Photomicrography

An Introduction
to Photomicrography with
the Microscope

FOURTEENTH EDITION

Eastman Kodak Company
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Due to wartime conditions and attendant limitations of essential materials and production facilities in general, some of the products mentioned in this book will in all probability be temporarily unavailable—at best, their delivery unavoidably delayed.

Also, some of the equipment illustrated and described here may have undergone or may undergo mild structural change without, however, affecting the products' efficiency in any way.

We know that your understanding and indulgence can be expected as long as present conditions exist. —APRIL, 1944

Introduction

PHOTOMICROGRAPHY is one of the fields of photography which attracts both the amateur who may be primarily interested in purely pictorial photography and the professional technical worker who may be very much of a specialist. Frequently, experts in the various sciences turn to this field as a means to an end when their education and experience have included little or no practice in photography. In either case, a general knowledge of the principles of this subject and of the characteristics of the materials used should prove both interesting and helpful. There is some tendency to carry over into this application of photography the restrictions that are necessary for general photography. Some procedures and choices of materials, however, become practical for photomicrography because it is usually done under conditions resembling those of a laboratory.

This book is offered as a text that presents a simple discussion of the pertinent fundamentals of optics and photography as well as practical instruction in the technique of photomicrography. No claim is made that all possible methods are included, but both more extensive and more advanced discussions of the subjects involved may be pursued in the references given in the bibliography and in the list of some of the publications of this company at the end of the book. Information concerning other technical and scientific publications by this company will be sent if the particular interest is described.

It is hoped that the tables included in the book together with the specific discussions of various methods, to which quick reference may be made through the index, will make this a useful handbook of photomicrography.

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CHAPTER I

Apparatus

THE APPARATUS arranged for photomicrography should fulfill two requirements. The apparatus should be free from vibrations, or it should vibrate as a whole; the source of light, condensing system, microscope, and camera should be capable of being centered into one axis with the plane of the plate or film parallel to that of the microscope stage. Unless the arrangement is temporary, it should be possible to fix all of these elements accurately in this position, although the camera should be removable to allow visual inspection through the microscope. It is well to have no physical contact between the microscope and camera, but to use a matte black lighttight connector between them. If the stand is vertical, a tube of black cloth can be easily made and is usually satisfactory. Elaborate apparatus is not a necessity and very good work can be done with simple equipment.

Elimination of Vibration

The ideal location for a photomicrographic outfit is, of course, in a place where there are no vibrations from the outside and it is directly part of a massive support. If this is not possible, the camera support must be stable and preferably supported separately.

If trouble from outside vibration is encountered, it is wise to mount the whole photomicrographic table or bench on a suitable absorbent for the vibration. This is better than mounting the microscope or even the microscope and the camera on such an absorbent, since the floating system should have as great an inertia as is conveniently possible. The best type of vibration absorbent varies with the source and type of the incoming vibration. Sponge rubber is often used and is helpful if it is sufficiently thick and is renewed frequently; piles of bath sponges represent the most effective type even if they are apparently flattened beyond usefulness by the weight. Horizontal vibrations seem to be best removed

by standing the table on multiple layers of some kind of fabric or metal sheeting. A most effective method for shielding from many types of vibration is to support a platform, carrying the whole table or bench, on softly inflated inner tubes of truck tires protected by a loose wrapping of canvas strips.

The act of making the exposure may cause vibration, and if a mechanical shutter is used, a cable release should be employed. After manipulating the microscope or drawing the dark slide of a plate holder, it is best to wait a fraction of a minute, when possible, before making the actual exposure.

PROFESSIONAL APPARATUS

When photographic plates and sheet films are used, the specialized professional materials discussed on page 88 are available. This is a decided advantage for technical work, of course, and professional equipment is made to use these materials.

The Kodak Precision Enlarger can be used as a vertical photomicrographic stand with professional materials by using the Camera Back Adapter in place of the Condenser Head and Lamp. The Bellows Assembly A and the Camera Back Adapter A take photomicrographs on 2¼ x 3¼-inch plates or films (as for the Recomar 18) with a bellows extension of 8 inches. The Bellows Assembly B and the Camera Back Adapter B use 3¼ x 4¼-inch plates or films (as for the Recomar 33) with a maximum bellows extension of 12 inches. In either case, the Microscope Adapter should be placed in the lens opening of the enlarger. Specific instructions are in the directions accompanying the enlarger.

If the exposures are likely to be shorter than a second, a good mechanical shutter such as the Kodak Supermatic Shutter should be mounted in the light beam. Care must be taken to locate the shutter in a plane where it will not be even approximately in focus with the microscope image. With short exposure times this gives the effect of uneven illumination which decreases toward the corners of the picture. The shutter, therefore, must be in an aperture plane of the system, i. e., near the substage diaphragm or at the eyepoint above the ocular.

When considerable technical photomicrography is to be done, it is undoubtedly wise to invest in a properly and solidly constructed piece of equipment especially designed for the purpose, such as is sold by the microscope manufacturers. Whereas a long optical bench has some advantages for general work, there are now many special de-

signs for particular purposes that might prove more efficient or convenient. Most specialized, yet in wide-spread use, is the apparatus for work by reflected light, especially designed for metallography. The simple principles previously discussed hold for such apparatus, and should be considered in judging it. Excellent direction booklets are usually furnished with such apparatus and should be carefully studied.

Only a first-class microscope is really suitable for serious and extensive photomicrography. The microscope should be very rigid and heavy, and it must be fitted with a mechanical stage. If the microscope which it is proposed to use is not fitted with a mechanical stage, it is desirable to fit one of the detachable mechanical stages which are on the market. If considerable photomicrography by reflected (incident) illumination is to be done, a microscope with a focusing stage is almost a necessity. For the photomicrography of metals with vertical illumination, specialized and complete apparatus is on the market, which almost universally utilizes an inverted microscope with the objective placed below the stage. Such apparatus is more convenient than the standard type of microscope with vertical illuminator. However, it should not be forgotten that as excellent work can be done with the latter apparatus, and it is often more adaptable to experimental or unusual problems.

APPARATUS FOR SIMPLIFIED PHOTOMICROGRAPHY

If a picture of a particular field seen through the microscope is desired when no regular photomicrographic setup is available, such a picture can be obtained merely by tilting the microscope so that it is horizontal and by blocking up almost any kind of camera (as discussed later) so that the optic axis of the microscope beyond the eyepiece also becomes the axis of the camera. However, a vertical stand on which the camera is suspended over the microscope is probably the most satisfactory arrangement unless a long bellows draw is required, and then an optical bench is preferable. The Stand Assembly of the Kodak Precision Enlarger furnishes a convenient vertical support, and any Kodak or similar camera can be fastened to it by means of a tripod screw so as to hang over the microscope. If the tripod socket on the camera has become at all loose, it should be sent to a camera shop for this simple repair.

A relatively inexpensive yet convenient setup can be made from a wooden board and the usual type of metal laboratory supports, although the vertical support rod must be of adequate diameter; that of the usual $\frac{3}{8}$ or $\frac{1}{2}$-inch laboratory rod is insufficient. The camera can be

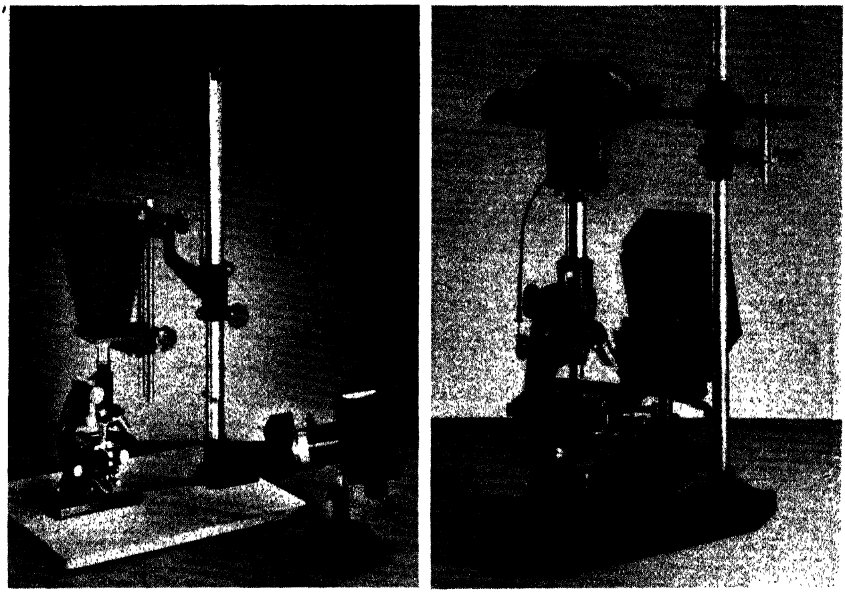


Fig. 1

supported on its face by the horizontal wooden board which has one or more holes cut through it to allow the camera bellows or other projections to drop through. (See illustrations above.) The board in turn is supported by a laboratory extension ring (such as is frequently used with flasks) which swivels around the vertical support stand and thus can be swung aside during the usual use of the microscope. The camera can be clamped into position by heavy rubber bands snapped over picture hooks.

The greatest simplification of all consists in the use of a roll-film camera since with it a darkroom is not even necessary. Not only is the camera loaded in daylight, but the film can be sent to a photofinisher for development and printing. A personal camera can be used with photomicrographs and general pictures on the same roll of film.

In general, photomicrography can be done with either of two types of camera, namely, one with or one without a lens. In utilizing most hand cameras, only the first method is practical.

Use of Camera with Lens

A compound microscope is designed, in general, for visual work with its image at infinity, i.e., with parallel bundles of rays emerging from the eyepiece. The eye is expected to focus this image on the retina while

the eye is relaxed, as when it is focused for distant objects. Therefore, if the camera with its lens focused at infinity, or in practice at 100 feet, is placed over a well set up microscope that has been visually focused, a good photographic reproduction of the image can be obtained by exposure of the film. It has been found, however, that most people when looking through a microscope strain a trifle with their eyes; therefore, it is best to focus the camera at 25 feet. There are many, particularly young people, who accommodate their eyes still more, and in some cases the degree of accommodation is so variable that it is impossible to predict the distance to set the focus of a camera.

There is a simple method which is so effective in obtaining the optimum results upon focusing that it probably should be used in all cases for photomicrography with a hand camera containing a lens. Briefly, this method consists of looking into the microscope during the operation of focusing through a telescope held to the eye. The telescope must contain a graticule upon which its eyepiece has been carefully focused at 100 feet by pointing out a window. If no such telescope is at hand, one can be made easily. One form consists in utilizing a 48-mm. microscope objective with its "back" toward the object and the other end pushed into a cork with a hole bored through it; this in turn is inserted into one end of a convenient tube. A microscope ocular with focusing eye lens and a microscope scale can be used if the field (lower) lens is unscrewed and removed; this is held in the other end of the tube by a washer. The camera lens should, of course, also be focused at 100 feet.

One further precaution should be taken. The distance of the camera should be so adjusted that the eyepoint of the microscope falls in the center of the front surface of the camera lens. This point can be determined by placing a thin sheet of paper above the eyepiece and moving it up and down until the circle of light is the smallest. The definition is best with this arrangement, and the quality of the lens is least important in this case. A single element achromat would be the best lens for this purpose, but a simple spectacle or meniscus lens is quite satisfactory. This simple method is optically excellent; its only objections are:

1. It takes a picture of the whole visual field whereas only the central portion is usually in focus. For this reason, it is wise to use only high-power oculars. The magnification will be less than the rated visual magnification of the compound microscope. With a lens of average focal length in a hand camera, the magnification will be about one-third of its rated value.

2. It takes a picture of the illuminated back lens of the objective and superposes that as a small flare spot on the picture. Raising the camera from the plane of the eyepoint causes this flare image to go out of focus rapidly; the field then becomes smaller on the film as it is limited by the lens diaphragm, and the definition becomes somewhat degraded.

The convenience of the method often outweighs these two disadvantages. With a roll-film camera, such as a Kodak 35 or Bantam, a complete record of a stained specimen or the polarization colors of some crystals is easily obtained with Kodachrome Film.

Use of Camera without Lens

The second type of camera, one without a lens, is used more frequently for photomicrography than one with a lens. In this case, the image is focused on a ground glass. It is even better to focus the image with a magnifying lens against a clear central portion of the ground glass bearing cross lines. This can be made easily by cementing a micro cover slip to the ground glass side with Canada balsam, after having first drawn a well-centered cross on the ground glass with a pencil.

When it is not desirable to have a permanent clear area in the ground glass of a camera, the cover slip can be held with cedarwood oil. The film plane of such a camera should be set up no nearer than ten inches from the ocular (except as discussed below). It should be rigidly supported so that the extended optic axis of the microscope intersects the cross line on the ground glass. (See section on "Centering," page 28.)

High-precision miniature cameras, such as the Kodak Ektra, constitute a class of roll-film cameras with removable lenses. Special accessories are available that make them efficient for photomicrography without the two disadvantages associated with camera lenses but with the advantages of roll-film cameras. With one type of accessory, focusing is done on a ground glass, which is put into the same plane that is occupied by the film during photography. For instance, a ground-glass focusing back is supplied for the Kodak Ektra. Another special type, which is inserted between the camera and the microscope, deflects the image beam so as to set up another image which is simultaneously in focus with that on the film. Ordinary cameras can be supported over the microscope by the Kodak Precision Enlarger by means of their tripod socket; however, with the Kodak Ektra, the tripod clearance head should be used as an intermediate adapter. Miniature cameras can also be supported by the wooden board arrangement discussed on page 10.

The 35-mm. Kodachrome Adapter, which is a sliding focusing panel, with the Kodak Precision Enlarger is a most satisfactory device for utilizing these films for photomicrography. The Kodak Precision Enlarger furnishes the entire support equipment, including the bellows, or the adapter and bellows can be supported by a piece of wood on a ring stand as previously described.

With miniature cameras there is a tendency to make the whole unit compact; this causes the film plane, with no lens intervening, to be within a few inches of the top of the ocular. The objectives of a compound microscope are designed to be used visually, as described on page 10, with the image plane at infinity. After a microscope has been visually focused, if more than a comparatively slight refocusing with the fine adjustment will not bring it into focus on the ground glass, further adjustment should be made by slightly pulling out the eye lens of the ocular. As a poorer alternative, the whole ocular can be pulled out which extends the tube length, but this is preferable to refocusing with the coarse adjustment. If the ground glass of the camera is sufficiently distant, this adjustment is unnecessary, as the minimum distance for this is rather arbitrarily assumed to be ten inches.

LIGHT SOURCES

Incandescent tungsten lamps are most frequently used as light sources for photomicrography because of their great convenience and their steadiness both of position and intensity. Even the common frosted bulb can be used, but unless certain precautions are taken it is rather unsuitable, chiefly because of lack of uniformity over its surface and the danger of glare due to its size. All illuminants should be well housed with the size of the aperture in the front of the lamphouse carefully controlled, and this type is no exception. A metal can or a box can be used if a hole is cut for the aperture and others for ventilation, opening at the back. In general, it is best not to use a reflector such as that provided in a desk lamp. The optical arrangement of Fig. 4,d, page 24, can be used, in which case the focal lengths and positions of the two lenses should be so chosen that the reduced image of the bulb just covers the required aperture of the substage iris diaphragm.

One of the simplest, yet most satisfactory, illuminants can be made by using, as the light source, a piece of finely ground glass illuminated from behind by such a bulb, with a variable diaphragm situated directly

against the ground glass and a condenser lens held at the proper distance in front of it. A series of diaphragms can be made from sheet metal, each piece containing a hole of different diameter. A diaphragm can also be made very easily from two pieces of sheet metal with horizontal V cuts in each to form a diamond-shaped opening of variable size. A Photoflood lamp is especially suitable for this arrangement, particularly when Kodachrome Film Type A is used (see page 132). By placing a resistance of 30 to 35 ohms in series with the lamp, its life is extended to that of normal lamps. During photography the resistance can be short circuited in order to produce the high intensity and correct illumination quality which are available only because of the lamp's short life of a few hours at such voltage.

Most of the illuminants sold for visual work with the microscope can be used, particularly for low-power work in a bright field. If considerable photomicrographic work is to be done, however, one of the lamps especially made for this purpose should be obtained. The more frequent use of narrow-band filters in photomicrography is one reason for the need of a more intense light source for photomicrographic than for visual work.

Whether an alternating or direct current electric supply is available will govern the choice of illuminant to some extent. Incandescent lamps with clear bulbs and bare incandescent wires usually are not suitable even when they are imaged in the objective plane by Köhler illumination (see page 24), although the small (6-8) voltage lamps with a concentrated filament are good. One of the best of the incandescent type, because of its intensity and its homogeneous image, is the ribbon filament lamp burning at 6 volts and 108 watts, which is furnished in a tubular bulb. Its chief disadvantage is for color photography (see page 135), since the illumination quality (or color temperature) from this type of lamp is unusually variable from one lamp to another even with the same current flowing through them. Great care must be taken with these, as with all low-voltage lamps, that so much voltage in the leads is not lost that the lamp is burning at a much lower voltage than is rated or assumed. Mercury illuminants, such as sold by the Bausch & Lomb Optical Company, when used with the appropriate filters (see page 70) provide the best source of monochromatic light.

For extensive photomicrography, there should always be an ammeter and possibly a voltmeter in the lamp circuit, together with a current regulator. This can be a variable resistance, but for A.C. supply, a

good autotransformer is advantageous, although more expensive. Such an arrangement is practically a necessity for color photomicrography. (See page 136 for discussion of use of the color temperature meter with incandescent light sources.)

The direct current carbon arc lamp is widely used for metallography and is unusually suitable for color photography (see page 133) except in those cases, chiefly at low magnifications with transmitted light, where the exposures are excessively short. In the latter case, use of the properly calibrated incandescent bulb (Photoflood or lamp at 3200°K.) is suitable when the arrangement discussed above is employed. The short exposure times that are obtained when an arc is used may require the use of a good camera shutter of the gear type, which is not frequently utilized in photomicrography. Nevertheless, these intensities and times, equivalent to camera exposures made in sunlight, have some advantages, not the least of which is the lessened danger from vibrations or other disturbances.

For color photography, a homogeneous light source is necessary. Therefore, carbons containing metallic salts in their cores (daylight carbons, etc.) should never be used for this purpose. Likewise, the relatively new tungsten arc cannot be used for this purpose because of the mercury vapor contained in the bulb to start the arc, which gives its discontinuous spectral illumination mixed with that from the tungsten electrode. This lamp, however, is excellent for black-and-white photomicrography, particularly for dark-field work or with vertical illuminators, since it has the highest intensity of all of the steady, concentrated light sources and is relatively uniform over the incandescent area.

For photomicrography with oblique, reflected illumination (see page 41), the problem is usually to get the beams of light at the objects with satisfactory control of direction and quantity because of limited working space. Special illumination devices for this type of work are now commercially available from several microscope companies. Such devices are enormously timesaving and convenient in comparison with the setup and manipulation of the ordinary type of lamps. For low-power work, they usually consist of either a ring reflector or a circle of illumination surrounding the objective; such a device can be constructed with flashlight or automobile headlight bulbs. For medium and high powers, special units consisting of an objective surrounded by a ring condenser are employed, the illumination from a lamp at one side being utilized by a proper optical system. These are not only convenient, but at higher

magnifications, where there is little working distance, it is impossible to obtain comparable results by other expedients. Directions for the operation of these devices can be obtained from their manufacturers.

Whenever a high intensity light source is used, such as a ribbon filament lamp, it is a wise precaution to place a heat-absorbing filter in the beam. This is to protect from the intense concentration of heat not only the objects but also the balsam-cemented objectives and the very thick lens of the modern high-aperture substage condenser. A two-inch layer of water is usually sufficient, but filling the cell with a 1% solution of copper sulfate acidified with a drop of sulfuric acid is more effective, though achieved at the cost of a little loss in a white light exposure with panchromatic film. A piece of special heat absorbing glass can also be used. These colored heat filters cannot be used for color photography of course, although compensating filters for them have sometimes been individually devised.

CHAPTER II

General Optical Principles

THE compound microscope, as it is termed in textbooks dealing with optics, consists essentially of two portions, the objective and the eyepiece. As illustrated in the diagrams on page 18, the objective would form a real and inverted image of the object in Plane II but the field lens of the ocular, F, intercepts the rays, forming the image in the slightly lower plane (III). This real image is examined with the eye lens, E, of the ocular acting as a simple magnifier and forming a virtual image (IV). Obviously the original object undergoes two stages of magnification, one by the objective and the other by the eyepiece. The magnifying power of the microscope is therefore the magnifying power of the objective multiplied by that of the eyepiece. The following tables of magnifications are taken from the catalogue of microscopes published by the Bausch & Lomb Optical Company:

Tube length = 160 mm. Projection distance = 250 mm. (10 inches).

ACHROMATIC FLUORITE OBJECTIVES		HUYGHENIAN EYEPIECES OR HYPERPLANE EYEPIECES					
Equiv. Focus in mm.	Magnification	5x	7.5x	10x	12.5x	15x	20x
48	2	10	15	20	25	30	Hyperplane only
32	4	20	30	40	50	60	
16	10	50	75	100	125	150	200
8	21	105	157	210	263	315	420
4L	43	215	320	430	537	645	860
4S	45	225	338	450	562	675	900
3	60	300	450	600	750	900	1200
1.9	97	485	727	970	1212	1455	1940
1.8	100	500	750	1000	1250	1500	2000

APOCHROMATIC OBJECTIVES		COMPENSATING EYEPIECES					
Equiv. Focus in mm.	Magnification	5x	7.5x	10x	12.5x	15x	20x
16	10	50	75	100	125	150	250
8.3	20	100	150	200	250	300	500
4	45	225	338	450	562	675	1125
3	62	310	465	620	775	930	1550
3	61	305	457	610	762	915	1525
2	90	450	675	900	1125	1350	2250
1.5	120	600	900	1200	1500	1800	3000

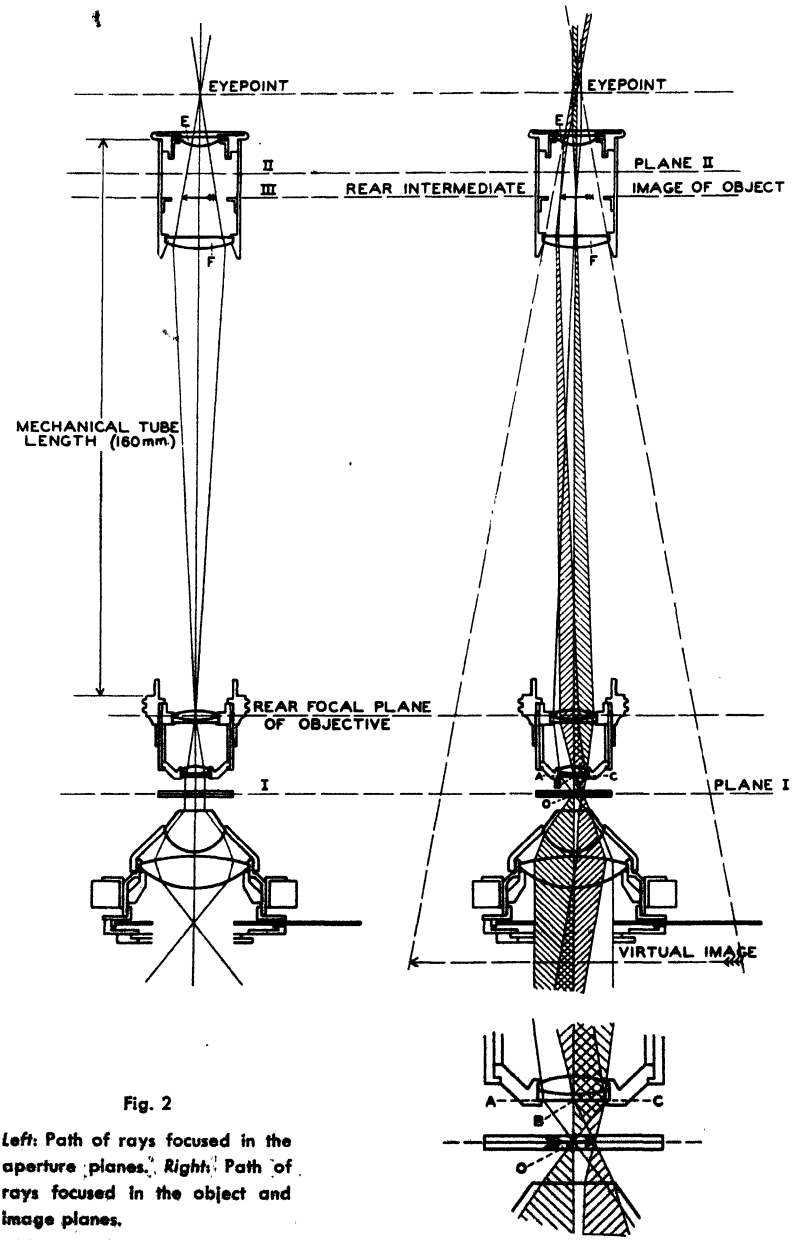


Fig. 2

Left: Path of rays focused in the aperture planes. *Right:* Path of rays focused in the object and image planes.

These magnifications are obtained on the ground glass when it is ten inches from the eyepiece of the microscope; if the camera extension is twenty inches (measured from the eyepiece) the magnification will be twice as much, and so on. It is obvious that with these optics and the proper tube length, the magnification in use can be obtained by multiplying the magnification for the proper objective and eyepiece in these tables by one-tenth of the camera extension measured in inches.

Now it is clear that we could get any degree of magnification required simply by using a draw tube of sufficient length or by using an eyepiece of high enough power; there would seem at first sight to be no limit to the fineness of detail which could be perceived with the microscope since we could easily use another microscope instead of an eyepiece and so get almost unlimited magnification. Unfortunately, while we could easily obtain any magnification in this way, the amount of detail which can be observed is quite strictly limited by certain optical laws and mere magnification of the image does not permit us to see more detail than is given by the construction of the objective. This limit to detail, or, as it is generally called, to the resolving power of a microscope, is fixed by the structure of light itself. Light is not a continuous flow of substance but consists of definite waves having a definite length which make a structure to the light so that we cannot hope to see things which are much smaller than this structure of the light itself. Therefore, by decreasing the wave length of the light used for illumination, the size of the ultimate structure that can be made visible by the microscope will be smaller.

Two of the most useful concepts for anyone using the microscope are those of the structure of the microscope image and the effect on it of the character of the illumination. A more detailed exposition of this subject should be pursued in such a text as that of C. Beck (see bibliography), which is one of the best in English on the optics of the microscope. The easiest and most successful method has been to study the imaging of a point object, since any object can be considered as a collection of points. An ideal microscope system would obviously recombine all of the light rays emanating from this object point into a true point image. The narrowest cross section of the image cone of light from the point with an actual microscope is a small disk which may already be familiar to users of ordinary cameras as the "circle of confusion" but which microscopists call the "antipoint" or "Airy disk." The distribution of light within the disk as well as its diameter is important; it is rarely uniform but normally

consists of a bright center surrounded by alternately dark and light rings of rapidly diminishing intensity, although the aberrations of the lens, tube length, etc., have an influence. It is evident that two points in the object that are so close together that their image disks largely overlap, will be indistinguishable as separate entities. Just how close their centers can be and still allow them to be seen as more than one antipoint is a disputed question. The least possible separation of two distinct antipoints is frequently assumed to occur when their first dark bands touch. There is evidence, however, that they can be still closer without appearing as one and that greater detail can sometimes be distinguished upon high magnification than formulas developed from the first assumption would allow.

Now the size of the antipoints, and therefore the minimum distance, d, possible between two distinctly imaged object points, becomes smaller the greater the angle of the light coming from the object point O (angle AOC Fig. 2) which the objective can take in and utilize. This angle of the extreme rays is called the aperture of the objective. The ability of the objective to image distinctly or to "resolve" two object points is proportional to the trigonometric sine of the angle, so the latter is used as the measure. Actually the sine of half of this angle or sine of angle AOB is used. It is usually termed sine u and is merely the ratio of distances $\frac{AB.}{AO}$

The behavior of light and what happens to it depends on the refractive index, n, of the space in which it travels and how it changes when it enters a new material such as a lens, so of course this is also a factor. Specifically, the wave length of the light decreases in inverse proportion to the refractive index. Therefore, when the object is immersed in a medium such as balsam or cedarwood oil whose refractive index is about ½ greater than that of air, the wave length of the light will be only ⅔ of the length it would have been in air, with consequent greater resolving power, as we have seen on page 19 for the same angle, u, from the object that is utilized by the microscope system. It will be noted that the method of illumination is not specified; this explanation applies equally to the cases of transmitted or reflected illumination. However, it is not enough to insure that the object is in a medium of high refractive index. To illustrate this, we may consider three cases: (1) where an object point, O, lies in air on a surface below an objective, (2) where O is mounted in balsam and covered with a glass slip but with an air space

between this and the objective, as illustrated in Fig. 3,b, and (3) where all of the intervening space between the object and the objective has a refractive index of about 1.53 although several materials, such as balsam, glass, and cedarwood oil may be present in layers. Such materials that are optically equivalent are most important in microscopy.

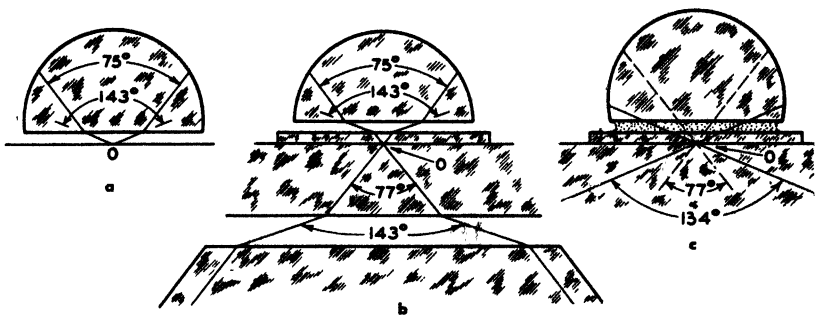


Fig. 3

Considering case (1), Fig. 3,a, the largest cone of illumination coming from O that could possibly be used by the objective would have an angle of 180° (u = 90°). Since a practical working distance between the object plane and the front surface of the objective must be provided, the widest cone that eventually actually passes through the microscope with dry objectives has an angle of about 143° (u = 71.5°) in the air space below the objective. Sine u is now equal to 0.95; the refractive index of the air, n, is equal to 1.0.

Considering case (2), Fig. 3,b, we note that the rays cross a flat boundary of different refractive index in the object space. According to the well-known law of refraction, the angle of the cone becomes greater as it enters the air space from glass or its optical equivalent. Specifically an angle in the glass of about 77° becomes 143° in the air, which we have seen is the maximum that the microscope system can collect. Conversely, if the object be illuminated through the objective, as it is in metallography, the cone angle in the glass lens of 77° becomes 143° in the air and is the maximum cone angle for identical reasons. (When illuminated from below, as when lying on a glass slide or condenser surface, the maximum angle of illumination is 82° since all light of greater angles is totally reflected into the glass, i.e., 82° is equivalent to 180° in air for this refractive index.)

We can see that rays leaving the object point in the balsam with an

angle of 77° (u = 38.5°) will apparently come from the point with an angle of 143° when they reach the objective. By the law of refraction, the sine of the angle of the cone leaving the object point (sine 38.5°) is 1/n*th* (or about ⅔) of that in the air, so that in spite of the fact that the wave length is 1/n*th* of that in case (1), the resolution will be the same in both cases. Now it is also obvious that if all the object space has a refractive index of 1.53 as in case (3) and if the angle of illumination collected by the objective from O is again 77°, the resolution will again be the same. With no intervening layer of air, however, it is no longer necessary to limit the cone to this angle; with a medium that is homogeneous with the glass, the rays go right through into the objective without any deviation and a cone of about 134° from the object point can be obtained in practice with immersion objectives. This cone obviously contains more light and has greater resolving power.

Since a given objective is invariably used with an object space of given refractive index, as a "dry," an "oil immersion," etc., it is convenient to combine the two factors of angle and refractive index into a single term, *numerical aperture* or N.A., which is defined as n sine u. Here, as we have seen for practical purposes, n, may be considered as representing the lowest refractive index in the space between object and objective. If the illumination beam is to be adequate for an immersion objective, the air between condenser and objective must also be replaced with the same immersion medium. When an objective is used at the same working distance, so that the distance BO in Fig. 2 is constant, as it usually is for a constant tube length and visual use (or with a fairly long bellows), then the numerical aperture is equal to the effective aperture of the back lens of the objective divided by twice the equivalent focus. Therefore, the relative proportion of the stated N.A. of an objective that is in use can be measured by observing the proportion of the back lens that is filled with light.

The ability of the objective to image fine detail separately, i.e., its resolving power, is greater when the N. A. is large as is the intensity of the illumination of the image. The depth of focus, however, decreases as the N. A. is increased as does the image contrast (see page 53). A compromise must often be made.

We have seen that the higher the N. A., or the shorter the wave length (*w.l.*), the greater the resolving power. The distance between the finest detail that can be separated by a microscope objective is equal to the value, $d = \frac{w.l.}{N.A. + n.a.}$ where n.a. is the numerical aperture of the con-

denser and ½ (N.A.+n.a.) is called the working aperture of the system. If a very narrow central pencil is used for illumination, the condenser aperture in use is negligible, but due to the diffraction from the object, a greater objective aperture may be in use than that of the illuminating pencil. Therefore, the finest detail that can be shown with sufficient eyepiece magnification and such narrow axial illumination is equal to $\frac{w.l.}{N.A.}$ The wider the pencil used for illumination, the greater the resolving power until a maximum is reached when the whole aperture of the objective is full of light and the condenser aperture used is equal to that of the objective. In this case the resolving power becomes equal to $\frac{w.l.}{2\ N.A.}$ This same limit may be reached when a narrow pencil enters the lens as obliquely as possible, thus utilizing the same condenser aperture. The wave length of light can be taken as being half 1/1000th of a millimeter, or 0.5 micron, which is about 1/50,000th of an inch, so that a lens in which the effective aperture of the back lens is equal to the equivalent focus and in which consequently the numerical aperture is equal to 0.5, can separate lines which are 1/50,000th of an inch apart if all the back lens is filled with light. With a narrow pencil the lines must be 1/25,000th of an inch apart in order to be resolved with this objective.

It will be noted that the magnification does not enter the determining expression for the resolving power, that is, with a given wave length (color) of light and with a given N. A. a certain degree of detail exists in the image whether it has been magnified sufficiently for the eye to see or not. Conversely, we can now see why increasing the magnification beyond a certain limit will not increase the visible detail, as stated on page 19. This will be discussed further under the title of "Selection of the Objective," page 25.

In order to make full use of high aperture objectives, it is necessary to arrange the illumination so that the full aperture of the objective is utilized, because if the beam entering the objective from the condenser is not of sufficiently wide angle, the high aperture of the objective is of no use (except for the light diffracted by the object) and only a portion of the possible resolving power will be obtained. The essential condition for obtaining full resolving power is that *the whole aperture of the objective is evenly filled with light.* The effective illumination for this purpose is either (1) "critical" illumination, or (2) Köhler illumination.

This term "critical" illumination is much discussed in microscopy,

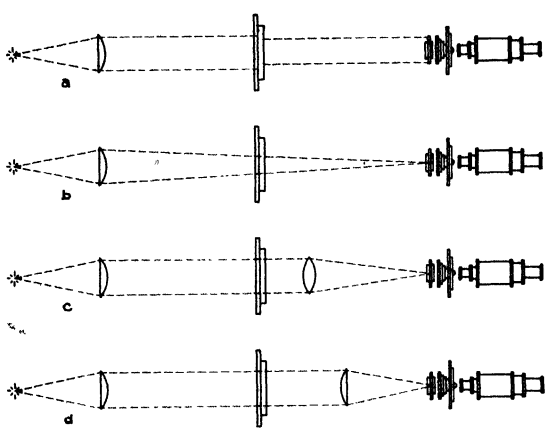


Fig. 4

but in reality it is quite simple both in its fundamental theory and in practice. The condition for obtaining critical illumination in theory is that all the waves of light reaching the object point and forming the image at any one instant of time leave the source of light also at the same instant of time and from the same point. This condition is fulfilled absolutely, of course, if the source of light is focused on the object, as is the case when a parallel beam from a collimator impinges on the substage condenser as illustrated in Fig. 4,a. An enlarged, real image of the light source may be focused on the specimen, if the primary source does not cover the field.

This type of illumination, which is usually used in metallography, requires that the source of light be absolutely uniform over its surface if an even illumination is desired. The alternative method introduced by Köhler floods the specimen with a uniform beam of light by imaging 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 imaging the lamp condenser in the plane of the object. In practice, an image of the light source is placed on the substage iris diaphragm as illustrated in Fig. 4, b, c, and d, by the use of one or two lenses between the source of light and the microscope condenser. This image should be sufficiently large to just fill the aperture of the condenser with light, the size of the image depending upon the focal lengths of these lenses. When using an opal bulb Mazda lamp or similar large source of light a good arrangement is shown at d, where the lenses are used to project upon the back

of the condenser a somewhat diminished image of the large source of light. Since the microscope condenser forms an image of the lamp condenser in the plane of the specimen, a diaphragm placed close to the latter lens will act as a field diaphragm excluding all light that is not being used. One of the chief advantages of the Köhler method of illumination is the ability to control easily and efficiently both the extent of the illuminated field and its aperture of illumination. The latter is controlled by the substage diaphragm. One of its disadvantages is that at high magnifications, only a small aperture can be used at the lamp condenser.

If the image of the light source just fills the opening in the condenser, then with a given aperture of objective and condenser the brightness of the image on the focusing screen depends simply upon the intrinsic brightness of the source of light and not at all on its size; for instance, a properly arranged 4-ampere arc gives the same brightness as a 30-ampere arc, because the increased current simply increases the area of the crater of the carbon and not its brightness.

Determination of the Necessary Aperture and Selection of the Objective

Obviously it is not worth while sacrificing anything to reproduce detail in the picture that is finer than the eye can see; on the other hand, the minimum distance that the microscope can resolve should not be magnified in the print too far above the limit of the eye to discern it. Although most frequently presented from the standpoint of avoiding excessive "empty magnification," the actual situation is liable to be the converse and involves the selection of the objective. The desirable magnification for a given specimen is usually known; the question then becomes, what N. A. is needed? This the microscopist should know by preliminary mental calculation.

The limit to the detail which the unaided eye can distinguish is a variable which depends on a number of factors including the brightness of the object, its distance, and its contrast. It can be assumed that a contact print will be viewed at optimum brightness, and at the generally accepted "normal" viewing distance of 10 inches (25 cm.). The contrast of the detail is very important and more of a problem; it can temporarily be assumed to be entirely that of black and white. In this case the average limit to vision, expressed as the angle subtended by the minimum discerned distance, is about one minute of arc, although it may average

less for groups whose eyes have been corrected by glasses. In rare cases, objects subtending less than 40 seconds visual angle may be perceived (*Seeing* by Luckiesh and Moss, cf. bibliography). At 10 inches the distance subtended by an angle of one minute is about 1/300 inch or 0.085 millimeters (85 microns). It would be safer to consider 75 microns as the maximum distance which is usually *not* distinguished by the unaided eye and since this value also gives a simpler factor for calculation, it will be used. Some idea of these distances and the personal limit to resolution at the highest contrast, may be gained from the patches below, which have been printed from half-tone screens. The separation *between* elements is given in the captions. It is very instructive to cover one-half of each patch with a transparent gray tint to lower the contrast.

Fig. 5

It is evident that if a magnification of 100× is desired in a certain photomicrograph, an antipoint of 75 microns in the image will be produced if the limit, d, of the microscope resolution of the specimen is 75/100 micron. Referring to the formula for resolution on page 22, the numerical aperture required will be: $\frac{\text{wave length.}}{2d}$ Assuming the simple figure of 0.5 micron for the wave length (which may be considered as the dominant one, visually, with the Wratten H Filter), the expression simplifies to: N. A. $= \frac{0.5}{2 \times 0.75} = 0.33$. Accordingly, a numerical aperture of 0.33 is required at 100 diameters magnification if detail is to be as complete as the eye can see under optimum conditions. Conversely, a greater magnification than 100× with a N. A. of 0.33 merely increases the size of the antipoints further above the ultimate limit of vision.

Practically, this limit is often exceeded and rightly so. In the first place the smallest dimension that the eye can distinguish becomes considerably larger when the contrast diminishes, as when it is desired to examine small detail with other portions of the specimen as a back-

ground (cf. *Seeing* by Luckiesh and Moss). Moreover, there frequently is no reason why the desired detail should not be magnified until it is "easy to see."

In ordinary pictorial photography, the photograph is commonly considered to be in good focus if the "circle of confusion" is no larger than 1/100 inch or 250 microns. The photomicrographer, who must usually maintain stricter standards of definition, may then consider this to be the upper limit for the acceptable diameters of his image antipoints under normal conditions. In the case of 100× magnification, the least distance resolved on the specimen must be 250/100 microns and the numerical aperture, determined as above, can be 0.10.

Let us formulate this for practical use. Let M represent the magnification of the print (including possible enlargement) and D stand for the acceptable diameter of the antipoints. Then:

$$\text{N. A.} = \frac{\text{w. l.} \times \text{M.}}{2\text{D}}$$

If w.l. be considered as 0.5 micron:

Case 1. Strictest standard of useful definition:

If D = 75 microns, the visual limit of resolution under optimum conditions,

Then N. A. = magnification ÷ 300

Conversely, the highest acceptable magnification = 300 × N. A.

Case 2. Lowest acceptable definition:

If D = 250 microns,

Then N. A. = magnification ÷ 1000

or the highest acceptable magnification = 1000 × N. A.

For other wave lengths, these values for the N. A. required may be multiplied by the factor $\frac{2 \times \text{w.l. (specified in microns)}}{}$. It is easy to see that considerably less numerical aperture is required by the short wave lengths of ultraviolet for a given magnification.

These simple mental calculations, which set the upper and lower limits, should be made before undertaking the photomicrography of any specimen. The definition needed, of course, will depend on the detail of the given specimen. It is useful to set up some standards where the definition, or the diameters of the antipoints, is known in each case.

CHAPTER III

Adjustment of the Apparatus

IT IS a great advantage in photomicrography to have the apparatus arranged on a permanently aligned optical bench although it is only necessary that the apparatus be rigid. If the microscope base can be clamped to an aligning plate, it can be removed for other work and returned into alignment with impunity.

Greater care must be taken in the adjustment and centering of the apparatus than may be necessary for routine visual inspection, since illumination defects, such as uneven lighting, are more evident in photomicrography.

The exact technique of centering a photomicrographic system depends somewhat on the particular equipment in use, especially whether or not each optical unit can be separately removed and centered. A technique is described in detail below for the adjustment of a setup of the microscope with a vertical axis. As the operator gains experience, he will undoubtedly modify this system to suit himself, but careful procedure through each step should result in satisfactory illumination. Obviously with a permanent setup, all of the steps need not be repeated each time. Centering of a horizontal setup is discussed on page 31.

Centering of Illuminant

Considering the frequent situation where the light source itself and its condenser are carried together in a housing, it is best whenever possible first to center the light source to the lens and to insure that the plane of the source is perpendicular to the optical axis. This is most easily done by lining up, from the rear of the lamphouse, the source itself and its images formed by the surfaces of the lens, using a smoked glass or combination of mutually exclusive color filters to reduce the light intensity. Since it is not possible to see through the source itself, it is necessary to line up the images first horizontally and then vertically, or vice versa.

These images can easily be differentiated from that formed by the glass bulb of a tungsten lamp by moving the lens. In case this method is impossible, the best substitute is to place a piece of paper against the front of the lamp condenser, focus the source on it as nearly as possible, and then center this image to the lens aperture. In either case, turn and tilt the lamp so that the light beam falls squarely on the microscope mirror, and focus the beam on the plane of the substage iris.

With the microscope, the first step is to determine its optical axis, usually from its mechanical axis, by determining two points on it, preferably at its two extremes. A pinhole centering eyepiece used in place of the usual ocular is invaluable for determining the upper point (and also for other uses as discussed below) and can be obtained from the manufacturers of microscopes. The determination of the lower reference point on the optic axis will depend on the type of the microscope. If the substage iris diaphragm is separately mounted and centered (as it is in the case of the so-called swing-out condensers), it forms a good reference aperture when closed down as far as possible. If the substage iris diaphragm cannot be used alone or is centerable with the condenser and if the objectives are fixed in position as with a revolving nosepiece, then make this determination of a lower reference point on the optical axis by focusing on a suitable test object, centering it with respect to the objective, then leaving it untouched through the subsequent centering procedure. This test slide should contain a point recognizable at both low and high powers. The most convenient is a center glass, such as is supplied by microscope manufacturers, which consists of a fine cross in the center of several concentric circles.

In either case, with the lower axial reference point in position, remove all lenses from the optic axis of the microscope. Put in the pinhole eyepiece which now determines the axis. Insure that the mirror bar is in its central position, then twist the mirror so that the bright round image of the lamp condenser is centrally located with respect to the test object or by closing the substage iris diaphragm around it. The correct angular tilt of the lamp, both vertical and horizontal, can now be accurately adjusted by being sure that the image remains symmetrical as the lamp condenser is focused back and forth. By focusing the lamp condenser back so that the source of light is in focus through the pinhole eyepiece, it can be finally and accurately centered with respect to the lens, if it could not be done from the rear of the lamphouse.

If the source of light is not mounted in a unit with a condenser, set it

up alone and tilt the mirror at the proper angle to center it before interposing the bull's eye lens. The work will be much easier if a small hole in a sheet of cardboard or metal is held close to the center of the light source if the latter is extended or does not form a sharp image. The correct tilt of the lens can be determined by equalizing the appearance of all the edges of the aperture image. This is most easily accomplished by placing a thin piece of paper or, better, a piece of ground glass in the substage plane and observing it by looking down the microscope through the pinhole eyepiece. If the mirror is untouched, after centering the source, the lens will be centered when the image is again central. Unless an optical bed is in use, one type of movement of the bull's eye lens affects all of the others so that it may take considerable experimenting to obtain correct alignment.

The light beam should now be focused again in the lower focal plane of the substage condenser, which can be assumed to be that usually occupied by the iris diaphragm. Seen through the microscope pinhole eyepiece, it should be a uniformly bright disk centrally located.

At this point the procedure will differ according to the type of microscope in use.

Centering of Objective and Substage Condenser

(1) Put on the stage a simple test object, such as previously described, unless it has already been done, or possibly the slide to be photographed if it is suitable for testing centering. If the microscope objectives are centerable, center the test object into the optical axis as seen through the pinhole without an objective.

(2) Put in a low-power objective and a low-power eyepiece. Focus on the test object. Center the objective if it is centerable. Otherwise, bring the test point into the center of the visual field by moving the slide. If a fixed condenser iris and a fixed objective do not align with the pinhole eyepiece, it is best to center the test slide into the visual field.

(3) Insert the substage condenser without disturbing the mirror, if possible, and move it up until the disk of light is the smallest.

(4) If the mirror has been undisturbed, center the condenser (if possible) until this disk of light is central with respect to the test object.

(5) If it was necessary to tilt the mirror in inserting the condenser, retilt it approximately correctly and close down the substage iris diaphragm. Substitute the pinhole eyepiece for the ocular, and focus on the edge of the iris aperture as seen through the objective. Center this aper-

ture with respect to the back lens of the objective, repeatedly retilting the mirror to give better central illumination, if necessary.

Replace the low-power ocular and use a lower-power objective than is to be employed later. Center the light disk by touching the mirror. All color fringes should be symmetrical.

Centering of Medium and High-Power Objectives

(6) If objectives are centerable, substitute them without touching the test slide, and insure that the center point is in the middle of the visual field. In centering these, it is probably easier to center them first in a preliminary way, with the pinhole ocular in the tube and the objective slightly above its normal focus, so that the test object can be observed.

With fixed objectives, the higher-power objective may not have the same center as the other if they were not purchased with the microscope stand. In such case, center the test point (cross) with respect to the high-power objective *by moving the test slide.* Swing back the lower-power objective without disturbing the slide, and center the system around this point by retouching the mirror to center the disk of light. If the difference is great, the objectives should be brought to the same mutual axis by the centering screws which are found on almost all revolving objective holders.

After the specimen is on the stage and the field to be photographed has been selected, it is often simplest to insert the pinhole eyepiece and set the camera over the microscope so that the spot of light is in the center of the ground glass.

Centering of Horizontal Bench

Where the photomicrographic outfit is set up on a horizontal optical bench, these results are better achieved in a somewhat different manner.

If the apparatus is of a standard commercial type from one manufacturer, the simplest and easiest directions will be found in the pamphlet supplied by the manufacturer.

If the camera is not attached to such an optical bed, one can proceed as previously described but moving the whole microscope into alignment instead of tilting the mirror. Then the camera can be added into this optical axis by centering the ground glass to the beam from the pinhole eyepiece as described above. If the apparatus is mounted on a bench, it is well first to center the light source, so that the beam of light is parallel and central with the bench and at the right height above it,

and then to interpose the microscope into this optical axis. There is usually some centered, unadjustable point, either the illuminant itself, or possibly the crossed lines in the center of the ground glass of the camera, which serves as a point of reference together with the line (and plane) formed by the optical bench. Some manufacturers furnish a centering gauge for their horizontal apparatus. If such is not available, it is convenient to have a couple of vertical pointers made on a stand fitting the bench and preferably with their tips in line with the horizontal optical axis. Each should have a double elbow with a horizontal portion extending in the opposite direction. The optical bed should be carefully leveled as should be the plate on which the microscope is to be clamped. The beam of light from the illuminating unit is then set into the correct vertical plane with the aid of the pointers or gauge. The lamp should be tilted to give a level light beam by insuring that the center of the beam is at equal heights from the bench at the lamp and at the other end of the bench, and then the beam can also be adjusted to the correct height. When interposing the microscope, proceed as described on the preceding page for the vertical microscope, but move the whole microscope to align, since the mirror is not used, and center the image or light spot on the ground glass instead of looking directly through the tube. For example, when possible first put in the microscope with no lenses in it and align with regard to the substage iris and pinhole eyepiece. It is far easier to align into the beam alternately, first the closed aperture of the substage iris diaphragm and then that of the pinhole ocular, than to attempt to do both at once.

Focusing the Substage Condenser

Having aligned the optical system, and with the slide to be photographed on the stage, the next step in obtaining efficient illumination is to focus the condenser correctly. For this purpose, insert a low or medium-power objective and place a cardboard pointer against the front lens of the lamp condenser, or close the lamp diaphragm if it is very close to the lens. Insure that the illuminant is focused in the plane of the substage iris diaphragm. Then focus the substage condenser until the edge of the cardboard or diaphragm is in focus in the microscope together with the image of the object. If the illuminated field is too small, substitute a longer focal length substage condenser or unscrew the top lens of the one in use. If it is necessary to use a condenser of too short focal length, it is better to have the specimen inside the focus.

Testing Illumination and Adjusting the Substage Diaphragm

Now insert the objective to be used and focus it. Replace the ocular with the pinhole eyepiece, and look down the tube at the back lens of the objective with the substage iris wide open. Unless the objective is an oil immersion type, or *has a greater numerical aperture than the condenser in use*, the back lens of the objective should be completely filled with light and appear as a round, evenly illuminated disk. If the objective is an oil immersion, then it is necessary to fill the space between the substage condenser and the object slide with cedarwood oil to fill the aperture of the objective (see page 22). If there are any irregularities from a perfectly illuminated disk, the whole setup should be re-examined including the matter of the correct cover glass thickness and tube length of the microscope.

A somewhat unevenly illuminated disk may be obtained at wide apertures if an ordinary insufficiently corrected Abbe condenser is used, since all of the light transmitted by it cannot be brought into focus in the same plane because of its great spherical error. An aplanatic condenser is corrected for such aberrations.

Strictly parallel light coming from directly behind an object, as it does with a very small aperture in the substage diaphragm, gives a pure silhouette effect; the image is dark and the background is bright. On the other hand, when the illumination comes at a high obliquity from wide condenser apertures, much light is diffracted and refracted from the object detail and sent into the objective, as in dark-field illumination (see page 47); this tends to make image details bright and the background dark and obviously reduces the contrast from bright-field lighting. This effect is enhanced by the greater proportion of the light that is totally reflected or scattered by the under side of the cover glass at high obliquities. Any condenser aperture greater than that of the objective gives pure dark-field effect with no component bright-field usefulness and should not be allowed in bright-field work. As the substage diaphragm is closed down beyond the maximum aperture of the objective, the component of this diffracted dark-field type image beam decreases, and the bright-field contrast of the image increases; at the same time, object points are represented in the image by brighter and larger diffraction disks so that resolution of detail decreases (see page 20). The nature of the object and the purpose at the time will determine how far the iris

diaphragm must be closed to obtain sufficient contrast or how far it may be closed and still resolve required detail.

With this in mind the actual procedure is as follows:

Gradually close down the iris diaphragm until its edges can be seen cutting down the diameter of the disk of light at the back of the objective. This is the maximum allowable aperture. Reinsert the ocular to be used and examine the image. Then close down the iris until necessary contrast is gained, but no further. Fortunately in photomicrography, some contrast can be gained in the photographic process.

Removing Glare

Remove the ocular and inspect the inside of the microscope tube. If any reflections are seen (aside from the upper part of the tube covered by the ocular), these should be reduced by covering with a matte black. Velvet is good and coffin paper is excellent if care is taken to remove particles that sometimes fall from it onto the back lens of the objective.

For best results, only the field of the specimen used in the image should be illuminated, since the light scattered by the object from external and uselessly lighted portions of the object degrades the definition in the used field of the image. Therefore a "field diaphragm," placed close to the front surface of the lamp condenser and of just sufficient aperture so that its image clears the field that is to be photographed, is much to be recommended. (See page 25.)

Correction for Short Ocular-Film Distance

Microscope objectives are calculated to be used at a definite working distance, which is obtained in visual use with the real image distance at infinity. When used in photomicrography to project a real image at short distances, this working distance is increased, and there is a decrease in the spherical correction, known as undercorrection, which is most marked with objectives of large aperture when used with low-power eyepieces. To remedy this, first focus visually through the microscope, and then bring the image into focus on the ground glass by extending the tube (or pulling out the ocular), possibly leaving the final small adjustment for the fine focus screw. The ideal adjustment, in order to focus on a close image plane, is to move out only the eye lens of the ocular, and with "projection oculars," this can be done.

CHAPTER IV

Low-Power Photomicrography

WHEN the desired magnification is low, the area of the specimen wanted in the final field becomes comparatively large and the image takes on a familiar aspect as when seen directly or with a hand magnifying glass. This somewhat influences the technique; the microscopist may even be willing to sacrifice some definition in order to gain greater depth of field or to obtain a field of sufficient area to include a whole mount. The usual objectives of a compound microscope have been computed to give the best possible central definition at the highest practical aperture for the grade of the objective. Since there has been no sacrifice for extent of field, the area of the latter is relatively small, being somewhat dependent on the kind of ocular used.

The compound microscope may be dispensed with and a photomicrographic objective may be used directly on the camera front as a simple microscope without ocular. These special objectives consist of photographic lenses of the usual fundamental design but computed for image distances of the order of photomicrographic bellows lengths, and are sold with various focal lengths as Micro Tessars, Mikrotars, Micro Summars, etc., according to the manufacturer. Their maximum aperture is about $f/4.5$, which may be considered to be equal to a N. A. of 0.1 for magnifications above 5 diameters, although they usually contain an iris diaphragm. It must be remembered, however, that as photographic objectives they have been designed to have a relatively large field (subtending about 18°) and therefore will have somewhat less central definition than corresponding microscope objectives.

Within a range of magnifications from about 25 to 50 diameters, the microscopist must choose between the larger fields furnished by the photomicrographic objectives and the higher definition of the compound microscope. There may be no detail in the specimen fine enough to be lost by the former method. At low magnifications the points at the

limit of resolution of the objective are enlarged so little that according to the formulas discussed on page 27 only a small aperture is required to maintain the image antipoints below the acuity limit of the eye (see discussion page 25). In fact, it is possible to stop down the objective considerably in some cases without noticeable loss to gain some of the advantages of depth of field and extent of field.

In the table below, the figures in bold type show the minimum apertures that may be used at various magnifications without losing discernible detail in the photomicrographs. This set of values assumes a wave length of 500 mμ and "the strictest standard of useful definition," with the antipoints 75 microns in diameter as discussed on page 27. The other set of figures is based on "the lowest acceptable definition." The notation, f/a, represents the aperture of the photomicrographic objective which is usually marked on mounts as f/5.6, etc.

Magnification	D in μ	N.A.	f/a	Magnification	D in μ	N.A.	f/a
1×	**75**	**0.003**	**75**	10×	**75**	**0.050**	**13.6**
	250	0.001	250		250	0.150	45.4
2×	**75**	**0.007**	**50**	15×	**75**	**0.050**	**10.7**
	250	0.002	167		250	0.150	35.7
3×	**75**	**0.010**	**37.5**	20×	**75**	**0.067**	**7.1**
	250	0.003	125.0		250	0.020	23.8
4×	**75**	**0.013**	**30**	25×	**75**	**0.083**	**5.8**
	250	0.004	100		250	0.025	19.2
5×	**75**	**0.017**	**25**	35×	**75**	**0.117**	**4.3**
	250	0.005	83		250	0.035	13.9
7.5×	**75**	**0.025**	**17.6**	50×	**75**	**0.167**	**2.9**
	250	0.008	58.5		250	0.050	9.8

It will be noticed that about the highest magnification that can be employed with the f/4.5 photomicrographic objectives and subjects of extremely fine detail without showing a discernible degradation is slightly less than 35 diameters.

When illumination by incident light is used, the light scattered by the specimen, unless it is polished, is usually depended upon to fill the aperture of the objective. The methods employed are discussed in Chapter V.

Illumination by Transmitted Light

With low magnifications by transmitted light, the problem is usually to obtain: (1) a field of sufficient extent, (2) absolutely uniform illumination, and (3) the requisite objective aperture completely filled with light. Unlike the technique with incident illumination, unless the specimen scatters a considerable fraction of the light, the prescribed aperture is utilized only by the deliberate and proper arrangement of the optical system. In most of the successful systems for such illumination, either the condenser lens of the lamp is imaged in the object plane and the light source in the objective, or vice versa; the former is usually preferable. Unless there is some medium, such as a ground glass, scattering light between the lamp and the object, after the optical system has been properly arranged it will be found that the size of the uniformly illuminated field varies inversely with the aperture of the objective that is filled with light. It can be seen that the sizes of the light source and its condenser are ordinarily the limiting factors in the size of the uniformly illuminated field obtainable with a given aperture. Normally an $f/1$ lamp condenser is the largest commercially available. With a lamp containing a 2-mm. ribbon filament, it is a rough rule that the diameter of the uniformly illuminated field, in millimeters, is equal to twice the f-value of the aperture, so that about a 9-mm. field in the object plane is the largest obtainable with an aperture of $f/4.5$ under the above conditions. It is well to form the habit of thinking of the size of the field as it exists in the object plane, since this value need only be multiplied by the magnification to give the size of any image field on the ground glass.

In low-power work with a compound microscope, the larger field size and lower N. A. are often obtained by unscrewing the top lens from the substage condenser; otherwise, a condenser of longer focal length may have to be substituted. The focal length of the usual condenser can be lengthened by placing the proper spectacle lens in front of it.

When photomicrographic objectives are used, an optical system introduced by A. Köhler is efficient and particularly convenient. The substage condenser is split into two lenses that are separately mounted. It is illustrated in Fig. 6.

A simple lens, III, with a focal length that is about equal to its distance from the micro-objective, IV, is chosen as an object condenser and placed as near to the plane, F, (object field) as possible. Another lens, II, is placed at such a position that it forms an image of the lamp condenser, I, in the object field; this is most easily done by placing a

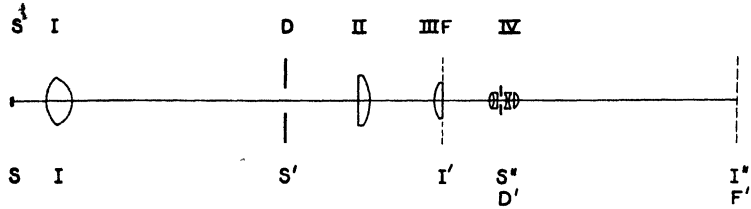


Fig. 6

paper pointer against the lamp lens, I, and obtaining a sharp image of it on the camera glass together with that of a specimen in plane F. A diaphragm, D, such as a separate iris diaphragm, should be placed in front of lens II at a distance equal to the focal length of the latter. It is convenient to have the two tied together with a rod so that they can be moved as a unit. An image of the filament, S, is then focused in the plane of the diaphragm, D. The photomicrographic objective, IV, should be observed from behind to insure that the prescribed aperture is completely filled with light. If the diaphragm, D, is closed down, an opening will be found where its image can be observed to be just equal to that of the objective. If the magnified filament image just covers this opening in plane D, the correct aperture of illumination will be achieved. If the aperture is made smaller, a refocusing of the optical system will provide a larger illuminated object field as discussed above.

It is easier to work with such a system when it is all on one axis as on an optical bench, but this is not necessary. The simplest way of aligning the system is first to establish the optical axis by the two ends of the system, i.e., the lamp and the center of the camera ground glass. The other elements are then correctly centered if the circle of illumination is again made central when each in turn is interposed. Possibly the most convenient order of insertion is, successively: the objective, the diaphragm D, the object condenser III, and the lens II.

As may be surmised, it is much more important that the lamp condenser be a corrected lens for low-power work than for photomicrography at high magnifications. Microscope lamps with $f/1$ condensers which are partially corrected for spherical aberration are sold by the microscope companies. Lens II can be a simple lens, although if a corrected lens, such as a large photographic or telescopic objective, is used, a better image of the lamp condenser is formed and the field is uniformly illuminated to its very edge. It must be of sufficient diameter not to limit the aperture of the illumination. Because of its position, its

f-value must be appreciably greater than that of the objective used, at least *f*/3.5 for an *f*/4.5 objective. Theoretically, this lens can have any focal length, but it is inconvenient if too short or too long. If extremely short, its position is close to the object condenser III, and such a small filament image is required that the lamp condenser lens must be focused further out than most commercial lamps will allow. The size of the field is also slightly reduced. Too long a focal length for condenser II causes the whole optical system to spread to an excessive length. A 6 or 8-inch (15 or 20 cm.) lens that is 2 or 2½ inches (5 or 6 cm.) in diameter is a convenient size. It is sometimes convenient to change it when a greatly different field size-aperture relationship is desired instead of displacing the lamp by a large distance. An individual object condenser III is needed for each photographic objective used. An ordinary spectacle lens whose diameter is larger than the object field is convenient.

If a larger illuminated object field at a high aperture is desired than is furnished by this system, it can be achieved without loss in illumination quality, but at a great loss in intensity, by interposing a ground glass in the system. A secondary light source of greater area can be set up by substituting an opal glass in position S and forming a slightly larger image of the filament with another lamp, or a ground glass can be placed in the plane of the diaphragm D. In the latter case this unit, including lens II, should be shoved as close to lens III as possible.

An entirely different and very simple method is to dispense with such a substage optical system and to place the specimen directly on a piece of opal glass, which is uniformly illuminated from behind and over a sufficient area. The scattered light fills the objective aperture, but a black paper diaphragm or similar device with a hole equal to the size of the field should be placed next to the opal glass in order to prevent unnecessary glare. There will be less contrast in the image with this system, however.

Vertical Illumination

Oblique Illumination

ODESSA—TEXAS METEORITE

CHAPTER V

Photomicrography by Incident Illumination

THE term "incident illumination" is used as opposed to the term "transmitted illumination." It concerns both reflected light as from opaque objects and scattered light as from colloidal particles in dark-field substage condensers.

Illumination by incident light may be divided into two distinct types: (a) non-specular or "oblique," (b) specular or "vertical." With the first type, smooth planes perpendicular to the optical axis will appear dark in the image and the object is generally darker than its background. For this reason it has sometimes been called "dark-field illumination by incident light," although this is not an obvious term if the object occupies the whole field as in the case of the meteorite in the illustration. This type corresponds most nearly to that used in ordinary seeing and ranges from very oblique light from a single source to oblique illumination from the whole azimuth (conical illumination), and also includes completely diffused illumination. Specular illumination, which is the mirror-like reflection of the source by a surface, results in an appearance that is the negative of the first type (see photomicrographs of Texas Meteorite). Therefore, a mixture of the two types tends to reduce the contrast of the image, as would a mixture of incident illumination and illumination by transmitted light. A controlled mixture of two of the above types is frequently useful if one of the two is definitely subordinate. In specular illumination, a polished surface perpendicular to the optical axis will reflect the maximum light, and any deviation therefrom will appear as dark detail. It is therefore in great use as "vertical illumination" for metallography and is described on page 44.

Illumination by Oblique Light

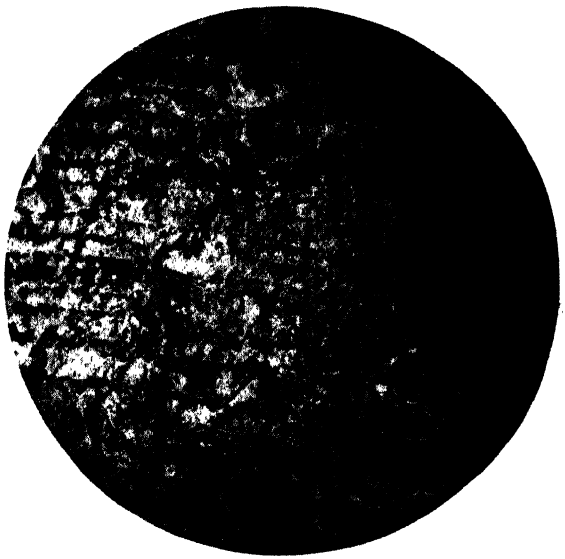
Since this illumination is most nearly similar to that used in ordinary vision, there is a possibility of giving the appearance of relief by restrict-

ing its azimuth and controlling its direction. On the other hand, there is a danger of obtaining deceptive shadows that obscure or can be mistaken for detail. The latter possibility is especially liable to occur when the magnification is sufficient to make the field an unfamiliar sight. Completely diffused illumination gives a very flat appearance. With this type of illumination, there is also the danger of flare from light which strikes the lens without coming directly from the field unless special precautions are taken.

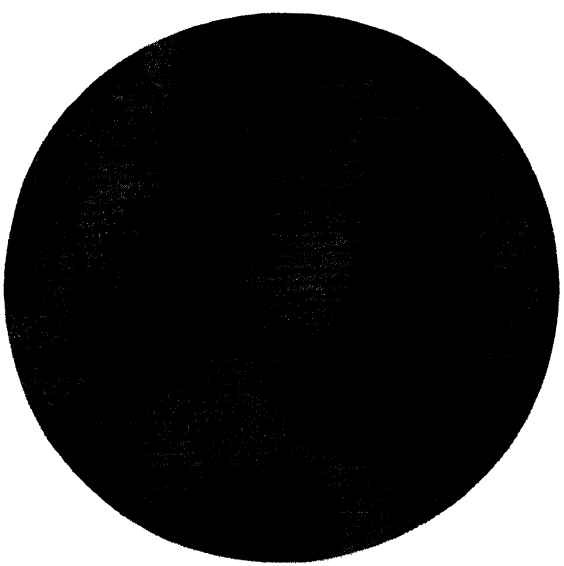
Light scattered in all directions can usually be depended on to fill the objective aperture so that only the limitations of the objective itself need be considered. The ordinary laws of resolution apply (see page 22). As with transmitted light, the simple photomicrographic objectives are most useful for low magnifications in place of the compound microscope.

The actual technique employed will depend chiefly on the working distance of the objective and hence on the magnification. With objectives of 16-mm. focal length (10×) or longer (lower magnification), it is possible to use simple or temporary illumination setups in ways that are not available for the higher-power objectives.

For very low-power work, it is convenient to use a number of goose-necked desk lamps with their reflectors grouped around the field, either symmetrically or not, according to the best appearance of the image. Where this does not prove suitable, the next obvious way is to direct the focused beams from several microscope illuminators on the object, using reflectors of mirror or paper in the beam on the opposite side of the field or by using opposing lamps. Sometimes it is best to interpose a piece of ground glass in the beam quite close to the object. If ground glass is not available, the same effect can be obtained by using Kodak Ground Glass Substitute, which can be poured over a sheet of ordinary glass, or by causing a fine precipitate of barium sulfate to form inside of the gelatin of a fixed-out photographic plate. A simple variation of this which uses only one lamp is as follows: Set up the lamp with a horizontal beam parallel to the stage as for vertical illumination but with the beam passing over the object and under the objective. On the other side of the object let the beam strike a plane mirror held in a laboratory clamp and inclined so as to direct the beam on the object. In front of the stage and in the beam is an ordinary piece of clear glass inclined so as to throw part of the beam up at a high angle. This beam is then directed back at the object by a mirror, the beam passing between the piece of clear glass and the microscope tube and hitting the object on the opposite side from



Unfiltered Tungsten



Kodak Pola-Screens, Crossed
Use of Polarizers With Oblique Incident Light To Reduce Specular Reflections
OPALIZED WOOD, 35X
Ultropak Objective N. A. 0.15 Wratten "M" Plate

the main beam. By coating the under side of the clear plate with dilutions of Kodak Ground Glass Substitute (or moistening a piece of ground glass with glycerine and water) the relative intensities of the two beams can be controlled.

In this general method of illumination, undesirable specular reflections sometimes occur on parts of the specimen, obscuring some detail or reducing the contrast in the image. This can usually be eliminated by putting a plane polarizer in the illumination beam or beams and another one in the image beam of the microscope or micro objective. One polarizer should be rotated with respect to the other in such a way that the specular reflection disappears. This method depends on the fact that the scattered light from the object is largely depolarized. The exposure factor will be very high, of course. Kodak Pola-Screens are especially suitable for this purpose since a light beam can be covered with them when its diameter is sufficient to make the use of calcite prisms prohibitive. The filter that is placed in the image beam must be cemented in glass of "A" quality to avoid excessive degradation of the image.

Another technique for illumination consists of placing the object in a cardboard box, the inside of which has been painted a matte white. The illumination beam passing through a hole in the side may fall either on one side of the box, whereby the illumination will be almost completely shadowless, or on the object, depending on the scattered light from the box to illuminate the shadows. Special care must be taken to protect the objective lens from direct light from the sides of the box.

Specifically designed devices must be used for incident light at relatively high magnifications, and at lower magnifications they are time-saving. They are discussed on page 15 under "Apparatus."

Specular (Vertical) Illumination

This method of illumination finds its chief use in the photography of metal specimens and minerals. Anyone doing a considerable amount of metallography should be familiar with the *National Metals Handbook* and the Standard Methods for Metallography specified by the American Society for Testing Materials. (See page 165.)

In vertical illumination, the illuminating beam is directed to the specimen as nearly as possible along the same axis as it is observed by the objective. Since the light source itself must be at one side, this usually involves directing a horizontal, focused beam to the optical axis and then deflecting it to the specimen by a reflector inclined at 45°.

Either a mirror or prism can be used with one edge on the optical axis, the image-forming beam returning in the other half of the aperture, or a thin glass slip can be used over the whole aperture, reflecting a fraction of the illuminating beam and also transmitting the image beam. For objectives of lower power than the 16-mm. (10×), the easiest and best method is to interpose an ordinary but clean micro cover slip below the objective. For 16-mm. objectives and those of higher power, it is necessary to utilize the objective itself as a condenser and place the reflector behind the objective by means of the so-called "vertical illuminator" especially made for the purpose. It is also necessary to use special "short mount objectives" (to bring the rear objective lens close to the reflector), which have been designed for use without cover glass. Directions for the adjustment of such illuminators can be obtained from the companies that manufacture them.

The mirror or prism gives far brighter images with much more contrast than the plane glass. The opaque reflector, however, which necessarily reduces the aperture and therefore the resolving power, should not be used for the high powers. The flare produced from the reflections of the upper surfaces of the objective lenses can be eliminated by an ingenious use of polarized light with the consequent improvement of image contrast*, which is not, however, available for the highest-power objectives. A vertical illuminator of novel design that utilizes somewhat the same principle is incorporated in a commercial metallographic apparatus.** The illumination beam is polarized by the introduction of a polarizer between the lamp and the vertical illuminator, and another polarizer is placed in "crossed" position in the image beam as previously described. In this case all of the light image will disappear together with the flare. If a thin piece of mica is interposed between the objective and the specimen and rotated in its own plane, a position will be found where the image-forming beam will be rotated through 90° and hence pass through the upper polarizer (used here as analyzer), but the reflections from within the vertical illuminator will still be blocked out.

In metallography "critical illumination," i.e., forming an image of the light source in the plane of the object, is frequently used in actual practice, especially when such a light source is used as the arc or incandescent ribbon tungsten filament. The method of focusing the lamp condenser in the object plane can also be used as it is in work with

*See paper by E. E. Jelley quoted on page 165.

**See paper by L. V. Foster, quoted on page 165.

transmitted light (see page 24). It is especially urgent in this type of illumination to control the excess light by an aperture diaphragm and, if possible, also a field diaphragm (see "Removing Glare" page 34). An aperture diaphragm at least is usually provided on the vertical illuminator.

Oblique illumination can be produced with vertical illuminators when the plane glass reflector is used by interposing a sector into one side of the illuminating beam but away from the plane where it would be imaged in the field. If a small opaque disk is inserted centrally in the beam, conical illumination is produced. This type of illumination at higher powers is most efficiently accomplished by the new illuminators for the purpose.

Up to 100 diameters no color filter may be necessary, except with colored specimens, but above that magnification filters are needed unless apochromatic lenses are used. With uncolored specimens like iron or steel the filter for the best optical performance of the microscope can be chosen. With achromats this is one transmitting apple green light so that for metallography the Wratten B+G Filters are usually used or the yellow-green glass fitted into commercial metallographic outfits, together with an orthochromatic plate, such as the Wratten Metallographic or the Kodak Commercial Plates. (See also page 65 for other green filters for achromats.) Some workers use the blue C filter and apochromatic objectives to gain resolution. Some work has been done in ultraviolet metallography, but at a wave length of 275 mμ the increased transparency of the metals to the ultraviolet causes the exposures to be very long unless an exceedingly intense source is used. Special short mount objectives can also be obtained for photomicrography with the 365 mμ line (see page 80).

For heat-tinted specimens, the K2 filter should be used with the panchromatic plate if the crater of a carbon arc is the light source.

Handling the Specimen

The suitable preparation of metal specimens for photomicrography involving the somewhat elaborate technique of polishing and etching is described in the *National Metals Handbook* and textbooks on the subject. The preparation of mineral specimens is discussed in the textbooks on mineralogy quoted in the bibliography.

Since any considerable movement of the objective would destroy the illumination adjustment, a microscope with a focusing stage is a convenience almost amounting to a necessity if a considerable amount of

work of this type is to be done. If a biological type of microscope is the only one available, it is often possible to adapt the substage condenser rack as a coarse focusing adjustment. The specimen can be mounted in modeling clay in a hollowed rubber stopper and inserted into the substage condenser ring. The flat surface of the specimen is then aligned correctly by pressing it against the back of a microscope slide which is held on the stage. More elaborate adaptations that allow lateral movement of the specimen can also be made.

The Le Chatelier type of inverted microscope, which is in common use in metallography, holds the plane face of a prepared specimen perpendicular to the optical axis by merely placing it on the stage. For the ordinary type of microscope, the specimen should be mounted so that the single plane polished surface will be parallel to the stage. Ordinary gas caps are suitable after the tops and bottoms have been planed parallel. The most common method is to use metal rings, somewhat larger than the specimen and with parallel ends. If the specimen has already been prepared, it is easily mounted by pressing it into a piece of modeling clay within the ring which is lying on a flat surface. The plane surface of the specimen should be uppermost, and it can be pushed exactly flush with the end of the ring by a flat surface such as a piece of plate glass. Sometimes, however, the specimen is mounted thus in sealing wax or a low melting alloy, and ground and polished in the mount. Care must be taken to keep the surface of the mounting medium sufficiently below that of the finally prepared specimen so that it will not smear over it. Ideally, the mounting ring or cap should be of the same kind of metal. In practice the ring or cap in which the specimen is to be prepared should not be appreciably softer than the specimen.

Where considerable metallography is contemplated, it would be wise to obtain a mounting press and use the plastic mounts that have practically displaced other methods.*

DARK-FIELD ILLUMINATION

When objects to be illuminated by incident light are small and occupy only part of the field, the method of dark-field illumination is used. If a beam of light is sent toward the microscope objective, as in illumination by transmitted light, but in such a way that it passes through the object field at a sufficient angle to the optical axis to miss the objective lens entirely, the object field, if optically empty, will appear entirely dark

*See article by L. L. Wyman, quoted on page 165.

through the microscope. Indeed, the objective can be inside a surrounding cone of light with its apex at the focus of the objective without changing the dark-field appearance. If now, some object is introduced into the field, it will stand out brightly against its background with an intensity dependent on its refractive index, reflecting power, size, and the quantity of light incident upon it. Dark-field illumination is particularly useful in two special cases, both depending on the enhanced contrast against the dark field as compared with bright-field illumination; first, where the specimen is colorless and the difference in refractive index between it and its transparent medium is low, as with living organisms; second, where the specimen is composed of very minute particles even somewhat below ordinary microscopic resolution. The first case, which is used to obtain contrast, is useful at any magnification, but is most typical at low and medium powers; the second case is often called ultramicroscopy and will be discussed as such.

Low and Medium Powers

If a substage condenser is used, the aperture of which is considerably higher than that of the objective, and the central cone of light is cut out, the aperture of which is equal to, or slightly greater than, that of the objective, the latter will be inside a hollow cone of light (see Fig. 7) provided that the condenser is properly focused; this is the essential con-

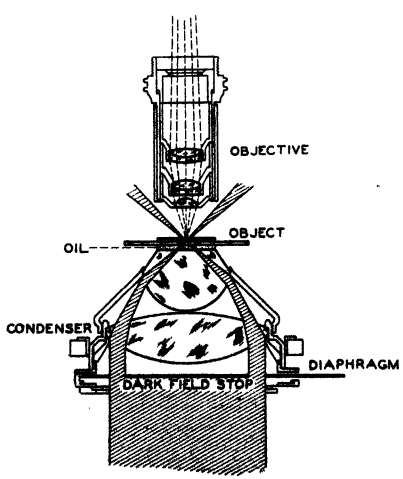


Fig. 7

dition as described above, i.e., that *no* direct light enter the objective. This is most simply achieved by inserting an opaque disk of sufficient area in an axial light beam set up as for transmitted light but rendered parallel as for Abbe illumination (see Fig. 7). Such disks are supplied by some microscope manufacturers as "dark-field stops." Except for objectives of the very lowest apertures, it is necessary to connect the condenser and the slide optically with some medium like immersion oil to obtain sufficient difference in aperture between objective and condenser (see Fig. 3). The condensers sold as "Dissecting Condensers" are suitable for dark-field photomicrography at low and medium magnifications.

The resolving power at high magnifications obtained by dark-field methods can be as high as that by bright-field. In fact, some of the recent advances in photomicrography have consisted of the application of short ultraviolet wave lengths to dark-field illumination. The methods of application are essentially the same as described below for substage ultramicroscope illuminators.

The Ultramicroscope

This instrument represents an application of the Tyndall phenomenon. When an intense beam of light impinges upon particles that are small with respect to its wave length, some of the light will be scattered and the presence of the particles can be detected even though their size is considerably below the limit of resolving power. Thus, the *detecting power* of the microscope, that is, its ability to show the presence or absence of a particle, is independent of its resolving power and can be made to extend to much smaller limits of size. Whereas the former depends on the three factors of wave length, angle, and refractive index in the object space, as discussed on page 19, the detecting power is dependent only on the intensity of the incident light and the contrast of the field, which in turn increases with the difference between the refractive index of a particle and its background. Since the wave length is not one of the factors limiting the detecting power of the microscope, particles can be seen that are below the resolving limit (see page 23) in size, but they will appear as points of light, and nothing can be directly ascertained as to their size, form, or color. Any objects above the resolving limit will be reproduced with the ordinary dark-field appearance as discussed above and according to the law of resolution.

The first form of this instrument, due to Siedentopf and Zsigmondy

(1903), is the slit ultramicroscope which is still in use. The sample of dispersed particles is illuminated by a beam of light coming at right angles to the axis of the observing microscope. The beam is focused in the field by an auxiliary microscope objective. A narrow slit which lies at right angles to both axes and in front of the illuminating objective acts as a diaphragm so that the width of the slit becomes the depth of the illuminated portion of the field and can be adjusted so that the latter just equals the depth of field of the observing microscope. In 1913 Zsigmondy improved the slit ultramicroscope by making both the illumination and observation objectives of immersion type; thus the brightness of the particles was increased about ten times. The slit ultramicroscope is most convenient to use visually since it requires no extreme cleansing of an observation cell due to the fact that the limit of the microscope field in all directions is a liquid boundary. However, only a very small fraction of the original illumination beam can be used by the observation microscope, partly because of the tremendous light loss at the slit and partly because of the small fraction of light scattered at 90°. Most of the light from a beam is scattered in a forward direction, and the greatest intensity of scattered light is observed just off the axis of the scattered beam. Therefore, the principle of utilizing a double hollow cone of light with the particles at its apex, as described on page 48, is by far the most efficient. The lower limit in size of detectable particles is dependent on the intensity of the illumination incident upon them, and this, in turn, is dependent on the intrinsic brilliancy of the light source, the size of the hollow cone of light (or the difference between the apertures of objective and condenser), and the perfection of its concentration at the apex within the visual field.

Reflecting condensers have been developed that are better than even the best of the usual types of refracting condensers with a central stop. They may be called substage ultramicroscope illuminators but are usually referred to directly according to their type, which may be either (1) Paraboloid or (2) Double reflecting (such as the Cardioid Condensers). The latter type (see Fig. 8), which appeared later, has the more concentrated focus but its accuracy makes it somewhat more difficult to adjust. Since the apertures of these condensers are

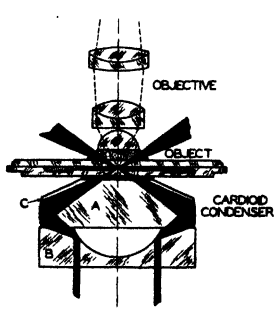


Fig. 8

very high, they must always have the proper immersion medium (usually cedarwood oil) between them and the slide. The maximum allowable objective aperture varies somewhat with the design, but usually it is about N.A.1.0. It is necessary to stop down ordinary immersion objectives with a funnel; special objectives containing an iris diaphragm can be used.

The direction sheets of manufacturers should be studied for the technique to be used with any particular condenser. The cardioid type is calculated for the lamp condenser (which is the imaged surface) to be from 10 inches to a foot away from the substage condenser. The light source should be focused at a considerably greater distance than this. The illuminating beam must cover the aperture of the condenser. It is often simplest to center the rest of the system first, using a bright-field condenser as described on page 28. This is especially helpful if it is possible to change condensers without disturbing the adjustment of the mirror. Then view the preparation with the dark-field condenser under a much lower power than will be used. If the condenser is out of focus, an illuminated ring will be observed, instead of a small bright disk. This must lie centrally in the field.

The tip of focus of the hollow cone from the modern dark-field condensers is very fine and is formed from a high angular aperture so that their position (focus) is sensitive to the location of the object plane, which is determined by the thickness of the microscope slide. This suitable slide thickness is stated by the manufacturer of the condenser and must be determined by a micrometer. Some dark-field condensers have a method of varying their focus to suit the thickness of the slide. Moreover, the medium to be examined must not be too thick or too concentrated, or the required detail will be lost in the general glare and brightened background. For high magnifications, the maximum allowable thickness of a suspension of particles or organisms is about 10 microns. For the same reason the slides and cover glasses must be scrupulously clean and free from scratches, and there must be no air bubbles in the immersion medium, for these all show up brilliantly and decrease the contrast. The slides and cover slips (or dark-field cells) for these condensers are usually made of quartz, partly because glass shows some fluorescence under intense illumination which decreases the background contrast, and partly because quartz will better withstand the drastic cleaning methods used. If the quartz cells become scratched, however, with repeated use, better results can be obtained with absolutely new, cleaned glass slides and cover slips.

In either case, for best results, the slides and cover slips should be cleaned by the following method or by another that gives equivalent results. If ordinary slides are used, they should be new and kept separated throughout the treatment so that they will not scratch each other. Some glass slides will not withstand the treatment with the etching which renders them useless for dark-field work. They should first be given a light dusting to remove possible grit particles on their surfaces, and if the slides or cells are not already free from obvious dirt, they should be given a preliminary cleaning with soap in soft or distilled water. They are then kept for several hours in a cleaning solution consisting of concentrated sulfuric acid and sodium bichromate or for a shorter time if the mixture is warm. They must not be allowed to remain indefinitely in this, for even the best slides become slightly etched. They are then transferred to water, or better, to a solution made up of four parts of a concentrated solution of sodium bichromate and one part of concentrated sulfuric acid, where they are kept until needed. Since chromic acid is absorbed by the glass, the slides must be given a very thorough washing under the tap, followed by a rinse in distilled water. After immersion successively in two jars of alcohol, the latter is then burned off. If platinum or similar forceps are not available, the acid must be poured off and the materials given a preliminary wash in the jars before handling. The slides should be held at a lower corner by the forceps to prevent the drainings from ruining the surface. Also much care must be taken not to contaminate the final storage or rinse baths. Forceps with glass tips are quite satisfactory. Cover slips should be cleaned in substantially the same manner. A technique as complete as this is necessary if absolutely clean dark fields are desired. When the train is once set up, it goes fairly easily.

CHAPTER VI

Image Contrast

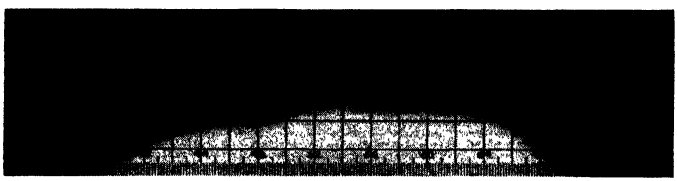
ONE of the most important factors in photomicrography is the contrast, or the brightness differences, in the image and also in the corresponding tones of the photograph. The contrast within the different specimens themselves varies greatly, as does the contrast between the structure to be shown and its background. Therefore the problem usually resolves itself into the rendering of intensified or diminished contrast. The contrast of the negative depends on both the image contrast and the photographic contrast of the plate. In photomicrography, both of these factors are under the control of the operator to a large extent. The control of contrast in the formation of the microscopic image is discussed now, the control of contrast in the photography of it later (see page 84).

Causes Preventing Sufficient Contrast in the Image

Dirty lenses or cover glass will spoil the whole image, covering it with scattered light ruinous to all contrast. Sometimes insufficient contrast may be produced by unsatisfactory lighting; too much scattered light from illuminated portions of the specimen outside of the field of view or a larger condenser stop than the objective will bear will produce it. A field diaphragm as described on page 34 will obviate the first difficulty. As discussed on page 33, in bright-field work the substage iris diaphragm should never be opened more than will admit light of the aperture of the objective in use. It is necessary to control the visual contrast of colorless objects by the aperture of the illumination, always remembering that as the substage diaphragm is closed down to gain image contrast it is obtained at the expense of resolving power. Therefore, a superior method of control of image contrast, applicable to all colored specimens, will be found in the control of the color of the light used for illumination, and it is for this control that a special system of filters (see page 64) used with plates sensitive to all colors has been devised.

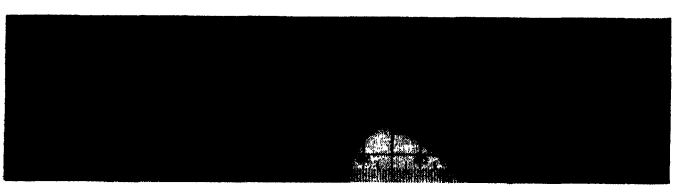
The Nature of Light and Color

When ordinary light is analyzed by means of the instrument known as the spectroscope, it is found to consist of a mixture of different kinds of light which, falling upon the eye, produces various sensations which we term colors. The analyses of daylight, arc light, and that from incan-



SPECTRUM OF PANCHROMATIC PLATE

ABSORPTION SPECTRUM OF ANILINE BLUE



TRANSMISSION BAND OF B AND E FILTERS

Filters used together to obtain maximum contrast in objects stained with aniline blue. These spectra were photographed on "M" Plate with High Intensity Tungsten Light.

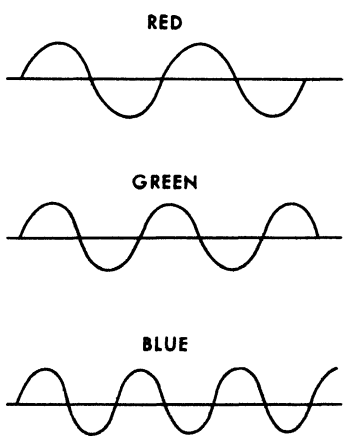
descent lamps show a continuous band of colors which appears to consist of three main portions, blue-violet, green, and red, the blue-violet passing through gradations of blue and blue-green to green, and the green through gradations of yellow and orange to red. If an object does not equally reflect or transmit all the different kinds of light of which the white light is composed, the light coming from it to the eye will be

more or less wanting in some constituents and will produce a sensation of color, so that a colored object is one which does not equally reflect or transmit all the constituents of white light but which "absorbs" some. The light which is absorbed is usually converted into heat and helps to warm the colored object.

If we analyze in a spectroscope the light reflected by a colored object, or transmitted by a colored "filter," we shall find that the continuous spectrum which is obtained with white light is replaced by one from which a portion is partly or completely missing. This missing portion appears as a black band, which is generally known as the "absorption band" of the color. If a particular object absorbs most of the constituents from white light so that only a small portion of the spectrum is transmitted, that portion may be referred to as the "transmission band."

As it is the light which is not absorbed that falls upon the eye, the sensation of color produced is the reverse of, or "complementary" to, the color which is absorbed.

If objects of various colors are examined, it will be found that a light blue object has an absorption band in the red, a purple object in the yellow, a magenta in the green, an orange in the blue-green, and a yellow in the blue-violet. Thus, a sensation of "light blue" is produced by a mixture of green light and blue-violet light falling on the eye, the red light being more or less absent, having been absorbed from the white light by the object, which appears to be colored light blue. In the same way, a sensation of "yellow" is produced by a mixture of green light and red light falling upon the eye, the blue-violet light having been absorbed.



Relative Wave Lengths of Red, Green and Blue.

Fig. 9

If the blue-violet light is deficient in the light source, the original light will appear yellow, as does gaslight for instance.

The Definition of Color

Light is known to consist of waves, and the color of the light is con-

nected with the length of the waves. The length of a light wave is the distance from the crest of one wave to the crest of the next, measured in very small units. (The millimicron (mμ) is one-thousandth of a micron (μ) and one-millionth of a millimeter (mm.), which in turn is about one-twenty-fifth of an inch.)

A wave of the darkest violet that we can see will be 400 mμ in length, a wave of blue-green 500 mμ, of bright green 550 mμ, of orange 600 mμ, and of deep red 700 mμ (see Fig. 9). Visible light, then, is composed of light waves, varying in length from 400 to 700 millimicrons which may be divided roughly into three portions: blue-violet, 400-500 mμ; green, 500-600 mμ; red, 600-700 mμ (see Fig. 10).

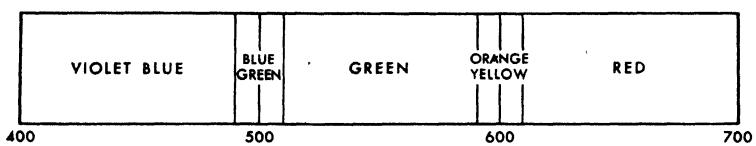


Fig. 10

The position of an absorption band may be defined by the length of the waves of light which are absorbed by it. We may speak of an absorption band as extending, for instance, from 600 to 640 units, meaning thereby a band absorbing those particular waves of red light, and therefore producing a blue color.

Color Produced by a Single Absorption Band

In order to realize the relation between absorption and the color of the object, it may be worth while to examine the effect of a single sharp absorption band in different parts of the spectrum. First consider a sharp absorption band in the extreme red, stretching from 640 to 690, and completely stopping all red light of those wave lengths. The remaining color will consist of all the blue-violet and all the green light, with some of the red. The actual visual effect of a mixture of the light left, what one might call the *residual* color, is a sky-blue. Imagine this band to shift so that the absorption is between 580 and 620; the residual color will be a light blue-violet because there is a great deal of red being transmitted and less green. If the band shifts into the yellowish-green, from 560 to 600, it will absorb a great deal of the green and no red, and the residual color will become a bluish-purple. As it shifts lower in the green toward the blue, this purple becomes a reddish-purple, so that

when the band is situated at from 520 to 560, the color will be magenta. As the band shifts toward the blue the magenta becomes more orange, and then as the band moves into the blue-violet the orange becomes a yellow and finally a lemon-yellow (see Fig. 11).

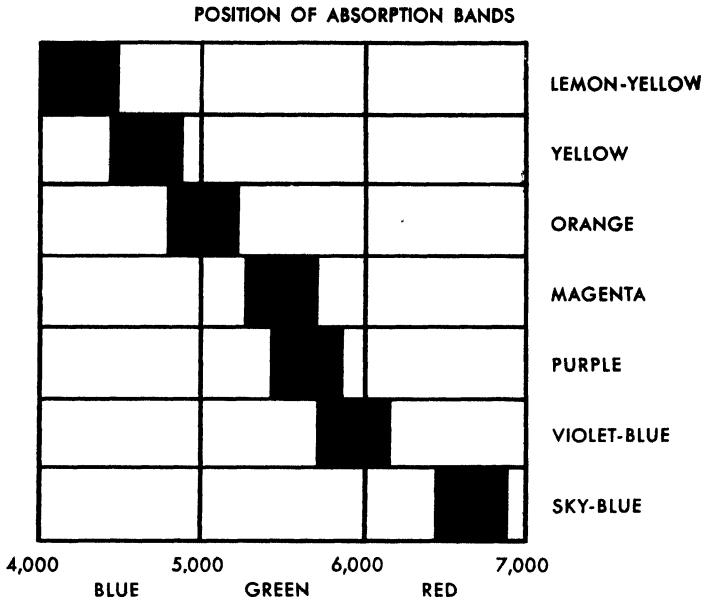


Fig. 11
CHART SHOWING RESIDUALS

Therefore, when anything is colored sky-blue, it means that it is absorbing the deep red; a violet-blue object absorbs the orange, a purple the yellow-green, a magenta the central green, an orange the blue, a yellow the blue-violet, and a lemon-yellow only the extreme violet. If a sky-blue object is looked at through a piece of yellow glass, it will be found to look bright green in color, so that a green color is produced by the absorption both of the red and of the blue, the blue object absorbing the red light and the yellow glass the blue light.

Natural colors generally do not show sharp absorption bands, though the absorption bands produced by the stains used in microscopy are mostly fairly sharp. The same rule holds true, however; if a magenta object in nature does not signify a clean, sharp absorption band in the green, it still means that that object absorbs far more of the green

than of any other color, and the rules deduced from theoretical residuals to natural colors can be applied. The first of these rules is:

If a color is to be rendered as black as possible, then it must be viewed, or photographed, by light which is completely absorbed by the color, that is, by light of the wave lengths comprised within its absorption band.

Demonstration of Absorption and Color Contrast

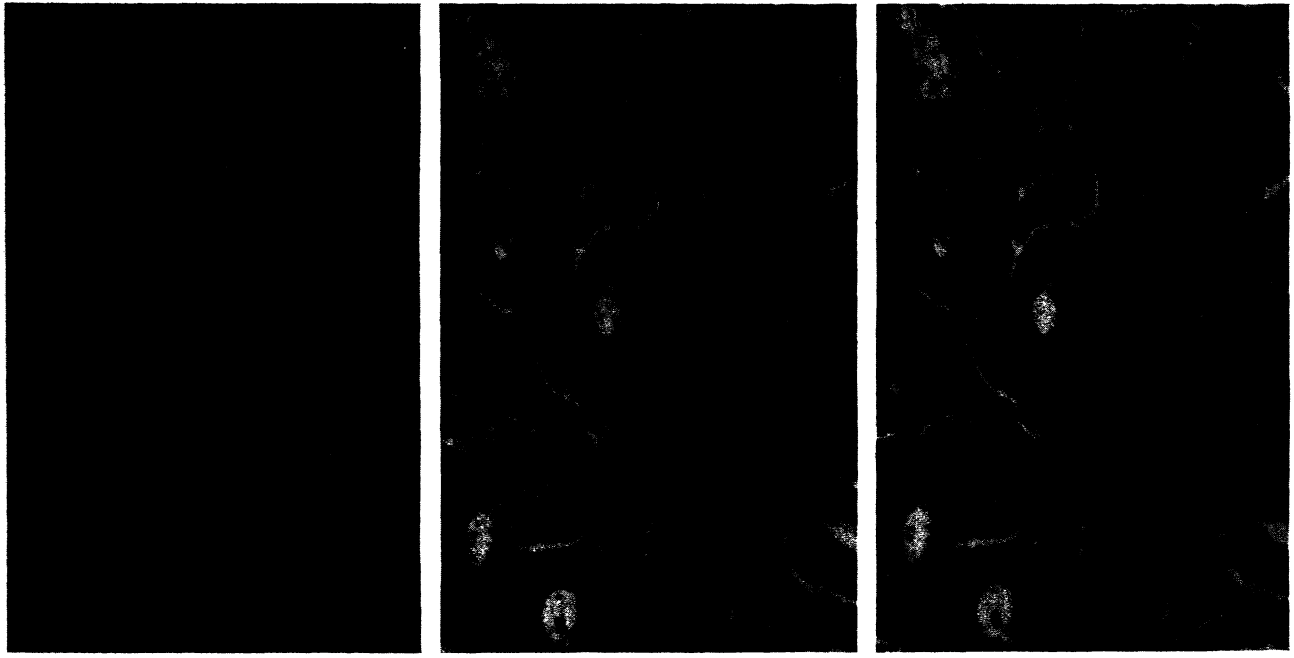
On pages 60 and 61 have been printed illustrations in two colors; the magenta ink used has a strong absorption in the green, and the blue-green ink has a strong absorption in the red.

On page 61 there are two little pockets containing a piece of green and a piece of red gelatin. The green gelatin transmits only light absorbed by the magenta ink, and if the picture is examined through this gelatin the red spots appear almost black. If, on the other hand, the picture is examined through the red gelatin, then the contrast between the red printing ink and the paper will disappear and it will be difficult to distinguish it from the paper on which it is printed, while the blue ink appears considerably darker. This shows us that the contrast presented by a colored object can be entirely controlled by altering the color of the light by which it is examined. A useful example is given by the photomicrographs of a section stained with acid fuchsin shown on the next page. This section appears pink, acid fuchsin absorbing light from 510 to 560. If the section is photographed by light of 510 to 560, which is completely absorbed by acid fuchsin, the maximum amount of contrast is obtained, and owing to an excess of contrast, the detail of the section is blocked up. Photographing at 570 on the border of the absorption band, we get a greatly lessened contrast, which for this particular section will give us the best result. If we photograph by red light of wave length 640, which is completely transmitted by the section, the contrast disappears and the results are flat and useless.

Contrast Within the Specimen Itself

The second rule for procedure deals with the case where contrast is required, not against the background but within the object itself.

A good case of this is the photography of an unstained section of whalebone; this is of a yellow color and shows ample detail to the eye, but it completely absorbs blue-violet light. If it is photographed only in the blue-violet light to which an ordinary plate is sensitive, then it

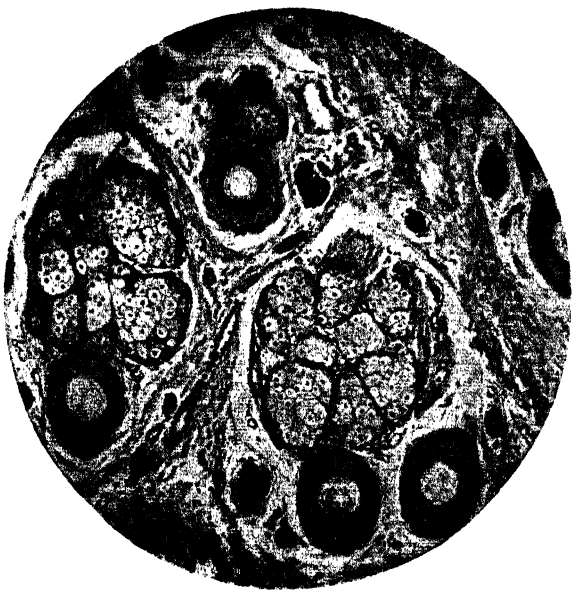


A B 62

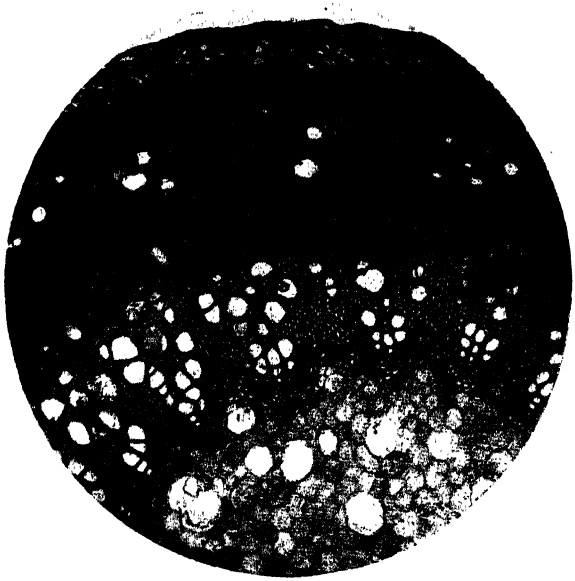
SECTION OF CAT DUODENUM, 325×

Stained with Acid Fuchsin

Photographed through Wratten filters (A) of same color as specimen, (B) with transmission band of filter wider than the absorption band of the stain, (No. 62) with transmission of filter included within absorption band of stain.



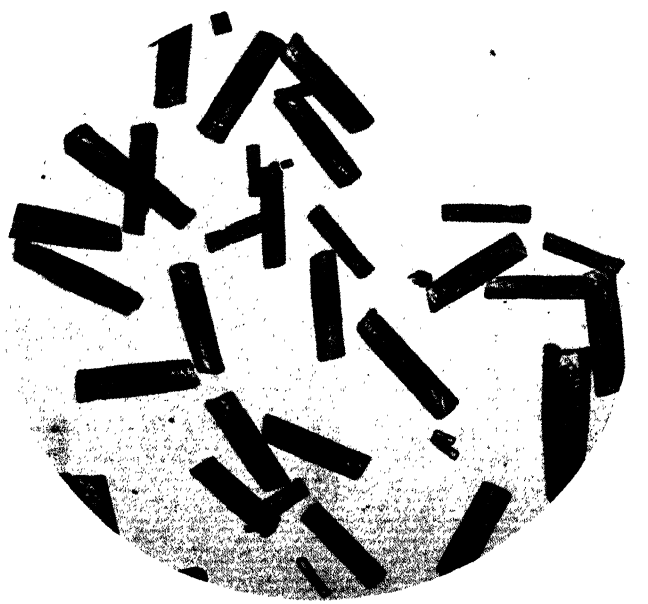
Horizontal Section of Human (white) Scalp, 75×
Wratten Panchromatic Plate with A (red) and H (blue-green) Filters



Ampelopsis stem 50×
"M" Plate with A (red) and B (green) Filters

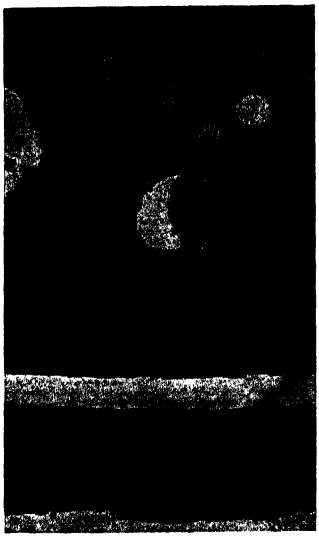
The two sections reproduced here in natural colors not only serve to illustrate the beautiful differentiation possible in the color reproductions made as described on page 158 but also are shown to illustrate the use of the filters as mentioned on page 58.

As Seen Through the Microscope

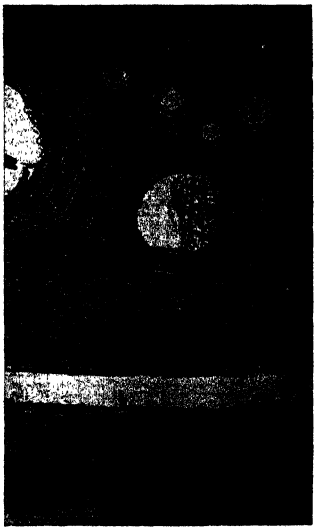


On "M" Plate with C Filter

BLUE COBALT MERCURIC THIOCYANATE CRYSTALS 125×



Photographed with Blue Filter
for Maximum Contrast



Photographed with Red Filter
for Maximum Detail

WHALEBONE SECTION

shows far too much contrast, appearing as a black mass without detail against the background and presenting an exaggerated example of the loss of detail which has already been noted in the acid fuchsin section photographed by light which it completely absorbs.

The proper procedure in this case is to photograph the object by the light which it transmits. The whalebone section, for instance, photographed by red light gives perfectly satisfactory results and shows ample detail in structure.

A class of microscopic objects which frequently requires this treatment is that of the usual insect preparations, which give the most satisfactory results when photographed by yellow, red, or infrared light.

Procedure for Choosing Color Filters

The best method of determining the contrast required by any object is to examine the object visually under the microscope, first by means of a combination of filters transmitting as completely as possible light of the wave length absorbed by the preparation, and then by other filters transmitting light less completely absorbed, until the degree of contrast obtained is satisfactory to the eye. As a guide to procedure, the next

section contains a list of the chief microscopic stains and of the filters which will produce the maximum contrast.

THE WRATTEN "M" FILTERS

In order to enable microscopists easily to control the color of the light which they use, a special set of filters known as the Wratten "M" Filters has been prepared by the Eastman Kodak Company. These are prepared originally as gelatin film stained with appropriate dyes selected for their brightness, sharpness of absorption, and stability. A complete description of all these filters, showing their spectral characteristics, will be found in *Wratten Light Filters.*

The "M" filters can be obtained either as gelatin film or cemented between two sheets of white optical glass, the cementing both protecting the filters and rendering them more transparent. The gelatin films require considerable care in handling and should only be touched with the fingers at the extreme corners. It is best to bind them between two pieces of glass. Since it is practically impossible to clean them other than by gently dusting them with a light brush, they should be carefully protected from dirt. The cemented filters should be treated with care equal to that accorded to lenses. They should be kept in their cases when not in use. No water or other solvent must be allowed to come into contact with their edges. The light source should never be focused on a light filter. A heat-absorbing cell or filter (see page 16) should always be interposed between a light filter and a high intensity illuminant.

Number	Name	Visual Color	Spectral Transmission
25	A	Orange-Red	From 590 mμ to red end
58	B	Green	From 480 mμ to 620 mμ
47	C5	Blue-Violet	From 370 mμ to 510 mμ
35	D	Purple	From 320 mμ to 470 mμ and from 650 mμ to red end
22	E	Orange	From 550 mμ to red end
29	F	Pure Red	From 610 mμ to red end
15	G	Strong Yellow	From 510 mμ to red end
45	H	Blue	From 430 mμ to 540 mμ
11	X1	Pale Green	For orthochromatic reproduction with tungsten light

By using these filters in pairs, the spectrum can be divided into approximately monochromatic portions. (See curves for "Absorption of Certain Pairs of Filters" in the back section of *Wratten Light Filters.*)

Visual Inspection Best Guide

As has already been explained, it is not possible to give instructions for

all cases. The best guide to the combination of filters to be used in any particular case is the visual inspection of the objects through the filters and, of course, the use of the combination with which the greatest contrasts or greatest detail, as the case may be, can be obtained. As a general guide, when contrast is required:

Use for	Blue	stained Preparations a		Red	filter
" "	Green	"	" "	Red	"
" "	Red	"	" "	Green	"
" "	Yellow	"	" "	Blue	"
" "	Brown	"	" "	Blue	"
" "	Purple	"	" "	Green	"
" "	Violet	"	" "	Yellow	"

When selecting a filter, care must always be taken that too much contrast is not obtained, or the result will be a choking of shadows and loss of detail. It can be seen from the following table that a considerable choice of dominant wave lengths is available.

Filters	Dominant Wave Length	Color
D and H	445	Violet
C " H	460	Blue
B " H	510	Bluish-Green
G " H	525	Pure Green
B " G	540	Yellowish-Green
B " E	575	Greenish-Yellow
A	600	Orange-Red
F	625	Red
A and D	670	Deep Red

Sometimes better results are obtained by the use of much wider regions of the spectrum, or possibly a narrow band may require an inconveniently long exposure time. On the other hand, the more nearly monochromatic the illumination, the better will be the image from an achromatic objective. The following Wratten filters are especially useful with achromats since they transmit the proper green light (given in the order of *decreasing breadth* of transmission band): No. X1, X2, B, B + G, 61, 62, G + H. Also see page 66. These can be supplied in all usual sizes from stock.

The spherical aberration of achromatic objectives is best corrected for the region of the spectrum transmitted by the B + G filters. Hence, when the specimen is colorless, as it frequently is in metallography, the illumination is restricted by these filters, or their equivalent, in order to use achromatic objectives most efficiently. Apochromatic objectives also give better definition when the illumination is restricted by a color filter.

Correct Brightness Rendering of Colors

It is sometimes desirable to photograph colored preparations, heat tinted metal specimens, objects under polarized light, etc., in such manner that the gray tones of the photographic print will appear the same to the eye as the corresponding areas of the original specimen with regard to their relative luminosity values. This subject is discussed at greater length in *The Photography of Colored Objects*, listed in the bibliography. It is, of course, necessary that "white" light be used for illumination and that the photographic material be sensitive to the whole visual spectrum, i.e., that it be panchromatic. Since the spectral sensitivity of panchromatic plates is not identical with that of the eye, a corrective filter should be used which will vary with the color distribution of the source of the "white" light.

Kodak panchromatic materials may be divided into two types, B and C, according to their spectral sensitivity, the C type having considerably higher sensitivity to red light. The type of each material is given on page 86. The corresponding filters to give the correct brightness rendering of the various colors are as follows:

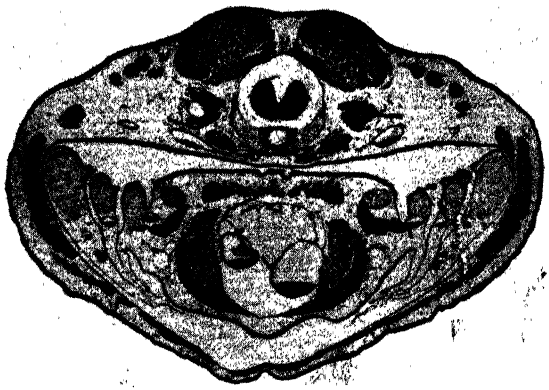
Panchromatic Type	Filter for Daylight or Carbon Arc	Filter for Incandescent Tungsten
B	K2	X1
C	X1	X2

Monochromatic Photomicrography

Chromatic aberration, even in excellently corrected lenses, is one of the principal defects that prevent the attainment of perfect definition in the image. Obviously this is eliminated when the illumination is confined to a single wave length, i.e., monochromatic light. Indeed, it can be shown by critical test,* that there is improvement in image quality when monochromatic illumination is substituted for light through a narrow color filter in photomicrography.

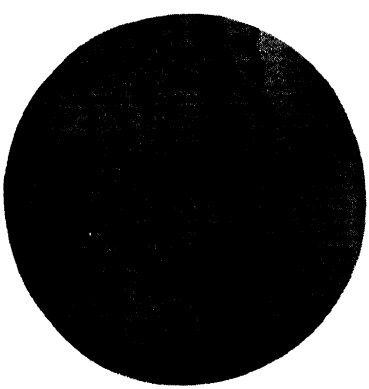
The simplest method for obtaining monochromatic illumination is to use a discontinuous source of light such as an electric vapor arc, which emits radiation only as a group of individual wave lengths which may be scattered through the spectrum, together with a color filter. This filter, in some well-selected cases, easily isolates a single wave length that is emitted without a closely similar color being present to interfere. The yellow light from a sodium arc has thus been isolated and used, but is not strictly monochromatic.

*A. Trivelli, *Trans. Amer. Micros. Soc.*, *49*, 258 (1930).

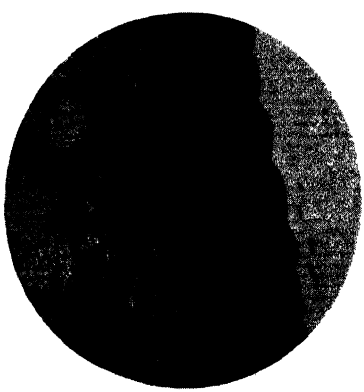


SECTION THROUGH HEAD OF LARVA OF NEWT
(Triturus), 10×

Hematoxylin-Eosin. Wratten X2 Filter, Tungsten Ribbon Filament,
32 mm. f/4.5 Micro Tessar, Wratten "M" Plate

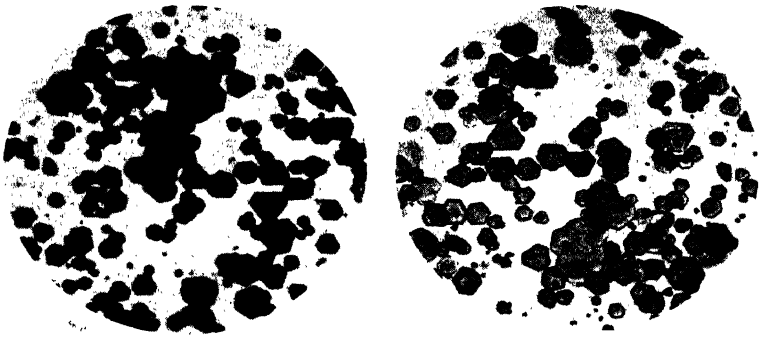


Wratten "M" Plate and F Filter



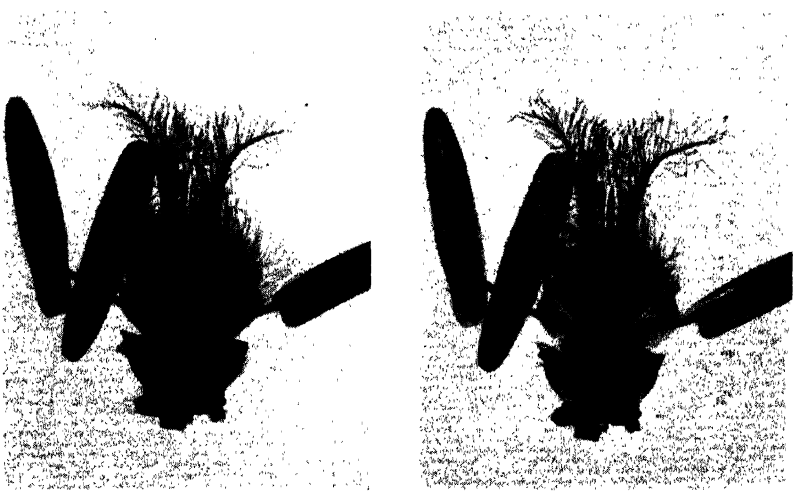
Wratten "M" Plate and B Filter

SECTION OF SKIN OF MAN, 15×
With Dye Injected Blood Vessels



On Ordinary Plate — On "M" Plate with B Filter

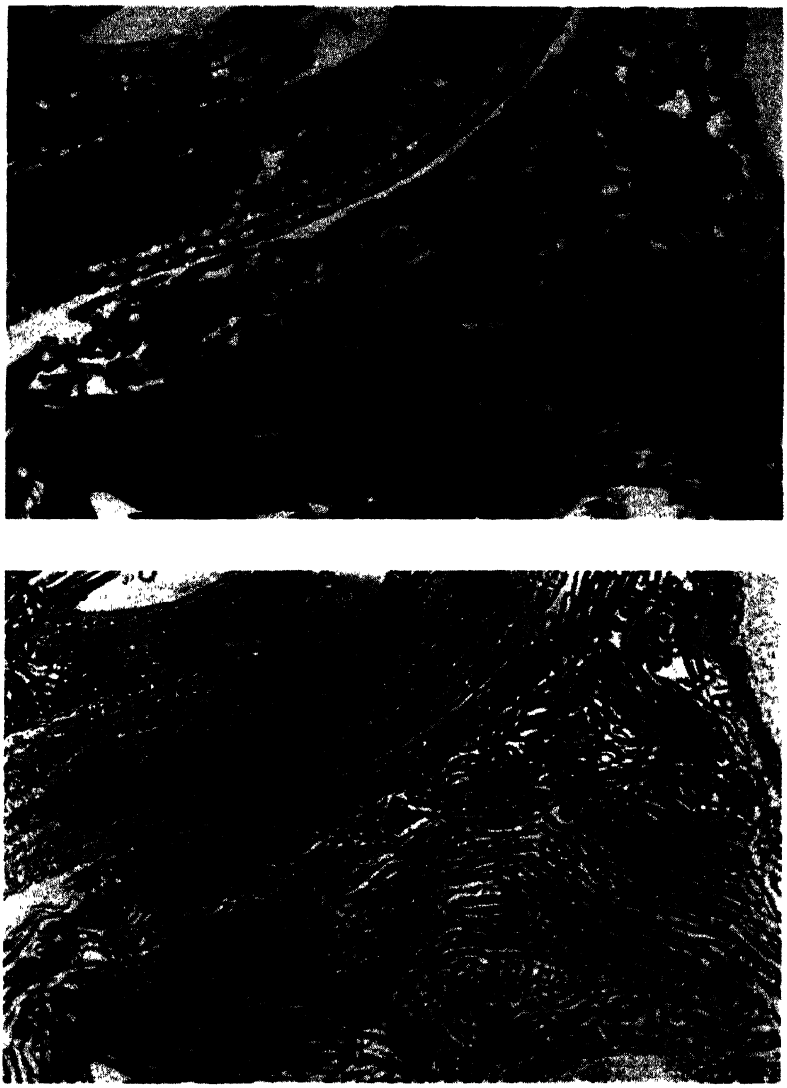
LEAD IODIDE PRECIPITATE, 125×



On Ordinary Plate — On "M" Plate with K2 Filter

Tungsten Ribbon Filament, 48 mm. f/4.5 Micro Tessar
Carmine Stain

WHEAT FLOWER (Triticum), 8×



LEAF BUD OF AESCULUS HIPPOCASTANUM, 150×
Above: On Ordinary Plate
Below: On "M" Plate with E Filter
Photographed with Tungsten Light

The most common and convenient source of filtered monochromatic illumination is undoubtedly the mercury arc. Monochromatic illumination for photomicrography is easily available when the appropriate filter or pair of filters from a selected series of Wratten gelatin filters is used with the capillary high intensity mercury lamps which utilize ordinary illumination equipment. (See page 80.)

The particular filter to be chosen will depend to some extent upon the requirements of spectral purity. Frequently, the inclusion of a very small amount of radiation of the wave lengths from adjacent spectral lines is unimportant compared with the considerable gain in illumination intensity that is obtained by using a filter of greater transmission. In the table below are given the wave lengths of the mercury arc which are most useful for photomicrography, together with the Wratten filters most useful in isolating them.

Color	Wave Lengths	Filters for Isolation	Trans-misson	Practically Useful Filters	Transmission Principal	Impurity	
Yellow	577-579			22(E_2)	19%	Red	(total)
Green	546	77A	68%	77	72%	577-9 612-672	0.5% 66%-83%
Blue	436	2A+34	46.2%	—	—	612 672	none 45%
Ultraviolet	365	18A	38%				

Although the No. 22, No. 77A and No. 77 filters transmit freely the red lines of mercury, these lines are very weak and rarely interfere. They do not interfere at all if orthochromatic plates or films are used. They can be practically eliminated by the superposition of a No. 57 or a No. 58(B) filter over the No. 77 or No. 77A. A No. 52 filter is preferable with the No. 22 if such a filter is needed, although the use of acidulated copper sulfate solution in a cooling cell is even better for this case. A concentration equivalent to a 15 per cent solution of $CuSo_4 \cdot 5H_2O$ in a 1 centimeter layer should be used.

The blue light of wave length 436 mμ is a powerful source. Although it is a deep blue, it can frequently be focused without undue difficulty and, with apochromatic objectives, affords the highest resolving power that can be visually obtained.

For use of the 365 mercury line which, although in the ultraviolet, can be utilized with visual focusing with the aid of special objectives, see page 76.

Microscopic Stains and Suitable Filters

Some of the chief microscopic stains and their absorption bands, as recommended by the Commission on Standardization of Biological Stains, with the filters recommended to obtain maximum contrast, are listed on the following page. Pages 64 and 65 show the method by which this table is constructed. A solution of aniline blue shows an absorption band stretching from about 550 to 620, which region of the spectrum is transmitted by filters B and E used together, so that if an aniline blue stained section is photographed by the aid of these two filters, the maximum obtainable contrast results.

It may be noted here that, except in the cases of the very dark combinations, these filters may be usefully employed in the visual examination of faintly stained preparations.

The exposure factors for Wratten "M" Filters and Wratten plates, particularly useful in photomicrography, are given on page 93.

Filters for Visual Microscopy

THE WRATTEN VISUAL "M" FILTERS are primarily intended for visual work with the microscope. The set, described below, usually consists of nine circular filters, which are the most generally useful.

NUMBER	COLOR	USE
78	Blue	A photometric filter (see page 135) made to convert the illumination quality of light from incandescent tungsten lamps of the common type to that which is visually equivalent to daylight. Such a filter is often employed for viewing colored specimens with their commonly accepted standard daylight appearance.
38A	Blue	A filter for increasing the apparent contrast in faintly stained yellow or orange preparations. Helps in the resolution of fine detail.
45A	Blue-Green	Especially useful when the highest resolving power visually possible is required, as in the study of diatom structure. It has no red transmission and its dominant wave length is at about 470 mμ.
66	Light Green	A contrast filter for use with pink and red-stained preparations. Preferred by some workers for general use in place of No. 78.
58	Green	A contrast filter for use with faintly stained pink or red preparations.
15 22	Yellow Orange	For increasing the contrast in blue preparations and for helping in the observation of detail in insect mounts by reducing the contrast between the preparation and the background.
25	Red	Contrast filter for use with preparations stained with Methylene Blue, Methyl Green, etc.
96	Neutral tint	A filter for moderating the intensity of the illumination. The density supplied transmits about one-tenth of the incident light.

Stain	Spectral Absorption †	Screens Recommended	Band Used
*Acid Fuchsine	530-560	B & G	510-600
*Aniline Blue	550-620	B & E	560-600
*Azure I	580-640	B & E	560-600
*Basic Fuchsine	520-550	B & G	510-600
#Bismark Brown	General in blue and violet	C	400-510
Brilliant Cresyl Blue	570-640	B & E	560-600
Brilliant Green	600-640	F	610-680
*Carmine	500-570	B & G	510-600
Congo Red	480-520	B & E	560-600
*Crystal Violet	550-610	B & G	510-600
Cresyl Violet	550-630	B & G	510-600
Eosine B	480-550	B & H	480-540
*Eosine Y	490-530	B & H	480-540
Ethyl Eosine	490-540	G & H	510-540
*Hematoxylin (Ehrlich)	Gradual through green	B & G	510-600
(Heidenhain)	560-600	B & E	560-600
Indigo Carmine	560-650	B & E	560-600
Janus Green	560-640	B & E	560-600
*Light Green SF	600-660	F	610-680
Malachite Green	590-640	F	610-680
Martius Yellow	380-450	C	400-510
*Methyl Green	620-650	F	610-680
*Methyl Violet	550-600	B & E	560-600
Methylene Violet	560-620	B & E	560-600
*Methylene Blue	600-620 & 650-680	A	590-700
Methyl Orange	430-500	C & H	420-510
Neutral Red	480-550	G & H	510-540
Nile Blue	560-650	B & E	560-600
Nigrosine	General with max. at 580-600	B & E	560-600
*Orange II	460-510	C	400-510
Orange G	470-500	C	400-510
Phloxine	510-550	G & H	510-540
Pyronine	530-560	B & G	510-600
*Safranine O	480-540	G & H	510-540
*Sudan III	General in blue and green with max. at 500	G & H	510-540
*Sudan IV		G & H	510-540
Thionine	560-610	B & E	560-600
Toluidine Blue	550-650	B & E	560-600

*The most commonly used stains are marked with an *. Gentian Violet belongs to these stains, but it is not officially recognized by the National Commission on Standardization of Biological Stains. It is a poorly defined mixture of violet rosanilins, nearly synonymous with Methyl Violet. The same filters as recommended for Methyl Violet may be tried.

†The spectral absorption of the stained object may differ from that of the stain in watery or alcoholic solution. Such object should therefore be investigated separately (see page 64).

#When photographing by transmitted light for insects and yellow sections generally, photograph for *contrast* with a C filter; for detail *in the* section with an F filter.

Wratten Visual "M" Filters are supplied in 33-mm. circles, thin enough so that several can be placed in the substage condenser ring. The glass used is of the same quality as that of cover glasses. Extreme contrast may be secured by combining the two filters that will transmit a very limited region of the spectrum.

The control of color contrast obtainable with this set of filters is indicated in the following table which gives the filters, and in three cases, pairs of filters, arranged in the order of their dominant wave lengths.

Filter	Dominant W. L.	Filter	Dominant W. L.
45A	470 mμ	66	550 mμ
38A	500	38A with 15	555
38A with 66	525	15	600
58	535	22	630
38A with 58	545	25	650

The Wratten Neutral Density Filter No. 96 is now available in eight varieties transmitting 50%, 25%, 10%, 5%, 3%, 1%, 0.1%, 0.01%, respectively, of the incident light.

RHEINBERG DIFFERENTIAL COLOR ILLUMINATION

By means of this method of illumination a difference in color may be secured between an object and its background or between two transverse sets of striations. This is a valuable aid in the correct interpretation of structure and often affords an increased ability to see the object. Incidentally, the effects obtained are of extraordinary beauty.

The Rheinberg method of differential color illumination is based on principles somewhat similar to those of dark-field illumination.

If a central stop or filter disk of a size to intercept all of the light entering a low or medium-power objective is placed in the condenser ring, then the background seen through the microscope will be dark or any desired color. The condenser must, however, pass a much wider cone of light which will be refracted by the object and, because of its great preponderance, cause it to appear white, or, if a peripheral filter is used, of that particular color. Thus, a slide of diatoms may be seen with the latter white on a dark background, vice versa, or with the two of different colors. Undyed cloth may be illuminated so that the warp is of one color and the woof of another, which principle may be extended to display finer transverse detail. Unless the colors of these filters are carefully selected with regard to their transmission spectra, the results will be of inferior quality. Mr. Rheinberg personally assisted in the selection of the Wratten set of disks and rings.

The colored central disks and the colored peripheral rings are supplied in the same mountings as are the Wratten Visual "M" Filters. They are thin enough to permit of two or more being inserted into the condenser ring and rapidly changed as may be required.

The central disk-stops used determine the color of the background which is seen.

The peripheral ring-stops determine the color in which the object is seen.

The set comprises: Central Disk-Stops: (1) Greenish Blue, (2) Blue, (3) Green, (4) Red, (5) Purple, (6) White Matte, (7) Black; Peripheral Ring-Stops: (8) Red, (9) Orange, (10) Blue-Green, (11) Blue; also (12) Red and Blue Sector Stop.

Instructions for Using Wratten-Rheinberg Filters

Objective: The objective should be of low or medium power. A 32-mm. or 16-mm. objective in general will be most suitable.

Condenser: Any condenser of standard size can be used. It should be used at full aperture and focused on the object.

Any form of the Abbe Condenser, which usually should have its top lens removed, is desirable. The central color disk in the condenser when viewed by looking through the microscope tube (without ocular or object) should completely intercept the light passed by the objective. This may be better seen by moving the condenser ring and stop in and out of position. Although the size of the central disk may slightly exceed the cone of light passed by the objective, it may be necessary to use a lower-power condenser or an objective of greater aperture if it greatly exceeds it. If, on the other hand, the color-stop does not completely fill the objective cone of light, a higher-power condenser must be used unless the aperture of the objective is cut down with a funnel or an objective of lower aperture substituted.

Illuminant: The light should proceed from a uniformly illuminated surface of some area. This may be suitably obtained by employing a strong artificial light source in a frosted bulb or behind a ground-glass screen.

The Filter Stops: To see the object white, or in its natural color, on a colored ground, the use of the appropriate central disk-stop only is necessary.

To see the object colored, but on a white ground, the white central disk-stop must be used in conjunction with one of the peripheral color ring-stops.

Color disk-stops can be employed in combination. Thus, by the use

of the greenish-blue and the green central disk together, a useful dark green background is secured.

The peripheral ring-stops can only rarely be employed usefully in combination.

The red and blue sector stop should always be used in conjunction with the black central disk-stop; it serves to illuminate striae or ridges which are transverse to one another in different colors, the whole object being seen on a dark ground. Care should be taken that either the red or blue sectors lie transversely to the ridges or striae of the object. If the direction of the latter is not known, or if it is desired to discover whether such striae exist, this can be ascertained by rotating the sector stop in the condenser ring.

Specimens Suited to Rheinberg Technique: *The refraction method of differential color illumination* is applicable to all objects which refract light or show differences of refractive power in their different parts. It lends itself to the study of mineralogical, botanical, and physiological preparations, to the study of living organisms and to commercial purposes, such as investigation of textile fibers, papers, etc.

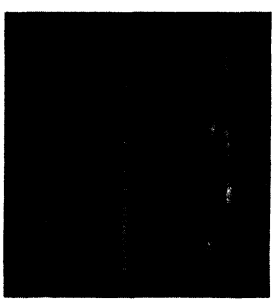
To name just a few suitable objects, available to everyone, which may be tried first, the following will serve: Slides of diatoms, foraminifera, polycystina, sections of bone, unstained sections of plants, living rotifers or water fleas, etc., any textile fibers.

For further information on the subject of Differential Color Illumination, those interested are referred to Mr. Julius Rheinberg's original papers in the Journal of the Royal Microscopical Society, August, 1896, and April, 1899, in the Illustrated Annual of Microscopy of 1898 and 1900, and in the Journal of the Quekett Microscopical Club, April, 1897, and November, 1897.

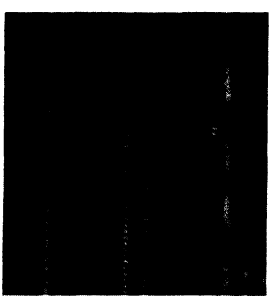
CHAPTER VII

Photomicrography with Ultraviolet Light

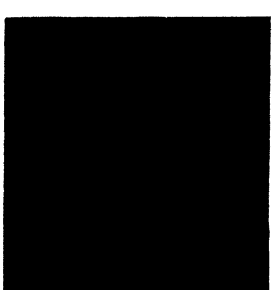
THE ADVANTAGES of the application of ultraviolet over visible light are: (1) Increase in resolving power. (2) Structural differentiation unobtainable, or obtainable only with difficulty, at longer wave lengths.



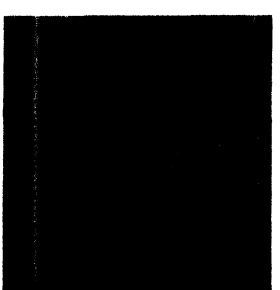
Yellow
(579-577 m μ)



Green
(546 m μ)



Violet
(436 m μ)



Ultraviolet
(365 m μ)

AMPHIPLEURA PELLUCIDA, 3000 ×
Mounted in Hyrax. Highly Oblique Illumination
Mercury Vapor Illuminant with Wratten Filters

The Effect of the Color of the Illumination on Resolving Power

As discussed on page 23, the distance between two lines which can just be resolved by a microscope objective is equal to half the wave length of the light by which it is observed, divided by the numerical aperture of the objective.

$$d = \tfrac{1}{2} \frac{w.\,l.}{\text{N.A.}}$$

We see, therefore, that the shorter the wave length of the light, the better the resolving power.

Thus, with a N. A. of 1.0 and an F filter, we shall be able to see 80,000 lines to an inch, with a B filter, about 95,000 lines to an inch, and with a C filter, 100,000 lines to an inch.

Thus, with the C filter, negatives can be made showing dots in the diatom *Amphipleura pellucida* which are 100,000 to the inch, while these are invisible with a filter of longer wave length.

The H filter will be found useful in visual work for increasing the resolving power, and if the illumination is sufficient even the C5 filter may be employed.

Use of Ultraviolet for Differentiation of Structure

The utilization of the differential absorption of the ultraviolet cited in case (2) is merely an extension of the control of contrast by the color of the illumination, discussed under "Image Contrast." As can be deduced from the principles discussed there, the region of the ultraviolet that will be most suitable to exhibit a particular detail will depend on the absorption bands of the specimen and will therefore vary with the specimen. Nearly all biological materials contain proteins that strongly absorb the very short wave lengths, becoming opaque below 250 mμ. While wave lengths as short as 199 mμ* have been used for photomicrography, the wave lengths 365 mμ and 275 mμ are most commonly employed and seem most useful. (See below for further discussion of this.)

In many cases, the staining methods commonly employed for histological work may be used for ultraviolet photomicrography. The absorption of 365 mμ and 275 mμ ultraviolet radiation by the usual stains in solution 1 cm. in depth is shown in the table on the next page.

The absorption is expressed in percentages of transparency. The

*Reference to B. K. Johnson, *An Ultraviolet Microscope for the Examination of Opaque Objects*, in Bibliography.

results; however, are not to be regarded as the same as when the stain is in histological sections, but an approximate indication can be seen as if the stain were used for ultraviolet micrography.

For photomicrography in visible light most sections are mounted in Canada balsam, and only in special cases is realgar or another medium used. Nearly all these substances are opaque to 275 mμ wave length, but many are transparent to 365 mμ wave length. A convenient method by which the absorption of the mounting medium can be roughly determined is by the use of an X-ray screen of barium platinocyanide, which, when exposed to ultraviolet radiation, gives a bright green fluorescence. If the mounting medium absorbs the radiation, the slide forms a shadow on the X-ray screen in the beam of the ultraviolet radiation.

The relative absorption of a 0.2-mm. thick layer of some of the common immersion and mounting media at various wave lengths is listed below. The absorption of balsam is a variable which increases markedly with the age of the mounted slide.

	Refractive Index No.	440 mμ	365 mμ	275 mμ
Cedarwood Oil	1.51	14	56	100
Sandalwood Oil	1.51	10	32	100
Glycerine	1.46	Very transparent throughout		
Balsam	1.52-4	11	82	100
Styrax	1.58	22	94	100
"Hyrax"	1.73	11	60	100

Much ultraviolet photomicrography has been done using the 275 mμ line from a cadmium arc. This requires expensive equipment, including a quartz optical system and monochromator, and also involves a very difficult technique not the least of which is the critical focusing of the invisible image. Since the single material, quartz, is used for the objectives, they are necessarily monochromats and have a numerical aperture of 1.25 using glycerine as the immersion fluid. Therefore, the maximum theoretical resolution is 0.11 micron (1 micron = 1/25,400 inch).

It was suggested in the Kodak Research Laboratories that it might be advantageous to utilize that portion of the ultraviolet spectrum where the wave length was just sufficient in length not to be appreciably absorbed by optical glass, thus not only saving expense over the use of a shorter wave length but also allowing the use of greater numerical apertures and achromatic corrections. This has been successfully developed* using the 365 mμ spectral line from a high intensity mercury

*A. P. H. Trivelli and L. V. Foster. See page 165.

ULTRAVIOLET ABSORPTION OF STAINS

Stain	Purity	Solvent	Dilution	Percentage of Transparency 365 mμ	275 mμ
*Acid Fuchsin	62%	H_2O	1:7500	68.	8.3
Aniline Blue		H_2O	1:5000	7.4	0.
Azure I		H_2O	1:50,000	48.	1.4
*Basic Fuchsin	82%	H_2O	1:20,000	72.	20.
Bismarck Brown	43%	H_2O	1:20,000	17.	11.
Brilliant Cresyl Blue	68%	H_2O	1:25,000	25.	2.9
Brilliant Green	94%	H_2O	1:40,000	66.	33.
Congo Red	89%	H_2O	Fades rapidly		
*Crystal Violet	91%	H_2O	1:50,000	76.	31.
Cresyl Violet		H_2O	1:20,000	40.	2.2
Eosine B	91%	H_2O	1:20,000	34.	3.4
*Eosine Y	83%	H_2O	1:20,000	48.	10.
Indigo Carmine	89%	H_2O	1:10,000	10.	0.
Janus Green	62%	H_2O	1:20,000	13.	1.4
Light Green SF		H_2O	1:20,000	63.	13.
Malachite Green	78%	H_2O	1:40,000	59.	25.
Martius Yellow		H_2O	1:20,000	9.5	2.3
*Methyl Green	56%	H_2O	1:20,000	55.	11.
*Methyl Violet	85%	H_2O	1:50,000	56.	21.
*Methylene Blue	88%	H_2O	1:50,000	55.	4.3
Methyl Orange	97%	H_2O	1:20,000	21.	3.0
Neutral Red		H_2O	1:20,000	35.	0.
Nile Blue		H_2O	1:30,000	35.	0.59
Nigrosine		H_2O	1:20,000	1.6	0.
Orange II	95%	H_2O	1:20,000	21.	4.0
*Orange G	83%	H_2O	1:20,000	20.	6.6
Phloxine		H_2O	1:20,000	42.	6.0
Pyronine		H_2O	1:30,000	40.	7.3
*Safranine O	94%	H_2O	1:30,000	57.	0.
Toluidine Blue	83%	H_2O	1:30,000	49.	0.52
Thionine		H_2O	1:40,000	51.	0.63
*Carmine°		$H_2O+Na_2CO_3$	1:5000	0.63	0.
Ethyl Eosine		$H_2O+C_2H_5.OH$	1:20,000	41.	4.2
*Hematoxylin	C.P.	$H_2O+C_2H_5.OH$	1:10,000	0.19	0.29
Methylene Violet		$H_2O+C_2H_5.OH$	1:10,000	45.	0.42
*Sudan III	87%	$H_2O+C_2H_5.OH$	1:10,000	4.6	3.2
*Sudan IV	90%	$H_2O+C_2H_5.OH$	1:10,000	23.	14.

*The most commonly used stains.

°Sodium carbonate has been used in 10% solution. The percentage of transparency for 365 mμ is 72, and for 275 mμ 46 in a 1-cm. solution.

lamp,* a set of specially corrected objectives,† and a pair of filters. The objectives are designed so that the green mercury line with wave length 546 mμ and the ultraviolet of 365 mμ are simultaneously in focus. Thus, it is necessary merely to focus the image using a mercury green filter such as Wratten No. 62 or No. 77A, substitute the filter No. 18A which transmits the 365 mμ mercury line, and make the exposure. The result is a sharp picture of the object revealing greater detail than could be seen with the visible light. At the highest aperture and adequate magnifications (1.7-mm. 1.3 N.A. objective) a very slight and previously calibrated adjustment of the fine focus must be made after shifting to the No. 18A filter in order to obtain the best definition. This final calibration is no greater than would have to be made when focusing in green and photographing in strictly blue light with the usual type of achromatic or even apochromatic objectives. The maximum theoretical limit of resolution with the 365 mμ line and an objective of N.A. = 1.30 is 0.14 μ as compared with 0.17 μ using blue light (wave length = 450 mμ).

Sandalwood oil is used as the immersion medium since cedarwood oil has too high an absorption. The mounting medium should be kept as thin as possible.

It is necessary to select carefully microscope slides of the maximum thickness that can be used with a correctly focused condenser so that capillarity will be sufficient to keep the very fluid sandalwood oil in place; this is most urgent with a horizontal microscope axis.

The photographic materials most suitable for ultraviolet work will be discussed on page 89. A bibliography on ultraviolet photomicrography will be found on page 165.

*Such as the Type H-4 capillary lamp which can be obtained from the General Electric Co. or in a housing for photomicrography from the Bausch and Lomb Optical Co.

†Bausch and Lomb Optical Company, Rochester 2, N. Y.

Green (546 mμ) Filter No. 77

Ultraviolet (365 mμ) Filter No. 18A

Transverse Section of LEAF BUD OF AESCULUS HIPPOCASTANUM, 100× (Horse Chestnut)
Photographed with Light from Mercury Arc

CHAPTER VIII

Photomicrographic Materials

Characteristics and Terminology of Plates and Films*

WHEN a photographic plate (or film) is exposed and developed, the tones obtained on the various areas of the negative can be measured and the values stated numerically. If each different tone of any photographic negative were to be measured and plotted on a graph against the amount of light exposure that produced it, the values would be found to lie along a curve that is characteristic for that photographic material and the treatment which it received (Fig. 12b). This curve is more completely and easily determined, however, by exposing a plate or film to a regularly increasing series of exposures wherein the light intensity is uniform over a conveniently large area but varies progressively with each adjacent area. Upon development and fixation this forms a "step tablet" (Fig. 12a) which is then measured on a photometer. The photographic effect is depicted graphically in Figure 12b by the vertical distance of a point on the curve from the exposure axis ABCD, i.e., as if the tablet in Figure 12a were turned vertically with the lightest tones at the bottom. It has been found that the exposure for a picture must be such that the effect of

Fig. 12a

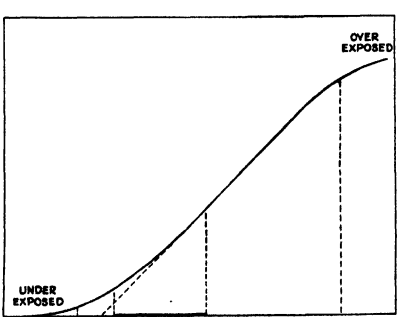


Fig. 12b

*A more extended discussion of this general subject will be found in the book *The Fundamentals of Photography.*

the lowest intensity will not be represented on the curve at a point where the slope is less than that corresponding to B in Figure 12b nor the effect of the highest exposure represented above a point corresponding to C.* Only when all of the exposure values lie within this range BC, which is a measure of the available exposure scale of the plate or film, can a print of excellent quality be made on photographic papers. Usually the available exposure scale is very much greater than is required to accommodate the brightness range of the camera image. The latter may be represented on the graph by m-n in Figure 12b. Variation in the time of exposure of the photographic plate to the camera image would, then, be equivalent to sliding m-n along the exposure axis. As the exposure is decreased, the darkest parts of the camera image must be represented by a progressively flatter portion of the curve so that equal differences in exposure produce less and less photographic effect until point m lies below B on the graph and the detail of low brightness in the microscope image will not be satisfactorily reproduced on the photographic print. If long exposures are given so that part of m-n is represented as beyond point C, correct rendering again is not obtained for the same reason, but this time highlight detail will be lost in the photographic print.

Now the relation between the *differences* between the tones of a negative, as compared with the *differences* in the corresponding exposures that produced them, is defined as the *photographic contrast* of the material. This is represented on the graph (Fig. 12b) by the slope of the straight line portion of the curve and is denoted as "gamma."

The logarithm of the opacity (defined as incident light ÷ transmitted light) of an area of a negative, which is defined as its density, D, is usually plotted against the logarithm of the exposure, E, which it received, to form the *characteristic curve* of the material (also called the H & D curve after F. Hurter and V. C. Driffield who originated it). Then the photographic contrast, or gamma, is represented by the tangent of the angle which the straight line portion of the curve makes with the log E axis, or $\frac{D_2 - D_1}{\log E_2 - \log E_1}$. If the brightness *differences* between the tones of a negative are the same as in the corresponding detail of the microscope image, the photographic contrast (gamma) is unity. (In the negative, of course, the tones are inverted.)

If each of a number of photographic plates or films of the same kind is given a range of exposures, then developed for a series of increasingly long times and the resulting tones measured and plotted as before, *a family of curves will be obtained* (Fig. 13). The photographic

*L. A. Jones and C. N. Nelson *Effective Camera Speeds of Photographic Negative Materials*, J. Phot. Soc. Amer. 7:10, Feb., 1941.

contrast, gamma, is higher the longer the development time, since a more heavily exposed area develops faster than an area of lighter exposure. As before, the range of tones of any particular negative is found by projecting m-n (representing the over-all brightness contrast of the image) onto the right curve, the slope of which (photographic contrast) is absolutely determined by the development given. Obviously, variations in development cannot be used satisfactorily to compensate for errors in exposure.

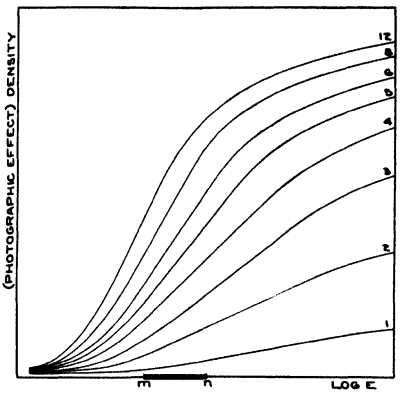


Fig. 13

If development of a negative is indefinitely prolonged, a limit to the photographic contrast that can be obtained is gradually reached, although the development fog continues to increase. This is shown more easily by plotting the photographic contrast, gamma, directly against the time of development (Fig. 14). The *limit* of the photographic contrast available upon increase of development time and also the *rate of increase* of contrast during development are determined both by the character of the plate and the nature of the development. On the other hand, the type of the plate (or film) will determine the limit of available contrast that can be obtained with any developer. This is illustrated in Figure 14. Curve A may be taken to represent the increase of photographic contrast when a plate of high available contrast, such as the Wratten "M" or Metallographic plate, is developed in a high contrast developer. Such a combination is necessary to achieve high contrasts within a reasonable development time and without appreciable fog. If a low or medium

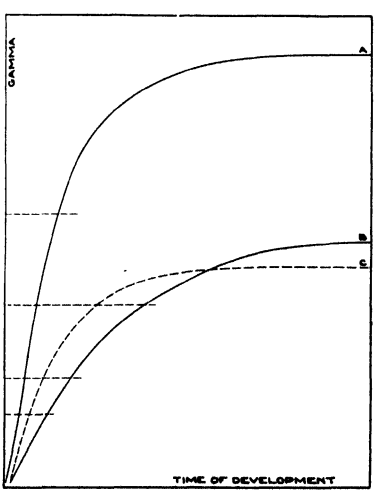


Fig. 14

photographic contrast is wanted for the negative, however, it can be seen from inspection of curve A that the contrast of the plate in the developer will be increasing so very rapidly at the short development times required, that it would be extremely difficult to obtain satisfactory consistency in the production of such negatives, and this combination becomes impractical. If a "soft" developer, such as Kodak D-41, is used with the same "high contrast" plate, the growth of contrast may be represented by such a curve as B. Obviously, small differences in development time will produce much less effect on the contrast of the negative in this case. An alternative procedure would be to use a medium contrast plate or film, such as the Wratten Panchromatic Plate, with its normal developer. Curve C might be taken to represent the growth of contrast in this case.

Selection of Materials

From the viewpoint of the photomicrographer the properties of a photographic plate of most interest are as follows:

1. Spectral sensitivity, or range of colors that will affect it.
2. Available Contrast.
3. Resolving Power.
4. Sensitivity or Speed.
5. Latitude.

All except the first of these items are so interrelated that it is impossible to obtain a plate with an extreme value of any one of these properties without accepting the others as they come. In general, it can be stated that plates or films with a high available contrast and a high resolving power will have a slower speed and shorter latitude than those plates in which these latter properties have been chiefly developed.

Contrast and resolving power are usually the most important of these properties to the photomicrographer. Fortunately he usually does not need to sacrifice for speed or latitude; as his subject is not moving, the illumination is under his control, and he has the opportunity to determine the correct exposure.

On the other hand, if the image is very faint as it may be in some high-power work with opaque objects, dark field, or in polarized light, etc., then materials of high sensitivity become an absolute necessity irrespective of the contrast desired.

In general, in selecting negative material for photomicrography the best procedure is to consider first the type of plate needed on the basis of contrast and the other correlated properties. Then select that type of plate (or film) which is sensitive to the color of the light to be used.

Many workers find it most advantageous to use a plate of high available contrast, high resolving power, and general sensitivity to all colors, such as the Wratten "M" Plate, for all kinds of photomicrography not requiring a material of extreme sensitiveness. This plate, made especially for photomicrography, can be used to produce negatives of a wide range of contrasts (standardized as low, normal, high, and extreme) by use of the appropriate development conditions which are given for that particular batch of plates by a card enclosed in each box. The Wratten Metallographic Plate is a similar type of all-purpose plate made especially for photomicrography, but can be handled by red light in the darkroom since it is not sensitive to that color. It is particularly suitable where a high contrast negative is desired and blue or green illumination is used, as is usually the case in metallography.

In general, only plates or films with an antihalation backing should be used for photomicrography. This is particularly important when the illumination is by transmitted light, since in this case the image detail is obtained by subtraction from the maximum density of the negative. In fact, even with such a backing the practical upper limit of the exposure latitude of a material is caused by the loss of resolving power rather than by poor tone reproduction as described on page 83. When materials are not regularly supplied with such a backing, they can be obtained backed on special order.

Photographic plates having only the original color sensitivity of the silver salts to ultraviolet, violet, and blue light are technically called "ordinary" plates. When the sensitivity range is extended by dyes into the green, the plates are "orthochromatic," while "panchromatic" plates are sensitive throughout the whole visual spectrum and sometimes even beyond into the near infrared. Panchromatic materials supplied by the Eastman Kodak Company are now of two types: B, Materials of extremely high color sensitiveness corresponding approximately to the color sensitiveness of the eye. These are conveniently termed "orthopanchromatic" materials. C, Materials of high total sensitiveness with extreme sensitiveness to the yellow, orange and red portions of the spectrum. These are conveniently termed "hyperpanchromatic" materials. This subject is more adequately discussed in the booklet *The Photography of Colored Objects* listed on page 167.

Information concerning plates or films with a wide range of properties may be obtained from the booklet *Eastman Films and Plates for Professional Use* procurable from any Kodak dealer. The photographic

specifications of most Kodak films are contained in the Kodak Data Book, *Kodak Films*.

A table of materials that are especially suitable for making separation negatives for color photomicrography is given on page 153 and includes processing recommendations. The latter may also be useful for making black-and-white photomicrographs that require negatives of relatively low photographic contrast.

The group of materials which are listed in the following table is sufficient in extent to include all of those usually required in photomicrography and is based on the criteria discussed on the previous page. The letter following the names of the panchromatic materials indicates the type to which these materials belong. Under R. P. is a number which gives the resolving power of the material in lines per millimeter when the contrast range within the subject is 30:1. This value increases somewhat with the subject contrast and is often quoted for a contrast range of 1000:1, but these values correspond more nearly to practice. The relative sensitivities or "speeds" of the materials are also given corresponding to an exposure time of 1/25 second. The values given are proportional to the inverse of the minimum exposure that will give a satisfactory reproduction upon properly printing the resultant negative. The criteria for this are discussed in the reference of Jones and Nelson (page 82).

The Kodak Speed is the best expression for the relative exposure required, when considering various photographic materials for the purpose of making a pictorial negative, and in general probably most photomicrographic negatives should be classed thus. The Weston or General Electric photoelectric meter settings for pictorial negatives in lists published by the Eastman Kodak Company are based on this speed with a safety factor included. In photomicrography with bright-field illumination, the proper exposure of the negative is frequently judged by inspection of the background (maximum) density, especially with such subjects as dispersed particles, some lacelike sections, and even some metal specimens. In this case, the White Card Weston Setting is a preferable criterion of comparative speeds. This value was devised for copying with process photographic materials and is based on the exposure required to give a specific and fairly high density in the negative with the recommended development. It is discussed further in the Kodak Data Book, *Copying*. Since the photographic efficiency of tungsten lamps varies with the temperature of the fila-

ment, and this affects the materials according to their color sensitivity, these values must be considered somewhat approximative.

	Tungsten					*Tungsten*				
	White Card Weston Setting	*Kodak Speed*	PLATES	*Recommended Wratten Safelight*	*White Light R.P.*	*White Card Weston Setting*	*Kodak Speed*	KODAK FILMS	*Recommended Wratten Safelight*	*White Light R.P.*
HIGH CONTRAST—VERY HIGH RESOLVING POWER										
Pan	16	20	Wratten "M" (B) (D-19)	3	70	10	12	Contrast Process Pan (B)	3	80
Ortho	16	20	Wratten Metallographic (D-19)	2	85	8	10	Contrast Process Ortho	1	120
Ordinary	4	3	Kodak Process	1	70					
MEDIUM CONTRAST—HIGH RESOLVING POWER										
Pan	2	40	Wratten Panchromatic (B)	3	55	20	400	Super Panchro-Press Type B	3	50
Ortho	2	40	Kodak Commercial	2	60	10	200	Super Ortho-Press	2	45
Ordinary	1.2	20	Kodak 33	1	45	1.2	25	Commercial	1	50
EXTREME SPEED										
Pan		320	Kodak Super Panchro-Press (C)	3	45		500	Tri-X Pan (C)	3	40
Ortho		200	Kodak Super Ortho-Press	2	45		250	Ortho-X	2	40
Ordinary		32	Kodak 40	1	35					

For photomicrography with roll film, as discussed on page 10, Kodak Plus-X Panchromatic Roll Film and Panatomic-X 35-mm. Film will probably be most generally useful. Both of these are Type B panchromatic films, with adequate resolving power when developed in such developers as Kodak DK-50 or D-76. Verichrome Roll Film is available as a high-speed orthochromatic film. For highest resolving power (170 lines per mm.) at high intensities of illumination, Micro-File 35-mm. Film (Pan B) should be used.

In considering the various speeds of the above materials, the color of the illumination that is to be used should be taken into account. For instance, if the Wratten "M" or Wratten Metallographic plates

are being considered for a case where the H filter is being used, the relative speeds for the purpose are obtained by dividing the white light speeds for tungsten of the two materials by their respective filter factors for the H filter with tungsten light (given in the table on page 93). Thus, the relative speed of the Wratten Metallographic Plate to such illumination is twice as fast as that of the "M" plate, instead of the two plates being the same speed. The further use of these speed values in conjunction with the exposure factors of color filters is illustrated on page 94.

For photomicrography in the ultraviolet, either at wave lengths of 365 mμ or at 275 mμ, "ordinary" materials can be used, although the Kodak Ortho-X Film will be found to be faster than the Kodak 40 Plate but with less contrast and resolving power. For photomicrography at 275 mμ, some gain in the exposure requirements may be obtained with the use of Eastman Spectroscopic Plates U.V. Sensitive, which may be obtained in materials furnishing any range of contrast. These plates, which are coated with a thin layer of fluorescent material, are described in the booklet *Photographic Plates for Use in Spectroscopy and Astronomy*, which will be sent upon request by the Eastman Kodak Company. Type 103-0 U.V. Sensitive may be most useful for this purpose.

CHAPTER IX

Exposure

Effect of the Intensity of the Camera Image

IT IS usual in pictorial and commercial photography to assume that intensity and time are equivalent factors in determining exposure, i.e., that $E = I \cdot t$ where E is the magnitude of the exposure, I is the intensity of the camera image, and t the time of exposure. If the image brightness is reduced eight times, by closing the lens diaphragm or otherwise, according to this rule the same quality of photographic negative as before can be obtained by prolonging the exposure eight times. This is a most useful rule when the change in the general intensity that is under consideration is not very great.* It is, however, a fundamental characteristic of all photographic plates and films that they do not obey this "reciprocity law," as it is called. Moreover, the departure from the values expected from the reciprocity law will vary considerably with the different plates or films. This is of great importance in photomicrography where large variations in intensity level may occur, from those of a fraction of a second as in low-power work to those lasting many minutes or even hours with a very dim camera image.

Obviously, the relative sensitivity of photographic materials, i.e., their relative "speeds," depends on the intensity level at which the measurements are made. Those given in the table on page 88 were measured at the "normal camera level" corresponding to a fraction of a second with a high-speed material. Speed and contrast are the two factors chiefly affected by this phenomenon. In general, it may be stated that the speed of a material will decrease if the intensity is very much lowered, and the negative contrast obtained for a given develop-

*L. A. Jones and J. H. Webb, *Reciprocity Law Failure in Photographic Exposure* J. Soc. Motion Picture Engineers, *23:* 142, Nov., 1934.

ment may increase. Therefore, if the exposure calculations given on page 94 indicate a very large exposure difference in the direction of a prolonged exposure, the exposure may need to be even longer for this reason. However, any intensity effect is already included in the filter factors given on page 93.

For a very low intensity of illumination with white light, as may occur when polarized light is used, etc., it will be found that Kodak Portrait Panchromatic (Type B) Film is about equally as sensitive as Tri-X Panchromatic Film and offers some advantage in resolving power and graininess. When the exposures threaten to be very long, one of these two films should be used; although if the illumination is confined in color to the blue or ultraviolet, Kodak 40 Plate will be more sensitive.

CORRECT EXPOSURE

By far the best system in photomicrography is to choose beforehand from the appearance of the image, etc., the contrast that is desired and develop to gain it, since it is possible in this case to determine the correct exposure. Satisfactory quality absolutely depends on correct exposure, and no two types of photographic plate or film can be truly compared unless each is correctly exposed.

In spite of the fact that the many factors involved in determining the exposure time make the correct calculation of its length a complicated process, many methods for such calculation have been published. Fortunately, with each worker some factors usually remain constant. The simplest method of arriving at the correct exposure consists in exposing areas of a strip of the film or plate to be used as described and illustrated on page 95.

The exposure varies directly as the square of the magnification. This factor alone is changed if only the eyepiece or bellows length is varied. The relative exposure factors are given in the following table:

EXPOSURE FACTORS FOR VARIOUS MAGNIFICATIONS

Magnification	*Exposure Factor*	*Magnification*	*Exposure Factor*
10	0.01	150	2.25
20	0.04	200	4
25	0.0625	250	6.25
50	0.25	500	25
75	0.5625	750	56.25
100	1	1,000	100

The exposure varies *inversely* as the square of the working aperture. The working aperture may be estimated from the N.A. marked on the objective by judging the proportion of the back lens that is filled with light or calculated by measuring the diameter of the Ramsden circle of light which is at the narrowest cross section of the beam emerging from the ocular (see page 20). The semidiameter of this disk × the magnification of the eyepiece ÷ the focal length of the objective = working aperture. If the back lens is completely filled with light, the N.A. of the objective may be taken. Exposure varies as $1/(\text{N.A.})^2$.

EFFECT OF NUMERICAL APERTURE

Equivalent Focal Length in mm.	*N.A.*	*Approximate Exposure Factor*	*Equivalent Focal Length in mm.*	*N.A.*	*Approximate Exposure Factor*
48	0.08	40	4L	0.65	2/3
32	0.10	25	4S	0.85	1/3
16	0.25	4	3	0.85	1/3
8	0.50	1	Oil 1.9	1.25	1/8

When objective and condenser form a complete oil immersion system, the loss of light by reflection is very much reduced, and the exposure may be decreased by about one-half. (Use factor 1/8 for 1.9-mm. objective under these conditions.) If the field is illuminated through the objective as in vertical illumination, this relation fails and the exposure varies little with change of objective at the same magnification.

The table on page 93 gives the factors by which the exposure time to white light should be multiplied when "M" filters (see page 64) are inserted in the illumination beam, individually or in certain useful pairs. The multiplying factors are specified according to the type of the spectral sensitizing of the plate or film. These factors are for Wratten or Kodak panchromatic and orthochromatic materials and will be useless if any other plates or films are used. The factors for the arc refer to the crater of the ordinary hard cored carbon arc. The spectral type of the materials most useful for photomicrography are given on pages 88 and 89. A more extensive list of Kodak and Wratten materials grouped according to spectral type can be found in the *Kodak Reference Handbook*, *Wratten Light Filters*, and the Kodak Data Book, *Filters and Other Kodak Lens Accessories*.

Filter	PAN. C		PAN. B		ORTHO	
	*Tung.**	*Arc***	*Tung.**	*Arc***	*Tung.**	*Arc***
A	3	4	4	5	–	–
B	8	9	6	8	5	8
C5	10	9	10	9	5	6
C4	24	20	24	20	12	15
E	2	3	3	4	–	–
F	6	8	8	10	–	–
G	2	3	2	3	4	6
H	14	17	14	17	7	12
X1	4	5	3	4	3	4
X2	5	6	4	5	4	5
K1	1.5	2	1.5	2	2	3
K2	1.5	2.5	1.5	2.5	3	4
A+D	40	50	60	75	–	–
B+E	75	90	80	100	–	–
B+G	9	12	10	13	7	13
B+H	100	120	90	110	70	110
C5+H	35	30	35	30	20	25
C4+H	120	100	120	100	60	75
D+H	600	800	600	800	400	500
G+H	350	400	300	350	220	350
40	5	6	4	5	3	5
61	10	12	8	10	6	10
62	60	70	50	60	30	50

*Tungsten source (3000°K.). **Cored carbon arc source.

In substituting one filter for another, it is merely necessary to adjust the exposure in accordance with the ratio of the factors of the two filters. For example, if a B+G filter has been in use with a carbon arc light and a Metallographic plate and it is desired to use the B filter alone, then the exposure would be changed by a factor of 8/13 or, in other words it would be decreased by about one-half.

Full tabular notes of all exposures should be kept. Although the nature of the object itself is one of the most puzzling variables, it will become increasingly simple to determine the correct exposure. Methods for the calculation of exposure time are useful for determining the approximate duration of exposure and especially for calculating exposures for other photomicrographs under changed conditions. Utilizing a correct exposure without filter (possibly by dividing by the exposure factor for the filter used) as a standard, the exposure for other microscopic conditions can be calculated with the aid of the tables by taking

into account the variations of the different factors affecting exposure: the photographic material, the magnification, the color filter, and the *working* aperture. One method calls for first taking a standard exposure for all specimens using a panchromatic plate such as the Wratten "M," no filter, and a standard magnification of 100 diameters with a standard objective such as the 8-mm. with N.A. of 0.50. The tables are based on this standard. Thus, if a section is to be photographed with tungsten light on an "M" plate with a 16-mm. objective, utilizing the full numerical aperture of 0.25, at 150 diameters, and using a B filter (factor 6), the exposure is calculated as follows, the standard exposure being 1/25 second from a previous trial:

Exposure = Standard Exposure × N.A. factor × magnification factor × filter factor = $1/25 \times 4 \times 2.25 \times 6 = 2$ seconds.

If it is then desired to use a Kodak Commercial Plate with the B+G filters, the relative tungsten speeds given in the table on page 88 may be used, since the correct exposure times of photographic materials to images formed by *white light* vary inversely as their relative speeds. (See also the discussion of the failure of the reciprocity law on page 90.)

The calculation is as follows:

$$\text{New Exposure} = \text{Exposure with "M" Plate} \times \frac{\text{B + G factor for Commercial Plate} \times \text{Speed "M" Plate}}{\text{B factor for "M" Plate} \times \text{Speed Commercial Plate}}$$

$$= 2 \times \frac{7 \times 20}{6 \times 40} = 1 \text{ second}$$

It should be remembered that if the balsam or other mounting medium is appreciably yellow, as in the case of old mounts or where a deep cell has been employed, the exposure, as calculated from above formula, will need to be considerably increased, unless strong yellow, green, or red filters are used.

Trial Exposures

As the exposure must necessarily depend largely on the object, a practical trial of the exposure should always be made when a new type of object is to be photographed. In making this trial it should be remembered that photographic exposures should be varied in a geometrical progression. One sometimes sees exposures for trial given as 5, 10, 15 and 20; such exposures are most misleading, as the effect of a change from 15 to 20 is very much less than the effect of a change

from 5 to 10. (See page 83.) If the exposure is guessed to be 15 seconds, then 5, 10, 20, and 40 seconds should be used in the trial.

The right way to make a trial exposure is as follows: A plate or film of usual size is taken and for reasons of economy cut lengthwise into a number of narrower strips, say two or four. Pull out the slide of the plate holder until the plate is uncovered, and expose the whole plate one unit of time, for example 4 seconds. Then push in the slide about an inch, and repeat the exposure for the same unit of time. Successively push in the slide to cover equal steps, exposing each step for twice as long as the previous one. The plate, in the above example, will then have had 4 seconds, $4+4=8$ seconds, $4+4+8=16$ seconds, $4+4+8+16=32$ seconds, etc. A convenient aid is to cut a piece of stout cardboard into a series of steps as illustrated below. With an arc, for instance, the exposure series might be 1/100, 1/100, 1/50, 1/50+1/25, 1/10, 1/10+1/5, 1/2, 1, 2, etc. seconds.

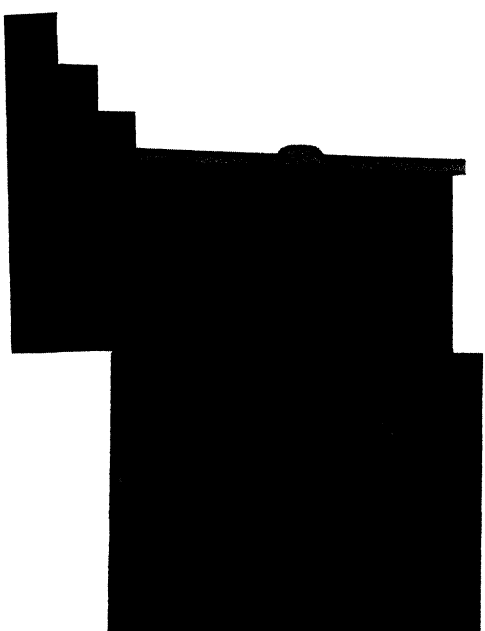


Fig. 15

Exposure Meters

The subject of the determination of the exposure in photomicrography with a photometer, including the photoelectric type, is not a simple one. On the other hand, since photomicrography is usually done under laboratory conditions, it is freed from some of the restrictions imposed upon general photography. The apparatus used for exposure determination need not be conveniently portable, and it can be connected to an electric power supply; moreover, it is convenient to examine the illumination in the image plane. Any method of adequately measuring this illumination is sufficient for the exposure determination with no further consideration than that involved in taking into account the sensitivity of the plate to it.

An exposure meter is probably most needed where the exposures are the longest, as when polarized light is used; there may be very little knowledge of the correct value in this case and a trial exposure is itself time consuming. A really sensitive meter is needed. For this purpose, there is probably nothing more satisfactory than a visual photometer that compares the illumination in the image plane with that from a standard light source. The principle of the old grease spot photometer can be, and has been, utilized here with satisfactory results. For this, a small area of translucent material, such as the Matte Acetate Sheet for binding Professional Kodachrome, is surrounded by a white matte reflecting area and the latter illuminated by a lamp, set up in such a manner that the illumination can be varied until that on the two areas is equal when the middle area is transmitting the image illumination.

There has been considerable interest in the use of photoelectric exposure meters for photomicrography and they do offer certain conveniences. Unfortunately the commercial hand type, in general use for photography, is not applicable when most needed. In low-power work with a simple microscope, such a meter can sometimes be used to measure the illumination in the image plane since the latter may be as much as 10 foot-candles, but it is impractical to use one in this position with a compound microscope. For example, in one case where the exposure was 5 seconds through a blue (C5) filter with a Wratten Metallographic Plate, the intensity of the brightest portion of the image plane was 0.13 foot-candle with no color filter in the beam. The simplest remedy is to hold the meter at a predetermined distance (see table on page 97) from the eye lens that will cause the illuminated circle of the image beam intercepted by the photometer

to be *larger* than the face of the photometer, and then calculate the brightness of the image plane. If all distances are measured from the eyepoint of the microscope, the illumination in the image plane can be found by *dividing* the illumination in the photometer by the *square of the bellows length*, and then *multiplying* by the proper *one of the factors* in the table. A card can be made which is placed against the eyepiece to determine the proper distance of the photometer and can bear the complete multiplication decimals for use at various magnifications with a given ocular. All measurements should be done with white light and the result multiplied by the proper color filter factor.

Distance of Photometer	*Bellows Length Measured in*	*Factor*
3⅛ inches	inches	10
10.0 centimeters	centimeters	100
17.32 centimeters	centimeters	300
7☆ inches	inches	50
22.36 centimeters	centimeters	500
10 inches	inches	100

In the case cited above, the illumination measured at 3⅛ inches from the eyepoint ("Ramsden disk") was about 2 foot-candles. For such levels of illumination, a photocell connected with a more sensitive galvanometer than those of the hand meters must be used. Such combinations are commercially available; the lowest scale generally has a range from 0 to 2.5 foot-candles. For somewhat higher levels of illumination, as at low magnifications or with an arc lamp at high magnifications, the usual hand meter can be used. (See page 99.)

In almost all practical methods, the meter measures the average amount of illumination over the whole of the microscope field or a considerable portion of it. Considering a specimen to be in the field, the value obtained is not a good measure of the optimum photographic exposure; in fact, this method of measuring the integrated light is particularly inapplicable to photomicrography in general. This can be illustrated by considering the case of a field of particles large enough to contain some detail in them. The meter reading with the above methods will be approximately proportional (or inversely so) to the area of the field that is covered, whereas the optimum photographic exposure might remain constant. Moreover, if the field is illuminated first by bright-field and then by dark-field illumination, the integrated

light from it will vary much more than will the correct exposures for the two cases.

In making negatives, the value of the correct exposure is proportional to the lowest brightness of an area within which detail is desired. This is frequently a very small dim area so that the direct determination of its brightness is difficult, if not impossible. Photoelectric photometers with amplification and measuring quite small areas have been made available commercially. The ideal photometer, however, would have to measure an area not larger than a square millimeter.

In making a photograph with a reversal film such as Kodachrome, however, the same criterion applies as in making a print, i.e., since the whites should look white but not at the expense of lost detail, the brightest portion of all fields is given the exposure that will cause it to be reproduced with the minimum density that will show detail. This is accomplished with negatives by varying the exposure during printing so there is less latitude for exposure errors with reversal materials. On the other hand, the fact that the correct exposure of reversal films is directly determined by the brightest portion of the field, greatly simplifies the exposure determination in photomicrography with exposure meters. With most specimens taken by transparent illumination, the brightest portion of the field is the unobstructed bright field itself. Obviously, the thing to do is to rack the specimen out of the field entirely and to determine the brightness of this field with a photometer. With oblique reflected light, it is often best to substitute a white object such as a piece of paper for the specimen for the exposure determination. In metallography, the equivalent technique is to use a polished mirror of the metal in question, although it is usually satisfactory to choose a field with relatively light etching for this purpose. In any case, this simple measure of brightness is the only one that is needed if measured in the image plane or adjusted to that.

The highlight method of determining exposure is so simple that it also proves to be the best method with negative materials. That is, it is actually both more accurate and less difficult to measure the highlight and estimate the brightness range of the image than to measure the shadow tone directly. In practice, this is done by merely classifying the images (subjects, illumination, etc.) into types and making a separate calibration for each very different type. For any one person this usually involves only a few cases.

In any case, the "speed" values given with a meter are of no direct

use; it is preferable to obtain the measure in illumination units, although any proportional values (such as f-values on a meter) can be utilized in the calibrations that must be made. It is best to have a definite set of exposure standards, one for each important difference in type of subject or illumination. These are made by making trial exposure steps, as described on page 94. It is then possible to set the meter at some constant but arbitrary value on the dial, use the proper meter setting for the plate or film, and read an exposure time value in seconds. This *time* must be *multiplied by the square of the bellows extension* and *divided by* the proper one of *the factors* in the last column of the table on page 97.

CHAPTER X

Processing and Printing

DEVELOPMENT

AFTER the plate or film has been exposed so that the developer is capable of reducing a portion of the silver halide to silver, the rest of the silver halide is still sensitive to light and further general exposure can cause it also to be developable. Moreover, the sensitivity of the residual silver salt will continue until it is dissolved away in the fixing bath, although this sensitivity will be lowered somewhat in the processing solutions and the activity of the absorbed developer is lowered by the acid. The ideal method is to handle the material in complete darkness up to this point, a procedure which is quite simple after practice. A most useful device, in this connection, is a foot pedal with which a safelight may be turned on temporarily in order to note the time or to transfer negatives from one solution to another. It is also very practical to have all timing measured by audible signals. Various devices are sold for this purpose; a metronome may be used to tick off seconds.

Handling such a material as the Wratten Metallographic Plate in absolute darkness is unnecessary, as it is also with other materials not sensitive to the whole visual spectrum, i.e., ordinary and orthochromatic materials. However, only such safelights as are recommended by manufacturers of photographic materials should be used.

Darkroom Illumination

AFTER scientific study of the ratio between safety and intensity of darkroom lights, it was found best to have darkroom lamps that would only reflect light and allow no light to leave the lamp directly. In order to fulfill this condition and, at the same time, to obtain a lamp whose safety could be relied upon, the Wratten Safelight Lamp

was designed together with the Wratten Safelights. When used with an electric lamp of the specified wattage, they will produce no perceptible fog on any of the indicated plates or films within three minutes if the latter are held at a distance of three feet. If a plate is held closer than this for examination, it must be remembered that the allowable time will approximately decrease as the square of the distance. Plates should not be held up immediately before them for inspection. The dark red Series 2 safelight should be used for all ordinary and orthochromatic plates and films. However, since panchromatic materials are sensitive to red light, the customary red darkroom light is obviously useless. Although it is, of course, safest to handle panchromatic plates or films in complete darkness, the Eastman Kodak Company has provided a special green safelight (Series 3) for use with them; although it is seemingly too faint for help at first, it is adequate when the eye is accommodated to it. This is because it is made on the following principle: Although the eye is sensitive to all colors if the light is sufficiently bright, it becomes relatively less sensitive to red and blue as the intensity of the light is diminished until with very faint light the green gives by far the brightest light. The Series 3 safelights use the light by which we can see the most with the least possible light, the only method applicable with plates sensitive to all colors. *No other safelights are safe for these plates or films in the slightest degree and red light especially must be avoided.* In the table of materials for photomicrography on page 88, the Wratten Safelight is listed that can be used with each in a Wratten Safelight Lamp as specified above.

Time-Temperature Development

While there are a number of different methods of photographic development, that most suited to photomicrography, where exposure is under complete control, is development for a specified time at a definite temperature according to the degree of contrast desired in the negative. This is the most satisfactory method that can be used with panchromatic materials since they would tend to fog if illuminated enough for inspection.

For timing development, either a clock with large hands or, more conveniently, a Kodak Interval Timer can be used. This timer which can be set for a given time and then started in the dark, gives a signal at the expiration of the time.

Development may be by either tray or tank. For routine work

when many negatives are handled, the latter is preferable. Kodak Sheet Films are flat so that they can be handled singly like plates. Since the back as well as the face of the film is coated with gelatin, neither side should be allowed to come in contact with anything during manipulation or while drying.

When a material of high available contrast is being developed in either a tray or a tank to a moderate contrast, it is wise to transfer it from the developer directly into a stop bath such as Kodak SB-1a described on page 109.

Tray Development

To develop a plate or sheet film in a tray, proceed as follows: Get the dishes, developer, clock, etc., ready and have a cover for the developing tray; the latter should be appreciably larger than the plate or film to be developed. It is wise to have the tray in the sink or in a larger tray so that sufficient agitation can be given without fear of the consequences if some developing solution is spilled. With the solutions at the proper temperature, pour the developer into the tray to a depth of about one-half inch, turn on the safelight, if used, and turn out the white light. Get out the sensitive material and note the time, or start the previously set time clock.

Immerse the plate or film quickly and evenly; this can be done by tipping up the tray on its long edge, dropping in the plate or film, and causing the developer to flow back in one quick wave by lowering the tray. Break any air bells that may form on the surface by rubbing gently with the fingers. Cover the tray immediately, and rock continuously and fairly vigorously for the time specified on the card enclosed in the box in order to gain the desired degree of contrast. This is best done as follows: Raise the left side of the tray ½ to ¾ inch, lower smoothly, and then immediately raise and lower the near side in a similar manner; continue agitating by raising and lowering the right side and then again the near side. These four operations constitute an "agitation cycle." Then quickly remove the plate or pour off the developer and rinse it well in clean water before transferring it to the fixing bath or a stop bath where it should be agitated for about 30 seconds.

The most uniform development can be obtained in the case of a tray by brushing the surface of the plate in the developer with a long-haired, soft, flat, wide brush. This method will require about 20 per cent less time to obtain the same contrast.

Tank Development

For tank development, a suitable developer is kept in covered tanks sold for the purpose; it is a great advantage to have it in a thermostatically controlled water bath. The plates or films are placed in special development hangers such as the Kodak Film and Plate Developing Hanger.

The following development technique for plates will be found satisfactory, especially with a high contrast developer. The plates in their racks are dropped vertically in a group into one end of the tank while the time is simultaneously noted or a timer started with the other hand. The emulsion side of the plates should face the end of the tank near which they are dropped. Immediately, move each negative to the other end of the tank, one after the other and as quickly as possible. Then grasp the top of the front hanger and move the negative energetically to the other end of the tank tapping one side of the tank on the way. It may be necessary to hold back the other hangers. Repeat this agitation successively with each negative until they have all been moved to the other end of the tank. Then quickly push the whole group of plates back to the other end of the tank. This whole operation should be repeated at the end of each thirty seconds (or other constant time interval). Thus each negative will be individually moved against its emulsion surface at equal periods which are independent of the number of negatives in the batch. At the end of the development period, all of the negatives are lifted together in their hangers out of the developer, drained from a corner, and dipped into rinse water or a stop bath before being transferred to the fixing bath.

Development by tank should last about 20% longer than that in a tray with the same developer, in order to obtain the same contrast.

DEVELOPING SOLUTIONS

Preparing Photographic Solutions

The only metals that should be allowed to remain in contact with photographic solutions are silver, 18-8 molybdenum stainless steel, Inconel, and Monel metal; glass, enameled steel, and hard rubber are also satisfactory for containers. If metals are used for containers, all joints should be welded. Separate containers should be used in preparing the developing and fixing solutions.

When procurable, *distilled water* should be used in mixing all solutions. With hard water a precipitate of calcium carbonate will form in

a developer, but this will do no harm provided that the solution is allowed to settle in a separate vessel and only the clear liquid drawn off for use.

Any scum that forms on the surface of either developer or fixing bath should be skimmed off daily with a sheet of blotting paper.

Further information on photographic chemicals and their handling is given in *Elementary Photographic Chemistry* and the Kodak Data Book, *Formulas and Processing*.

Selection of Developer

The developer formula recommended by the manufacturer of the plates or films should be used with his own product together with the recommended development times. Formulas for all Kodak Films and Plates can be obtained from any Kodak dealer or they will be sent upon request by the Eastman Kodak Company. Such formulas are also given in the two references quoted above.

Wratten "M" and Metallographic plates can be used exclusively in photomicrography; they produce negatives throughout the range of desired contrasts by the choice of the appropriate one of the developer formulas, according to whether a comparatively low or very high contrast is desired. (See page 84.) For those who wish to use only films as negative materials, Kodak Contrast Process Panchromatic Film can be used instead of the Wratten "M" Plate and Kodak Contrast Process Ortho Film instead of the Wratten Metallographic Plate for "high" or "extreme" contrast, with the developer formula Kodak DK-50 as further specified below. Although these films were primarily designed for use in copying, they can be used in photomicrography to yield negatives of exceedingly high resolving power and unusual latitude for their contrast, although particular care must be taken in using them for lower gammas than those normally encountered in process work, since they develop extremely rapidly. Therefore, particular care should be exercised to immerse the films evenly and quickly and to agitate them strongly yet uniformly. Immediately after development, they should be transferred very quickly to an acid stop bath such as Kodak SB-1a where they should be well rinsed with strong agitation before they are transferred to the fixing baths.

For "normal" and "low" contrasts, a film developing at a more usual rate must be selected. Either Kodak Super Ortho-Press Film or Super Panchro-Press Film, Type B, will be satisfactory for this purpose when

used as specified in the table below. The following development conditions will yield satisfactory results with these films.

Contrast	*Approx. Gamma*	*Kodak Developer*	*Time of Development at 68°F. (20°C.) (Tray)*		
			Kodak Contrast Process Films Ortho and Panchromatic	*Kodak Super Ortho-Press*	*Kodak Super Panchro-Press Type B*
Very high	3.0	DK-50	4 min.		
Very high	3.0	DK-50 (1:1)	6½ min.		
High	2.0	DK-50 (1:1)	3½ min.		
Normal	1.2	DK-50		7½ min.	8 min.
Low	0.8	DK-50		4 min.	4½ min.

In spite of the range of selection of plates and films afforded by the table on page 88 for use with a wide variety of specimens and working conditions, it is most efficient to restrict the number of developer formulas required in the darkroom. Three fundamental developer formulas can be used most satisfactorily for practically all photomicrographic work. All three of these developers, Kodak D-19, D-76, and DK-50, which are described below, can also be obtained in packaged form prepared so that they need only be dissolved in the proper quantity of water according to the enclosed directions. Moreover, all of them have proved to be excellent for use in either tray or tank without change in concentration, and they keep remarkably well. The developer D-76 should be modified for photomicrography as described below; this is very simple to do, either with the packaged developer or from the developer as made up according to the formula.

Kodak Developer D-19 is recommended for use with Wratten "M" and Metallographic plates where an extreme pictorial contrast (about a gamma of 3.0) is desired. It also represents an excellent developer for any of the materials listed on page 88 with which a high contrast is available. The course of development can be represented by such a curve as A of Figure 14 (page 84).

Kodak Developer D-76 is a slow working developer whose rate of development with such materials as the "M" and Metallographic plates can be described by a curve such as B in Figure 14, so it is particularly useful, after a slight modification, as Kodak D-41, for obtaining low (below a gamma of 1.0) and normal contrasts with these materials. By another modification, consisting principally of the

addition of the particularly well-suited alkali Kodalk (made and sold by the Eastman Kodak Company), a developer, Kodak D-42, can be made whose rate is comparable to such a developer as Kodak DK-50. It is recommended when a high pictorial contrast (about a gamma of 2.0) is desired with the "M" or Metallographic plates. Kodak D-42 can also be used as one of normal rate with the other materials listed in the table on page 88. With those of medium contrast and speed, its rate of development will resemble that described by curve C in Figure 14. Where considerable work with these materials is done, it probably would be preferable to use the regular developer DK-50 which will frequently be prescribed in the direction sheets for these materials; typical curves describing the rate of development with this developer and some of these materials will be found in the Kodak Data Book, *Kodak Films*.

Kodak Developer D-19

FOR RAPID DEVELOPMENT OF FILMS AND PLATES

	Avoirdupois / *U. S. Liquid*	*Metric*
Water, about 125°F. (50°C.)	16 ounces	500 cc.
Elon	32 grains	2.2 grains
Kodak Sodium Sulfite, desiccated	3 oz. 90 grains	12¾ ounces	96.0 grams
Kodak Hydroquinone	128 grains	1 oz. 75 grains	8.8 grams
Kodak Sodium Carbonate, desiccated	1 oz. 265 grains	6 oz. 175 grains	48.0 grams
Kodak Potassium Bromide	73 grains	290 grains	5.0 grams
Cold water to make	32 ounces	1 gallon	1.0 liter

Dissolve chemicals in the order given.
Use without dilution for either tray or tank.

Kodak Developer D-76

	Avoirdupois / *U. S. Liquid*	*Metric*
Water, about 125°F. (50°C.)	24 ounces	96 ounces	750 cc.
Elon	29 grains	116 grains	2.0 grams
Kodak Sodium Sulfite, desiccated	3 oz. 145 grains	13¼ ounces	100.0 grams
Kodak Hydroquinone	73 grains	290 grains	5.0 grams
Kodak Borax, granular	29 grains	116 grains	2.0 grams
Water to make	32 ounces	1 gallon	1.0 liter

Dissolve chemicals in the order given.
Use as modified by directions following for either tray or tank.

Kodak Developer D-41

For slower rate

	Avoirdupois U. S. Liquid	Metric
To Kodak Developer D-76 Solution	32 ounces	1 gallon	1.0 liter
Add *Kodak Benzotriazole (0.2% Stock Solution)	1¼ drams	5 drams	5.0 cc.

Use without dilution.

Kodak Developer D-42

For normal rate

	Avoirdupois U. S. Liquid	Metric
To Kodak Developer D-76 Solution	32 ounces	1 gallon	1.0 liter
Add Kodalk	145 grains	1 oz. 145 grains	10.0 grams
and *Kodak Benzotriazole (0.2% Solution)	2½ drams	1¼ ounce	10.0 cc.

*Kodak Benzotriazole (0.2% Stock Solution) is supplied in an 8-ounce bottle ready to use or the solid chemical is supplied in several sizes.

Use without dilution.

Kodak Developer DK-50

FOR PROFESSIONAL FILMS AND PLATES

Stock Solution

	Avoirdupois U. S. Liquid	Metric
Water, about 125°F. (50°C.)	16 ounces	64 ounces	500 cc.
Elon	37 grains	145 grains	2.5 grams
Kodak Sodium Sulfite, desiccated	1 ounce	4 ounces	30.0 grams
Kodak Hydroquinone	37 grains	145 grains	2.5 grams
Kodalk	145 grains	1 oz. 145 grains	10.0 grams
Kodak Potassium Bromide	7 grains	29 grains	0.5 gram
Water to make	32 ounces	1 gallon	1.0 liter

Dissolve chemicals in the order given.

Use without dilution for either tray or tank.

Development Recommendations

The following recommendations for the development of Wratten "M" and Metallographic plates can be used only as a general guide. If results of greater accuracy are desired, the material should be calibrated with the developer in question under actual working conditions. As stated on page 90, the contrast obtained for a given time of development will increase as the time required for the exposure increases. Therefore, a somewhat shorter time of development will be needed if the proposed exposure time is considerably longer than was given in the case of the standard exposure as discussed on page 94.

Kodak Developer	*Contrast*	*Approx. Gamma*	*Time of Development in Minutes at 68°F. (20°C.)*			
			Tray		*Tank*	
			Metallographic	*"M"*	*Metallographic*	*"M"*
D-41	Low	0.8	3½	4	4½	5
D-41	Medium	1.2	5½	5½	7	7
D-42	High	2.0	4½	4½	5½	5½
DK-50	High	2.0	6	5	8	6½
D-19	Extreme	3.0	8	4	10	5

It is recommended that, whenever possible, the developer solutions be brought to a temperature of 68°F. (20°C.) and maintained there. The most satisfactory results are obtained between the temperatures of 68°F. and 70°F. However, the time of development required to obtain the same contrast at any other temperature between 55°F. and 75°F. can be obtained with the aid of Figure 16 as follows. Locate as a point on the chart the time of development which is known to be satisfactory, such as the recommended time at 68°F. Draw a new diagonal line parallel to that corresponding to the developer concerned. The desired time can then be read from the intersection of this new line with the proper horizontal temperature line.

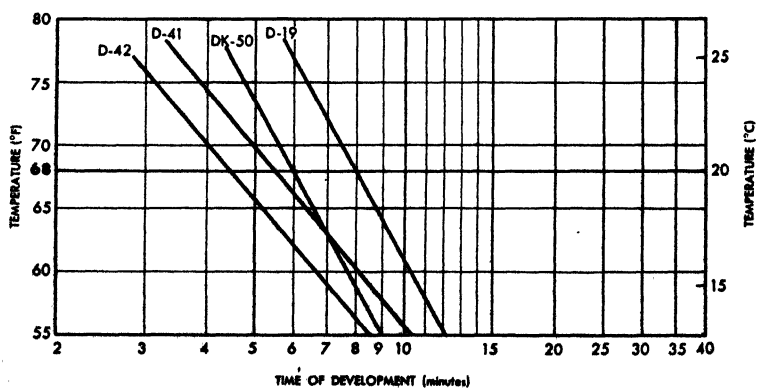


Fig. 16

Tropical Development

Development at temperatures above 75°F. (24°C.) should be resorted to only when it is impossible or very difficult to control the temperature

of the photographic solutions by artificial cooling. In this case a special technique is necessary.

Kodak Developers D-19 and D-76, whose formulas are on page 106, can be used up to 85°F. (29°C.) by adding 1 ounce 290 grains (50 grams) of desiccated sodium sulfate to 32 ounces (1 liter) of developer. If crystalline sodium sulfate is used, the amount added should be 3½ ounces (105 grams) for the same volume of solution. This concentration of neutral salt will prevent excessive swelling of gelatin and reduce the development rate. The development time at 80°F. with this amount of sodium sulfate will be approximately the same as it would be at 68°F. without the salt. With Kodak DK-50, add sodium sulfate, desiccated, 3 ounces 145 grains (100 grams) to maintain a constant time of development at 80°F.

When development is completed, rinse the film or plate for 1 or 2 seconds only and immerse in Kodak Hardening Bath SB-4 for 3 minutes before putting it into the acid hardening fixing bath. Then wash for 10 to 15 minutes in water not over 95°F. (35°C.).

Kodak Hardening Bath SB-4

FOR USE AT 75° TO 90°F. WITH FILMS AND PLATES

	Avoirdupois U. S. Liquid		Metric
Water	32 ounces	1 gallon	1.0 liter
Kodak Potassium Chrome Alum	1 ounce	4 ounces	30.0 grams
*Kodak Sodium Sulfate, desiccated	2 ounces	8 ounces	60.0 grams

*If it is desired to use crystalline sodium sulfate instead of the desiccated sulfate, then use 4 ounces 290 grains per 32 ounces (140 grams per liter) when mixing the formula.

Agitate the negatives for 30 to 45 seconds when first immersing in the hardener, or streakiness will result. Leave them in the bath for at least 3 minutes between development and fixation. If the temperature of the developer is below 85°F. (29°C.), rinse for 1 to 2 seconds in water before immersing in the chrome alum hardener bath.

Further details on tropical development can be obtained on request from the Eastman Kodak Company.

Kodak Stop Bath SB-1a

FOR FILMS, PLATES, AND PAPERS

	U. S. Liquid		Metric
Water	32 ounces	1 gallon	1.0 liter
*Kodak Acetic Acid, 28%	4 ounces	16 ounces	125.0 cc.

*To make approximately 28% acetic acid from glacial acetic acid dilute three parts of glacial acetic acid with eight parts of water.

This bath can be used under normal temperature conditions as an intermediate rinse between rapid developer and fixing bath to check development instantly and to prevent uneven spots and streaks when negatives or paper prints are immersed in the fixing bath.

Fixing

Eastman X-ray Fixer (a prepared powder) may be used or, if preferred, the following formula which is especially satisfactory for use with highly alkaline developers such as Kodak D-19.

Kodak Fixing Bath F-10

FOR USE AFTER HIGHLY ALKALINE DEVELOPERS

	Avoirdupois / *U. S. Liquid*		*Metric*
Water, about 125°F. (50°C.)	16 ounces	64 ounces	500 cc.
Kodak Sodium Thiosulfate (Hypo)	11 ounces	44 ounces	330.0 grams
Kodak Sodium Sulfite, desiccated	¼ ounce	1 ounce	7.5 grams
Kodalk	1 ounce	4 ounces	30.0 grams
*Kodak Acetic Acid, 28%	2¼ ounces	9 ounces	72.0 cc.
Kodak Potassium Alum	¾ ounce	3 ounces	22.5 grams
Water to make	32 ounces	1 gallon	1.0 liter

*To make approximately 28% acetic acid from glacial acetic acid, dilute three parts of glacial acetic acid with eight parts of water.

Dissolve the chemicals in the order given, taking care that each chemical is dissolved completely before adding the next.

Agitate the films or plates when they are first placed in the fixing bath and at intervals until the fixation is completed.

Films or plates should be fixed properly in 10 to 20 minutes in a freshly prepared bath. Wash thoroughly and *wipe each negative carefully* before drying. The bath need not be discarded until the fixing time becomes excessive, that is, over 20 minutes.

When the time to clear a plate or film has been increased, through use, to twice that of the fresh fixing bath, the bath should be discarded. *By thoroughly rinsing the films or plates between development and fixation, the useful life of the fixing bath is prolonged.* Stains and other troubles may largely be avoided by frequent renewal of the fixing bath. Always use *fresh, strong, and clean* fixing baths. Old and dirty ones are certain to produce trouble.

Washing

After fixation if the plate is given a separate preliminary rinse, it should be satisfactorily washed after twenty to thirty minutes in clean,

rapidly running water or five changes of water for three minutes each; the sufficient period varies with the plate. For thorough washing, the water in the tank should be replaced about six times every half hour.

The pamphlet *Stains on Negatives and Prints*, which will be sent upon request, will be helpful if trouble is experienced from fog, spots, or stains on negatives. Water spots sometimes form on the surface of negatives where small drops of water have collected before drying. Drops of water sometimes fall out of the film clips, after the film has dried, and produce such spots unless the water is shaken out of the clips before the film is set up to dry. The surface of the negative should be wiped off with a Kodak Photo Chamois or a moist sponge upon removing it from the wash water unless it is rinsed in water containing a wetting agent such as the Kodak Water Spot Preventive. Directions for use of this solution are given on the bottles. In general, the negative should be immersed in a 1:100 dilution of this solution for about two minutes, drained, and then allowed to dry.

MAKING PRINTS AND SLIDES

Photomicrographic prints are usually made by contact since the desired magnification can be made in the negative. A glossy paper is usually most suitable for the purpose, such as Velox Glossy or Azo F, and the gloss may even be increased by drying on a ferrotype plate. The papers are graded according to their contrast, the lowest numbers being used for the negatives of highest contrast, and vice versa; grade No. 2 of either paper is most suitable for a normal negative. Since the contrast of the final negative is the product of the photographic contrasts of the negative and the printing paper and since the low contrast papers such as Velox No. 1 have the longest scale for tone reproduction, it is well to make the negatives so that grade No. 1 or No. 2 can be utilized if the specimen has a long range of detail. This method is especially valuable in photomicrography because of the unusual control that is available in making the negative. On the other hand, where high contrast is desirable, such as work with particle size, pearlitic structure, etc., grades No. 3 or No. 4 will be most suitable, even with negatives of high contrast.

Kodak Developer D-72 is recommended for use in making prints on either Velox or Azo papers by contact or on Kodabromide by enlargement. It can be obtained in prepared form in a package or prepared from the following formula.

Kodak Developer D-72

FOR PAPERS, FILMS, AND PLATES

Stock Solution

	Avoirdupois U. S. Liquid		Metric
Water, about 125°F. (50°C.)	16 ounces	64 ounces	500 cc.
Elon	45 grains	180 grains	3.1 grams
Kodak Sodium Sulfite, desiccated	1½ ounces	6 ounces	45.0 grams
Kodak Hydroquinone	175 grains	1 oz. 260 grains	12.0 grams
Kodak Sodium Carbonate, desiccated	2¼ ounces	9 ounces	67.5 grams
Kodak Potassium Bromide	27 grains	110 grains	1.9 grams
Water to make	32 ounces	1 gallon	1.0 liter

Dissolve chemicals in the order given.

For use with any of the three printing papers noted above, take stock solution Kodak D-72, 1 part; water, 2 parts. Develop about 60 seconds at 68°F. (20°C.) for optimum results.

It is especially important to wash the prints thoroughly to reduce the residual hypo in the paper fibers, or the prints may turn brown or spot with age. One hour of efficient washing should be sufficient. An efficient test for residual hypo in the paper using alkaline potassium permanganate is described in the Data Book on Kodak *Papers* and in the *Kodak Reference Handbook* (see page 167). Hypo can be eliminated almost completely from photographic papers by treatment with the Hypo Eliminator HE-1 which is completely described in the section on Chemicals and Formulas of the latter publication. This is particularly recommended whenever photomicrographs are being made to keep as permanent records of importance.

For transparencies or lantern slides, Kodak Lantern Slide Plates are satisfactory. Further details can be obtained from the Kodak Data Book, *Slides* (see page 168). However, methods of color photography are especially suitable for photomicrographic transparencies; the appearance of the original specimen can be very closely reproduced.

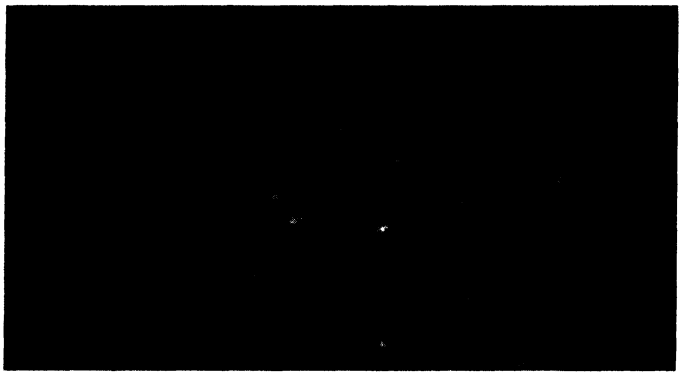
CHAPTER XI

Motion Photomicrography

THE USE of the motion-picture camera can be a great aid in the study of living organisms, both to picture moving subjects and to obtain a continuous record of growth or other change, since regulation of the camera speed permits the rate of the action under observation to be apparently either slowed down or made faster. It is also useful in the study of certain types of chemical or physical change. The action of many hours or even days can be condensed to a viewing time of one minute. In this manner discoveries have been made that otherwise had escaped all notice.

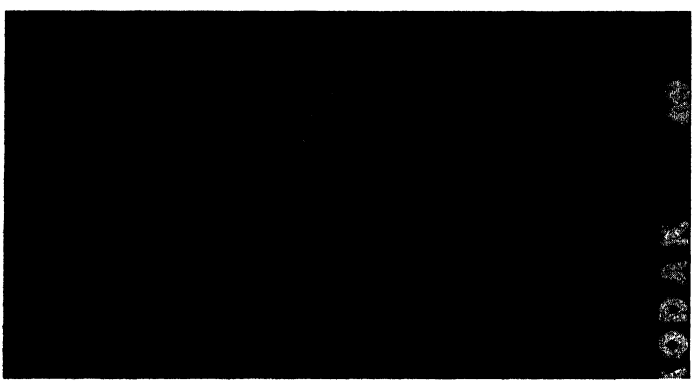
APPARATUS

As in still work, the transitional region between ordinary photography and those magnifications requiring a compound microscope is important. The usual camera lenses are most useful in this case, but they must be held rigidly beyond the usual distance from the camera. It must be remembered when estimating the exposure that the *effective* aperture of a lens used in this manner will be less than its rated aperture when used for objects near infinity. When the lens is held more than two focal lengths away from the film, it will be acting as a simple microscope (magnification greater than one) and it should be reversed, unless the lens is perfectly symmetrical, so that the back of the lens (for ordinary photography) is facing the object. A series of extension tubes can be furnished for use with the Ciné-Kodak Special and with Magazine Ciné-Kodaks that hold their lenses at distances which allow successive field sizes to be obtained, down to a field width of about one millimeter with a 25-mm. lens and 16-mm. camera. A ring adapter for the ordinary photomicrographic objectives can be obtained for those cases where the microscopist already possesses the latter. The Ciné-Kodak Special possesses a reflex finder with which any image



LIVING PROTEUS VULGARIS, 900×

Dark-field illumination to show flagellae. Motion picture on hypersensitized panchromatic film. Light source—high intensity arc.



LIVING PROTEUS VULGARIS, 900×

Dark-field illumination to show flagellae. Motion picture on hypersensitized panchromatic film. Light source—high intensity arc.

that would be focused on the film can be viewed between operations and with which the image can be accurately framed and focused on a ground glass as in still photomicrography. The focusing finder of the Magazine Ciné-Kodak serves the same purpose. The detailed directions for the use of such a system can be obtained by writing to the Eastman Kodak Company.

In ordinary photomicrography, one picture is usually all that is desired for any one setup of conditions which, therefore, need only remain satisfactory and constant for the duration of one exposure time. In motion photomicrography, however, a succession of pictures is desired, and conditions must remain satisfactory for a considerable length of time. These include not only conditions for critical illumination but also freedom from vibration, maintenance of critical focus, etc. Moreover, there is at least one machine in the system that is operating with intermittent motion, and there is usually a motor included which is acting as a source of vibration.

It is obviously necessary that there should be no physical contact between the microscope and the camera; in fact they should be mounted on completely separate supports. The whole apparatus must be designed for stability.

The individual research workers in Europe and America have usually found it necessary to design their own apparatus for motion photomicrography with the professional size (35-mm.) film, although it is now possible to purchase such equipment. The descriptions of several of these setups have been published (see bibliography, page 166). With the advent of the 16-mm. reversal process it has become possible for all cases, such as in research work where a large quantity of identical prints are not wanted, to accomplish the same results in a much cheaper and also easier way.

The Viewing Device

It is essential in motion photomicrography that the operator have the opportunity of focusing the microscope and of selecting the proper field of the microscope slide without interrupting the photographic process. The best device for accomplishing this two-fold purpose is what is known in optics as a "beam splitter." A beam splitter makes it possible to utilize part of the light which comes through the eyepiece of the microscope for visual inspection while the remainder of the light is used to form an image on the film. The field seen through the

visual eyepiece should contain a pair of cross hairs as well as lines that indicate the limitations of the camera field; really more than the camera field should be seen through the visual eyepiece so that selections can be made during photography. This piece of glass bearing the cross lines, which is at the focus of the top eye lens, should be so placed that when the image is sharply in focus in its plane, it is also most critically in focus on the film. Unless the eye lens, through which the cross lines and also the aerial image are observed, is made adjustable for focusing on these lines, very few observers will obtain exactly the best focus on the film. Moreover, if the eye lens is made quite high powered (making the visual images larger than their apparent screen size), it will be found that the focus obtained on the screen is sharper.

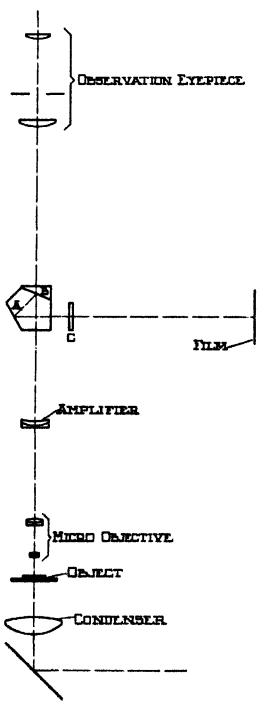


Fig. 17

The light lock between microscope and camera should open in the same direction as the optical axis of the microscope, because with a horizontal light lock between a vertical microscope and horizontal camera axis, there is the extreme inconvenience of removing the camera each time the height of the microscope tube is changed a little. In the latter case, where the main microscope beam is bent at right angles by the beam splitter, the whole observation unit should be attached to the camera and not rest on the microscope.

Figure 17 illustrates the optical system of the beam splitter viewing device which was originally designed for equipment for photomicrography with a Ciné-Kodak.* It can be adapted to other cameras that have removable lenses such as Ciné-Kodak Models E and K, and the Ciné-Kodak Special, Fig. 18, although this must be done by the factory. Since the Ciné-Kodak Special also has a reflex finder which is useful in low-power work, this camera, thus equipped, is especially suited to motion photomicrography. This is particularly so, due

*See Bibliography, article by C. Tuttle. This device can be obtained from the Bausch and Lomb Optical Company.

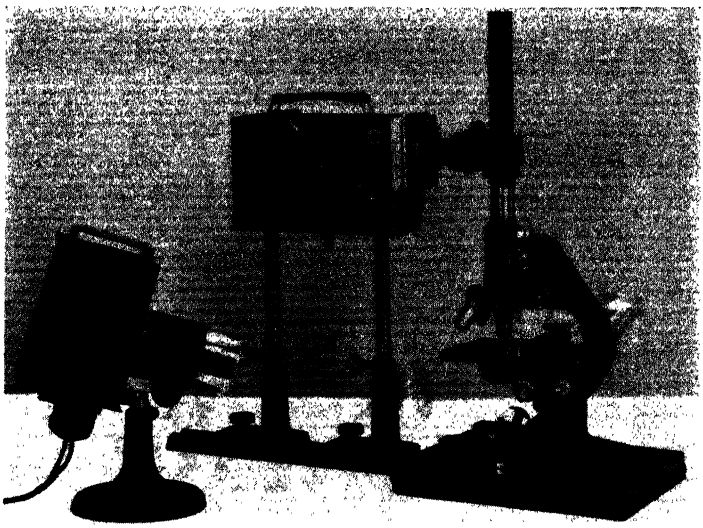


Fig. 18

to the fact that lower primary magnifications on the film are used for motion photomicrographs than for still photomicrographs as discussed on page 121. Consequently, many subjects that would be taken with a compound microscope in ordinary photomicrography are photographed more advantageously with the simple microscope (enlargement) for motion pictures.

With this viewing device, a negative lens amplifier takes the place of the regular microscope eyepiece and projects the microscope image under low magnification on the film of the motion-picture camera after reflection at the pentagonal prism A. The surface between prisms A and B is coated with a thin transparent deposit of platinum which allows about 10 per cent of the image-forming light to pass through to the observation eyepiece. About 90 per cent of the light is reflected onto the film in the camera.

Most of the preparations of which one wishes to make motion pictures are best handled on a horizontal stage, and the equipment should be designed with this point in view. A smoothly working mechanical stage is essential.

Use of Camera with Lens

Cameras whose lenses are not removable can be used for motion photomicrography as they can for still photomicrography and with the same restrictions (see page 10). A simple yet effective split beam viewing device can be made that employs the advantages of focusing with a telescope set for infinity. If the camera is fixed so that the axis of its lens is a continuation of that of the microscope, and a microscope cover glass which has been selected for flatness is inserted between the two so that its plane is at 45° to the axis, a small fraction of the illumination beam will be reflected at right angles. A telescope containing a graticule, on which the equivalent of the film frame can be marked, is focused on a distant view and then, at the same setting, clamped so as to view the horizontal image beam from the microscope. If the camera lens is focused at infinity, this will allow the picture to be focused accurately and framed. The whole unit should be enclosed in a black box.

The Camera Support

A rigid, stable support for the camera, which is massive with respect to the bulk of the camera, is an essential, for no vibration from the camera or its driving mechanism must be transmitted to the microscope. At the same time, it must be possible to move the camera easily and smoothly in a vertical direction in and out of the light lock, and to provide easy accommodation for the different heights of the microscope tube with different optical systems. Moreover, it should be possible to move the camera out of the way easily for direct visual work and yet be possible to put it back quickly into its exact optical position. It also should be possible to level the camera with respect to the microscope.

When working with high magnifications it is desirable to place the microscope and the camera support on different tables. It is also desirable to use a microscope in which the adjustment is stiff enough to prevent accidental jars from spoiling the focus.

The Camera Drive Unit

In order to utilize all of the advantages offered by motion photomicrography, the operator should be able to select any one of a variety of taking speeds. On the other hand, when only pictures of motile organisms such as protozoa are contemplated, the usual type of spring-

driven motion-picture camera can be utilized satisfactorily, although it is advantageous to have available the use of half and double speeds as are available with the Ciné-Kodak Special and Magazine Ciné-Kodak and the Ciné-Kodak Model E. Many changes, for instance the growth and division of cells, take place so slowly that they are imperceptible when viewed in the ordinary way. This is where "time-lapse" photography becomes valuable in speeding up the rate of change as it appears on the screen.

Two types of apparatus may be utilized to obtain time-lapse photography. One type is intermittent, wherein a timing device in conjunction with a relay-controlled mechanism makes a series of single-frame exposures of constant duration with a preassigned interval between them. This method has the advantage that the photographic exposure need not be changed when its rate is changed, so that the illumination can remain the same. Care must be taken that no vibration is caused in the camera by the necessary sudden starting and stopping of the mechanism.

The other type of mechanism consists of a motor drive with a variable speed reduction unit. This must also be carefully planned to eliminate the effect of the vibrations emitted. One excellent method is to mount the camera drive unit separately on a cushioned system and to connect it to the camera with a sliding tube and universal joints. This continuous-drive method has the advantage that very feeble illumination can be used at the slow rates of photographing growth, etc., since the exposure period extends over approximately half of the elapsed time. This may be an important advantage when working with preparations which may be injured by high light intensities. When the exposure rate is to be changed, neutral densities whose transmission factors are equal to the changes of speed must be ready to be inserted into or withdrawn from the illumination beam. The split beam device is provided with such densities.

The construction of a number of camera drives for "time-lapse" motion photomicrography has been described in the literature (see bibliography). Accessories are supplied for the Ciné-Kodak Special whereby it may be driven with the intermittent type of variable time lapse. With the Electric Time Lapse Outfit, it is possible to choose exposure times with a duration of 1/100 second, 1/50 second, 1/20 second, or any time from 1/4 second to 6 seconds, while the interval between the beginning of the successive exposures can be set at values between 1/4 second and 24 hours as long as these intervals are at

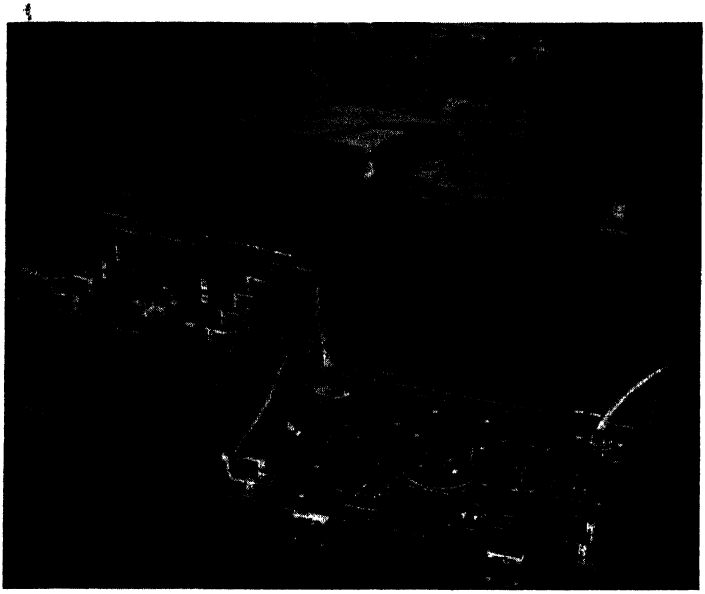


Fig. 19

least equal to the exposure times. If the interval is less than six seconds, the Interval Timer box (shown at lower right in Figure 19) is not needed in the outfit, and any interval time within the range (1/4 second to 6 seconds) can be chosen. As illustrated, the Interval Timer has a dial of 24 units divided into quarters; by means of a scale switch these units can be chosen for the intervals as either seconds, minutes, or hours. The outfit can also be used to give remote control by hand over the exposure so that the operator need not remove his eye from observation of the field while starting or stopping the mechanism.

The continuous-drive method can be obtained with a 16-mm. camera by utilizing the Ciné-Kodak Model E with *f*/1.9 lens (or with this replaced by the split-beam device) and a special motor drive. To change the speed of the camera, a pair of gears must be removed and another pair substituted although this operation is done quite quickly. Further details can be obtained from the Eastman Kodak Company.

In experimental work it is often necessary to determine the rate of growth or the time period of some change. This can be done by counting the frames or measuring film lengths if the camera is driven at an accurately constant and known speed. The accuracy of a synchronous

motor can be depended on to within one-half of one per cent. About the most satisfactory method is to include a picture of the face of a clock or watch in one corner of the frame that has been photographed simultaneously by an independent lens system, usually with the aid of a prism. The simplest method of accomplishing this is to utilize the prism finder for viewing the image through the film to photograph this image through the base from behind the film with those cameras that are equipped with such a finder. This includes most cameras for 35-mm. film. A suitable mask open only in one corner must, of course, be in the rear beam and the opposite type of mask in front of the film. A film must be used that does not have an opaque antihalation backing. For professional cameras using 35-mm. film, all of the normal negative materials are available since the clockface can be exposed right through the gray tinted base without difficulty. Eastman Release Safety Positive Film (Type 5301) and Fine Grain Release Safety Positive Film (Type 5302) are available with a clear base and, although only blue sensitive, are sometimes used for photomicrography of growing organisms when high contrast and resolving power are needed and the relatively long exposure times make the exposure problem simple.

Where films with opaque antihalation backing are used, such as the 16-mm. reversal films, the clock must be photographed from the front with the aid of a small prism inserted between the microscope and the camera.

It is sometimes desirable to slow down the rate of action on the screen as in the case of rapidly moving protozoa. A number of 16-mm. cameras can be driven at the rate of 64 frames per second. In this case it is often a problem to obtain sufficient illumination (see page 123), so that it is usually best when possible to slow down the rate of free moving cells by introducing some viscous medium, such as gelatin or clarified gum arabic, into the specimen cell. For this purpose the gum arabic solution should be mixed with raw egg white, boiled, and then filtered.

OPTICAL REQUIREMENTS

Photomicrographers who are accustomed to working with plates up to 8 x 10 inches in size may need a word of caution regarding the amount of magnification desirable in motion photomicrography. Magnifications of the order of 5,000 diameters or more which are common in still photomicrography are impractical when one is working with

the limited image area provided by the motion-picture film frame.

Magnification should be kept at a minimum to include as large a portion of the object slide area as possible and to decrease the light intensity required for exposure. The criterion of minimum magnification is, of course, the final screen image. The ideal result is a picture in which all of the detail which is resolved by the microscope objective is distinguishable on the screen.

There are in this connection several points to consider. (1) The resolving power of the eye of an observer when viewing the projected picture. (2) The magnification obtainable upon projection of the film. (3) The resolving power of the photographic emulsion. (4) The resolving power of the various objectives to be used.

Under good projection conditions it is possible for a person twenty feet from the screen to distinguish detail made up of lines or dots separated by about 5 mm.

A 16-mm. film image can be enlarged on projection about 100 diameters (40-inch screen) without difficulty. It should be possible then to see on the screen the image of detail which is separated on the film by only 0.05 mm. Since the resolving power of the Ciné-Kodak emulsion is of the order of 50 lines per millimeter, a separation of 0.05 mm. for the finest detail should result in a good film image of this detail.

If the magnification of the optical system of the microscope and viewing device is sufficient to separate the finest detail resolved by the objective by 0.05 mm. on the film, an increase of magnification has no advantage unless the film is to be shown before an audience larger than can be accommodated within twenty feet of the 40-inch screen.

The table shows the magnifications required to render the resolved detail with a typical set of objectives, assuming that the aperture of the objective is filled with light. See pages 27 and 36 for a further discussion of the resolving power needed at given magnifications.

The three negative lens amplifiers supplied with the motion photomicrographic equipment are designed to correct the objective for the actual image distance—a factor which is set invariably by the location of the viewing device relative to the Ciné-Kodak film. These three amplifiers supply, respectively, 3×, 5×, and 7.5× magnification of the objective image.

These amplifiers, which are an optical substitute for the more usual oculars, are corrected for use with achromats. Equivalent compensat-

TABLE SHOWING MAGNIFICATION REQUIRED TO SEPARATE RESOLVED DETAIL BY 0.05 MM.

Objective Focal Lengths	*N.A.*	*Resolving Power* $\lambda = 500\ m\mu$.	*Total Magnification Required*	*Objective Magnification (Tube length = 160 mm.)*	*Magnification Required of Amplifier*
2.0 mm.	1.30	520	260	90	2.9
3.0 mm.	1.40	560	280	61	4.6
3.0 mm.	.80	320	160	62	2.6
4.0 mm.	.95	380	190	45	4.2
8.0 mm.	.65	260	130	20	6.5
16.0 mm.	.30	120	60	10	6.0

ing negative amplifiers for use with apochromatic objectives can be obtained.

For the usual cases it will hardly be necessary to use an amplifier of greater power than 5.0×.

As a matter of fact, one can profitably use a lower-power amplifier than the one indicated in the last column if it is not necessary to record the highest resolution of which the objective is capable. When photographing with wave lengths shorter than 500 mμ or when producing film for a large audience to view on a small screen, greater amplifier magnification than those indicated may be desirable.

Illumination

With transmitted light and normal or subnormal taking speeds, the problem of getting sufficient exposure usually is not a serious one. A 108-watt, 6-volt ribbon filament lamp with an $f/1$ aspheric condenser will be found sufficient for most purposes. This lamp can be operated at an overload in amperes of about 15 per cent for short periods; thus its photographic effect with the ciné panchromatic films is increased by 200 per cent. The Electric Time Lapse Outfit for the Ciné-Kodak Special has provision for operating the lamp intermittently, with the illumination period lasting about 8 seconds during which time the exposure is taken. For exposure intervals longer than 12 seconds, this should conserve the life of the lamp. For slow taking speeds with growing organisms, the light must frequently be reduced; it is usually best to reduce the illumination with a color filter, such as one of the green series given on page 65, since it simultaneously improves the image and is less harmful to the organisms. The incandescent tungsten

lamps also lend themselves well to devices to switch the light off when the camera shutter is closed, such as a mercoid switch tilted by a rotating cam.

When a carbon arc is used for transmitted light illumination, the image of the crater is usually focused in the apertures of the microscope system (Köhler illumination, see page 24); therefore, the flickering of the arc will produce bad frame-to-frame variations in illumination. It is frequently necessary to use an arc, however, with camera speeds that are normal or faster. In fact, it probably is not possible to take dark-field motion pictures of motile organisms at high magnification and at normal camera speed without the use of a special high intensity arc (see reference on subject in bibliography).

Dark-field illumination is especially suitable for motion photomicrography. The subject is discussed under that title on page 47. The light scattered into the objective in dark-field illumination, and hence the photographic exposure, is very much a function of the refractive index of the object and its reflecting power. The protoplasm of single cells or carbon particles in Brownian motion allow but a small percentage of the incident light to be used. Dark-field illuminators vary greatly in efficiency. It does not seem probable that sufficient exposure can be obtained with any light source so that normal camera speeds can be used with a slit ultramicroscope containing minute particles.

The possibilities of success in taking pictures at a specified camera speed may often be explored in a preliminary way by substituting a still camera for the motion-picture camera and taking a series of exposure test strips as described on page 95. It is wise to extend the test to insure that one step has sufficient exposure, even though the length of time required may be ridiculous in relation to the movement of the specimen, in fact, even if the moving objects are lost entirely on the test film. The factor by which the image intensity must be changed can then be calculated, including the possibility of substituting a more intense source of light. The crater of an ordinary 4½-ampere carbon arc may be considered to have a brightness of 160 candle power per square millimeter. The high intensity arc (such as that sold by the Sperry Gyroscope Company) is of a different type, taking special carbons and having a rotating anode. The 12-inch 12,000 candle-power arc, which operates at 38 amperes and 40 to 45 volts across the arc, has an intrinsic brilliancy of about 505 candle power per square millimeter. The intensity from a heliostat varies

according to external considerations, but a value of about 1000 candle power per square millimeter may be assumed as an average value with an unobscured sun.

Exposure Trials

The processing of Ciné-Kodak film is done at a large number of laboratories well distributed geographically over the world. The technique of producing the reversed (positive) Ciné-Kodak image is too complicated to be undertaken by the photographer himself.

It is advised that when a roll of the exposed 16-mm. film is sent in for processing, the box should be very plainly labeled PHOTOMICROGRAPH so that it will be specially handled.

Since, however, the sending of film to the processing laboratory involves some delay and needless loss of material if the exposure is greatly in error, it is recommended that the motion photomicrographer make and develop trial exposures on short lengths of film. It is further recommended that when considerable work is to be done a set of exposure standards be set up. This can be done by exposing at one end of a roll of film sent for processing a series of a few frames to increasing light intensities as described below, and upon the return of the film, mounting this strip opposite one exposed identically but processed by the operator. In fact, a separate standard of this type will probably be required for each entirely different type of subject or illumination, such as dark-field and bright-field.

Most of the variables by which the exposure is ordinarily regulated are fixed in motion photomicrography by conditions peculiar to the individual subject. The time of exposure is fixed by the camera speed which is determined by the speed of movement of the subject. Other conditions are fixed with equal rigidity by the demands of resolving power and magnification. The operator has only one variable under his complete control, i.e., the intensity of the light source. This factor can be regulated roughly by the choice of a light source of approximately the correct wattage. The intensity of the beam can then be more accurately regulated either by controlling the electrical input to the source or by the use of Wratten Neutral Density Filters placed in the beam. If a set of such filters is available, this method offers the better means of controlling the intensity. Changing the current through the lamp by means of the rheostat does not offer much of a range of photographic exposures, unless the current range below the rated

amperage is used, since an increase of about 15% (such as from 16 to 18.5 amperes in a ribbon filament lamp) only increases the photographic exposure by a factor of 2.0.

Exposure trials can be made then either by interposing neutral tint filters which vary in transmission by approximately 50 per cent increments (i.e. the series of transmissions 50%, 25%, 10%, 5%, etc.) or by reducing the lamp current in steps of 6 per cent.

Having set up the apparatus according to the instructions which accompany it, the operator can proceed with exposure trials. The camera is loaded with a roll of film and a few feet of protecting film leader are run off. The trial exposures need be only a few frames in length. The camera must be unloaded in the dark. The exposure trial strip should be removed and the cut end rethreaded on the lower reel before closing the camera.

The test strip may be developed in Kodak D-19, given on page 106, for 5 minutes at 68°F. (20°C.) to give a negative image. It should be possible to recognize a bad over or underexposure by this means; there should be a noticeable deposit of silver over the thinnest portion of the negative. However, a preferable procedure is to process the film strip with the Kodak Direct Positive Film Developing Outfit.

However, in the case of Ciné-Kodak Super-XX Panchromatic Film, it will be necessary to prolong the first development to 17 minutes. This will result in positive pictures which are more accurately and easily judged and which lend themselves to the formal exposure standards recommended above.

It will probably be necessary to swab off the backing with a piece of cotton or squeegee, after the rinse following the development, and before putting into an acid bath, such as a stop bath or fixer.

After having set up proper exposure standards, the exposure can be determined by a photometric measurement as described on pages 96 et seq. Since Ciné-Kodak Film is a reversal material, the remarks made concerning Kodachrome Film in this section and in the discussion on page 137 also apply to this case.

The viewing device of the Ciné-Kodak equipment is supplied with a series of filters set in a revolving mount (see Fig. 18, page 117). The filters are marked "0.3, 0.6 and 0.9." The lightest filter transmits half the light, the second, one-quarter, and the third, one-eighth. The purpose of these filters is to provide means for balancing the light needed for visual inspection. For the slower picture speeds it will be

found that the requisite intensity is too low for accurate visual focusing. It is better in this case to moderate the light intensity by interposing one of the filters in the revolving mount. The use of these does not affect the visual intensity.

It is necessary to use one of these filters only when it is found as the result of exposure trials that the intensity required for photography is too low for clear vision of the field in the observation eyepiece. As a means of controlling exposure, the reduction of intensity before the light passes through the object slide is to be preferred.

MOTION PHOTOMICROGRAPHY IN COLOR

The Kodachrome Process makes it possible to obtain the results of motion photomicrography in the natural colors of the specimen. Kodachrome Film merely needs to be loaded into the camera in the same manner as is done for black-and-white photography.

The general discussions concerning photomicrography with Kodachrome Film in the following chapter should be read since they are also applicable here. In motion photomicrography, the exposure time is determined largely by the speed of the camera which in turn is usually determined by the subject and the purpose of the photography.

For work at normal speeds a carbon arc is undoubtedly the best light source; the illumination emitted by the 4.5-ampere carbon arc is correct for Kodachrome Film, Type A with no photometric filter. Special care must be observed to prevent arc flicker, however, since this affects successive frames and makes a poor appearance on the screen. If the weather is damp, the carbons should be heated to 600°F. (315°C.) in an oven and then kept over a desiccant. The 10-ampere automatic arcs are very advantageous in this respect. A Wratten Filter No. 86C should be inserted in the beam when this arc is used with Type A Film. If the results seem slightly yellow, the following filter system should be substituted: a 10.0-millimeter layer of a 3% solution of sodium nitrite plus an amber Eastman Color Compensating Filter CC14. (See page 147.) It will easily allow further modification to suit necessary compensation for the particular optical system.

For growth work an incandescent tungsten lamp with the proper photometric filter as determined with the aid of the Eastman Color Temperature Meter will be most useful. Kodachrome Film, Type A should be used.

CHAPTER XII

Color Photomicrography

PHOTOMICROGRAPHS in color can, of course, be made by the usual processes of color photography. Because photomicrography is most frequently done in a laboratory or under conditions which can be rigidly controlled, and because the subjects are stable and remain in position, some methods and controls are available which are not practical for general photography. On the other hand, there are some problems which are peculiar to photomicrography in color, such as the unnatural absorptions of many biological stains, the critical requirements for reproduction where the shade of color is used for identification or diagnosis, as are the constituents of some metal alloys, or the difficulty in including a gray scale in the object field as a control. These will be discussed on page 138 et seq., with methods for overcoming some of them. Motion photomicrography in color was discussed in the chapter, "Motion Photomicrography."

Full-color photography is based upon the phenomenon that any color can be matched for the human eye by mixing, in suitable proportion, light of three specially selected colors. These are known as primary colors, and they are usually blue, green, and red. All full-color photographs involve the making of a separate black-and-white negative (at least temporarily) by each of the three primary colors. This is done by means of tricolor separation filters or with selectively sensitized emulsions. A positive image is then obtained from each negative, either by photographic reversal or by printing on a new piece of sensitized material.

Photographic color processes are classified as either "additive" or "subtractive" according to the way in which the positive image in color is formed. In an additive process, each of the positive pictures is viewed by light of the same color as that by which its negative was made. Naturally, all three positive images must be viewed simultaneously and in register. The combination of the blue, green and red not absorbed by

the positive pictures should add up to white light. The positive pictures are usually black and white.

In a "subtractive" process each of the final positive images is composed of a dye or pigment whose principal absorption lies in the region which is transmitted by the taking filter, and whose transmission is mostly of the color absorbed by that filter. In a full-color process these images would be minus blue (yellow), minus green (magenta) and minus red (blue-green or cyan). These component positive images must be superposed in register, and viewed against white light, as for instance, the light reflected by underlying white paper or a projection screen. An area containing no dye or pigment in any of the images would, of course, be white. If there were an equal amount of the colored images through which the white light must pass, the area should appear gray or black. Different colors are provided by varying amounts of yellow, magenta, and cyan dye or pigment.

It must be realized that in any full-color process, at least three separate pictures must be made, and viewed simultaneously and in register.

From the standpoint of the user, the methods of color photography can best be classified as (1) the integral tripack, (2) the screen plate or film, and (3) the separation negative methods. In the first method, a single exposure takes the three pictures simultaneously on one film or plate having three superimposed emulsion layers. A typical example is Kodachrome Film, which has three emulsion layers, one sensitive to each primary color. In the second method, the three taking filters are arranged as a pattern of fine lines or minute patches covering the surface of the film or plate. Exposure is made with the filter elements toward the lens. If the film is reversed to a positive, the filters become viewing filters, a color transparency results, and there are no registration difficulties. This was among the first of the practical color processes. The oldest method is the third, in which the negatives are separately exposed, simultaneously or in succession (especially in photomicrography), and separately processed. Separate color positives are made and superimposed. The making of excellent color prints by such processes demands experience and skill. This process allows a great degree of control, and is capable of accurate reproduction. It is discussed in more detail on page 150 et seq. and in the books listed on page 166.

INTEGRAL TRIPACK PROCESSES

KODACHROME

The advantages of the integral tripack process, represented by Kodachrome, are: simplicity in use, convenience, and speed of operation. The advantages are so marked that it may be the only method that is practical in a given situation. The question arises whether these advantages can be utilized in most color photomicrography. It is true that frequently special problems are involved, and that special arrangements are necessary to overcome them. Usually this can be done more easily and quickly than by the alternative of resorting to the slower and more clumsy three-color separation negative method, especially if there is a considerable amount of work to be done (cf. page 150).

It may be necessary to resort to the separation negative method in some cases where accurate reproduction is more important than the saving in time and trouble possible with an integral tripack process. Usually, however, there is no commensurate gain in quality of the results of the older method to justify the extra time and labor involved. The procedure of making a photomicrograph in color, especially one at low magnification, may be almost as simple as that of making it in black and white. The range of tolerance for incorrect illumination varies widely with the nature of the subject and the requirements at the time. If the third color is relatively unimportant, or absent, particularly if it be blue, the correctness of the illumination quality itself will be relatively unimportant.

One essential condition is common to all integral tripack processes. Since the three component tricolor pictures are taken simultaneously, the three filter factors for them are predetermined in the manufacture of the film; for optimum color reproduction, the quality of illumination specified by the manufacturer must be used.

Anyone who intends to do an appreciable amount of color photography should become familiar with the modern terms for expressing illumination quality, and its control with filters.* The quality of an illuminant is frequently specified in terms of its "color temperature," which is the temperature, in degrees Kelvin (°K.), at which a "black body" or "perfect radiator" must be burned in order that its emitted radiation will have the same spectral energy distribution as that of the

*A more detailed discussion is given in the articles by R. S. Estey and H. P. Gage, etc. quoted on page 166.

illuminant. The degree intervals are the same as that of the Centigrade scale, but the zero is about −273°C. A "color temperature" of an illuminant usually is not its true temperature, but merely the temperature of a black body that would radiate the same light quality. The term has come into common use in photography. Actually the spectral quality of illumination frequently varies much from that of a black body, and careless use of the term "color temperature" is to be avoided; sunlight and daylight differ appreciably from it but are often specified in terms of equivalent color temperature. The light from incandescent tungsten filaments and carbon arcs is fairly close to "black body quality" (see page 126).

A photometric filter is one which converts the quality of an illumination from one color temperature to another; the term is also applied to filters transforming light to sunlight or daylight quality. There are no perfect photometric filters; the two component liquid filters of Davis and Gibson* are probably the best. However, photometric filters such as the gelatin filters of the Wratten No. 78 or 86 series are much more convenient to use and are usually satisfactory. It is wise to use a relatively light photometric filter if possible, since its errors will be of less consequence.

Kodachrome Film is available for miniature cameras, which are often used in photomicrography, and as sheet film for the usual type of photomicrographic cameras. Complete discussion of the available types and their characteristics is given in the Kodak Data Book, *Kodachrome, Photography in Color.***

The table on page 132 gives the types of Kodachrome Film that are available, together with the type of illumination with which they must be used to give satisfactory reproduction of color.

Duplicates of Kodachrome still transparencies may be obtained as enlargements, as the same size, or as reductions to almost all of the regularly listed Kodachrome Film sizes, by ordering through a Kodak dealer who sends them to the Eastman Kodak Company, Rochester 4, New York. Duplicates of 28 x 40-mm. originals will be supplied only in the 24 x 36-mm. size, but so that the entire picture will be included within this size mask unless the picture has been previously masked to that size. Photomicrographers are frequently in the peculiarly advantageous position among photographers, however, in that they may have a stable object that is relatively permanently imaged in the focal plane of

*See Bibliography, page 166.
**See Bibliography, page 168.

Kodachrome Type		*Correct Illumination Quality*	*Cameras*	*Picture Size*
Name	*Designation*			
Regular	K135	Daylight	35-mm. Cameras	24 x 36 mm.
Regular	K828	Daylight	Kodak Bantam	28 x 40 mm.
Type A	K135A	Photoflood (3450°K.)	35-mm. Cameras	24 x 36 mm.
Type A	K828A	Photoflood (3450°K.)	Kodak Bantam	28 x 40 mm.
Professional Daylight Type	Sheet	Daylight	Professional	Usual sizes for sheet film. See page 168
Professional Type B	Sheet	3200°K.	Professional	Usual sizes

their camera, and also have special conveniences for determining the proper exposure conditions to obtain the color photograph. In such case, it is simpler to make a series of duplicate pictures at once in the camera.

Illumination for Kodachrome Photomicrography

Since few of the usual photomicrographic illuminants are directly suitable for Kodachrome photomicrography, one of two methods must normally be adopted in setting up the illumination: (1) the proper lamp for one of the types of Kodachrome Film must be chosen and converted to an efficient illuminant for photomicrography; or, (2) the quality of the light from one of the normal photomicrographic illuminants must be converted to that which is correct for Kodachrome Film. In either case, but particularly with the second method, an ammeter or voltmeter and a variable rheostat or autotransformer should be included in the lamp circuit if much work is to be done. A reasonably stable line voltage is very desirable for all photography in color.

The first method is particularly suitable to Kodachrome Film, Type A, since a satisfactory illuminant can be made very simply from Photoflood Lamps for which this film was designed, by the method described on page 13. The studio Mazda lamps, for 3200°K., made for Kodachrome Film, Type B, are not so adaptable to this method, although they can be used. For most subjects, especially at low magnifications, Kodachrome photomicrography by this method is very simple; expo-

sures are made as directly as they would be for a black-and-white photomicrograph. The optical system, however, does introduce some defects in the color balance, particularly at medium and high magnifications (see page 139). If this occurs, and excellent reproduction is desired, the second method, which employs the same lamp that has been used for general photomicrography, is preferable.

Particularly suited for Kodachrome photomicrography is the carbon arc lamp using carbon electrodes with cores of amorphous carbon, such as is widely used in metallography. Only with the arc is it possible to set up a specified illumination quality (color temperature) in any laboratory without a specific calibration of the individual lamp. This is not true of incandescent filament lamps except in those few cases, such as the Mazda lamp for 3200°K., where the lamp has been especially made to produce a given color temperature. With a carbon arc, it is only necessary to know the proper current, voltage and carbon diameter to reproduce the quality of another arc using the same carbons, but the lamp itself need not be of the same manufacture. There is, moreover, less variation of quality with variation of current than in the case of most tungsten lamps, particularly with a relatively high current for the size of the carbons, as with the 10 to 12-ampere arcs.*

Although the arc is somewhat susceptible to drafts and hence to the extent to which it is enclosed, this variable also decreases with increase of current density, that is, with increase of current or decrease of the anode carbon diameter. The carbon arc does emit an excess of ultraviolet radiation just beyond the visible caused by the formation of the gas cyanogen, but the spectrum beyond the blue (λ 450 mμ) is very closely that of a black body.** In fact, the presence of this excess ultraviolet and far blue has given the ordinary carbon arc a reputation for having a color temperature much higher than corresponds with most of its visual spectrum, and has led to the choice of Kodachrome Film, Daylight Type, for use with the arc without modifying filters. This is incorrect, as can be seen from the following data.

Unfortunately, specification of the color temperature of the carbon arc is not a simple matter.* It is usually defined as the result of visual measurements, whereas the ultraviolet radiation is indistinguishable by Kodachrome Film from blue light. A filter to reduce the excess

*H. G. MacPherson, *The Carbon Arc as a Radiation Standard*, J. Opt. Soc. Am., *30:* 189, 1940.

**See page 131 for the definition of *black body quality* and the use of *color temperature* as a measure of illumination quality.

ultraviolet or to eliminate it entirely should normally be used with a carbon arc for Kodachrome photomicrography, but the resultant truncated spectrum is still difficult to specify accurately as a color temperature. The carbon arc, particularly with direct current, must therefore remain valuable because its unusual reproducibility at a specified amperage, voltage, and carbon diameter eliminates the need for the individual calibration of the lamp. It is rarely useful directly with a calculated photometric filter. However, the table below is helpful in giving perspective regarding the quality of the arc light (the "color temperature" of sunlight can be considered as 5400°K.) and in ranking the various arcs in use in photomicrography. The color temperature quoted was determined by visual comparison of the crater of the arcs as imaged by an achromatic lens with the light from a calibrated tungsten lamp. On the whole, Kodachrome Film reacts as if the illumination were at this color temperature, or a little higher, when the ultraviolet is reduced to the normal amount for an incandescent body. A filter which will do this consists of a solution of sodium nitrite of about 30 grams per liter (1 oz. per 32 fl. oz.) in a 10-mm. layer.

Amperes	Anode Diameter	Color Temperature
4½ D.C.	8 mm.	3645°K.
5 D.C.	8 mm.	3680°K.
5 D.C.	6 mm.	3750°K.
10 D.C.	8 mm.	3820°K.
10 A.C.	6.4-6.4 mm.	3475°K.

The color quality of the illuminants in use for visual microscopy varies widely, and the particular habit that has been formed with any one illuminant establishes the standard of color balance that is considered acceptable for colored photomicrographs. This is most strikingly illustrated by the case of those microscopists, including most medical workers, who are accustomed to a high intensity tungsten lamp with a thick bluish glass "Daylite" filter. Such individuals usually consider that the photomicrographs obtained with Kodachrome Film, Type A, and the D.C. carbon arc at 4.5 amperes with no ultraviolet-absorbing or photometric filter are satisfactory for color balance. These pictures appear too blue to anyone accustomed to a microscope illuminated with incandescent tungsten or even with actual daylight. In all cases, the final picture quality should be judged by projection or by viewing with an illuminator of quality similar to that of the Kodachrome Illuminator.

The 5-ampere D.C. arc with 8-mm. carbons will normally be most satisfactory with an ultraviolet-absorbing filter, such as a 10-mm. layer

of a solution of sodium nitrite, 30 grams per liter (1 oz. per fl. oz.). The color temperature is too high for Kodachrome Film, Type A, and should be reduced by a negative photometric filter such as the Eastman Color Compensating Filter CC14.

Kodachrome Professional Film, Type B, can also be used very satisfactorily with the carbon arc although the color temperature of the illumination must be reduced to a somewhat greater extent, and the excess ultraviolet removed from the spectrum. The gelatin photometric Wratten Filter No. 86C has about the correct conversion power as determined visually, but the equivalent of the Wratten Filter No. 2A is incorporated in it, causing it to remove all of the ultraviolet and violet from the radiation. This makes the photograph too yellow (but see page 144). On the other hand, the amber photometric Eastman Color Compensating Filters CC13, CC14, and CC15 transmit the near ultraviolet and therefore require some auxiliary absorbent such as the sodium nitrite solution. The Eastman Color Compensating Filter CC15, together with the same nitrite filter quoted above, constitutes the proper combination for use with the 5-ampere arc with 8-mm. carbons for Kodachrome Film, Type B. However, as discussed on page 139, the proper filters may depend somewhat on the equipment, especially with a compound microscope.

Although well adapted for its common use in metallography or dark-field work, the carbon arc lamp is objectionably intense for bright-field illumination, especially at low and medium powers. The illumination may be suitably reduced by means of a neutral density gelatin filter film (Wratten No. 96) although this will probably introduce a slight yellow tinge, which is negligible if a filter completely absorbing the ultraviolet is being used. Some commercial glass neutral filters are rarely even approximately gray photographically although they may be excellent visually. They can and have been used with compensating filters. A very practical method, and one which is especially appropriate for work at low magnifications, is to illuminate a piece of ground glass from behind with the arc and then to use this glass as the light source by placing a focusing condenser in front of it. The ground glass must have a diaphragm against it although this may merely be a hole in a piece of black paper. It is also wise to place a water cell behind the ground glass.

The various forms of incandescent tungsten illuminants for photomicrography, such as the ribbon filament, can also be used for Kodachrome photomicrography by utilizing the second method on page 132.

The chief difficulty is the fact that they cannot be depended upon to reproduce the same color temperature from lamp to lamp even when burned at the same amperage, and there is an appreciable change of quality at a given amperage with age which is absent in the carbon arc. Therefore, some method of determining their color temperature is a necessity unless individually calibrated lamps can be obtained. Such a method is available with the use of the Eastman Color Temperature Meter. (See page 168.) This instrument measures the quality of the light, assuming that its spectral distribution corresponds to that of an incandescent black body at some color temperature, by determining the ratio of illumination at two separated wave lengths (of green and red color). In practice, the user merely points the instrument (Fig. 20) at the light source, sees a different color in each half area of a circle upon looking through the eyepiece, and turns the knob until the whole circular area has the same color. The color temperature of the illumination is read directly on the dial.

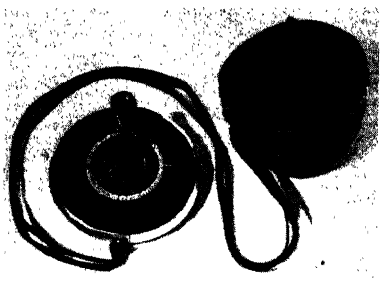


Fig. 20

The difficulty of the change with age, which tungsten lamps undergo, can be reduced by burning them at a somewhat lower current rating and by always using a given and relatively pale photometric filter with them. For instance with Kodachrome Film, Type B, and a ribbon filament lamp, a Wratten Filter No. 78C should be used. When calibrating the lamp for this work, set the dial of the Eastman Color Temperature Meter at 3200°K., and adjust the current through the lamp until the two fields of the meter are equal when the lamp is viewed through this filter. With this filter the lamp will probably last for many weeks of satisfactory use, although it should be checked occasionally for quality difference due to aging; in fact, it is wise to avoid using a brand new lamp for color photography, but rather, to burn it a few hours, since the greatest rate of change occurs initially. On the other hand, after a grayish coating of evaporated tungsten has formed on the bulb to an appreciable extent, the color balance will no longer correspond to that of a clean bulb of the same apparent color temperature since the film acts as a color filter.

Illumination consisting of a mixture from several sources differing in quality, or that from a source with a discontinuous spectrum, as that from vapor lamps, is not generally suitable for color photography. Within this category is the otherwise excellent tungsten arc (see page 15). However, a compensating filter combination can be procured for use with this arc and Kodachrome Film, Type B, with which satisfactory color reproduction can usually be obtained. Since it is not a corrective filter, there will be some subjects which cannot be well reproduced. This filter combination consists of the Wratten Filter No. 2A plus a Corning didymium glass filter (No. 5120), 2.0 mm. in thickness.

Determination of Exposure

Trial exposures (see page 94 et seq.) will probably be necessary at first, but they need not be repeated on Kodachrome Film if definite step-exposure standards are set up.

Because exposure meters are particularly adapted for use with Kodachrome Film (page 98), frequent exposure trials will be unnecessary if a suitable meter is used. Such a meter must be standardized, however, for each new *type* of subject and illumination. Unfortunately also they cannot be used for very low intensities.

Information is frequently requested as to the relation of the speed of Kodachrome Film to that of some negative film or plate, such as the "M" plate. For both theoretical and practical reasons, a reliable comparison of speeds cannot be made between an ordinary negative material and a material used for making a reversal positive. Therefore, a reversal material that can be processed immediately by the photomicrographer must be chosen for the trial-exposure tests and for setting up a standard. As an exposure standard for use with Kodachrome Roll Film (K828 or K135), Kodak Direct Positive Panchromatic Film (35-mm.) should be used. This will give a black-and-white positive after it is processed with the chemicals that can be purchased from a Kodak dealer. This film is about six times as fast as Kodachrome Film, Type A. For cameras taking sheet film, by far the simplest and fastest method is to use Kodak Super Speed Direct Positive Paper, which has a speed about equal to that of Kodachrome Film, Type B, with its proper illumination. Its processing is exceedingly rapid and simple when done according to the directions enclosed with the boxes of paper. This material has, however, the disadvantage that it is only orthochromatic so that all red areas appear underexposed; this may be very serious

with specimens stained with eosin, safranine, fuchsin, or other red stains. In such cases, it is probably simplest to use the Direct Positive Panchromatic Film with the strips cut so that they can be held in a film holder.

It is worth while to set up definite exposure standards in the following manner: With a suitable test specimen in the microscope, make a trial exposure series, as described on page 95, but use the appropriate black-and-white reversal material; process it according to directions. Then immediately repeat the exposure series with the Kodachrome Film, allowing the proper exposure factor between the two materials. The Kodachrome Film should then be sent for normal processing. Upon its return, the two tablets should be kept together, preferably in a permanent mount with steps having the best exposures opposite each other. It is then always possible to estimate how some new trial-exposure test on the black-and-white film would look on Kodachrome Film, by comparison first with the black and white of the standard and then its counterpart in color.

SPECIAL PROBLEMS WITH THE INTEGRAL TRIPACK METHOD

Although probably the majority of photomicrographs, especially those at low powers, can be made on Kodachrome Film as simply as on black-and-white after the correct illuminant has been chosen, there are some problems which are peculiar to photomicrography with Kodachrome Film. Just when they become serious depends principally on the nature of the work, chiefly, on whether an accurate reproduction of color must be made or whether an equally good differentiation of colors is sufficient. The latter is usually the case. In any event, the enormous conveniences of the integral tripack method make it worth while to overcome these difficulties rather than resort to the more elaborate separation negative method.

Reproduction of Biological Stains

There are certain aniline dyes that are not reproduced well by a three-color process, such as Kodachrome, owing to the sharp limits of their spectral absorptions and their positions in the spectrum. Unfortunately, these include some of the most commonly used biological stains, such as eosin and fuchsin. Fortunately, however, both of these cases can be corrected by the same method, that is, by the use of a very dilute neodymium filter. This particular filter is valuable because it is stable and

effectively corrects the trouble with the addition of only a negligible general pink hue; in fact, apparently this filter can be left permanently in the illumination system without interfering with the color photography of other subjects. Theoretically, colors exist with which it should give trouble.

A liquid filter of a neodymium salt is very satisfactory. The soluble salts of neodymium are too hygroscopic to handle, so the material should be obtained as neodymium carbonate C.P.* A stock solution is made up as follows:

	Avoirdupois U. S. Liquid	Metric
Neodymium Carbonate C.P.	110 grains	6.0 grams
Glacial Acetic Acid	2 grams	7.0 cc.
Water to make	4 ounces	100.0 cc.

40 cc. per 100 cc. (2 ounces per 5 ounces) of the stock in water in a cell 10 millimeters (0.4 inch) thick will allow satisfactory reproduction of these stains. For absorption cells of other dimensions along the illumination beam, divide the 40 cc. by the thickness of the liquid in centimeters (or by the thickness in inches and multiply by 0.4).

Didymium glass filters, such as the Corning No. 5120, should also be satisfactory but an equivalent absorption corresponds to a very thin piece of glass, less than 2.0 mm. thick. Because of the considerations discussed below, this thickness might prove satisfactory, however.

Color Defects and Their Compensation

In the usual photography with Kodachrome Film, as in a studio, after the illumination has been adjusted so as to be correct for the particular type of film, such as by using a Mazda lamp for 3200°K. with Kodachrome Film, Type B, the lamp simply is made to shine directly upon the subject and the picture is taken. In photomicrography, however, a more or less elaborate optical system is interposed between the illuminant and the subject. In order to approximate the simpler condition and keep the same quality of illumination until it reaches the ground glass, not only must no color absorbent be present, including absorption in the near-ultraviolet, but the optical system must be well corrected throughout, including the lamp condenser. Such a system is rarely used, of course; particular trouble is encountered from variable absorption in the ultraviolet and blue-violet, both of which Kodachrome Film repro-

*This can be obtained from the Maywood Chemical Co., Maywood, N. J.

duces alike as blue since the normal amount of ultraviolet for a given color temperature is included in the color balance of the film. In practice, the field imaged on the ground glass usually has a greenish cast that increases at higher powers. Since the illumination no longer corresponds to that of a black body, the Color Temperature Meter cannot be used to help restore the correct illumination (see page 136). Fortunately, the defects can be compensated for by selection of appropriate filters and excellent Kodachrome reproductions obtained. Moreover, if such a compensatory filter system is to be employed, all of the defects can be grouped together and we do not need to be troubled concerning the individual causes except as they may become variable and affect the choice of filter.

Systems of Color Compensation

There are three general systems of compensation which can be used successfully. Any one of these three might be the simplest or most practical for a given illumination. Since the compensation is chiefly due to the optical aberrations and glass absorptions of the optical system, there will be some variation in the best prescription from one case to another.

An outline of these three methods is given in the following table. It is assumed that the original illuminant is correct for the particular integral tripack film in use—such as a quality of 3200°K. for Kodachrome Film, Type B, *at the lamp*. The compensation filters can be considered to be variable filters, since they will vary with the nature of the illumination defect, although they should remain relatively constant when used with one equipment. The defects in color balance and the remedies for it can be divided into that which affects the blue-yellow (minus blue) balance, and that which affects the green-magenta (minus green) balance. This is because only two of the three color components are needed for a complete adjustment of color balance. The departure from a satisfactory color balance in the final picture may involve more than the lack or excess of one color component, but if that due to a single component, such as blue or green, is predominant it alone may be obvious. In such a case, only when this is nearly remedied will another general hue be evident. If compensation is needed at all, it will usually include elimination of the greenish cast mentioned above; indeed in some cases, only the magenta filter may be needed.

Since the biggest variable factor affecting the color balance, espe-

Method	*Compensation Filter (Variable as needed)*		*Advisable Ultraviolet Absorbent*	*Original Defect*
	No. 1	*No. 2*		
I	Yellow Absorbent for ultraviolet and blue	A. Magenta or B. Green	Variable for compensation (Filter No. 1)	1. Excess ultraviolet and possibly blue (blue cast) 2. A. Green cast or B. Magenta cast
II	Blue (or blue-green) Primary color filter	A. Magenta or B. Green Primary color filter	Complete, such as Wratten 2A	1. Yellow cast 2. A. Green cast or B. Magenta cast
III	Photometric filter {A. Amber B. Bluish or Variation of the lamp current	A. Magenta or B. Green	Complete, such as Wratten 2A	Any color defect of small amount

cially from one individual equipment to another, seems to be the degree of absorption of the short wave lengths (ultraviolet into the blue), it is recommended that this region be eliminated by an appropriate filter whenever compensation is to be used. Such a filter, therefore, is included in Methods II and III. The Wratten Filter No. 2A is suitable. The use of another filter for this purpose will alter the compensation required to restore a neutral balance.

Since the compensation is principally due to the optical system, the amount required will differ among individuals, and liquid filters are therefore the most practical. The exact concentration required is determined as described below whenever the quoted filter is not satisfactory.

A solution of neodymium salt can be used as the magenta filter, but it is not well suited for the purpose and requires too great a concentration of salt in solution. Its chief advantage is its stability since, unfortunately, there is no really stable magenta dye. Moreover, it is almost impossible to obtain an identical color in a dilute dye filter by successive weighings from a commercial dye sample. Therefore, the Eastman Kodak Company will supply a stock dye solution for use in making up the magenta filter by simple dilution. Direction sheets, describing typical cases requiring compensation and specifying the dilutions, are included with the solution, which is unusually stable for a magenta filter, and

will keep indefinitely in its brown bottle. The properly diluted magenta solution can be put into the water cell that is usually in the illumination system, although any increase in its thickness from the standard 1.0 centimeter cell must be taken into account by *diluting* the solution in proportion to the thickness of the cell in centimeters; i.e., the solution must be diluted 3.2 times (10 cc. to 32 cc.) if used in the Pyrex glass cells 32 mm. thick, frequently sold with Bausch & Lomb illuminants. The filter solution must be protected from the light of an arc except during exposure, or preferably an alternative plain water cell should be substituted except during color photography. The ultraviolet absorbent, such as the Wratten Filter No. 2A, should be placed in front of this liquid filter.

A relatively concentrated stock solution of a blue liquid filter also will be supplied by the Eastman Kodak Company.* In general, the same considerations apply to it as to the magenta filter, except that Pyrex glass containers must be used for it. The effect of both the magenta and blue filters on Kodachrome Film is nearly directly proportional to the concentration of the coloring matter in solution. The two filter solutions may be mixed together in the same cell in any proportion. If two cells are available, particularly if they are 10.0 mm. thick, it is more convenient to keep the two solutions separate, at least until their correct composition is well established.

The dye solutions are very dilute as used, and it is convenient to have a secondary *working stock solution* 100 times more dilute than the original, i.e., in which 10 cc. of the original stock have been carefully transferred with a pipette into a 1000 cc. flask and the volume made up to 1000 cc. with distilled water. It is very simple to dilute this to the required concentration, but it should be done carefully.

A few cases have been encountered, principally in metallography, where a dilute green filter is required for filter No. 2 rather than a magenta filter (see table, page 141). A solution of nickel sulfate containing some sulfuric acid is very satisfactory for this purpose; not only is it very stable, but it acts as a true green to Kodachrome Film, i.e., the magenta component of Kodachrome Film is unaffected while the other two are equally affected. The following is a convenient stock solution:

*Stock bottles of both the magenta and blue color compensating liquid filters for Kodachrome photomicrography may be obtained by writing directly to the Kodak Research Laboratories, Eastman Kodak Company, Rochester 4, N. Y.

	Avoirdupois U. S. Liquid	Metric
Nickel Sulfate (as NiSO 4.6 H_2O) . . .	365 grains	20.0 grams
Sulfuric Acid, conc.	20 minims	1.0 cc.
Water to make	4 ounces	100.0 cc.

The bottle should be tightly stoppered, preferably with a clean rubber stopper.

As noted above, the adjustment of the magenta-green balance is common to all three compensation methods, and most frequently a dilute magenta filter is needed. The concentration required will, of course, vary with the optics, etc., in use; a very common dilution, however, for medium and high magnifications seems to be one equivalent to a 10 mm. thickness of the liquid magenta filter made by dilution of from 35 cc. to 50 cc. of the magenta *working stock* up to 1 liter (i.e., approximately 1 to 1⅔ oz. per 32 oz.). Typical examples are quoted in the instruction sheet that accompanies each stock bottle of the liquid filters.

Compensation Method I

Method I consists of reducing an excess of ultraviolet or blue in the illumination beam with an absorbing filter that can be varied until the desired blue-yellow balance is obtained. If we consider all of the actinic spectrum of shorter wave length than green to be blue, because Kodachrome Film is affected as if it were so, and the complementary filter to be yellow, whether or not its absorption extends into the visible, then Method I consists simply of adjusting the blue-yellow balance by controlling the blue in the illumination with a variable yellow filter. There is a real advantage, however, in choosing a filter that absorbs at progressively longer wave lengths with increase of concentration, rather than one absorbing more strongly throughout the ultraviolet and blue with increase of depth or concentration.

Method I is probably the most suitable one to use with the 4.5-5.0-ampere D.C. carbon arc, especially with Kodachrome Film, Type A, since the principal defect in this case is the excess of ultraviolet, and an absorbing filter such as sodium nitrite solution will be in the beam even though no further correction is needed. Sodium nitrite solution can be used as a variable filter to adjust the blue-yellow color balance to fit the particular case. It must be remembered, however, that to obtain equal increments of effect on the yellow component of Kodachrome Film, the concentration of sodium nitrite in solution must vary in geometric ratio

(0.5 per cent, 1 per cent, 2 per cent, 4 per cent, etc.). A stock solution of 500 grams dissolved in a liter (1.046 lbs. per 32 oz.) is very practical. A variable thickness of a Noviol 0 (Corning No. 3060) glass filter can be used with an arc lamp for the same purpose, but in this case, the effect is proportional to the thickness of the filter.

The correct filter concentration or thickness will depend upon the particular conditions of use. No specific recommendations can be universally valid, although the filter specifications found for the commercial apparatus used in the examples quoted here should prove helpful in the majority of cases.

EXAMPLE 1. A satisfactory color balance was obtained for Kodachrome Film, Type A, with the 4.5-ampere D.C. carbon arc as illuminant at medium and high magnifications, when a centimeter layer of a 4 per cent solution (40 grams per liter) of sodium nitrite was used. No magenta filter was needed. *

Use of the 4.5-5.0-ampere arc with Kodachrome Professional Film is discussed under Method III.

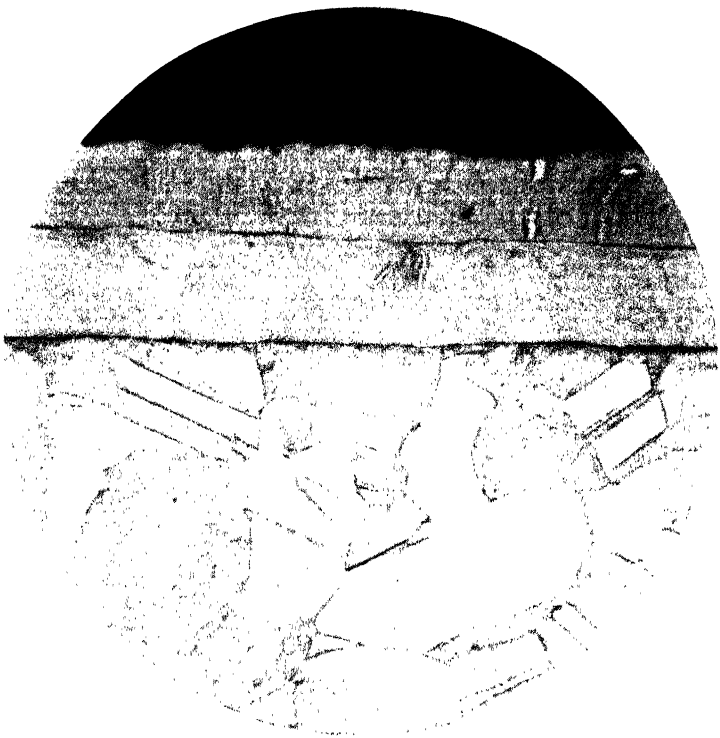
Compensation Method II

Method II is probably the most generally advantageous with the various photomicrographic illuminants in common use. As shown by the table on page 141, it consists of controlling the blue-yellow balance with a variable blue filter, i.e., one that absorbs the green and red components of the illumination. This method assumes a deficiency of blue and that the Kodachrome pictures would have a yellow cast. The magenta-green balance is also adjusted by a magenta or green variable filter as previously discussed.

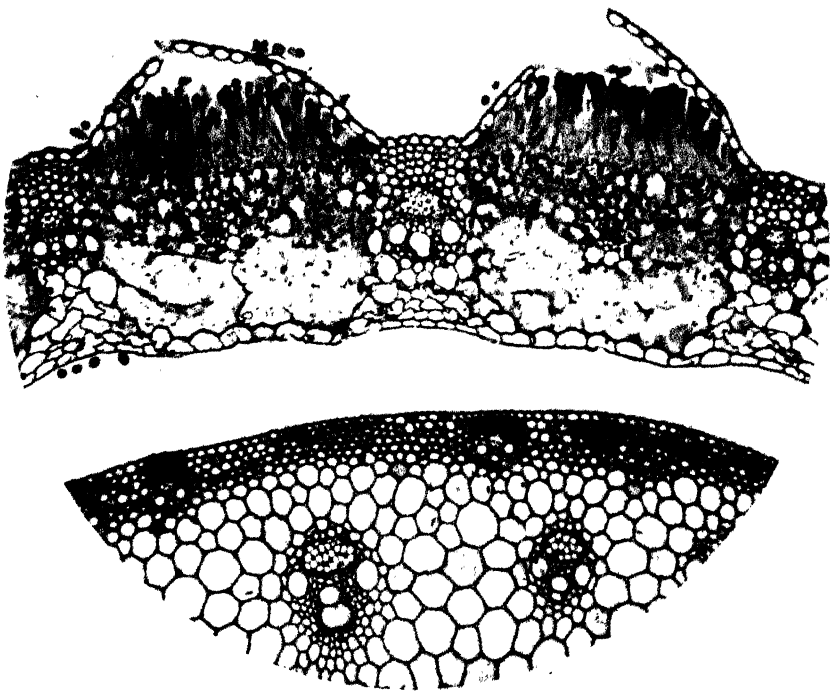
Irrespective of the original composition of the illumination, the introduction of a Wratten Filter No. 2A in the beam to remove all of the ultraviolet, will usually cause the Kodachrome reproduction to become too yellow and will therefore make Method II directly applicable. This is often an advantage.

This method of compensation, with two primary color filters, might consist of the use of dilute filters of two of the three subtractive primaries, namely, cyan (minus red) and magenta (minus green); each filter would then affect only its own Kodachrome color in the final balance. Two filters of the additive primaries, such as blue and red, could also be used to bring the colors to the desired balance. In the latter case, each filter would affect two of the Kodachrome colors at a time, leaving the third relatively unchanged. In practice, it has been found best to use a blue

*A neodymium filter was required when making photomicrographs of certain stained specimens as discussed on page 138.



HIGH SPEED COPPER ON BRASS PLATED OVER WITH NICKEL, 500X
Etchant: Ammonia and Hydrogen Peroxide
Reproduced from a Photomicrograph on Kodachrome Professional Film, Daylight Type
Made according to Example 3, page 146



PUCCINIA GRAMINIS 100X (Rust on Wheat Showing Spores)
Conant's Quadruple Stain
Reproduced from a Photomicrograph on Kodachrome Professional Film, Type I
Made according to Example 2, page 145

liquid filter that affects the cyan and magenta components equally and thus directly compensates for excess yellow, together with a magenta liquid filter to compensate for the prevalent greenish cast. These two liquid filters have already been described (page 141). The rarely used green filter, nickel sulfate solution, acts in an equivalent manner to that of the blue filter as an additive primary, but as compensation for its complementary magenta. Method II then becomes complementary to Method I.

EXAMPLE 2. The compensation required by this method for one arrangement for photomicrography with Kodachrome Film, Type B, at medium power (150X) with transparent illumination, was as follows, each liquid filter being in a 10.0-mm. layer: a Wratten Filter No. 2A, plus a solution of the blue filter diluted with distilled water from the *working stock* from 125 cc. to 1.0 liter, plus a solution of the magenta liquid filter diluted from the *working stock* solution from 35 cc. to 1.0 liter (see page 142). A ribbon filament was used to produce illumination of 3200°K. through a Wratten Filter No. 78C; the lamp condenser was colorless, but a simple aspheric lens of *f*/1 aperture was used. An achromatic substage condenser with its top element screwed off was used, together with a 16-mm. apochromatic objective. A different substage condenser would change somewhat the requirements for compensation. This would have to be determined as described on page 148.

The exact compensation requirements may change a little for any change in the optical arrangement, including an appreciable change in magnification, since this usually involves different optics.

Compensation in Metallography by Method II

In metallography, a carbon arc may well be used as the illuminant because of its special suitability for color photography (see page 133) and its frequent availability for this work. A filter must be included to eliminate the excess ultraviolet. Since the biggest variable for color balance, particularly from one apparatus to another, lies in the ultraviolet and violet transmission of the optical system, this spectral region is removed with an ultraviolet-absorbing filter such as a Wratten No. 2A. Rebalancing to a neutral for Kodachrome Film is effected by removal of the excess yellow with the liquid blue filter. Determination of the exact amount of the excess ultraviolet of the arc is avoided.

If Kodachrome Film, Daylight Type, is used with a 10-ampere carbon arc, the Davis-Gibson* photometric filter No. 18 is by far the best available to convert the illumination of the arc to sunlight quality; this is a two-component liquid filter. Since compensation for the spectral defects of the optical system is usually necessary for metallography with Kodachrome Film, the difference in color quality between the Davis-

*See Bibliography, page 166.

Gibson filters and less perfect filters can be included in the general compensation balance. Gelatin filters cannot be recommended for use with this arc, but daylight glass photometric filters are available. Moreover, since substitution of such a photometric filter will alter the required composition of both compensation filters, the thickness of the glass filter is not critical; in fact, it is probably preferable to choose a thicker filter than would be used for equivalent color temperature conversion, thus reserving the reduced quantity of liquid blue filter for adjustment of the exact balance for the particular equipment in use. Somewhat more magenta filter will probably be required to balance the defect of the glass filter. This partially involves the use of the principle of Method III, although the photometric filter is a constant factor for every individual.

EXAMPLE 3. In a case where a 10-ampere D.C. carbon arc was used for metallography together with a 60-mm. *f*/1 simple aspheric lamp condenser, a plane glass reflector in the vertical illuminator, and a 4-mm. apochromatic objective, Kodachrome Professional Film, Daylight Type, was used. Excellent color rendition was obtained with 4.7-mm. thickness of Corning Daylite Glass (Corning No. 5900 filter) plus a glass cell, 10 mm. thick, containing a dilution of the blue filter *working stock* from 80 cc. to 1.0 liter, and a similar cell containing a dilution of the magenta filter *working stock* from 60 cc. to 1.0 liter.

When Kodachrome Professional Film, Type B, is used with carbon arcs, the color temperature of the illumination must be lowered somewhat to suit that for which the film was designed (see page 132). In this case compensation Method III may be the simplest to use.

Compensation Method III

Method III is based on the fact that by variation of the color temperature of the illuminant, produced by deliberate variation of the current through it, the blue-yellow balance of Kodachrome photography is primarily affected, so that with the addition of the proper magenta (or green) filter complete compensation is available. The magenta component of the film is somewhat affected by such variation in lamp current, however, so that readjustment of the concentration of the magenta compensator is necessary after any marked change is made for a new blue-yellow balance. Variation of the lamp current can only be used over a small range in practice, but within the range this method can be most useful.

Under Method III we can also classify the use of a photometric filter as the compensating filter. This may be particularly convenient since there is usually a photometric filter in the beam from an illuminant used for color photography, and the choice can be made so as to provide the

correct illumination at the image plane, instead of using one that corrects the illumination directly in front of the lamp. The effect of variation of most photometric filters, such as the Wratten gelatin filters or the Corning Daylite Glass Filter (No. 5900) in its various thicknesses, is the same as the effect of changing the color temperature with the lamp current; the principal change is one of the blue-yellow balance but the magenta-green balance is also affected. This system is not as simple to apply, therefore, as Method II. However, where only slight compensation is needed, it may be very useful. The Eastman Color Compensating Filters (page 168), which may be considered as dilute photometric filters, can be used for this purpose together with a magenta filter. The blue series (No. 3-No. 6 inclusive), would be used where the color temperature needs to be raised, whereas the amber filters (No. 13-No. 15 inclusive) would be used where the color temperature is already too high. With the 4.5-ampere D.C. carbon arc, the 2 per cent sodium nitrite will probably be in use as an ultraviolet filter.

The "conversion power" of a photometric filter is a measure of the range of color temperature through which the quality of the transmitted light is transformed. It is calculated by subtracting from the reciprocal of the original color temperature that of the transmitted light and multiplying by one million (10^6). For example, with a Wratten Filter No. 78C. *

$$\frac{10^6}{2360} - \frac{10^6}{2500} = 423.7 - 400 = 23.7$$

This filter is said to have a conversion factor of 23.7 mireds. The advantage of this system is that this conversion power is a characteristic of the photometric filter and can be applied to obtain any other color temperature. For example: At what color temperature should a tungsten lamp be burned to produce 3200°K. through a Wratten Filter No. 78C.?

$$\frac{10^6}{3200} = 312.5; \quad 312.5 + 23.7 = 336.2$$

$$\frac{10^6}{336.2} = 2974°\text{K}.$$

When burned at this temperature an incandescent filament lamp will have an appreciably longer life than if it is burned directly at 3200°K. This is especially true of such a lamp as the ribbon filament.

Method III is useful when compensation is needed where Kodachrome Film, Type B, is being used with a 4.5-ampere D.C. carbon arc. Although this situation is probably most likely to occur in metallography, the principles apply where transmitted illumination is used; in either case the color temperature normally would be reduced to 3200°K. and the excess ultraviolet removed. Therefore the simplest compensation for color defects introduced by the optics, etc., probably is to alter the pho-

*_Wratten Light Filters_, page 14, Eastman Kodak Company, 1940.

tometric (Eastman Color Compensating) filter, although the choice may not give 3200°K., and add the proper amount of magenta compensating filter (or rarely a greenish filter). When an arc is used, a gelatin filter should be placed in the image beam, but this can be a satisfactory procedure since in photomicrography the image rays are nearly parallel and the eyepoint is in a true aperture plane. However, either an A quality glass filter or a selected clean area of gelatin filter film should be used.

EXAMPLE 4. (Metallography) Where the 4.5-ampere arc was used for metallography over a rather wide range in magnification of apochromatic objectives, the proper set of filters for a gray balance consisted of a 2 per cent solution of sodium nitrite in a 10-mm. cell, an Eastman Color Compensating Filter CC 13 and 35 cc. of liquid magenta filter *working stock* diluted to 1.0 liter with water, and also in a 10-mm. cell. On the other hand, some objectives were tried that introduced further yellow color and required individual compensation.

EXAMPLE 5. When this same arc was used for transmitted illumination with a ground glass acting as a secondary light source and with a 2-mm. apochromatic objective and achromatic substage condenser at 1500X, the same compensation filters proved to be the optimum.

Other examples are included with the direction sheets that accompany the bottles of liquid compensating filter.

Criteria for Correct Compensation

If compensation is needed, and the filter combinations cited above or given with the directions supplied with the stock filter solutions are not sufficiently accurate, then the proper concentrations of the color compensating filters can be determined in the following fairly simple manner. This assumes that previously taken pictures or tests have given some knowledge of the nature of the illumination defect, i.e., whether the illumination is too green, too yellow-green, etc.

The method consists merely of exposing the Kodachrome Film to white light illumination, as is obtained with the specimen racked out of the field, with those filters in place that are assumed to compensate illumination defects correctly, so that a medium density is produced, or much better, so that a series of density steps are produced by the method frequently used for the determination of exposure. (See page 94.) Such a series should then be repeated on the same piece of film, but with other estimations of the correct nature of the compensating filters. The film must then be sent for normal processing.

With the sheets of Kodachrome Professional Film, probably the best method is to use the black paper separators that come in the boxes of

film and cut from them a group of masks, each of which isolates a narrow strip of film surface for exposure at the series of times to produce the density steps; each strip represents a somewhat different illumination quality as with a varying dilution of the magenta filter. Six or seven such strips can easily be put on a 5 x 7-inch sheet of film. With 35-mm. or films for Bantam cameras, each frame must represent an exposure step.

In order to insure that the illumination tested represents that which will be used, the photomicrographic system should be set up and focused with some specimen in it as usual. Then if transparent illumination is being used, the specimen should be racked out of the field, leaving the background clear but with the same focus. With reflected light a piece of a matte white surface can be substituted for the subject, whereas in metallography a first surface aluminum mirror is the only object that can be presumed to be neutral to the whole spectrum.*

On the return of the film, the strip or group of frames showing the best neutral gray obviously represents the best filter combination. An excess in the concentration of a filter gives the gray a tinge of its hue. By purposely extending the test somewhat beyond the expected range of concentrations, it is usually possible to estimate the correct mixture by a judgment of the results even if the exactly proper proportions were missed in the trial.

It is worth while to use one of the new colorless immersion oils for objective and condenser in place of cedarwood oil, and even to embed specimens intended for color photomicrography in one of the new colorless plastics, such as butyl methacrylate or polystyrene, instead of balsam.

KODACOLOR

The negative-positive process, Kodacolor, which has made color photography and color prints available to the amateur for outdoor pictures with roll-film cameras, can also be applied to photomicrography. Kodacolor Film is supplied in 6-exposure rolls for use with ordinary roll-film cameras. The apparatus arrangements discussed on pages 10-13, therefore, can be used. It is absolutely essential that the extra film at the end of each roll, supplied for use in processing control, be neither exposed nor fogged.

Since Kodacolor is also an integral tripack process, it is necessary, as described on page 132, to insure that the illumination quality is that for

*First surface aluminum mirrors on microscope slides may be obtained from Evaporated Films Co., Inc., Ithaca, New York.

which the film was made, i.e., sunlight. Most of the remarks applying to the use of Kodachrome Film, Daylight Type, in photomicrography also apply to this case. The two-cell liquid filter No. 15 of Davis and Gibson (see page 166) is the best photometric filter for conversion of the illumination of either the Photoflood Lamps or a 5-ampere carbon arc to sunlight. The 5-ampere arc with 8-mm. anode plus the Davis-Gibson filter No. 15 should provide the proper illumination for this film. As discussed on page 146, however, if compensation filters are to be used, it is more convenient to use a solid photometric filter such as the Wratten No. 78 or No. 78AA (or the Corning No. 5900 Glass Filter with arc lamps) and to alter the compensation appropriately.

After exposure, the film should be returned to a dealer for processing of the color negative, together with an order for the number of prints desired. The short dimension of all Kodacolor prints is about 2⅞ inches; the longer dimension is determined by the dimension of the Kodacolor negative from which the print is made.

THE SEPARATION NEGATIVE METHOD

In the separation negative method of color photography, separate records are made of the reds, greens, and blues in the original subject. These records are termed *color-separation negatives*, and are obtained by making exposures through tricolor separation filters. In the case of negatives made directly from the original subject, the Wratten A, B, and C5 Filters (Nos. 25, 58, and 47) are commonly used; in the case of negatives made from Kodachrome transparencies, "sharp-cutting" filters are used, the Wratten F, N, and C4 (Nos. 29, 61, and 49). Color-separation negatives make it possible to obtain three positive color images which, when superimposed, form the reproduction.

The separation negative method is capable of very accurate color reproduction. It offers a great degree of freedom, since any reasonable variation in the quality of the illumination can be compensated for by merely altering the relative exposures through the color filters. There is even greater possibility of control in the processing by this method, since each print can be reworked or remade until it fits its component part in the color balance. This may be expensive in time and labor, but a positive control exists in the form of the scale of gray tones that can accompany each picture (see Fig. 12a, page 82). In ordinary photography, such a scale is usually placed within the camera field, near one

edge. In photomicrography, the method discussed on page 154 must be used. If there has been no local distortion in exposure or processing, the whole picture will normally be in the best color balance for the filters and dyes in use, when this gray scale is correctly reproduced. This is a critical adjustment, and frequently the reproduction of the specimen may be considered satisfactory when the gray scale still contains some hue. Indeed, if there is a stain on the specimen that is difficult to reproduce, a deliberate alteration from a gray scale balance may be preferable.

The scales on each of the negatives should be identical, although slight differences can be compensated for in printing. In the finished color print, if the scale differs from gray by a uniform hue, at least one of the three prints was incorrectly exposed. If the color changes along the scale, the three prints do not all have the same contrast. When there is a great amount of this work to be done, or when the method* is being resorted to in order to obtain very accurate results, a densitometer should be used. With this instrument, it is possible to measure the gray tones accurately, to plot them against the relative exposure that produced them, and thus to ascertain the exact nature and amount of correction needed. The colored scales on each component print can be measured as gray scales by balancing them with their complementary color filters.

If the gray scale on the print appears to be a color wedge, with an increase of color along it, the contrasts (gammas) of the separation negatives are not the same. If there is considerable difference between them they should be made over, particularly since this is usually a very practical matter in photomicrography with a stable specimen.

The modern masking method is particularly well adapted to the separation negative method of color photography. This remark calls for more explanation than can be given here. Briefly, there are no colored pigments or dyes actually available for making the three-colored components of a picture that do not absorb light outside of their own spectral domain and thus degrade the hues of the final result.

A method** has been devised by which the same result can be obtained as if the ideal coloring matters were available. It consists of combining, in register, with each negative, a print from at least one of

*For details of this method, see references to the Eastman Kodak Company and D. A. Spencer in the Bibliography (page 166); the chapter on this subject in *The Photography of Colored Objects* will also prove useful.

**A description of a masking method for printing in color from separation negatives, together with complete directions for its application, will be sent upon request to the Eastman Kodak Company.

the other two separation negatives and making the final prints through these combinations. Whereas theoretically it should always be used with the dyes or pigments actually available, in practice, its improvements may not be noticeable if there are no highly saturated (tinctorial) colors present in the picture. If there are, its results are sometimes startling. It is very frequently needed for the photomicrography of stained specimens. It probably is not practical to employ the masking method without adequate means for sensitometric control, i.e., without a gray scale and densitometer. The method of putting the gray scale on the negative is discussed below.

The technique for making the separation negatives themselves in general is similar to that which is normal to the photomicrographer who is used to exposing through color filters and developing to a definite and constant contrast. The Wratten Tricolor Filters, the red A (No. 25), green B (No. 58), and blue C (No. 47) are the most satisfactory for general use. The same panchromatic material should be used for all three negatives, but they should each be given the individual development that will produce equal photographic contrast (gamma). This will, in general, be somewhat different for each negative because of the different illumination color. The development directions specified by the manufacturer should be closely followed. The proper development for three-color negatives with suitable Kodak materials is given in the table on page 153. In order to maintain dimensions accurately for registration of the images in printing, the negatives should be fixed, washed, and dried at the same rate and for the same time. Temperatures in excess of 125°F. should be avoided in drying.

Since color contrast is now an added factor in the reproduction, the contrast of the negatives and their prints should, in general, be considerably lower than would be used in photomicrography in black and white. A material is desired which develops well to a relatively low gamma, and one with which a very long latitude is obtained by exposure through each of the three filters, as shown by the three component curves (see page 82). With this method of color photography it is worth while to adjust the exposure carefully in making each negative so that the image is reproduced on the straight line portion of the characteristic curve. With some materials, particularly when they are used with certain developers, the same shape of the characteristic curve is not obtained when they are exposed through each of the three color filters. Such materials or developers are not suitable for three-color work.

The materials listed below have the characteristics that are desirable for color photography and allow choice of plate or film. In most cases Kodak Tri-X Panchromatic, Type B, Plate and Kodak Tri-X Panchromatic Film are most suitable because of their unusual latitude and soft gradation. Their resolving power is more than adequate for contact prints which are usually used for photomicrography. If somewhat slower materials of higher resolving power and greater contrast are desired, Wratten Panchromatic Plates or Kodak Super Panchro-Press, Type B, Film with the noted developers will be more satisfactory:

Material	*Kodak Developer*	*Approx. Gamma*	*Time in Minutes at 68°F. (20°C.) in Tray*		
			Blue	*Green*	*Red*
Kodak Tri-X Panchromatic, Type B, Plate	DK-50	0.8	6.5	4.0	3.5
	D-76	0.6	8.0	6.0	5.5
Kodak Tri-X Panchromatic Film	DK-60a	0.8	7.0	5.5	4.5
	DK-50	0.6	7.0	5.5	4.5
Wratten Panchromatic Plate	D-11 (1:1)	1.1	4.0	3.5	3.0
Kodak Super Panchro-Press, Type B, Film	DK-50	1.0	11	8	7
	DK-50	0.8	7	5	5

Enclosed in each box of these plates and films is a card which gives the relative ratio of the exposure times through the three Wratten Tricolor Filters to sunlight and incandescent tungsten lamps.

The exact value of these factors, however, depends upon various conditions, and particularly upon the spectral composition of the light. That of a tungsten lamp, for instance, varies widely with small differences in voltage, current, or type of lamp. It will, therefore, pay a worker wishing accurate reproduction to determine his own filter ratios, using the values on the card as a basis. In general, the light from the high intensity tungsten sources used in photomicrography is whiter (contains relatively more blue) than the normal lamps.

The method for determining the precise filter factors is relatively simple. A panchromatic plate or film of the type to be used should be loaded in each of three plate holders, or a longitudinal one-third strip cut from a single plate or film can be used in each holder. Putting one of the tricolor filters in the illumination beam, make a series of step exposures in the microscope camera by pushing in the draw slide after

doubled time intervals as described on page 95, but with no specimen in the field. Since the shortest exposure time should be about that which produces a just perceptible density on the step "negative," it may be necessary to reduce the level of the illumination by closing down the condenser or objective aperture. The illumination across the field must be uniform. The other two plates or film strips should then be exposed in turn, each through one of the other two filters but multiplying the whole exposure series of each by the filter factor as given on the card from the plate box or in the table on page 93. Each exposed plate or film strip should then be developed to the same gamma for its appropriate time as specified in the table on page 153. It then becomes a relatively simple matter to estimate the correction to the exposure times that should be given to make the three density-step series identical. For instance, if the steps of any strip are identical with those of the succeeding steps of the next strip, the exposure times of the first series should be doubled. Another test with the adjusted exposure times should be made as a check. In fact, this process could be repeated as a successive approximation series.

For a complete discussion of the subject, including that of the exact determination of these ratios in one or two trials with a densitometer, see *Photography of Colored Objects*, page 90 (1938).

Exposure of Gray Scale Control

Usually the gray scale cannot be inserted into the area of a microscopic field. In very low-power work it may be possible and, if so, it is the simplest method. Very small steps of gray can be made either as a transparency on film or on paper by exposure and development in a non-staining developer. Otherwise, the scale must be impressed on the photomicrographic negatives by separate exposure; this requires care since this image must have the same characteristics as if the original scale had been in the field, but it is worth while.

A transparent gray scale, each step differing from the next by a density of 0.15, should be made, or a Kodak Photographic Step Tablet obtained from a Kodak dealer. This should be placed near the bottom end of the plate or film in the plate holder; the rest of the sensitive surface should be masked, using the black paper separators, cut to correct size, that are included in boxes of unexposed film. They can be kept against the emulsion surface of the film without producing fog; this is not true of most kinds of paper. In photomicrography by transmitted illumination,

the specimen is removed from the field and the control strip exposed in the camera with the plate holder set only halfway down into position so that the strip is in the center of the illuminated field. The illumination must be identical with that to be used later for the negative, including the same color filter and exposure filter factor. In metallography the light beam should be reflected from a first-surface aluminum mirror, and in oblique lighting from a matte white surface such as a good grade of paper.

After exposure through the gray scale, this area should be masked with the black paper, and the negatives exposed as usual and in proper order to the previously unexposed area of the plate.

Two-Color Separation Negative Method

The two-color separation negative method is considerably simpler in practice than the three-color method, particularly since the hues of the finished picture do not depend upon the exact exposure balance of the component negatives or the prints from them. Since most of the consideration concerns the printing of the two-color negatives, this method is discussed in the section on two-color printing (see page 157).

COLOR PRINTS

A number of processes exist for making prints from separation negatives, either as transparencies or on paper. In each case the techniques are sufficiently specialized, that a photographer should study the proper literature (see page 166) before undertaking any one of them.

A particularly satisfactory and relatively simple method that is applicable for using separation negatives in making three-color prints, either as transparencies or on paper, is afforded by the use of Kodak Wash-Off Relief Films together with the tricolor (A-B-C) dyes that can be obtained for them from Kodak dealers. The films have a yellow dyed silver halide emulsion and are exposed by contact or projection through the back, but developed and fixed in the usual manner. The resultant silver image is then bleached in a special bath which hardens the gelatin locally in proportion to the density of the image. The soft gelatin is removed with warm water. After fixation in hypo and subsequent washing, the films are ready for dyeing. The three dyed films may either be dried and superimposed in register to make a color transparency or used to make imbibition prints on paper. Complete instructions for the process will be furnished upon request to the Eastman Kodak Company.

It is in printing from separation negatives that the masking method, described briefly on page 151 and in detail in the reference, is applied. It is usually worth while in making a three-color reproduction of biological stains.

A simplified method of producing transparent prints for lantern slides with a two-color method is discussed in detail on page 157. It uses the fact that when gelatin, previously soaked in a bichromate solution and dried, is exposed to a strong light, the exposed portions become insoluble so that a gelatin relief image may be produced by dissolving the unexposed gelatin in warm water. This relief image may already bear a color in the form of pigment, or it may be dyed, but in each case the depth of the relief will determine the intensity of the color.

Minicolor and Professional Kotavachrome Prints

Just as the introduction of Kodachrome Film enormously simplified the process of obtaining color transparencies, the same principle has simplified the production of color prints. Anyone who has made a Kodachrome photomicrograph of 35-mm. or Kodak Bantam camera size may obtain color prints on a white cellulose acetate base and enlarged twice, 5X, or 8X, by merely giving it to a Kodak dealer with an order. Such prints made by the Eastman Kodak Company are known as Kodak Minicolor Prints, and they can be made from either glass slides or K135 or K828 Kodaslide transparencies. Unmounted Kodachrome transparencies will first be cut and mounted.

Prints in color may be obtained from pictures on Kodachrome Professional Films as Kotavachrome Professional Prints, also made by the Eastman Kodak Company. These are also on a white cellulose acetate base and are made by the Kodachrome Process. Kotavachrome Professional Prints have excellent color saturation, which is an important advantage in the reproduction of biological stains. The smallest print that is made is the 8 x 10-inch size; other degrees of enlargement may be obtained in the standard paper sizes up to a maximum size of 11 x 14 inches. Each order should be accompanied by written directions including any instructions for selection of a portion of the picture area for reproduction.

In checking print quality, it is exceedingly important to view the transparency and print simultaneously by light of the same color quality, and at balanced levels of illumination. If the transparency is viewed by strong daylight from a window and the print by weak tungsten light,

there will naturally be great differences in both color rendering and brightness.

One satisfactory method of comparing a color print with the Kodachrome original consists of projecting the transparency in a darkened room, and holding the print underneath a lamp behind the projector. An even simpler method is to place the print under a lamp and to view the transparency by light reflected from a sheet of white paper on the table underneath the same lamp.

Care of Minicolor and Kotavachrome Prints

Minicolor and Kotavachrome Prints contain dyes which are as stable as possible, consistent with their other requirements, but as dyes may, in time, change, the prints are not warranted against change in color. Prolonged exposure to bright daylight, and particularly to direct sunlight, should be avoided. The prints, therefore, should not be displayed for long periods of time in or near windows, or in other locations subject to direct sunlight. For the greatest degree of stability, Minicolor Prints should be kept in an album or folder in order to protect them against continuous exposure to light.

TWO-COLOR PHOTOMICROGRAPHY

Since most specimen slides for the microscope, such as sections, contain only the two colors of the stain and counterstain, they can be adequately represented by two-color photography. This is not only simpler and less time consuming than the general methods of three-color photography, but the reproduction of the correct colors is not so sensitive to the relative exposures of the negatives. The basic principles are not only simpler but different, since in this method the selection of *the two taking filters* varies to *match the absorptions* of the two colors of the specimen, while *the two stains* for the print are selected to *match the colors* of the stains of the specimen; in fact, sometimes the identical biological stains can be used. Thus, correct hue reproduction is at least assured, and is not entirely dependent on other considerations, such as a neutral gray balance, etc., as in three-color work. For example, suppose a section is stained with safranine and light green SF. First, a negative should be made that records only the red component of the image, that is, so that those portions stained only with green will appear entirely transparent and the portions stained with safranine will have their appropriate densities in the picture (or clarity on this negative). This is done by

photographing it through a filter transmitting within the absorption band of the red stain, as discussed on page 58 and specified in the table on page 72, namely the green Wratten G + H filter combination. Likewise, another negative must be made of the green image using a deep red filter, such as the Wratten F, which will cause the safranine portion apparently to disappear. Some parts of the specimen, of course, will be stained with both stains and so appear in both negatives. A print is now made from each negative on a lantern slide in the simple manner described below, so that the positive images each consist of clear gelatin of thickness proportional to the original densities. If the print of the red image, from the negative taken with the green filter, is dyed the same color as the original safranine, and the other print also is dyed the same color as the original light green SF, then when the two prints are superposed, face to face in register, the colors of the original will be reproduced. The illustrations on pages 60 and 61 of this book are facsimile reproductions of photomicrographs made in this way. It should be noted that in botanical specimens especially, the cell structure may have a rather variable yellow density, independent of the biological stains, which will somewhat modify the hues obtained locally in the reproduction. This is especially noticeable with such a dye as light green SF which may seem too blue in portions of the final picture. This can only be remedied adequately by use of the three-color process. The panchromatic plates and films discussed on page 153 are also suitable for making the separation negatives for the two-color method.

The choice of filters is of critical importance. The principle, as discussed above and on page 58, is to choose the complementary color that is most completely absorbed by one stain and transmitted by the other. The table on page 72 will help, but sometimes slightly different filters may be preferable to make the other component less visible. This is best decided by visual trial under the microscope.

Hematoxylin and eosin undoubtedly represent the most common zoological stain and counterstain combination, but it is an especially difficult one for standardization by the method of two-color reproduction. The absorption of eosin is a sharp single band as given on page 72. Hematoxylin stain has a broad absorption gradually decreasing in the blue but with a rather sharp absorption limit in the red. The broad peak of absorption, and also the long wave length limit, shifts toward the red (long wave lengths) with increasing alkalinity (pH). At the same time the absorption in the blue is increased so that in slightly acid

solution, when it has an absorption peak at λ 550 mμ, its appearance is similar to a dull magenta, whereas when alkaline, with its absorption peak at λ 600 mμ, it seems more like a blue-gray. This probably accounts for the quite different appearance obtained with different techniques and sometimes even with different tissues within the same specimen. In the latter case it may be necessary to take the picture by the general method of three-color photography to represent the field adequately. The most commonly used methods, such as Delafield's, seem to leave the color as it is when stained directly from the alkaline solution in spite of the nature of the formula. In any case, the filters to be used can remain the same, but the dye for the reproduction of the hematoxylin must be altered to match the appearance of the specimen. The Wratten A Filter (No. 25) will cause the image colored only with eosin to disappear; it is included in the "M" set. Where much work is done, the Wratten Filter No. 23A is slightly preferable. The Wratten filter combination B + H will give the maximum contrast for the eosin image; the fact that it will also record the hematoxylin image in this case will rarely be found to interfere with making an excellent reproduction. The selection of the dyes for the gelatin print is discussed below.

Making the Slides in Bichromated Gelatin

Printing in bichromated gelatin depends upon the fact that the exposure of such a layer to a strong light renders it insoluble in the exposed portions so that a relief image may be produced by dissolving the unexposed gelatin in warm water. When this relief image is stained with a suitable dye, the depth of the relief determines the amount of dye taken up.

Kodak Lantern Slide or Kodak Process Plates are sensitized for this method of printing by bathing for five minutes in a 2½ per cent solution of ammonium bichromate containing 5 cc. of 0.880 sp. gr. ammonia to a liter. This operation may be carried out under artificial light, since the plates are only slightly sensitive while wet. The temperature of the bath must not be above 65°F., or reticulation of the gelatin is very likely to occur. The bath may be used repeatedly if it is filtered before use. Very old photographic plates such as those after expiration date may prove unsuitable, since the gelatin may have hardened too much with age.

It is neither necessary nor desirable to remove the silver salts from the plates before sensitizing or exposing, since, although the silver is not used to form the image, it performs a useful function in limiting the penetra-

tion of the printing light and enabling a print of proper gradation to be made.

When the plates are removed from the sensitizing bath, they are rinsed for two or three seconds in clean water to remove excess bichromate solution and are then carefully drained in a rack until the surfaces are completely free from streaks. The glass sides are then cleaned with a wad of cotton squeezed out in clean water, and drying is hastened by a gentle current of dust-free air from a fan. The current of air must not be violent, or streaks of uneven sensitiveness will occur. An even current that will dry the plates in about an hour is satisfactory. Drying must take place in darkness or in a safe orange light.

The plates will remain good for several days if stored face to face in plate boxes.

The plates must be exposed *through the glass* in order that the hardened relief image will adhere to the glass. Also, one of the negatives must be reversed so that the slides can later be registered face to face. That is, this negative is arranged with the gelatin side toward the light source. Thus, we shall have, in printing this negative, first the negative image layer, then the negative glass support, in contact, then the lantern plate glass, and finally the bichromated emulsion. For the other negative, we shall have first the negative glass, then the negative image layer, then, in contact, the lantern plate glass, and last the bichromated emulsion. The exposures are made by means of essentially parallel light so that the thickness of the glass between the two films will not cause an indistinct image to be formed. For this reason, daylight cannot be used. The light of an arc is most convenient, the arc being placed at least at 18 inches distance, and an exposure of about three minutes being given for an average negative. The arc in a projection lantern may be used by removing the projection lens and exposing through the condenser, which must be clean. The naked arc may also be used.

The unhardened gelatin is dissolved, and the relief image thus developed by rocking the plates in trays of water at about 120°F. This operation is continued until all soluble gelatin is removed. Water directly from the faucet must not strike the plates at this point or the image may be damaged by its force. A washing away of the highlight detail indicates underprinting; too thick an image indicates overprinting.

The plates are next rinsed in cold water, the silver salts removed in acid hypo, and the plates washed thoroughly. They are then ready for staining.

In any of the operations, while the plates are wet, the delicate image must not be touched with anything but the liquids.

The bichromated gelatin method described is undoubtedly the simplest technique for making color prints from separation negatives, the only disadvantage being the very low light sensitivity of the bichromated gelatin compared with the more usual photographic materials, and their especial lack of sensitivity to tungsten light. If a carbon arc lamp is not available, the best procedure is to make the two prints on Kodak Wash-Off Relief Film, which is designed and normally used for three-color printing, using a tungsten bulb for printing through the backs of the films. Ordinary exposure and development of the film are followed by a tanning bleach, after which relief images are obtained on the films by washing off the unhardened gelatin. Detailed directions for color printing with Kodak Wash-Off Relief Film can be obtained upon request from the Eastman Kodak Company.

The prints which have been previously dried (if both were treated alike) should be dyed in clean trays, containing no iron, which are preferably of glass or white enamel to insure efficient inspection. The dyes should usually have a concentration of about 1% by weight. Only a small amount of control is obtained satisfactorily by varying this factor, since the prints should all be dyed with frequent agitation until no more dye is taken up by the print. Usually 30 minutes is a satisfactory period. Variation of the time as a controlling factor will only increase the risk of uneven dyeing. However, with the various dyes that may be used in this process, considerable control of the relative contrast of the two prints is necessary. For instance, with the reproduction of safranine and light green SF, or even more with the biological counterstain fast green FCF where the two certified green stains are being used to dye the gelatin reliefs, it will be found that the green stain will readily build up to a much greater contrast than will the azo rubine used to represent the safranine. The control should be chiefly obtained by varying the acidity of the dye bath where making it up to 1% acetic acid by addition of glacial acetic may be considered the normal amount, although the dye concentration may be reduced by half or even a quarter in this case. Addition of more acid will increase the strength of the dyeing and decreasing the acidity will lighten the final color, but the different dyes will vary much in the degree to which they are sensitive to this treatment. With strong dyes that behave as does the fast green FCF in dyeing gelatin, it may be necessary to omit the acid entirely or dye from a

buffer salt solution. After the dye bath some of the dye can be rinsed out with water or even very dilute ammonia to decrease the color contrast.

In this process the dyes for the gelatin prints are chosen with respect to the particular microscopic stain or color of that portion of the specimen which they are expected to match. If the microscopic stain itself is suitable, it is the ideal choice. The gelatin stain should be a water-soluble acid dye that is reasonably stable to light. The sulfonated (acid and water-soluble) derivatives of the basic tri-phenylmethane dyes (such as basic fuchsin and crystal violet), which are so much used in microscopy, have practically the same color as the microscopic stains but are usually unstable to light. They therefore are only available if the slide is to be used for a single occasion. By a little experimenting, it should be possible to find suitable gelatin dyes corresponding to most of the microscopic stains used in one's own field. The color of the commercial dye nigrosin varies through about the same range as hematoxylin and a very suitable shade may be selected. This will be acceptable for the color transparency, but it does not transfer well to make a two-color print by the Kodak Wash-Off Relief Process. A dye mixture is necessary for this case. The following list is merely intended as a suggestion for imitating some of the most used stains:

Stain	*Suggested Dye*	*Color Index Number*
Aniline Blue	Aniline Blue (Water soluble)	707
Methylene Blue	Toluidine Blue O	925
Crystal Violet	Wool Violet 4BN (Nat. Aniline & Chem. Co.)	698
Delafield's Hematoxylin	Water Soluble Nigrosin	865
Eosine Y	Eosamine G (General Dyestuffs Corp.)	119
Safranin O	Azo Rubine Extra (Nat. Aniline & Chem. Co.)	179
Carmin	Rose Bengal	777
Basic Fuchsin	Fast Fuchsin G (Nat. Aniline & Chem. Co.)	29
Orange G	Orange G	27
Sudan III	Croceine Scarlet MOO (Nat. Aniline & Chem. Co.)	252
Picric Acid	Quinoline Yellow	801
Light Green SF	Light Green SF Yellowish	670

Many of the dyes suitable for staining the gelatin plates can be obtained from the Kodak Research Laboratories, Eastman Kodak Company, in small amounts such as would be useful in photography. Bachmeier and Company *also sell dyes in small batches for photographic use.

A few experiments will soon show which dyes will give the best

*438 W. 37th St., New York City.

results. If necessary, the dyes can be washed out in dilute ammonia, and other dyes tried.

Sometimes the unstained specimens, especially cross sections of botanical subjects, have a yellow color which will alter the hue of the stained mount so that it cannot be satisfactorily reproduced with the otherwise correct stain, counterstain, or their substituting dyes. This may occur when the yellow color itself is hardly evident. For example, in the reproduction of a cross section of a young stem stained with safranine and light green, the reproduction of the green on the slide by the use of light green (cert. dye) itself, was definitely poor, particularly in the portions which were stained by both components. When an equal amount of the yellow "C" dye of the Wash-Off Relief set was added to the green dye bath, the reproduction became satisfactory.

After dyeing, the relief prints should be rinsed in at least two changes of ½% acetic acid to remove surplus dye and to set the dye. They should never be allowed to dry when wet with drops of colored solution.

The two prints should be superposed in register while still wet upon the bottom of a white tray by sliding one over the other and then lifted in register for examination before an illuminator. After drying, they are again brought into register, held together with a clamp (carefully!) and the edges bound with lantern slide tape.

Further details of the technique of this process will be found in the directions for the Kodak Wash-Off Relief Film which are very similar at this stage.

If Kodak Wash-Off Relief Films have been used, they can easily be trimmed to size after registering and before binding between lantern slide glasses. Where two lantern slide plates have been used, it is worth while to insure that the edges of the plates are well aligned when the registration is good to avoid a tendency in time to mechanical alignment at the expense of registration. This is not difficult in photomicrography since the specimen is stable, but it may require some consideration from the time the negatives are being made. If possible, they should be about the same size as the lantern slides (i.e., 3¼ x 4½-inch negatives) and it may be worth while to expose the two negatives successively using the same plate holders.

The directions for using Kodak Wash-Off Relief Film will be found very helpful for the staining and finishing of the relief prints since the techniques are very similar at this stage.

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See page 167 for the list of some of the publications of interest by the Eastman Kodak Company.

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ON SALE AT KODAK DEALERS

FUNDAMENTALS OF PHOTOGRAPHY by *Dr. C. E. K. Mees*. An authoritative and brief account that will prove interesting to readers with or without scientific training. Such a book should be read by all serious workers in photography.

ELEMENTARY PHOTOGRAPHY by *Neblette*, *Brehm*, and *Priest*. An excellent textbook on theory and practice for the beginner.

THE PHOTOGRAPHY OF COLORED OBJECTS. This valuable book, dealing with reproduction in both monochrome and color, is of special interest to the photomicrographer.

ELEMENTARY PHOTOGRAPHIC CHEMISTRY. A text on the chemistry of photography and a handbook on the preparation of photographic solutions.

KODAK REFERENCE HANDBOOK. In multiring binder with easy index systems for quick reference, this forms a complete handbook for general and professional photography. It contains many specification sheets of Kodak materials.

WRATTEN LIGHT FILTERS. The characteristics, including spectra, of over one hundred color filters are given.

KODAK FILMS. A Data Book giving the physical and photographic properties of negative films. Specification sheets are included for most of the films of interest in photomicrography.

KODAK DATA BOOK, COPYING. Some of the discussion and data on copying techniques are of direct interest for photomicrography. It also contains specification sheets of commercial and process films not included in the previous reference.

INFRARED PHOTOGRAPHY WITH KODAK MATERIALS. A discussion of the theory and practice of infrared photography is included as well as the specifications of the materials.

KODACHROME—PHOTOGRAPHY IN COLOR. A Data Book on color photography with Kodachrome Film, invaluable for workers in this field.

PHOTOGRAPHY WITH KODACHROME PROFESSIONAL FILM. A practical guide for all users of Kodachrome sheet film.

KODAK DATA BOOK, SLIDES. This includes direction sheets for making and showing slides in both black and white and color as well as the specifications of the materials.

DATA BOOK ON KODAK PAPERS. Discusses the technique of choosing and using the paper to obtain high quality prints, and gives the specifications of the more popular contact and enlarging papers.

KODAK DATA BOOK, FORMULAS AND PROCESSING. A Data Book presenting a comprehensive list of Kodak formulas, and discussing principles and procedures for processing films, plates, and papers.

KODAK LENS MANUAL. On the use of Kodak and Ciné-Kodak lenses, supplementary lenses, range finders, and shutters. Includes lens specifications with depth of field and field size tables and four pages of useful optical formulas.

Information will be sent upon request concerning films, plates, and papers sold by the Eastman Kodak Company and formulas for processing them. Advice may be obtained concerning materials for the construction of photographic processing apparatus which will be useful in the darkroom. Information is also available concerning supplies and apparatus for photography including those for extremely accurate control of photographic processes such as the Eastman Densitometer.

Alphabetical Index