Chapter 1

Light Microscopy

R. G. E. MURRAY and CARL F. ROBINOW

Light microscopy is made easy, interesting, and useful for bacteriological purposes if at least four different kinds of instruments are readily available, permanently set up for work, and maintained in good order. These are (i) a rugged, well-equipped, but uncomplicated microscope for "encounters of the first kind," (ii) a phase-contrast microscope, (iii) an optimally equipped and adjusted microscope for high resolution of detail and the best of photomicrography, and (iv) an instrument equipped for fluorescence microscopy. Many modern microscopes are equipped to combine two or more of these functions and are very satisfactory if the transitions are easy to accomplish. An additional instrument, a stereoscopic "dissecting" microscope, is valuable for undertaking the isolation of bacteria from nature on agar plates when colonies early in growth are small and close together.

A variety of excellent microscopes are available. Those from first-rank manufacturers are all of comparable quality, and offer a more than adequate range of accessories. The differences involve mostly the support structures, and the choice is likely to be based on convenience factors and personal preferences. Other choices may involve technical requirements, and it is always advisable to make direct comparisons of the possible instruments and the alternative accessories for a given material under given conditions of work. The really sophisticated instruments are expensive. With care, good work can be done with first-class optics fitted to a general-purpose or even a student microscope stand. However, it is hard to equal the modern instruments from the best manufacturers. It is not necessary to be specific about manufacturers. What matters is an awareness that a good microscope (whatever its age), a discerning choice of components, and a knowledge of how to use them enhance the results and the pleasure derived from the work, save time, and improve accuracy.

The descriptions that follow are based on an old-fashioned basic microscope with separate light source, mirror, and adjustable and centerable components. The optical principles are no different for the enclosed modern instruments with substage illumination, which seem simpler to use but can suffer from similar problems. The use of poorly adjusted and poorly illuminated microscopes is so widespread that most scientists are no longer aware of what microscopes can attain and are astounded when given a demonstration of the best resolution obtainable with even a basic microscope when it is used in accord with the principles outlined below (see sections 1.1 and 1.2).

1.1. BASIC MICROSCOPY

The microscopes assigned for general use in the classroom and in the research or routine laboratory can be of variable vintage, quality, and condition. The applications to the basic requirements of bacteriology are simple, and the questions answered with its help are as follows: Are there objects of bacterial size and staining properties? Are they consistent in size and shape? Are the bacteria gram positive or gram negative? Is there more than one kind of organism present? . . . and so on to more sophisticated questions. These primary questions can usually be answered without attaining the highest resolution, so a simplified procedure suffices as long as the user remembers that higher resolution demands improvement by adjustments at almost every step.

The beginner would be well advised to have at hand a fully illustrated manual for microscopy, of which there are many available (4, 6, 9, 18).

1.1.1. Instrumentation

The basic microscope (Fig. 1) should be equipped with the following.

1. Oculars (eyepieces) of at least 10× magnification. Prolonged observation is more comfortable with the use of a binocular system. The oculars fit into the microscope tube, which may be fixed or adjustable in length (to 180 mm, or whatever tube length the manufacturer recommends). Observations on bacterial structure at high resolution are assisted by higher-magnification (12× or 15×) compensating oculars (see section 1.2.2). Another circumstance in which high-power (15×) oculars assist vision is when scanning growth on agar plates with a 10× objective.

2. Low-power, dry objectives (e.g., 10× and 40×). These are essential for looking at growing cultures and for identifying rewarding areas for study at high magnification, according to the nature of the preparation. Ideally, the objectives (including the oil-immersion objective) should all be parfocal, i.e., close to being in focus when exchanged by the nosepiece carrier.

3. An achromatic oil-immersion objective (90× to 100×). This is essential to bacteriologists for observing the finest details in specimens.

4. A stage with a mechanical slide-holding device or with simple spring clips. If a mechanical stage is provided, it should be easily removable so that petri plates may be examined at low power.

5. A substage condenser of the "improved Abbe type." This is essential for use with oil-immersion objectives, because it provides the quality and quantity of light they require. It should have an iris diaphragm (aperture diaphragm) and a movable filter carrier incorporated below the lower lens. Some condensers have a movable top element (essential for work with high-power objectives) that tilts out of the way for purposes of low-power examination. A condenser that has three lenses, is aplanatic, and, when under oil, has a numerical aperture of 1.3 to 1.4 is preferable when high-resolution as well as general use is expected (see section 1.2.1).

6. A light source, either built-in and of limited adjustability or (preferably) external and movable. In the latter case an adjustable two-sided mirror (flat on one face and concave on the other) is fixed under the condenser and directs the light up the optical axis of the microscope by tilting. There are many lamps from which to choose. The simplest form, a frosted bulb behind a "bull's-eye" lens, is adequate for much work. However, the use of a lamp with a "collector" lens, capable of projecting a sharp image of the light source and provided with a filter carrier as well as an iris diaphragm (field diaphragm), results in greatly improved images of details in stained preparations, especially when Koehler illumination (see section 1.2.7) is used. A frosted-glass diffusing screen held in the filter carrier of the condenser can be of value for low-power microscopy, especially when a three-element condenser is used.

1.1.2. Operating Steps

Before operating the microscope, realize that two basic kinds of bacteriological preparations are used for simple microscopy: a specimen under a coverslip, usually a wet mount (see Chapter 2.2.1.1), or a stained smear on the slide surface (see Chapters 2.2.1 and 2.2.4). The former preparation is suitable for examination with all objectives. Start with a dry objective, either low power (10×) or "high-dry" (40×) to see whether the specimen is rewarding and to select an area for study or to detect motility. Then use an oil-immersion objective (90× to 100×) for more definitive observation. A stained smear is usually not suitable for study with dry objectives (although they may be used to locate the bacteria when the smear is very thin) because the smear must be covered with immersion oil to provide a clear and translucent image of the bacteria. The steps described below assume that a progression in levels of magnification will be used, although one can go directly to using an oil-immersion objective.

Supposing that a slide preparation is to be examined by use of the simple illumination source described above, the steps in operating the basic microscope are as follows.

1. With the eyepiece and 10× objective in place, the condenser lens fully up and almost level with the stage, and the aperture diaphragm of the condenser fully open, tilt the mirror to provide illumination up the optical axis. Adjust the objective by using the coarse control wheel to focus the light field, and then center it with the mirror. Use the *flat* mirror for most microscopy; the concave mirror is used only for low-power microscopy when a condenser is not being used.

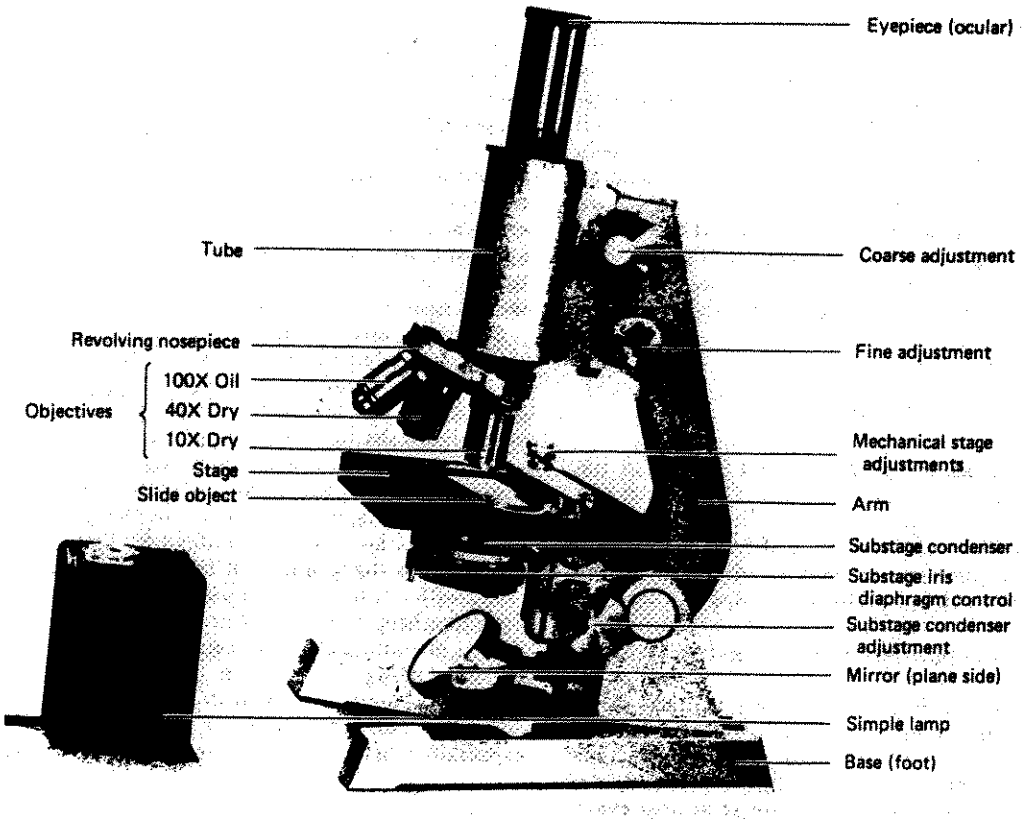

FIG. 1. Basic microscope and its parts.

2. Place a suitable slide on the stage, and focus the objective to get a sharp (not necessarily well-illuminated) image of the specimen.

3. Improve the illumination by adjusting the position of the condenser and the aperture diaphragm to get the best image and appropriate contrast. The simplest way to determine the optimum condenser position is to remove the eyepiece and look down the tube at the visible illumination of the back of the objective lens. While looking, adjust the position of the condenser to give the fullest and most homogeneous illumination of the objective. Any lack of concentration can be recognized and adjusted by tilting the mirror. Reduce glare (light scattering from lens mounts) by closing the aperture diaphragm of the condenser (if available) just enough to impinge on the bright disk. This will approximate "critical illumination"; i.e., the light source is centered and focused in the plane of the specimen. This whole procedure applies to *all* objective lenses as long as they are first focused on the specimen. Alternatively, or to check that the critical illumination is properly adjusted, place a wire loop against the lamp or against the frosted glass of other illuminators (the effective light source) while the objective is in focus on the specimen. Then adjust the condenser to get the sharpest image of the wire loop on the specimen.

4. Replace the eyepiece to examine the specimen, and make any final adjustments for the best illumination and focus.

5. Turn the nosepiece to any other objective desired. Many microscope makers provide parfocal objectives (i.e., the focal position for one is nearly identical to that for all the others). Clearance is sufficient for the dry lenses. If the lenses are from different manufacturers or otherwise mismatched, it is wise to raise the tube a little before bringing into place and focusing the oil-immersion objective, which has a small focal distance (often 0.2 mm). The clearance will be even smaller when the specimen is mounted under a coverslip (which should be no. 1 thickness).

6. If going direct to oil immersion, apply a drop of oil over the specimen on the slide before placing it on the stage. If the specimen has been scanned for a rewarding area with a lower power, it is convenient to apply the oil drop to the slide in place with the nosepiece turned halfway to the correct objective and before swinging the oil-immersion objective into position.

7. Using and focusing the oil-immersion objective takes a little practice and care even if the objective has a spring-loaded front end to protect both the lens and slide from the ham-fisted operator. While looking from the side, lower the objective slowly onto the oil drop and down just a little more so that the lens almost touches the slide. Using the coarse focusing wheel and watching in the eyepiece, look for the specimen while raising the objective *gently*. When the specimen is found, use the fine focus wheel. Adjust the illumination as above, if needed. Focus with care; contact between the objective

and the slide can crack the slide and damage the lens mounting. Adjust for critical illumination as described in step 3 above.

8. On a binocular microscope, adjust the eyepiece tube that has a knurled ring for adjusting tube length so that both eyes are in exact focus.

9. If the image is hazy, moves, or is only dim and not sharp, there is something wrong. It may be necessary to clean the front lens of the objective (with lens paper *only*). A common cause of hazy images is bubbles entrapped in the immersion oil (take out the eyepiece and look down the tube; the bubbles are then easily identified). To remove the bubbles, raise the objective, turn the nosepiece, wipe off the oil with lens tissue (the bubbles usually come up onto the objective), and then turn back the objective and refocus. A poor image may also be caused by having accidentally closed the diaphragm, moved the lamp, or moved the mirror. See section 1.1.7 for other troubleshooting suggestions.

1.1.3. Operating Rules

Remember the following simple rules of microscopy and practice them.

Rule 1. The focused image requires the best possible quality and quantity of light.

Rule 2. If there is too much light, it is best to reduce it with a neutral-density filter or with a voltage controller. The light can also be moved farther away, but then the condenser must be refocused. With oil-immersion (high-aperture) objectives, avoid reducing the illuminating aperture (by lowering the condenser or closing the aperture diaphragm more than 10% of the opening), although this stratagem is helpful for low-aperture dry objectives and for looking at living, unstained cells.

Rule 3. If there is too little light for the objective used, either there is something in the way, the alignment has shifted, or a more appropriate illumination system (lamp and condenser) is needed.

Rule 4. The specimen must be part of a (nearly) homogeneous optical system and therefore must be mounted in oil, water, or another optically appropriate medium. No details can be discerned in dried, stained films of bacteria viewed with dry objectives.

Rule 5. Keep all lenses clean and free from dust, fingerprints, noseprints, and the grime from oily eyelashes (with lens paper *only*).

1.1.4. Comfortable Microscopy

Long hours at a microscope are needlessly tiring if the microscopist cannot sit comfortably and upright while looking into the instrument. Inclined eyepiece tubes are no help if the table or chair height is inappropriate and causes stooping and straining. Binocular viewing also contributes to restful study.

Chairs (including stools in teaching laboratories) should be adjustable. For the very tall, a block of wood can be cut and used on a table of fixed height to raise the microscope to just the right position. The whole object of these adjustments is to allow the microscopist to look down the tube with minimal deflection of the neck or back. A comfortable position minimizes strain and the misery that can result.

1.1.5. Immersion Oils

Immersion oils are designed for use with oil-immersion lenses and provide an environment of the correct refractive index for the front lens of the objective. They also generate a homogeneous system, approximating the refractive index of glass, and they "clarify" the dried cells of bacteriological slide preparations by permeating and embedding the stained cells.

The commercially formulated oils recommended by microscope manufacturers have been tested to be sure that they do not have acidic or solvent effects on lenses or their mountings; substitutes are not recommended and may be a false economy. Certain properties can be chosen for special purposes (5), such as "very high viscosity" oil to fill wide gaps or to stay in place on horizontal or inverted microscopes, and "low-fluorescence" oil for fluorescence microscopy. For general purposes use immersion oil of a fairly high viscosity (e.g., type B, high-viscosity oil of 1,250 cP from Cargille Laboratories Inc., Cedar Grove, NJ 07009), which stays more or less where it is placed and creeps less than low-viscosity oils. The only problem with this level of viscosity is that bubbles are easily entrained, but this is avoidable.

Oil-immersion objectives should always be cleaned after use or, at least, at the end of a day's work. Use lens paper moistened with xylol or another appropriate solvent as recommended by the lens manufacturer. Ethanol and methanol are recommended by Wild-Leitz for its lenses, but xylol is also safe to use. The modern nonoxidizing oils are less of a problem than the traditional cedarwood oil (which hardens, oxidizes, and leaves annoying residues), but they still slowly alter and may also creep where not wanted. *CAUTION:* Modern immersion oils are safe, but before 1972 nondrying oils were often formulated with polychlorinated biphenyl compounds, which are now believed to be carcinogenic and toxic, so look out for old bottles!

Never use more oil than necessary; usually 1 drop will do. A good "oil bottle" will help to reduce mess; e.g., use a glass bottle with a broad base and an applicator (glass rod or wire loop) attached to the cap. Among the best is a double-chambered bottle in which the stopper is formed into a vessel for oil and the base is made to hold the solvent for cleaning. Squeeze-bottles tend to generate bubbles unless used carefully.

Oiling the condenser to the slide, which is necessary for highest resolution with the oil-immersion objective (see section 1.2.1), can be a messy procedure. It demands a high-viscosity oil and some simple precautions both when mounting and when demounting a preparation, as follows:

1. Place a drop of high-viscosity oil on the top lens of the condenser, and then lower it below the stage level. Avoid bubbles in the oil drop.
2. Place a drop of oil on the underside of the slide below the area you wish to examine.
3. Set the slide down on the microscope stage with the drop central in the hole, and hold the slide there while adjusting the mechanical stage or clips to keep the slide in place.
4. Raise the condenser so that drop meets drop and until the top condenser lens is fully covered with oil.
5. Place a drop of oil over the specimen, and focus with the objective. Then adjust the condenser to give Koehler illumination (see section 1.2.7).

6. When demounting the preparation, lower the condenser before picking up the slide. Lateral movement or wide scanning may lead to oil being deposited or creeping under the stage. Remember this, and clean the stage underside when cleaning the top lens (with lens tissue!) after use.

7. Only condensers with integral or screw-on top elements are suitable for oil immersion. Movable "swing-out" top elements on some "universal" condensers (i.e., those that can be slid aside with a lever for low-power work) must be oiled with great care or not at all because subsequent cleaning is difficult.

8. Cleaning condensers after use is the same as for objectives. Remove the condenser from its substage mount to avoid (or clean up) oil that has flowed around the condenser housing. Remove excess oil with lens tissue, and then clean the lenses with fresh lens tissue moistened with xylol or the appropriate solvent recommended by the microscope maker.

1.1.6. Care and Cleaning

Microscopes are remarkably tough and durable. With reasonable care and protection from the elements (particularly the hostile acid-laden air of some laboratories), a microscope will last a lifetime or more. Take care of the microscope as follows.

1. Protect the microscope from dust and grit. Put a dust cover over the microscope when it is not in use; the best is a rigid, transparent, and all-enclosing glass or plastic "bell." Do not allow dust to accumulate anywhere; it drifts into the lenses and mechanisms.

2. The moving parts, especially the rackwork and gears, should be cleaned and treated with new grease at long intervals (the grease for model-train gears from a hobby shop works well). Do not use thin oil on gears or bearing surfaces; the tube or condenser may then sink by its own weight.

3. Clean the stage regularly, and mop up any spills.

4. Keep the lenses clean, and, especially, clean up after a session of oil-immersion work. *Never* use the finger instead of an appropriate lens tissue (see below).

5. Do not attempt to repair an objective lens or the fine adjustments. These are best left to professionals. Good microscopes deserve a professional cleaning and adjustment every 20 years or so! Microscope dealers usually have or can recommend a repair facility.

6. Keep the tube closed at all times with an eyepiece, and keep all objective mounts filled or plugged, to minimize dust in the tube.

The "old soldiers" among microscopes can be cleaned, repaired if necessary, and put back to good use. The brass bodies on ancient ones (How elegant they are! How fine their lenses may be!) can be rubbed down with a lightly oiled cloth or one lightly moistened with tarnish remover and then a dry cloth. Modern black finishes should just be kept clean.

Clean lenses make for a dramatic improvement in image quality. Optical surfaces need special care in cleaning, and some general rules apply.

1. Keep a good supply of lens tissue (lens paper), a soft brush (artist's watercolor brush, 0.6 cm), and a nebulizer bulb with a short narrow rubber tube attached near the microscopy area. Keep them scrupulously clean and in a dust-free box.

2. Nonadherent dust can be easily removed by using the brush, puffing with the rubber bulb, or wiping with lens tissue after breathing on the optical surface.

3. Finger marks, other adherent grease and dirt must be polished off with lens tissue doubled over a finger and barely moistened with xylol or the recommended solvent (see section 1.1.5). Do not flood lenses with solvents, and *never* use (without specific recommendation) alcohol, ether, or acetone for fear of penetrating the cement between lenses. If the lens has a raised mount, clean the edges with moistened lens tissue wrapped around an applicator stick.

4. If xylol is not effective, try using tissue moistened with distilled water.

5. The usual way of cleaning oil from objective and condenser lenses is to wipe most of it away with lens tissue and finish with tissue barely moistened with xylol. Oil and grease can also be efficiently removed with a freshly broken piece of polystyrene foam (common packing material) by pressing it against the objective front lens and rotating it (H. Pabst quoted by James [9]). The foam has lipophilic properties. Xylol dissolves the foam, so do not use both.

6. Never take an objective apart to clean the components, because the interlens distances are critical. Dust can be puffed from the back lens or lifted with a superclean brush.

7. Dust on oculars is a constant problem and produces dark spots which move around as the lens is rotated. The eyepieces are quite simple in construction (top and bottom lenses with a fixed diaphragm between), and both lenses unscrew. If cleaning the external top and bottom surfaces does not remove a spot, there may be particles on the inner surfaces. Be careful of "bloomed" lens surfaces (antireflective coating which appears iridescent blue), and use a brush or tissue with care.

8. *Keep fingers off optical surfaces!*

1.1.7. Troubleshooting

The list of technical problems, causes, and remedies shown in Table 1 is taken (with permission) from the excellent book by James (9). For additional advice, consult other books (see section 1.9), an experienced microscopist, or an instrument technician to identify the problem. If the problem is optical, work along the light path in systematic order from the lamp and then through the condenser, specimen, objective, and ocular. Adjust at each step, and determine the effect of adjustment on the problem; this is where Table 1 is useful.

1.2. HIGH-RESOLUTION MICROSCOPY

The "research microscope" comes into its own for the resolution of fine detail at high magnification. Its use is not called for in determining the outcome of a Gram test, but it will help to settle questions about flagella and matters requiring the perception of fine detail; also, high resolution allows the sharpest photomicrographs. For such purposes it is essential to pay close attention to the quality of the optical components. Achieving high resolution as well as freedom from chromatic and spherical aberration requires attention to basic principles.

Table 1. Common problems in light microscopy, and their causes and remedies[a]

Problem	Possible causes	Remedies
Coarse adjustment is too stiff	Mechanism adjustment was faulty	With many stands, adjust simply by moving the two coarse-control knobs in opposite directions
	Dirt in rackwork	Clean and apply new grease
Tube or stage sinks spontaneously under its own weight (image drifts out of focus)	Incorrect adjustment of rackwork	As with first remedy
	Lubrication with too-thin oil	As with second remedy
	Faulty adjustment of focus control	As with first remedy
Micrometer movement is blocked to one side	Fine adjustment at the end of its travel	Bring a 10× objective into position with the revolving nosepiece, set the fine focus control at the middle of its range, and then focus with the coarse adjustment
Drift of focus with the slightest movement of the fine adjustment (especially with oil-immersion objectives)	Objective insufficiently screwed into the revolving nosepiece	Seat the objective fully into the nosepiece
	Surface of the coverslip stuck to the objective by the layer of oil	Use less viscous immersion oil; clip specimen firmly
Veiled, spotty image	Dirt or grease (i) on the eyepiece (spots move when the eyepiece is rotated in the tube), (ii) on the objective, (iii) on the coverslip (spots move when the specimen is shifted), or (iv) on any surface of the illumination apparatus	Clean where necessary
Sharply focused spots or specks in the image which change and disappear when the condenser is moved up and down	Dirt (i) on the light source, or on the diffusing screen in front of it with critical illumination, (ii) on the cover plate of a built-in lamp, or (iii) on a filter near the cover plate with Koehler illumination	Clean where necessary; when the contaminated surface cannot be reached, change the focusing of the condenser slightly or tolerate the problem for the sake of resolution
Hazy image, which cannot be brought sharply into focus	Wrong immersion medium (oil instead of air, air instead of oil, air bubble in oil)	Use correct immersion medium
	Transparent contamination on objective front lens	Clean where necessary
	Coverslip too thick, or too thick a layer of mounting medium	Use a correct coverslip or an appropriate objective
	Irregularly distributed remnants of immersion oil on the coverslip when using high-power dry objective	Clean the coverslip with dry cloth or paper tissue; beware of solvents, as some may weaken or dissolve mounting medium
	Slide upside down on the stage (only with high-power objectives)	Invert the slide; make sure a label is not stuck to the wrong side of the slide
Object field partially illuminated	Filter holder partially in the light path	Move the filter holder
	Objective not clicked into position	Reposition the objective
	Condenser (or swing-out lens) not in the optical axis	Realign the condenser
Object drifts diagonally when focusing	A lens is not centered in the optical axis	Check concentration of the lenses at all points in the optical path
Object field unevenly illuminated	Mirror not correctly in position	Reposition the mirror
	Condenser not centered (with critical illumination)	Realign the condenser
	Irregularity in light source and/or diffusing screen (with critical illumination)	Move the condenser slightly up and down; use ground glass in front of the light source
Drift of cloud across the field; after this, image out of focus (oil-immersion)	Air bubble in the immersion oil; oil in image space with a dry objective	Wipe off the oil from the specimen, and set up anew; clean the slide and objective carefully
Sharply delineated bright spots in the image	Transverse reflections in the interior of the microscope (often sickle or ring shaped)	Try another eyepiece; use correct Koehler illumination
	Longitudinal reflections in the tube, causing round light spots	Use lenses with antireflective coatings; change the combination objective-eyepiece

(Continued)

Table 1. *(Continued)*

Problem	Possible causes	Remedies
Unsharp bright spots in the image	Contamination at a lens surface, on upper or under side of the object; or air in immersion oil of the condenser (differentiate as explained before)	When localization and removal of the contamination are not possible, reduce the effect by opening the condenser diaphragm somewhat more

[a]Reprinted from reference 9 with permission.

1.2.1. Resolution Requirements

Resolution (R) is the shortest distance between points of detail which will still appear as a distinct gap in the images, visual or photographic. $R = \lambda/2NA$, where λ is the wavelength of the light used and NA is the numerical aperture, a measure of the light-gathering power of the objective. The purpose of a good research microscope is to help make R as small as possible. The smallest values of R require not only an objective of high NA but also a condenser of equivalent quality (see section 1.2.3) and the use of short-wavelengths light). Unfortunately, light of the most effective wavelength (namely, UV light of about 365 nm) is not perceived by the eye and does not pass through glass. Indirect methods of microscopy, requiring a microscope with lenses (lamp, condenser, and objective) made of quartz (permeable in various degrees to UV light), take advantage of the short wavelength of UV light, as is the case for fluorescence microscopy. The best results with visible light are obtained in the green/yellow region of the spectrum because it is the wavelength to which the eyes are most sensitive (close to the mercury green spectral line) and for which microscope objectives are designed to transmit with a minimum of aberrations. So much for the λ part of the expression for resolution.

The NA of oil-immersion objectives is usually a value of 1.30 or 1.32, and the best ones may be 1.40. The numerical aperture represents the sine of one-half of the angle described by the cone of light admitted by the objective lens, multiplied by the refractive index of the medium through which light passes on its way to the lens. The maximum value of the sine function cannot exceed 1.0, but the refractive index of the medium through which light passes on the way from the condenser to the objective can be raised to that of optical glass (i.e., 1.5150) by the use of immersion oil. It is this fact that allows lenses of NA of greater than 1.0 to be filled with light. Provided that certain other conditions are met, the resolving power of the costly objectives with NA of 1.30 to 1.40 engraved on their glittering housings can be utilized to the fullest.

1.2.2. Objectives and Oculars

High-aperture achromatic or apochromatic objectives are spherically correct for one or three colors, respectively, in the center of the field of view (where most of the work is done at high magnification). The two kinds of objectives work almost equally well, price notwithstanding, especially when narrow-band-pass (interference) filters are used.

The objective best suited to the task in hand should be complemented by an ocular of commensurate quality. What is needed for apochromatic oil-immersion objectives are compensating eyepieces. "They are designed to correct the lateral chromatic error of magnification inherent in objective lenses of high corrections and high apertures"; experience amply proves the soundness of the additional advice of Shillaber (15) that "the compensating ocular should be of high power, preferably 15× or 20×. The ability of apochromatic objectives to take a high-power eyepiece should be fully utilized; otherwise, the fine detail that they are capable of bringing out in a photomicrograph is likely to be lost."

For those who wear glasses with major corrections, it is convenient and helpful to buy high-eyepoint oculars which are made so that glasses may be worn while observing.

1.2.3. Condensers

Another important station in the path of the light from lamp to object to eye is the substage condenser. Objective NA values of 1.30 to 1.40 will be fully utilized only if the condenser NA is at least as high. Such condensers are usually also achromatic or aplanatic and should match the optical properties of the objectives described above.

Remember the role of the refractive index in the expression for NA: the full resolving power of an objective of high NA is utilized only when the medium through which light passes between the condenser and the lens system of the objective is homogeneous and glasslike all the way. Therefore, the maximum resolution of an oil immersion lens requires oil between the condenser and the object slide as well as between the slide and the objective lens. The condenser iris diaphragm (aperture diaphragm) restricts the beam and thus affects the NA attained (see section 1.2.7).

1.2.4. Mirrors

A mirror is used to reflect the beam of light along the axis of the condenser. The adjustable mirror issued with old-style microscopes has a plane side for work with a condenser and a concave side for work at low powers without a condenser. The plane mirror is made by depositing metal on the underside of an optically flat disc, does well in everyday work, and is durable. However, it is not the best for optimum illumination, because the front and back of the glass that covers the reflecting metal produce separate and overlapping reflections of the light source or of the opening of the field diaphragm when the Koehler procedure is followed, thus adding undesirable scattered light to the final image. Instead, use a "first-surface mirror," which lacks protective coverings and gives but a single reflection.

1.2.5. Filters

The light used for microscopy is modified in intensity or wavelength by the use of filters. Neutral-density filters (aluminized optical flats) allow the brightness to be adjusted for comfortable vision. Color filters restrict the wavelength used. Two kinds of color filters are available: expensive "interference filters" of narrow band pass consist of two half-silvered glass plates facing each other across a precisely measured gap; Wratten filters consist of colored gelatin mounted between two 5-cm^2 flats of optically polished glass. Wratten filters deteriorate with time because air creeps in from the sides, but they come in a wide range of colors and densities and are valuable accessories. A red filter (Wratten no. 29) or an orange filter (Wratten no. 22) is helpful in making dense structures transparent or enhancing structures stained with blue dyes. A green filter (Wratten no. 11 or no. 58B) is the most generally useful because it is the kind of light to which the human retina is maximally sensitive and because many commonly used histological stains are red. Their contrast is greatly enhanced when the light is green, the complementary color.

1.2.6. Light Sources

Optimum performance of a highly resolving objective depends on optimum illumination. The field of view must be evenly filled with light of a brightness sufficient for photography. This requires a more complex lamp than that effective for ordinary observation. The light from the usual coiled filaments is unevenly distributed over their surface and therefore over their image unless diffused by ground glass. The best structureless, uniformly bright sources of light are small mercury or zirconium arcs and the less expensive conventional projection lamps with tungsten bead or ribbon filaments that can be imaged to fill the condenser. The Koehler system of illumination below is designed to provide the appropriate quality of light and requires that the source be focused in the plane of the object being viewed. Therefore, the microscope lamp should be equipped with a lens system to project a sharp image of the light source into the entrance plane of the substage condenser. In front of this lens, there should be an iris diaphragm, the so-called field diaphragm (see below), which can restrict the area of illumination without interfering with the quality of the light. There should also be a carrier for filters.

1.2.7. Optimum (Koehler) Illumination

The Koehler system of illumination is useful because it provides appropriate illumination of the field for high-resolution microscopy and makes good use of high-quality optics. It is achieved by focusing an image of the filament or light source at the level of the condenser diaphragm (i.e., the lower focal plane of the condenser), when the condenser is in the correct position relative to the specimen. Under these conditions, an objective lens in focus on the specimen will be fully illuminated whatever the size of the source. Effectively, this applies to every part of a source, so that there is even illumination across the field of view even when a coil filament lamp is used. The principle can be applied usefully to both high-power dry and oil-immersion objectives.

With eyepiece, objective, and condenser of matching optical quality and with a first-surface mirror receiving filtered green light from a bright and homogeneous source, the research microscope is ready to be set up in the best way for work and needs only a rewarding specimen preparation to show its worth.

The steps allowing achievement of Koehler (nearly optimum) illumination for an oil-immersion objective are as follows.

1. With a specimen slide in place, switch on the lamp and adjust the condenser to give the smallest bright spot on the specimen. This approximates the focal position of the condenser.
2. Hold a small white card against the underside of the condenser, and, observing in the mirror, focus the image of the lamp filament on the card and move the lamp so that the image covers the opening of the condenser. Alternatively, focus the image on the closed condenser (aperture) diaphragm. If the physical arrangement is awkward, use a small mirror for observation or approximate the focal position on a card placed over the mirror.
3. Insert appropriate filters in the light path, and fully open all diaphragms.
4. Oil the condenser with 1 drop of high-viscosity oil on the top lens, and raise it to meet a similar drop on the underside of the slide (see section 1.1.5).
5. If necessary, scan the preparation with a low-power objective to identify a rewarding area, using reasonable centration and illumination (section 1.1.1), and mark it by the coordinates provided on many mechanical stages.
6. Apply oil to the top of the slide (see section 1.1.5 for precautions), change to the oil-immersion objective, and focus on the specimen. A fuzzy or moving image may indicate bubbles in the oil; check by looking down the tube after removing the eyepiece.
7. Adjust the field (iris) diaphragm of the lamp to a minimum opening, and bring it into view with the mirror. Focus the image margin with the substage condenser in the plane of the specimen (i.e., so that the objective and condenser are both in focus). Readjust the field diaphragm so that its image just impinges on the field of view and centers exactly with the mirror. The optical alignment is then correct.

Note: If the margins of the field diaphragm give an uneven color fringe when using white light, the condenser needs centration. To center it, turn the two adjustment knobs to give movement toward the red side, centering the aperture with the mirror, and stop when the color fringes are symmetrical.

8. Open the field diaphragm until it just impinges on the margin of the field of view. This prevents illumination of the specimen outside the field of view, which scatters light into the image plane. The field diaphragm does not affect resolution in the area illuminated and can be left in view.
9. Removing the ocular (which should be 12× or 15×), look down the tube and restrict the condenser aperture diaphragm to abolish glare or reflections. This restriction should not be more than 1/10 the diameter of the fully illuminated back lens; excess restriction lowers resolution.

10. Replace the eyepiece, and insert filters appropriate to observation or photography (section 1.2.5).

The same principles apply to adjustment of the illumination provided by modern integral substage systems or to incident-light systems (3). However, the centration of the light for these systems is attained by adjusting the position of the bulb in its mount with centering screws. The filament image is focused by positioning the lamp forward or backward in its sleeve, because the diaphragm and the lenses are fixed in place. The point of reference for alignment is usually an integral field diaphragm.

The same principles apply also to dry lenses, but oil immersion of the condenser is then unnecessary as long as the condenser is of adequate quality.

1.3. DARK-FIELD MICROSCOPY

Dark-field microscopy provides a useful means of looking at wet mounts of unstained specimens and detecting very small structures by reflected and diffracted light, revealed like motes of dust in a beam of sunlight or like planets and moons in a night sky. It is performed by illuminating the specimen with a hollow cone of light such that only the light diffracted by objects in the field of view is transmitted up the microscope tube to the eye or camera; the beams forming the cone of light focused on the specimen are at too low an angle to be captured by the objective. The result is a field of bright objects (spirochetes, bacteria, particles, organelles, etc.) against a dark background. It is an appropriate technique for all powers of objectives, but it has some requirements that impose the need for specialized condensers for use with oil-immersion objectives.

Low-power (10×) and "high dry" (45×) objectives can be used in a dark-field mode by using an ordinary condenser equipped with a central patch stop in its filter holder. This patch stop allows light paths only in the periphery of the condenser and so attains a hollow cone of light effective at a particular condenser position. This means that the NA of the condenser must be considerably greater than that of the objective being used in order to attain an appropriate illumination angle, and the condenser diaphragm must be completely open. The cone of light accepted by an objective limits the usefulness of patch stops, so they do not work for objectives with an NA greater than 0.60 to 0.65. A simple version of low-power dark-field microscopy with 10× and 45× objectives can also be attained by using a phase-contrast condenser (see section 1.4); it is useful but does not provide the highest-quality image. Few problems are encountered when dry objectives are used with a condenser particularly designed for dark-field microscopy, and an excellent image can be expected.

Oil-immersion objectives (which collect light at an angle proportional to the NA) require specialized condensers of high NA, which must be oiled to the slide so that an effective cone of light can be produced. These reflecting condensers consist of either a paraboloid mirror with a central stop, or, more effectively, a central spherical reflecting surface which reflects much of the light entering the condenser to a cardioid (curved) peripheral mirror on the perimeter, reflecting a very low-angled beam in the form of a cone (refer to texts for optical diagrams).

However, when these condensers are used, most oil-immersion objectives do not provide an adequately dark ground without the presence of devices to restrict scatter from the margins and mountings of their lenses. Excellence requires an objective with an adjustable diaphragm in the back focal plane or restriction in the back focal plane by use of a "funnel stop" (a metal tube with a hole in the lower end) that fits inside the objective to block transmitted rays scattered from the periphery of the objective.

Intensive light is necessary to show very thin or very small objects. Sunlight from a heliostat has been used to show bacterial flagella in action.

It is sometimes difficult to get a good dark background when there is poor centration of the condenser relative to the objective. This requires some trial and error to gain symmetrical illumination. It is helpful to find the best setting for the condenser by using tongue or cheek scrapings mounted in water under a coverslip; these large epithelial cells are easy to find. All the precautions about oiling the condenser and slide (section 1.1.5) must be followed. But given a well-aligned microscope, all that matters then is the intensity, geometry, and quality of the cone of light with an appropriate objective.

The focus of the condenser is crucial in dark-field microscopy. The apex of the cone of light *must* be at the level of the specimen, which is also the plane of focus of the objective. Above this focal point is a dark conical space, in which the front lens of the objective is placed, symmetrical to the dark conical space formed below the specimen above the condenser. When the dark-field condenser is brought up to the slide, with immersion oil making a complete contact, a bright ring of light is projected on the specimen. As the condenser is raised further, the light comes to a bright intense spot, which must be close to the best focus. Because the high-aperture condensers for oil-immersion microscopy have a very short focal length, it is important to use a thin slide (ca. 0.8 mm thick) so that the condenser can focus through it on the specimen. Standard slides (ca. 1.2 mm) may be too thick, thus making high-resolution dark-field microscopy impossible.

For many years, dark-field examination of exudates and media was the accepted method for demonstrating spirochetes. It is impressive to be able to see *Leptospira* spp. in low-power dark ground by using 10× objective and 15× oculars, but it is even more when they appear in all their glory under oil immersion at an effective NA of 1.3. It is a pity that these beautiful images are seldom seen nowadays because of the convenience of phase-contrast microscopy.

A procedure for oil-immersion dark-field microscopy is as follows.

1. See that the microscope and illumination system are fully aligned, as for bright-field microscopy.

2. Replace the condenser with the dark-field condenser, and apply 1 drop of high-viscosity oil to the top lens.

3. Make a wet mount of the specimen on a clean, thin slide. If it is very clean, it is helpful to make a small wax pencil mark under the coverslip for preliminary focus.

4. Raise the condenser to meet the specimen slide, and observe the ring of light. Focus this to give the

smallest spot. An asymmetrical ring of light above or below focus indicates that the condenser is not properly illuminated.

5. Focus the oil-immersion objective on the specimen. The objective should be equipped in the body with a funnel stop or with an iris diaphragm controlled by a knurled ring. In the latter case, start with the diaphragm half closed and make final adjustments later for maximum darkness of the field.

6. Move the specimen slowly to a useful field, and make final adjustments in the condenser focus for maximum brightness of the reflections from the specimen.

1.4. PHASE-CONTRAST MICROSCOPY

Phase-contrast microscopy is a system for gaining contrast in a translucent specimen without the help of stains (9, 14, 19) and has the advantage of using high-resolution optical components. Stained specimens or biological material with opaque areas form images in a microscope because the various components transmit different amounts of light, so that amplitude and wavelength are modified by absorption and scattering in the specimen. Most cells and their components do not cause enough amplitude modification to give useful contrast in the image. However, their materials are translucent and have different refractive indices from neighboring structures and from the mounting medium, and this causes different degrees of phase retardation in the light beam passing through a structure, compared with beams that have passed through other material or only through the mounting medium. The purpose of the phase microscope is to take advantage of changes in phase, converting these into amplitude differences to form an image with enhanced contrast.

An instrument that is permanently set up for phase-contrast microscopy is preferable to one that provides alternating service with ordinary optics on the same stand. Phase-contrast objectives are not suitable for the best bright-field work, despite their high quality. It is better to have objectives dedicated to each purpose. Phase-contrast microscopy demands much more light than is adequate for the examination of stained specimens with ordinary optics. A green filter should be used to reduce unavoidable chromatic aberration.

One of the advantages of phase-contrast over the less-available dark-field microscopy is that the former uses the full aperture of the objective lens. Therefore, the best of phase-contrast objectives should be used. This in turn implies the use of a substage condenser with a suitably high aperture and of 12× to 15× compensating eyepieces, which fully utilize the fine resolution that the objectives are capable of giving. Medium-power (40× or 60×) oil immersion phase-contrast objectives are available and provide crisp, rewarding images for work with large cells. For the very best results, the condenser and slide must be connected by oil when high-power (90× or 100×) objectives with numerical apertures higher than 1.0 are used. However, an oiled condenser is not required for all applications. A dry condenser is adequate for observing the shape and form of bacteria and their spores and for checking motility.

Phase contrast works because a phase annulus is inserted at the lower focal plane of the condenser (to generate a cone of light) and because a phase plate is incorporated in the back focal plane of the objective. An image of the annulus will thus be formed on the phase plate. Some light beams are diffracted by the specimen (e.g., a living cell) to go through all of the objective field, and some go directly through the specimen and the phase plate ring; both take part in forming an image. The phase ring in the objective is a flat ring-form groove in an optical flat plate; its size fits the cone of the direct light beams generated by the condenser annulus. The groove is formed so that the phase ring is less thick glass, causing at least a 0.25 wavelength difference in phase retardation compared with a direct (non-diffracted) beam. The phase difference of the diffracted and nondiffracted beams, some of which are also retarded by passing through a structure in the specimen, causes either destructive interference (dark contrast) or constructive interference (bright contrast) when the beams form the image. Therefore, it is essential to center the annulus plate in the condenser with respect to the analyzer plate in the back focal plane of the objective. This is achieved by imaging both with a focusing eyepiece (or "telescope") and by centering the visible rings by using the two adjusting knobs on the sides of the condenser. The bright image of the annulus should be completely enclosed by the grey annulus of the phase plate when centration and condenser positions are correct; complete filling of the annulus is not essential.

1.4.1. System Assembly

1. Set up the microscope as for Koehler illumination (section 1.2.7) without the annulus in place (use the "bright-field" position for the usual rotatable carrier in the condenser of a phase microscope), and adjust the phase objective to focus on a specimen.

2. Revolve the condenser carrier to bring the appropriate annulus into register below the condenser for the objective in use.

3. Insert a focusing telescope instead of the eyepiece and focus on the grey ring image of the phase plate, which has distinct inner and outer edges.

4. Look in the telescope for all or part of a very bright ring of light, the image of the annulus, which has to be brought into register with the objective phase ring, seen as faint rings for each side of the groove in the phase plate. Achieve centration either by manipulating the condenser centration knobs or by nudging the annulus with a knurled ring on the carrier (systems vary according to manufacturer).

5. Replace the telescope with the eyepiece, adjust the fine focus, and regulate the light intensity (by using a transformer) for comfortable viewing. The image should not shift asymmetrically on focusing; if it does, recheck step 4.

6. If the image is unsatisfactory, check that all surfaces are clean, that the specimen is not too thick, that there are no bubbles in the oil if you are using immersion, and that the correct annulus is in place. The image quality deteriorates if any part of the direct beam falls outside of the annulus in the objective phase plate or is not fully concentric. Accurate focusing of the condenser is essential.

1.4.2. Specimen Preparation

The specimens for phase microscopy should be as thin as possible and must be mounted in a fluid or gel

and under a coverslip to give a homogeneous background to the images. For general work, a clean standard slide and coverslip are satisfactory.

When intracellular detail of living organisms is wanted, attention must be paid to the refractive index of the medium in which the cells are mounted. This is most easily provided by dissolving gelatin or bovine serum albumin (15 to 30%) in the medium. According to the concentration used, this equalizes or brings closer together the refractive indices of the contents and medium, since phase retardation is proportional to the refractive index multiplied by the light path. The disturbing bright halos that usually surround dark-contrast images are then reduced or abolished, and the cells are more grey than black. As an additional benefit, the cells then show internal structures that are not easily discerned in life, e.g., nucleoids and granules other than the obvious lipid droplets, or developing endospores. When the mounting fluid and an adjacent structure are identical in refractive index, the structure will disappear; this technique of immersion refractometry can be used to measure the density and mass of structures (13).

1.4.3. Phase Condenser for Low-Power Dark Field

The condenser phase plate is, essentially, a form of patch stop, and so, given suitable geometry, it should produce dark-field images with ordinary low-power objectives. The annulus for oil-immersion phase commonly gives a reasonable semblance of dark-field optics (section 1.3) with an ordinary 10× objective and sometimes with a 45× objective, after a little fiddling with centration and the position of the condenser.

1.5. INTERFERENCE MICROSCOPY

Interference microscopes have definite applications in bacteriology for discerning the structure of cells. Such microscopes are expensive, which makes them less available, and their operation and the interpretation of the images obtained is best learned by practice with experts. Like phase-contrast microscopes, they attain contrast in images of translucent specimens by detecting phase changes induced in light that traverses cell components with different masses and refractive indices. The two systems differ in that the interference microscope develops separate object and reference beams, rather than forming an image from the direct and diffracted elements of a single beam from a condenser annulus as in the phase-contrast microscope (9, 14, 17, 19).

Interference microscopes provide superior images of the internal morphological details of cells without the halos that surround cells when viewed by phase-contrast microscopy. A great advantage of the former is that phase changes may be measured to provide quantitative cytological data. Furthermore, the lack of an annulus avoids deterioration of the image by optical effects from cell structures. The lenses are of high quality, are used to their effective numerical aperture, and will operate in either transmitted- or epi-illumination modes. The quality of light and the adjustment of the microscope to give critical or Koehler illumination at the outset are important.

The Nomarski-type interference microscope uses polarized light (white or monochromatic) from a filter at the source to fill the condenser through a Wollaston (birefringent) prism. The prism generates two polarized beams at right angles, each filling the lenses. The resulting cone of light traversing the specimen and illuminating the objective is a complex of these two beams; in effect, they are very close together, in parallel, and uniform. They act as object and reference beams according to what they traverse in the specimen. The beams are recombined above the objective by another Wollaston prism, allowing interference (constructive or destructive) to take place. The plane-polarized beam is recaptured by an "analyzer" polarizing filter, which is usually in a fixed orientation (this means that the polarizing filter at the source has to be rotated to give the appropriate orientation). If white light is used, with appropriate manipulation parts of the image will contain interference colors which are related to the amount and sign of the phase change. This information and photometric measurements will allow generation of mass data.

In some interference microscope systems (including the Nomarski type), the image has a pseudo-three-dimensional appearance, which is a consequence of an angle of shear between the beams.

1.6. FLUORESCENCE MICROSCOPY

Fluorescence microscopy employs all the principles of optics described for the research microscope (section 1.2). The differences in practice and design relate to generation and transmission of wavelengths of light suitable to the excitation of fluorochrome stains and of natural fluorescence in the specimen. The secondary emitted wavelengths are detected as an image of a fluorescing object. Because the excitation process usually requires short wavelengths in the near-UV or blue range, the lamp (a high-pressure mercury vapor arc lamp) and any lens between the lamp and object must be made of material (quartz) appropriate for passage of that range of wavelengths. A first-surface mirror is essential (to avoid an interfering glass layer). The immersion oil for the objective and condenser must be nonfluorescent (e.g., a special synthetic formulation or sandalwood oil). Quartz slides and coverslips must be used. Good advice on details comes from specific (10, 20) and general texts.

Most important are the light source and the arrangement of filters in the light path, which vary in mechanical arrangement among manufacturers (who provide appropriate sets of filters for a specific fluorochrome), but all meet the following demands.

1. The lamp must be able to excite the fluorochrome in use, and two types, of differing utilities, are available: (i) high-pressure mercury-vapor arc lamps produce a large proportion of UV rays and the widest spectrum of wavelengths, so that any fluorochrome will respond to the illumination; and (ii) quartz-halogen-tungsten filament lamps provide light rich in the blue end of the visible spectrum, so that there is a more limited response of fluorochromes, requiring other excitation wavelengths.

2. There must be filters between the lamp and the specimen. The first, when using UV light, is a (long-

wave) heat-absorbing filter. Then there is one filter or a combination of filters designed to pass the wavelength required for excitation and to absorb most other wavelengths. The combination may be narrow-band interference filters passing the blue (490-nm) rays capable of exciting fluorescein isothiocyanate.

3. There must be blocking or barrier filters between the objective and the eye. These have to absorb the exciting radiation, which may be a major component, except in the case of dark-field or epi-illumination systems. UV rays are damaging to the eyes, and a "stop" or barrier filter is an important protection. Also, longer (colored) wavelengths may be emitted by the specimen apart from those due specifically to the fluorochrome. Consequently, a suppression filter will assist in selecting the wavelength for the intended image.

1.6.1. Fluorescence Systems

The illumination systems for fluorescence microscopy take several forms: (i) transmitted light through a substage condenser; (ii) dark-field illumination through a specialized substage condenser; and (iii) incident illumination (epi-illumination) through a specialized objective, in which case the objective acts as both condenser and objective. The second and third systems have the advantage of an effective dark-field system, because the optical path is such that a direct beam does not go to the eye and there is minimal interference with the detection of the weaker fluorescent light from the specimen. In the third system, which is the best, the objective acts as the condenser and suffers no centration problem; therefore, the weak fluorescence suffers minimum attenuation from specimen thickness. The exciter beam is reflected down to the objective from a side or rear port in the microscope tube by a beam-splitting "mirror" or prism that reflects the exciting wavelength but transmits visible light back from the objective to the eye.

The cells or materials to be examined react with fluorochrome stains (e.g., xanthenes, acridines, and quinolines [see Chapter 2.1.4]), with substrates that release fluorochromes inside a cell (e.g., fluorescein diacetate), and with antibody conjugated to a fluorochrome (see Chapters 2.4.4 and 5.5.6) to allow localization or recognition.

The manner of using the microscope and its filters may be unique to the instrument, so consult the manual provided by the microscope manufacturer.

There is only a small light response (less than 1%) in fluorescent emission. Also, only a small proportion of the specimen usually fluoresces, and the responses of most fluorochromes tend to fade with time. In brief, the light level emitted is low. Therefore, it is more effective to use incident illumination (epi-illumination), if available, rather than transmitted light. Therefore, heed the following advice.

1. Work in a dark or well-screened room.
2. Do not use any unnecessary lenses or filters (no ground glass).
3. If there is an auxiliary lens, check the position that gives the most light. Open the condenser (aperture) diaphragm completely, but close the field diaphragm to the best position, as for bright-field microscopy (section 1.2.7).
4. Use eyepieces of low magnification (8×), because the intensity of fluorescence perceived decreases exponentially with the total magnification.
5. Use high-speed and high-contrast films for photomicrography, either color or black-and-white (see Chapter 28.3.3.1).

1.6.2. Confocal Scanning Microscopy

The elaborate equipment (2,16) required for confocal scanning microscopy, utilizing an intense beam of light from a laser, is designed to scan the sample by illuminating and imaging one very small area at a time in a single focal plane of the specimen. It is confocal because the scanning and the image are both attained through the objective. An epi-illumination system focuses a small spot of light at a plane in the specimen. This illuminated spot is imaged through a conjugate aperture, which accepts only the direct beams (but not the diffracted beams) for forming the image. The specimen is scanned through the objective by a moving beam producing a series of spots of light or aperture images. A raster scan allows the synthesis of a complete image in a detector system, and it is displayed on a monitor. Only structure that is in focus will form an image. This imaging system will operate either with direct imaging by visible light or with fluorescence imaging by UV light.

There are two major forms of the scanning equipment. One form involves tandem multiple-aperture arrays, and the other involves a regulated movement of a very fine laser beam. The principle is straightforward, but the equipment is very specialized and requires its own instruction manuals and expert users.

The advantages of this method of microscopy are that it can be used at high magnification with the best of epi-illumination objectives (including oil-immersion) to study large objects that scatter a lot of light in other modes of microscopy. It can focus on the structure of a surface and, particularly, can allow examination of structure within large cells. As far as bacteria are concerned, a major use is the study of the interactions of bacteria with or within eucaryotic cells.

Information is increased by using confocal scanning microscopy as a form of fluorescence microscopy; photomultiplier imaging reduces the problems with dim signals, and the confocal system evades fluorescent interference from features above and below the plane of focus. Because images can be generated at known depths in the specimen, a computer correlation of a stacked series of images generates three-dimensional information about the specimen.

1.7. CELL MEASUREMENT

It is important to determine the magnification imposed on bacterial cells either in projections for drawings or on photomicrographs. Equally important, a range of dimensions may be needed as part of experiments or for the description of cells for purposes of classification. Although a number of measuring techniques may be applied, they all must be based on a measurement standard provided by a stage micrometer; the use of the stage micrometer is clearly described in texts (11, 15).

1.7.1. Stage Micrometer

A stage micrometer is a slide on which a number of lines have been ruled by a grating engine or deposited photographically to show a precise scale. Usually, there are 10 parallel lines at 0.1 mm (100 μm) spacing and 10 parallel lines at 0.01 mm (10 μm) spacing. Image the micrometer slide with the same care as given to a specimen. Project the image on the ground glass of a photomicrographic camera for direct measurement, or record the image on film under the same conditions as for photomicrographs. A number of measurements of the appropriate intervals are then used to generate either the magnification on the film, the length required for a bar scale to apply to micrographs, or a set of measurements of the cells under study.

1.7.2. Eyepiece Graticule Micrometer

An eyepiece graticule micrometer is an optically flat glass disc, about 12 mm in diameter, with an arbitrary but accurately proportioned scale engraved on it. The scale is usually in numbered "units," each with 10 divisions. The graticule disc is placed in the plane of the intermediate image inside a Huygenian eyepiece, which has a diaphragm at the plane of the intermediate image where the graticule disc can be rested (access is obtained by unscrewing the top element of the eyepiece). In more complex eyepieces the diaphragm is below the lower field lens, and so there are fewer problems with parallax.

Each graticule must be calibrated for each eyepiece-objective combination to be used. With a stage micrometer and an appropriate set of ruled lines in focus, rotate the eyepiece so that the graticule scale is normal to the rulings and note a number of coincidence points. Then, it is easy to derive a statistical measurement of the graticule unit in micrometers to two decimal places. Final direct measurements against the real specimen should not be considered more precise than one decimal place.

There are graticules ruled in squares for counting cells (see Chapter 2.1.4) or in circles for rough sizing of cells. When counting, relate the squares to the specimen area and count the cells lying within the squares and touching two of the sides. In this application, it is advantageous to have a special eyepiece allowing focus on the grid rulings by rotation of the top lens.

1.7.3. Eyepiece Screw Micrometer

The eyepiece screw micrometer is a specialized eyepiece (sometimes called a filar micrometer) for direct measurements after calibration against the rulings of a stage micrometer. The units are on a wheel (with or without a vernier reading scale) to be read against a fixed mark, and the wheel drives a hairline across the field allowing a measurement in calibrated units by difference. As with an eyepiece graticule micrometer, the cursor line and the object must be in exact focus to minimize distortion by parallax.

1.8. PHOTOMICROGRAPHY

All types of light microscopy can make exact records on film whatever the wavelength of light being used. The technical requirements are important, in all cases, so that the most faithful record may be obtained. There are excellent books available that amplify and explain the requirements (3, 4, 6, 8, 13, 15, 18). Chapter 28 deals with all aspects of photography in bacteriology, including photomicrography (28.3.3).

Three types of photomicrography apparatus are in together with general use, as follows.

1. A roll-film camera back and shutter (usually for 35-mm film), together with an integral device that includes the ocular and a beam-splitting prism with a side viewing arm to allow focusing, forms the working unit. The whole device rests on the microscope tube.
2. Integral cameras have been developed by a number of microscope makers and come complete with a built-in light, an exposure meter, and timing controls. These cameras are usually adequate and produce effective routine photomicrographs.
3. An old-fashioned light-tight bellows is sometimes used. It is located on a stand on which a microscope can also be placed. A light-excluding sleeve is fitted at the bottom of the bellows and meshes with its mate, which slips onto the top of the microscope tube. A plate carrier is fitted at the top of the bellows and can be made for either cut film or instant (Polaroid) negative film.

In all cases the success of photomicrography and the resulting photographic print depends on:

1. A first-class specimen preparation, appropriately mounted and exactly focused.
2. Good optics, properly aligned and optimally (Koehler) illuminated (section 1.2.7) to give the best possible image, and a suitable choice of color filters (section 1.2.5).
3. A camera-microscope assembly that is free from vibration.
4. Appropriate photographic materials and processing technique appropriate to giving optimum grain size with adequate contrast and grey scale (see Chapter 28.2.2 and 28.3.3).
5. An appropriate final magnification, attained by printing with an enlarger.

The camera-microscope assembly can present some problems, because fixing photographic devices on the microscope tube tends to allow the transmission of vibrations. Partly, this is because pressing the shutter release removes the beam splitter before activating the shutter, and each action can generate persisting vibrations. When a shutter integral to the camera is used, it should be of the iris diaphragm type, and a focal-plane shutter must be avoided. Always use a cable release to actuate the shutter (see Chapter 28.3.3). Shutters for the timing of exposures are best put in the light path between lamp and microscope. Vibration is no problem with the bellows cameras as long as the light-tight collar around the eyepiece allows the camera and the microscope to be independent and not to touch each other. Vibrations must be kept to a minimum; a sturdy, heavy table that is not attached to a wall is a help.

Film for black-and-white photomicrography should be a panchromatic film of ASA 60 to 100 for general purposes (to keep the exposures at a manageable level). For phase-contrast photomicrography, a faster film (ASA 300 to 400) is needed because the light levels

are much lower. An exposure meter allows repetition of values established by test (see Chapter 28.2.1).

If an old bellows-type photomicrographic camera is available, acquire it because it is the best apparatus for the highest quality work, even though it is not the most convenient to use.

When exposing negatives and printing micrographs, think about the final magnification and the detail to be shown. Use film processing that enhances contrast with reasonably fine grain (see Chapter 28.2.2.4), as specified by the manufacturer. Grain in a developed negative dictates that you should not enlarge the film more than 2 to 2.5 times in printing. Good microscopy of most ordinary bacteria allows a total magnification of ×1,500, which is needed for fine detail and can be attained on the film (see section 1.6.1. for the determination of magnification). The larger images taken on a bigger area (4 by 5 in. [ca. 10 by 13 cm]) using cut film in an old-fashioned camera, for which enlargements of 1.25 to 1.5 times are usually sufficient, are better than higher enlargements of much smaller images on 35-mm film. If the micrographs are not as sharp and as good as they appeared in the properly adjusted microscope, try again. Focusing is critical, whatever the camera used. The trick with bellows cameras is to use a plain glass insert and to focus on the image obtained with a focusing magnifier adjusted to focus on a mark on the inside of the glass. The traditional ground glass and black hood can still be used effectively.

Prints should be enlarged to a format that allows easy visibility of the important detail, with the object occupying most of the frame.

1.9. REFERENCES

Books on light microscopy and photomicrography, ranging from encyclopedias to paperbacks, are readily available. However, books can give only basic principles. Satisfying microscopy is best learned through direct instruction and practical experience, not a small part of which is the art of preparing worthwhile specimens. No single book is directed toward microscopy for bacteriologists, but the principles are the same for all users. The list presented below is representative of the books available.

1. **Barer, R.** 1968. *Lecture Notes on the Use of the Microscope.* Blackwell Scientific Publications, Oxford.
   The advice of a master microscopist.
2. **Boyde, A.** 1990. Confocal optical microscopy, p. 185–204. In P. J. Duke and A. G. Michette (ed.), *Modern Microscopies.* Plenum Press, New York.
   A description of equipment and applications.
3. **Bradbury, S.** 1976. *The Optical Microscope in Biology.* Edward Arnold, London.
   A short paperback book that deals clearly with matters of resolution and modern forms of microscopy.
4. **Bradbury, S.** 1984. *An Introduction to the Optical Microscope.* Oxford University Press, Oxford.
   A short handbook with simple explanations of lens systems.
5. **Cargille, J. J.** 1975. *Immersion Oil and the Microscope.* Technical reprint 10-1051. R. P. Cargille Laboratories, Cedar Grove, N.J.
   Another excellent booklet produced by a manufacturer.
6. **Culling, C. F. A.** 1974. *Modern Microscopy—Elementary Theory and Practice.* Butterworth & Co., London.
   Another short paperback book.
7. **Eastman Kodak Co.** n.d. *Photography through the Microscope.* Eastman Kodak Co., Rochester, N.Y.
   An example of the booklets produced by manufacturers involved in aspects of microscopy. These booklets are obtainable from such firms and their agents in updated versions and are generally excellent.
8. **Engle, C. E. (ed.).** 1968. *Photography for the Scientist.* Academic Press, Inc., New York.
   General aspects of scientific photography, including photomicrography.
9. **James, J.** 1976. *Light Microscopic Techniques in Biology and Medicine.* Martinus Nijhoff Medical Division, Amsterdam.
   A fine book on theory and practice, with emphasis on the latter. It provides good advice on special and advanced techniques, including phase-contrast, interference, dark-field, polarization, and fluorescence microscopy.
10. **McKinney, R. M., and W. B. Cherry.** 1985. Immunofluorescence microscopy, p. 891–897. *In* E. H. Lennette, A. Balows, W. J. Hausler, and H. J. Shadomy (ed.), *Manual of Clinical Microbiology,* 4th ed. American Society for Microbiology, Washington, D.C.
11. **Mollring, F. K.** 1981. *Microscopy from the Very Beginning.* Carl Zeiss, Oberkochen, Germany.
    Another excellent booklet produced by a manufacturer.
12. **Quesnel, L. B.** 1971. Microscopy and micrometry, p. 1–103. *In* J. R. Norris and D. W. Ribbons (ed.), *Methods in Microbiology,* vol. 5A. Academic Press Ltd., London.
13. **Quesnel, L. B.** 1972. Photomicrography and macrophotography, p. 276–358. *In* J. R. Norris and D. W. Ribbons (ed.), *Methods in Microbiology,* vol. 7B. Academic Press Ltd., London.
    Quesnel's two articles are useful resources for optical details and practical advice on microscopy and photomicrography for microbiology in particular.
14. **Ross, K. F. A.** 1967. *Phase Contrast and Interference Microscopy for Cell Biologists.* Edward Arnold, London.
    An excellent explanation of the theory and practice of phase-contrast and interference microscopy. Also practical discussion of photographic techniques applied to microscopy.
15. **Shillaber, C. P.** 1944. *Photomicrography in Theory and Practice.* John Wiley & Sons, Inc., New York.
    Nothing is likely to replace this classic text, which deals exhaustively but readably with the properties of objective lenses, oculars, and condensers. It sets out the practice of good illumination, weighs the advantages of different mounting media, and deals with both theoretical and practical bench microscopy; however, it antedates phase microscopy.
16. **Shuman, H., J. M. Murray, and C. Di Lullo.** 1989. Confocal microscopy: an overview. BioTechniques 7:154–613.
    This review includes an example of three-dimensional reconstruction.
17. **Slayter, E. M.** 1970. *Optical Methods in Biology.* John Wiley & Sons, Inc., New York.
    A source book for the theoretical bases of most forms of microscopy and for analytical processes including diffraction, spectroscopy, and related optical techniques. It is concerned with principles and not practice.
18. **Smith, R. F.** 1990. *Microscopy and Photomicrography—a Working Manual.* CRC Press, Inc., Boca Raton, Fla.
    A professionally illustrated procedural manual with minimal theory. Useful for a beginner with no experience.
19. **Spencer, M.** 1982. *Fundamentals of Light Microscopy.* Cambridge University Press, Cambridge.
    A useful general survey.
20. **Wang, Y.-L., and D. L. Taylor (ed.).** 1989. *Fluorescence Microscopy of Living Cells in Culture. Part A. Fluorescent Analogs, Labelling Cells, and Basic Microscopy.* Academic Press, Inc., New York.
    A volume with helpful technical advice.