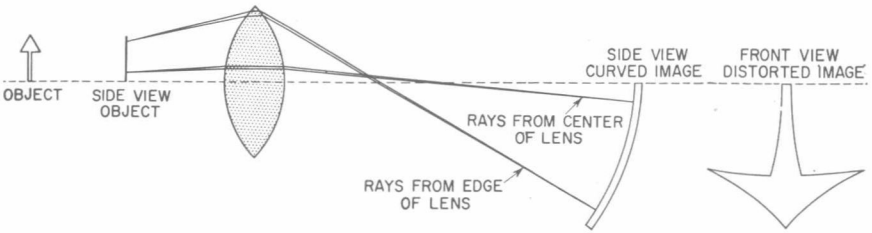


Fig. 3. Diagram to show the causes of spherical aberration. Rays passing through the edge of the lens are bent more than those passing through the center. The rays passing through the edge of the lens therefore come to a focus nearer the lens, with the result that the image is curved. As those portions of the image that are nearer are larger, it follows that the image is distorted.

diverging from the top part of the object to the top part of the lens strike the lens at a relatively large angle and are therefore bent more than the rays striking at a low angle. It follows that they go a relatively shorter distance before converging to form the upper part of the image. It is obvious that this effect is proportional all over the lens so that the image seen in side view is curved. Actually, since the surface of the lens is part of a sphere, the image is also part of a sphere and this effect is known as "spherical aberration."

Now, the size of an image is dependent on the relative distance of the image from the lens. Hence spherical aberration produces not only an image that is curved but also an image that is distorted in shape when it is cast on a flat surface. This distortion is shown, exaggerated for the sake of clarity, in the front view of the image seen at the far right of Fig. 3. Spherical aberration is the result of refraction. Dispersion produces "chromatic aberration."

Figure 4 shows exactly the same setup as Fig. 3 but rearranged to show the cause of chromatic aberration. Pencils of light diverging from the object are dispersed as they go through the lens. The red components are bent least and therefore travel a relatively long distance before con-

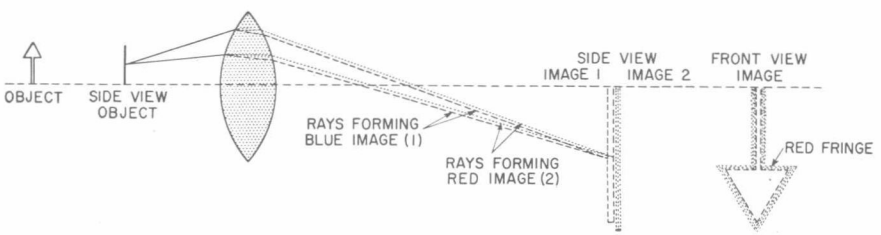
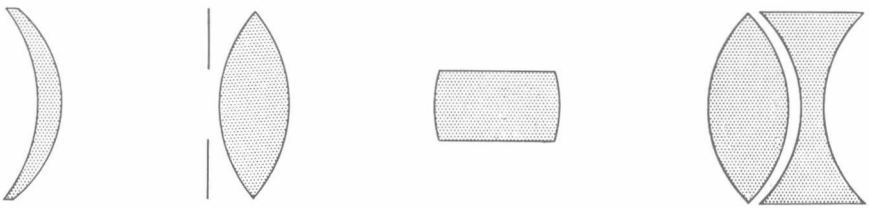


Fig. 4. Diagram to show the causes of chromatic aberration. Light is dispersed (see Fig. 2) as well as refracted by the lens. The blue components are bent more than the red components, so the blue image is nearer the lens than the red image. There is, therefore, a red fringe around the image seen in front view.

verging to form a red image. The blue components are bent most and therefore travel a relatively short distance before converging to form a blue image. When these two images are seen in front view, the central portion, in which all the colors are superimposed, still appears white. The red image is, however, larger than the blue image and sticks out around it. The edges of this image therefore show up as a color fringe around the outside of the white image.

Correction of Aberrations. Spherical aberration is theoretically easy to correct since it is caused by the difference in distance between the



Figs. 5, 6, 7, and 8. Methods of correcting aberrations. The meniscus (Fig. 5) diminishes the difference in thickness between the center and the edge. An iris diaphragm in front of the lens (Fig. 6) or a "Coddington" lens (Fig. 7) produces the same effect at the expense of the aperture. The action of the doublet (Fig. 8) in diminishing chromatic aberration is discussed in the text.

center and the edge of a lens. The easiest method (Fig. 6) is to place a diaphragm in front of the lens so that only the center is used. The same effect (Fig. 7) is obtained from a cylindrical piece of glass with a lens face ground on each end. This, which is to all intents and purposes the center cut from a larger lens, is often sold as a hand "magnifying glass." The last commonly used method (Fig. 5) is the so-called "meniscus lens" in which one face partially compensates for the other. Combinations of all three of these ways of correcting shape distortions are used in microscope objectives.

The correction of chromatic aberration is much more difficult. The only solution so far discovered makes use of the fact that the relation between "index of refraction" and "dispersion" differs in different kinds of glass.

Take, for example, the combination shown in Fig. 8. If the positive lens at the left is made of a glass of high refractive index and low dispersion, it will bend the light a great deal and separate the colors very little. The negative lens on the right, if made of a glass with a lower index of refraction, will not bend the light out as much as the one on the left bends it in, so that an image will be formed. But if the negative lens on

the right has a very high dispersion, it will pull the colors proportionately closer together even though it does not bend the light so much. Hence all the colors will come together at the same point and produce a colorless or "achromatic" image. Moreover, this image will be relatively free of spherical aberration since the thick edge of the negative lens balances the thick center of the positive one.

Unfortunately all this is a theoretical dream. Glasses of very high refractive index and very low dispersion—or vice versa—do not exist. All lens design is a compromise, sometimes involving as many as six kinds of glass, each bending or separating or compressing light in varying amounts. Lens designers early learned to incorporate fluorite as a substitute for one of the glasses, and during World War II many synthetic nonsilica glasses were developed. But the perfect lens is still in the future. Designers effect the best possible compromise between reality and theory in the light of the specific requirements of a microscope objective. These requirements must next be examined.

REQUIREMENTS OF A MICROSCOPE OBJECTIVE

Resolution. The most important part of a microscope is the objective. Every other part of the instrument is designed to help the objective produce the best possible image. The best image is not the *largest*—it is the *clearest*. There is no purpose in looking at an object through the microscope unless we arrive at a better understanding of its structure. Mere size is no aid to understanding. A simple black dot the size of a pin's head is just as understandable as a simple black dot an inch in diameter. What we want to know from the microscope is whether the pinhead-sized dot is a simple dot or whether its smallness conceals a pattern. The ability of the microscope to reveal this pattern is known as "resolution," and resolution is therefore the most eagerly sought characteristic of a lens. It is obvious that a lens with chromatic and spherical distortions will not resolve satisfactorily, but there is more to resolution than the correction of aberrations.

This elementary book is no place to present involved mathematical arguments about the cause and nature of resolution. There is, however, a very simple analogy that will serve to introduce the subject. Suppose that you have in your hands a sheet of material and that you do not know whether it is woven from fine fibers or rolled from a structureless plastic. You would immediately try to pull your hands sideways to spread any fibers that might be present. Nothing would be gained by plucking the sheet toward you. It is the same with lenses. If they are to resolve closely spaced lines and dots, they must spread out the image. They do this by spreading light rays—and the wider the spread, the greater the

resolution. The spread is measured in terms of the widest angle from which the lens can bring rays to a focus and form an image. Resolution is measured as the number of lines or dots per inch that can be separated. "Angular aperture" is thus a potent factor in resolution.

The effectiveness of angular aperture is, however, limited in practice by the wavelength of the light used. Again it is necessary to ignore theoretical arguments as to the structure of light rays in favor of a practical analogy. Suppose that light is propagated as a series of waves of varying wavelengths. Now mentally transpose these waves to the surface of the ocean. A liner will leave a perceptible wake—or, in optical terms, shadow—as it passes through even the largest waves. A child's toy boat will leave a wake of only the tiniest of ripples. Light rippling past an object on its way through a microscope to our eyes follows just the same rules. No object smaller than the waves of light can create a disturbance in the waves that is perceptible to the eye. It follows that the shorter the wavelength of light, the greater the possible resolution.

It must be reemphasized at this point that resolution, from the practical point of view, is a measure of crispness or clarity. People often fail to see how the number of lines or dots per inch that can be separated, or resolved, is a measure of the sharpness with which larger objects can be seen. Actually everything is seen against a background of something else. Sharpness and clarity are just measures of how well the object is separated from, or resolved against, the background.

Wavelength, resolution, and angular aperture have very simple relationships. In the first place it must be obvious that the angle of the cone of light that can enter any lens is dependent on the refractive index of the medium in which the lens is working. This dependence, or "numerical aperature," is expressed by the relation

$$\text{N.A.} = i \sin \theta$$

where θ is one-half the angle of the entering cone of light and i is the refractive index of the medium surrounding the lens. Since i for air is 1, and since sin θ cannot be greater than 1, it follows that no lens working in air can have a theoretical N.A. greater than 1.

The relation between N.A. and resolution is just as simple for

$$R = \frac{\text{N.A.}}{\lambda}$$

where λ is the wavelength of the light and R is the number of lines that can be separated. R or λ can be given either in inches or millimeters. Some books prefer to express resolution in lines per inch and wavelength in millimeters, in which case the relation becomes