Handbook of Basic Microtechnique

THIRD EDITION

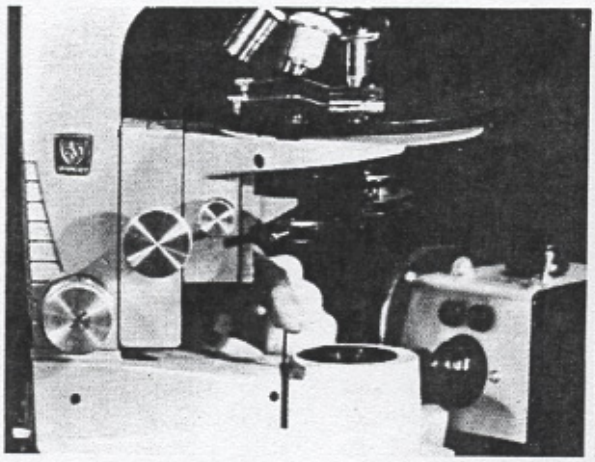
PETER GRAY, Ph.D., D.I.C.

Department of Biological Sciences
University of Pittsburgh

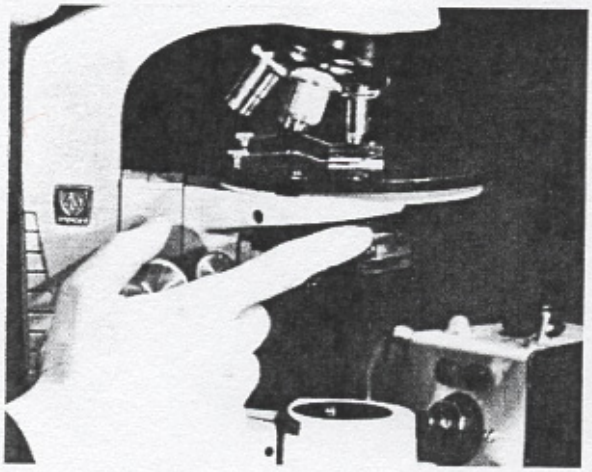
McGRAW-HILL BOOK COMPANY

New York
San Francisco
Toronto
London

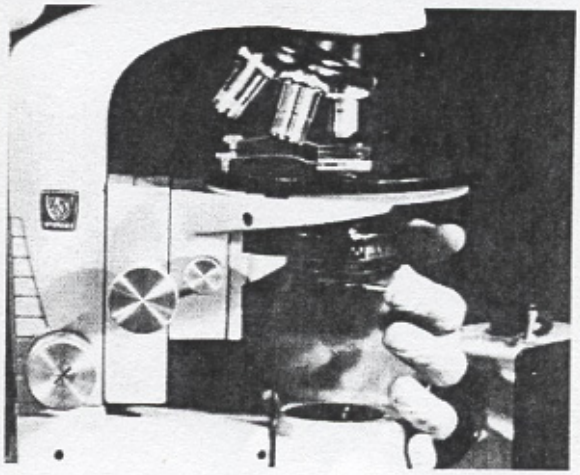
2-18



2-19



2-20



2-21

Figs. 2-18, 2-19, 2-20, and 2-21 | Setting up a medical school microscope with built-in illuminator. Fig. 2-18. The field iris is closed after the ×10 objective has been focused on an object. Fig. 2-19. The image of the field iris is focused with the aid of the substage condenser. Fig. 2-20. The N.A. of the substage condenser is matched to that of the objective with the substage iris. Fig. 2-21. Turning in the supplementary lens for use with a ×3.5 objective.

THE RESEARCH MICROSCOPE

Specifications. A description of a research microscope, of which a typical example with its illuminant is shown in Fig. 2-22, is included in this elementary book for two reasons. First, such microscopes are commonly set up for class demonstrations, and the student should certainly understand something about any instrument that he may look through. Second, there is a real justification for the use of these complex instruments even in elementary photomicrography, the subject of the next chapter. A first-class research microscope usually has most of the following features.

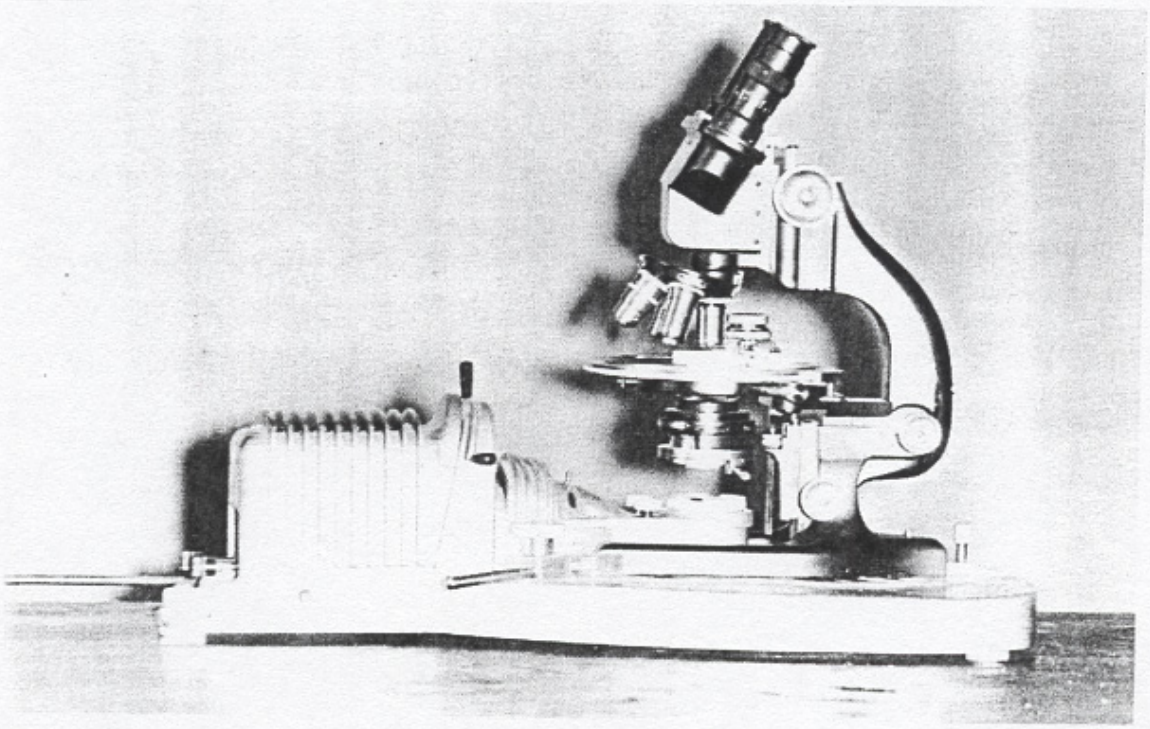


Fig. 2-22 | A research microscope and suitable illuminant. The microscope is an American Optical Company model No. 5, and the illuminant is an American Optical (formerly Silge and Kuhne) Ortho-Illuminator.

Stand. There are aš many kinds as there are manufacturers of research microscopes. The stand carries the tube, the stage, the substage, and the mirror. Most contemporary microscopes have a fixed tube and are focused by moving the stage.

Tube. Research microscopes rarely have a monocular body, which was at one time required for photography. Most contemporary stands have an inclined binocular body (Fig. 2-22) when used exclusively for visual observation or a trinocular body (Figs. 2-27 and 3-19) when used for photography.

In most research microscopes the turret, which should carry four lenses, is attached to the body, not to the tube.

Stage. The stage of most research microscopes is both rotating and centerable. The first of these features is highly desirable since it permits the orientation of material both for observation and for photography in cameras which cannot be rotated. The second feature not only permits the stage to be centered but also permits fine adjustment of the rectangular rack-and-pinion movement of the built-in mechanical stage. It is quite common to have venier graduations for both the rectangular

and rotating movements, but these are of more interest to geologists and metallographists than to biologists.

Substage. The substages of research microscopes are extremely complicated and consist of the following parts: (1) a device to permit substage condensers to be interchanged, centered, and focused; (2) an iris diaphragm, either mounted to permit lateral motion or supplemented by a separate lateral diaphragm; and (3) some fitting which will permit a supplementary lens to be placed under the condenser when low-power objectives are to be used. This is also found on some routine instruments (Fig. 2-21).

The two main methods of meeting these requirements are shown in Figs. 2-23 to 2-26. Figures 2-23 and 2-24 show the substage of a Bausch and Lomb DDE stand. The condenser, about to be inserted in Fig. 2-23, is mounted on a rectangular plate that slides in a dovetail slot. The iris diaphragm is mounted on a swing-out arm and has been swung out in Fig. 2-24 to show the rack-and-pinion that controls lateral motion. This rack-and-pinion is provided with a click stop to indicate the centered position, and the utmost care must be taken that the iris is clicked home before the substage is centered. The supplementary lens, also mounted on a swing-out arm, is shown out in both figures.

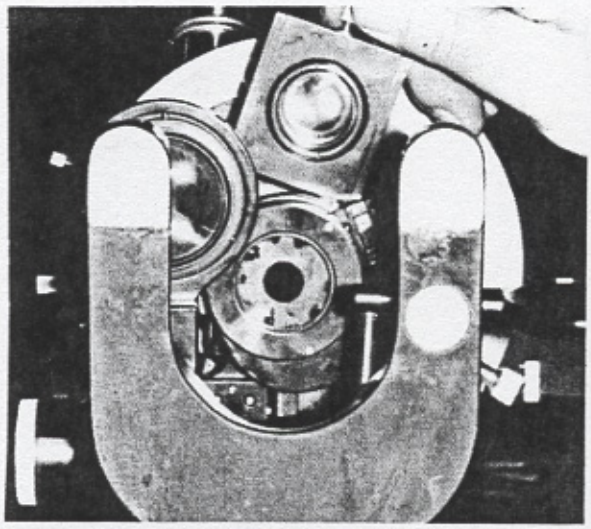
Figures 2-25 and 2-26 show the fork-type substage mount favored by the American Optical Company. The iris diaphragm is built into the condenser, and the whole unit is centered by the two screws shown in use in Fig. 2-25. This arrangement is less flexible, and also a great deal less trouble, than the mechanism preferred by Bausch and Lomb. The supplementary condenser lens, shown being inserted in Fig. 2-26, is mounted in a dovetail slide. This is a great deal more trouble to use than the swing-out lens on the Bausch and Lomb substage.

Both substage mounts are provided with rack-and-pinion focusing, and the particular American Optical model shown has, in addition, a fine adjustment. In Fig. 2-26 the coarse adjustment is the nearest milled head on the right of the figure. The fine adjustment is the small milled head in the left center of the figure.

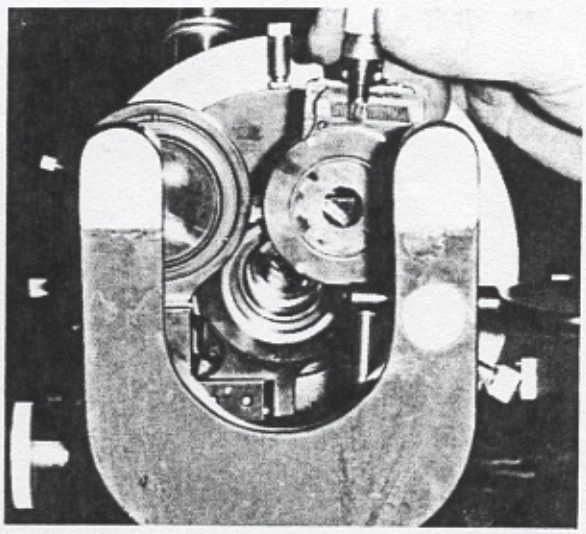
Mirror. The only requirement of the mirror on a research microscope is that it be flat and easy to remove or turn aside. It is, of course, unnecessary with built-in, or substage, illuminators.

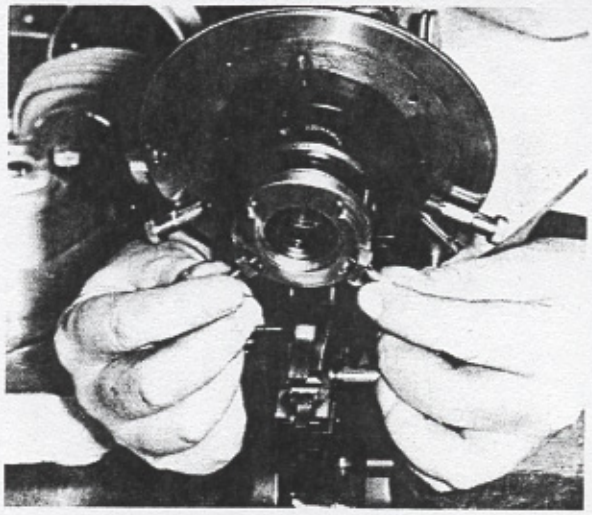
Objectives. The choice between apochromatic and achromatic objectives, the difference between which was explained in Chapter 1, depends on the use to which the microscope is to be put. Achromatic objectives have many advantages. They are cheap, accurately parfocal, of long

2-23

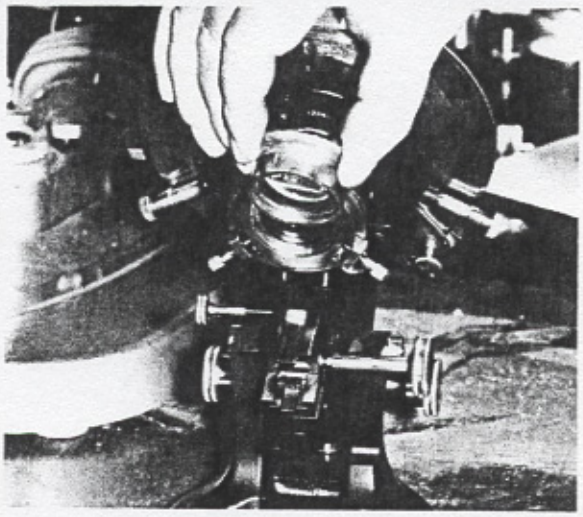


2-24





2-25



2-26

Figs. 2-23, 2-24, 2-25, and 2-26 | Substages. Figs. 2-23 and 2-24 illustrate the Abbe-type substage on a Bausch and Lomb model DDE. Fig. 2-23 shows the condenser being inserted on a dovetail slide. In Fig. 2-24 the substage iris has been swung out to show the rack-and-pinion lateral motion. In both Figs. 2-23 and 2-24 the supplementary condenser lens used with low powers has been swung out to the left. Figs. 2-25 and 2-26 show the fork type of substage mount on an American Optical Company model No. 5 microscope. In Fig. 2-25 the fingers are working the substage centering screws. The larger screws just above the hands are the stage centering focus. In Fig. 2-26 the supplementary condenser for use with low powers is being inserted in the dovetail slide. Notice the substage fine-adjustment control just below the left-hand condenser centering screw.

working distance, and of sufficiently high N.A. for most purposes. Apochromatic objectives have only the advantage of high resolution, more usually required for photographic than for visual work. It is necessary that all four lenses on the turret be in the same class since special (compensating) eyepieces are required for apochromatic objec-

tives. The image given by an apochromatic objective with a regular eyepiece is far inferior to that given by an achromatic objective with the same eyepiece.

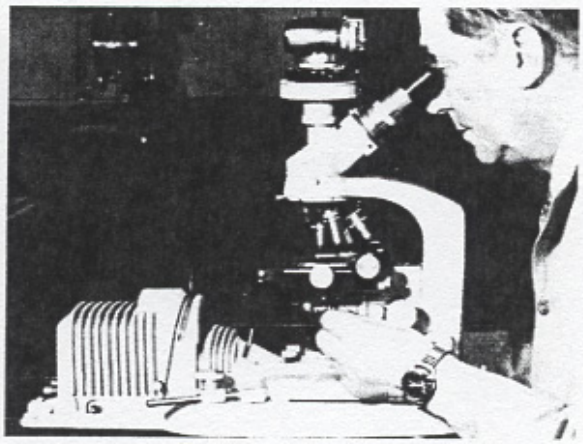
No matter what class of objective is chosen, the instrument should be provided with ×10, ×20, ×40, and ×90 (oil-immersion) objectives. A ×3.5 objective is even less practical on these instruments than on a medical microscope. In the apochromatic range there is often a choice between a ×90 N.A. 1.3 oil immersion and a ×95 (or ×100) N.A. 1.4 oil immersion. Only in the rarest circumstances does the 7 per cent increase in the theoretical resolution of the N.A. 1.4 justify the increased cost. Those who must have such a lens, because almost all of their work lies in the examination of objects at the extreme limit of the resolution of the optical microscope, would do well to substitute a ×80 (3-mm) oil immersion for the ×10 lens on the turret. These lenses have a field of view almost twice that of the N.A. 1.4 and are used to search the field for, and center, the object for subsequent examination under the high-resolution lens.

Oculars. These must be purchased as matched pairs. Although ×10 and ×15 are all that most people use, under ideal conditions of illuminating and centering the image made by an N.A. 1.4 apochromatic immersion lens will stand examination under a ×20 ocular. There is no point in using a ×20 ocular with lower-power objectives. A ×20 objective, for example, with a ×20 ocular gives a far less satisfactory image than a ×40 objective with a ×10 ocular.

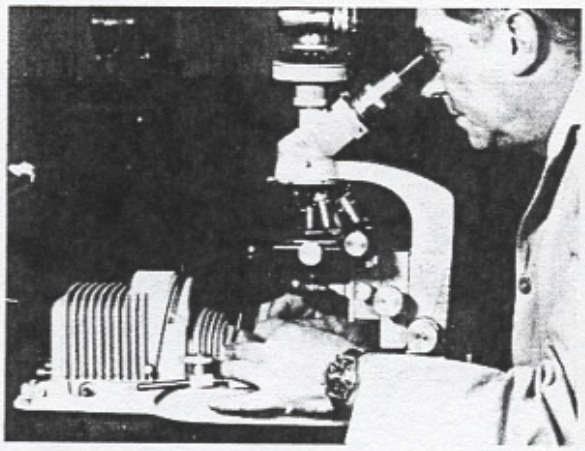
Substage Condenser. This is just as important a part of the optical system as the objective. Nothing should be considered except an N.A. 1.4 achromatic condenser.

Illuminating System. The lamp described for use with the medical microscope (Fig. 2-8) can be used with a research microscope. It is not nearly so convenient, however, as the Ortho-Illuminator shown in Fig. 2-22 and in Figs. 2-27 to 2-32, which the author regards as the greatest advance in microscope illuminators of the last half century. This device permits automatic Kohler illumination with almost effortless ease in the control of centering and of light intensity. Many microscopes with illuminating systems built into the stand give effortless Kohler illumination and ease of centering, but the intensity can be controlled only by a rheostat—a situation wholly disastrous if color photography is to be attempted.

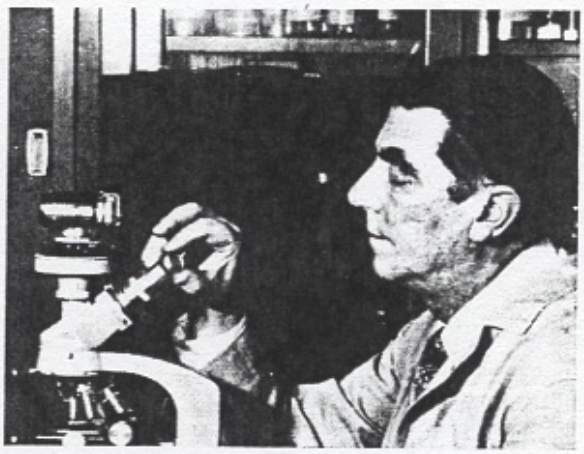
2-27



2-28

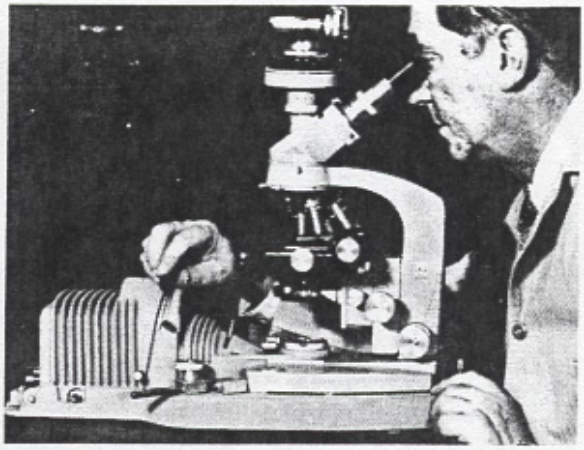


2-29



2-30

2-31



2-32

Figs. 2-27, 2-28, 2-29, 2-30, 2-31, and 2-32 | Setting up a research microscope on an Ortho-Illuminator. Fig. 2-27. The microscope is placed over the illuminator and moved until the ×10 objective is filled with light. Fig. 2-28. The substage iris is closed and the ×10 objective focused on the iris. The substage centering screws are then used to center this iris. Fig. 2-29. The substage iris is opened, the field iris is closed, and the ×10 objective is focused on the field iris. The field iris centering screws are then used to center this iris. Fig. 2-30. A pinhole eyepiece is substituted for one of the regular eyepieces. Fig. 2-31. The lamp centering screws are then used to bring the image of the filament, which is now seen through the pinhole, into the center of the field or view. Fig. 2-32. The intensity selector is moved until the desired intensity is obtained.

Setting Up a Research Microscope. There is no point in trying to use a research microscope that has not been properly adjusted. Many workers obtain worse images from these extremely expensive systems than can be obtained from a medical microscope simply because they do not know how to cope with the multiplicity of adjustments necessary. The two vital operations are centering the substage condenser and centering the light source. It is assumed that the reader is completely familiar with the operations described earlier in setting up a medical microscope.

Centering the Substage Condenser

1. Set the microscope approximately in place over the Ortho-Illuminator (Fig. 2-27) or set it up in front of a lamp as shown in Fig. 2-8.
2. Make sure, if the substage iris is of the type shown in Figs. 2-23 and 2-24, that it has been clicked into a central position. Then close the substage iris as far as possible and rotate the ×10 objective into position.
3. Focus up and down, using, if necessary, both the coarse adjustment of the microscope and the rack of the substage, until the substage iris is in focus. Make sure by opening and closing it that you are looking at the substage iris, and not at some other iris in the system.
4. Use the condenser centering screws (Figs. 2-25 and 2-28) to bring the image of the iris into the center of the field.

The substage condenser is now centered with the objective system, provided the iris is properly centered with the condenser. Failure to find an image of the substage iris is sometimes caused by using too powerful an ocular, and a ×2 or ×5 ocular is an extremely useful accessory to have available if a ×10 apochromatic objective is being employed.

Centering the Field Condenser

1. Open the substage iris and rack the condenser up to the top of its travel. Leave the ×10 ocular in position.
2. Close the field iris (the lever seen in the dark slot just in front of the microscope on the Ortho-Illuminator) and use the coarse adjustment of the microscope to focus an image of the field iris. Make sure that you are actually looking at this iris by opening and shutting it.
3. Center the image of the field iris either
   a. On the Ortho-Illuminator by using (Fig. 2-29) the two screws lying just in front of the field iris, or
   b. On a detached lamp by adjusting with the mirror and, when necessary, moving the lamp.

The objective, the field iris (which is presumed to be centered on the field condenser), and the substage iris (which is presumed to be centered

on the substage condenser) are now in line. It now remains to get the illuminant onto the same optical axis.

Centering the Light Source

1. Make sure that the image of the field iris is still in focus.
2. Replace one of the oculars with a pinhole ocular (Fig. 2-30). This device is most conveniently screwed to one of the clamp screws of the Ortho-Illuminator but must be purchased separately if another light source is used. On the Ortho-Illuminator the color selector must be set to zero and the intensity to maximum.
3. Open both the field iris and the substage iris.
4. Peep gingerly into the pinhole. If, as is almost certain, the glare is blinding, either slip a neutral density filter into the system or cut down the light emission with a rheostat.
5. The eye will now see a sharp image of the lamp filaments. Center these either
   a. On the Ortho-Illuminator by moving (Fig. 2-31) the two screws at the back of the lamp housing, or
   b. On a separate lamp by moving the lamp, tilting the lamp, and adjusting the mirror. This will probably throw the field iris out of line, as can instantly be seen by looking into the other barrel of the microscope, which still carries a normal ocular. It is now necessary, looking in each barrel alternately, to tilt the lamp, move the mirror, and move the lamp until both field iris and illuminant are centered. This infuriating procedure is what has caused most microscopists to abandon separate lamps.
6. Clamp the microscope in position on the stage of the Ortho-Illuminator. Those using a separate lamp will find it well worthwhile to provide themselves with a stage to which both microscope and lamp can be securely clamped.

Everything is now lined up, and it only remains to place a slide on the stage, set up Kohler illumination in the manner described for the medical microscope, and control intensity of illumination. On the Ortho-Illuminator (Fig. 2-32) this is done by rotating into position a disk of the required intensity.

All this laborious business, which stems from having a centerable substage condenser, is necessitated by the fact that the lenses in even the best turrets are not perfectly concentric. When, therefore, the $\times 20$, $\times 40$, or $\times 90$ lens is rotated into place and the substage refocused, it will be found that the image of the field iris is no longer perfectly central. It should be brought back to center by moving the substage with its centering screws. This movement will be of the slightest—if it is not, a complaint to the manufacturer should at once be made—and the slight off-centering of the field iris and light source will not be of importance. Never recenter the image by shifting the field iris, as it is of much more practical importance that the substage condenser be lined up with the objective than that the field condenser be lined up with the substage

condenser. In fact, once the system has been clamped, no centering screws except those of the substage condenser should ever be touched unless the whole system is to be relined.

The ideal system, of course, is one in which each objective can be centered over the substage condenser after the latter has been lined up with the field condenser and the light source. Such systems exist—Figs. 1-15 to 1-17 could not have been taken without one—but they are altogether beyond the scope of this book. It might be added that they usually have to be lined up from scratch before each use, a procedure that takes about 3 hr.

Only a few operations with the research microscope remain to be described.

Setting Up an N.A. 1.4 Immersion System

The reader who followed the discussion in the last chapter will realize that this N.A. is obtainable only if the condenser, as well as the objective, is working in oil. Therefore:

1. Line everything up as previously described.
2. Set the slide on the stage and get in Kohler illumination with a ×40 objective. Find the area to be studied.
3. Remove the slide, rack up the tube, and place a drop of oil on the top lens of the condenser.
4. Refocus the slide with the ×40 objective and then refocus the substage condenser. Rack up the tube and make sure that the whole surface of the top lens of the condenser is joined to the slide with oil. It sometimes happens that there is too great a gap between the condenser and the slide. Remove the slide, lay a couple of coverslips on the oil on the condenser, add oil to the top of the coverslips, and replace the slide. This compensates, in effect, for having used too thin a slide. There is no cure for too thick a slide.
5. Place a drop of oil on the slide. Swing the N.A. 1.4 lens into position, lower it into contact with the oil, and then focus it.
6. It will usually be necessary, in practice, to shut down the substage iris slightly —to, say, N.A. 1.3—to reduce glare.

It may be necessary, after examining one field with this lens, to change slides and search the new slide with a ×10 lens to find the required field. The image of the field iris will of course be minute, and that is why the supplementary lens shown in Fig. 2-26 is provided. Swing or slide it into position, and the whole field of a ×10 lens will be illuminated. A similar lens is available for an Ortho-Illuminator.

Most N.A. 1.4 condensers will adequately fill the field of a ×20 objective. If much critical work is to be done with a ×10 objective it is best

to have an N.A. 1.0 Abbe condenser, which can be exchanged for the N.A. 1.4. For occasional critical work, the top lens of the N.A. 1.4 condenser may be removed. The last operation to be described is centering the stage.

Centering the Mechanical Stage

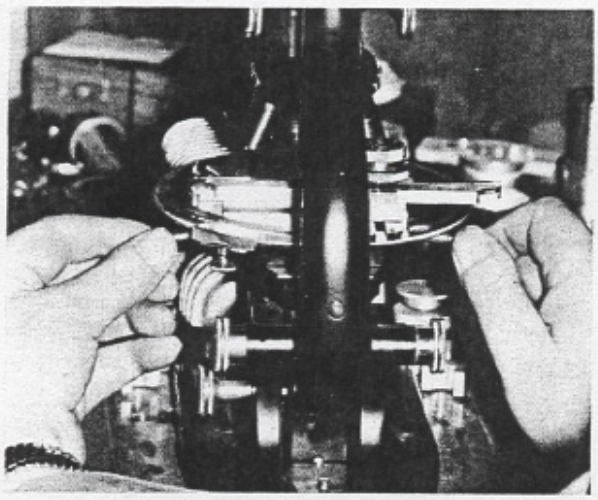
This is usually necessary only when the microscope is to be used for photography.

1. Set up the ×10 objective.
2. Place a slide carrying crossed hairs on the stage and center it with the rectangular rack movement.
3. Unlock the rotating movement (Fig. 2-34). This extremely important step is often neglected, with consequent damage to the instrument.
4. Rotate the stage. The cross will describe the arc of a circle. Estimate the position of the center of this circle and try to bring this center into the center of the field with the stage centering screws (Fig. 2-33).
5. Use the rectangular rack movement to bring the crossed hairs back into the center of the field. Again rotate the stage. This time the crossed hairs will describe the arc of a much smaller circle.
6. Repeat steps 4 and 5 until the cross rotates on its own center.

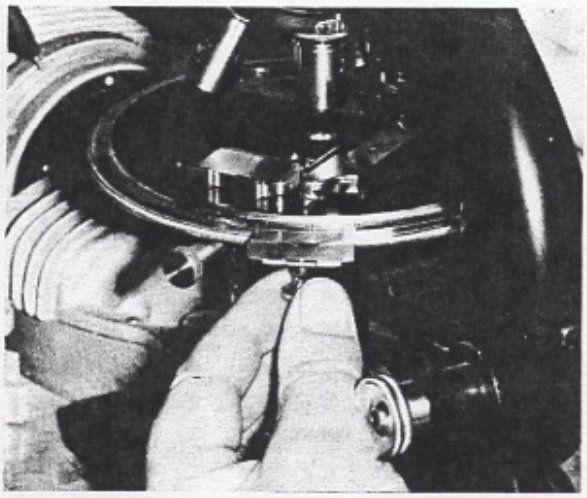
Setting Up a Phase-contrast Microscope. A student with no previous experience in phase-contrast microscopy should read the account of this at the end of the last chapter. It is vitally necessary to get a phase contrast set up exactly right, and it is impossible to do this without some knowledge of the theory involved.

Figs. 2-33 and 2-34 | Centering the rotating stage.

2-33



2-34



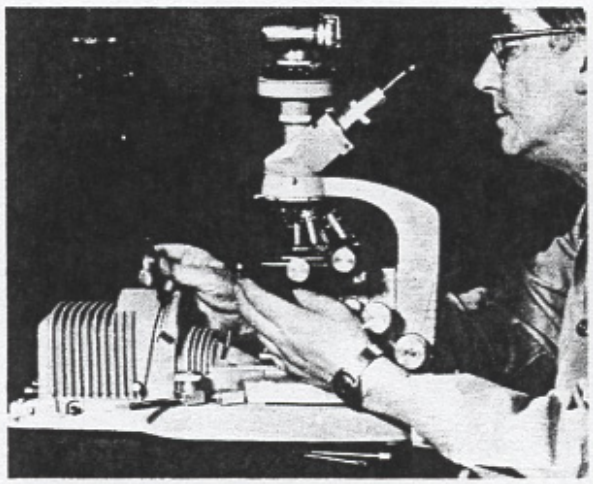
Each phase-contrast objective has to have a matched condenser since the annular disk underneath the condenser has to match the phase-contrast plate at the back of the objective. For this reason, the objectives and the condensers of the same manufacturer must always be used. Phase-contrast condensers, with their annular stops, are mounted in a turret that fits under the substage exactly as the objectives are mounted in a rotating turret on the front end of the microscope tube. In addition to the phase-contrast condensers, there must be a regular condenser, without an annular stop in it, which permits the whole substage system to be centered to the light. There are, therefore, two separate sets of substage centering screws. The regular substage centering screws, which in microscopes with interchangeable substage arrangements are used to center either the regular substage or the phase substage turret, are built into the microscope. The centering screws for the annular stops are built into the phase condenser turret itself in most microscopes, or else, as in the current American Optical models, they are activated by special probe handles which are inserted as required.

A convention which is at present accepted by most manufacturers of microscopes is that the regular condenser in a phase turret is labeled 0, the condenser matched to a $\times 10$ objective is labeled A, that matched to a $\times 20$ objective is labeled B, that matched to a $\times 40$ is labeled C, and that matched to the oil-immersion objective is labeled D. Compared with regular objectives, all phase-contrast objectives have very low numerical apertures, so that the phase-contrast condenser is never oiled to the slide. The apparatus is set up in the following steps.

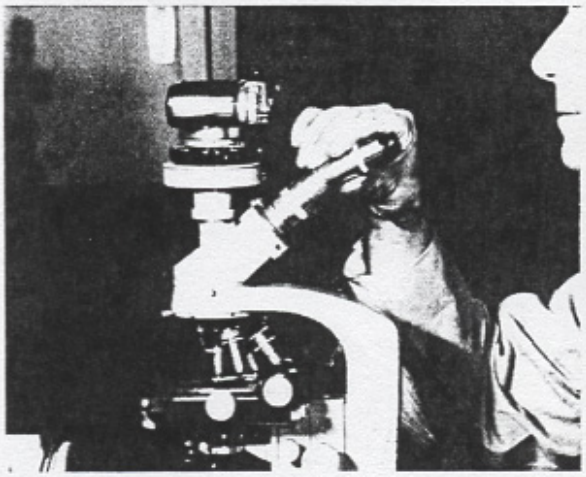
Centering the Phase Substage Turret

1. In the unlikely event that the microscope being used for phase-contrast microscopy does not have a built-in lighting system, a light should be set up and centered with a regular condenser, using the technique described under Setting Up a Research Microscope earlier in this chapter.
2. Insert the phase turret in place of the regular condenser and rotate it until the zero condenser—that is, the one that has no annular stop—is in position. In almost all microscopes (Fig. 2-35), the number zero can be read only with the aid of a mirror.
3. Close the iris diaphragm of the phase substage turret and use the regular substage condenser centering screws to center the image of this iris through a $\times 10$ objective (Fig. 2-36).
4. Check that the light is centered by using a pinhole ocular in the manner described under Centering the Light Source earlier in this chapter.
5. Rotate the phase objective which is to be used into position on the nosepiece and use the mirror to observe the rotation of the substage phase turret until the

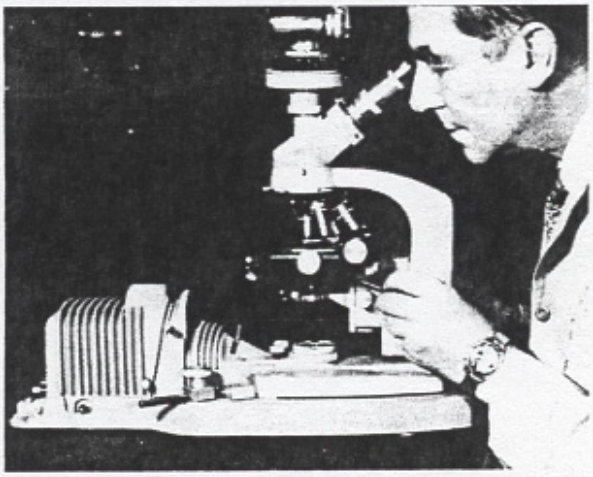
2-35



2-36



2-37



2-38

Figs. 2-35, 2-36, 2-37, and 2-38 | Setting up phase-contrast illumination. Fig. 2-35. The phase condenser turret is rotated until the notation 0 can be read in a mirror. Fig. 2-36. The image of the substage diaphragm is now used to center the substage condenser and to set up Kohler illumination. Fig. 2-37. A telescope ocular is placed in the tube and focused on the phase plate. Fig. 2-38. The annular stop is now centered against the phase plate.

condenser is matched to the objective. Insert a telescope eyepiece (Fig. 2-37), which comes with the phase equipment, and focus this on the annular phase plate on the back of the objective. This phase plate is seen as a dull ring of light. A much brighter ring of light, of exactly the same size, will also be seen. This is the image of the annular stop belonging to the condenser. If the two rings are not of exactly the same size, the wrong substage phase condenser has been rotated into position.

6. Use the annular stop centering screws (Fig. 2-38) to superimpose the two rings of light.
7. Remove the telescope and reinsert the regular eyepiece; the microscope is now ready for use.

Most people center all the phase plates against all the objectives when the microscope is first set up for phase-contrast work. This is less trouble than interrupting one's observations every time a new objective is swung into position.

SUGGESTED ADDITIONAL READING

Birchon, D.: "Optical Microscope Technique," London, Newnes Educational Publishing Co., Ltd., 1961.