

Proper Alignment and Adjustment of the Light Microscope

Optical microscopes can be powerful tools in biomedical research and diagnosis if properly aligned and adjusted. This is essential for optimal image quality and accurate quantitative measurements. Video cameras, digital cameras, and electronic image processing can improve visibility of structural detail resolved by the microscope optics in comparison to viewing by eye. However, the quality and accuracy of the image still depends critically on proper microscope alignment and adjustment.

This unit presents protocols for alignment and adjustment of a typical research compound light microscope for transillumination and epi-illumination imaging modes typically used today in biomedical research. The transillumination light modes include bright-field, phase-contrast, and differential interference contrast (DIC). The primary epi-illumination mode is fluorescence microscopy.

The described procedures are for alignment of a research upright microscope (Fig. 4.1.1). The procedures are applicable to inverted microscopes that have similar imaging and illumination light paths to the upright microscope. In either case, the specimen image is produced by an objective lens and the image is projected either to the eye with an eyepiece or to a camera with (and sometimes without) a projection lens. One lamp attached to the back of the microscope provides light for transillumination of the specimen through a condenser lens (Fig. 4.1.2 and Fig. 4.1.3). Another lamp attached to the back of the microscope provides light for epi-illumination of the specimen through the objective using a mirror in a filter cube to bring the illuminating light into the objective light path (Fig. 4.1.4). For both the transillumination and epi-illumination paths, there are field diaphragms for controlling the specimen region illuminated and condenser diaphragms for controlling illumination from the condenser. The lamps, the field diaphragm, and the condenser diaphragm, as well as the condenser and the objective, must be properly focused and centered for the best image formation. In addition, each different mode of image formation has special optical components that also require alignment and adjustment for optimal performance.

The first section in this unit (see discussion of Major Components of the Light Microscope) describes the location and basic functions of the most important features of the upright compound microscope. The second section (see discussion of Basic Imaging and Köhler Illumination Light Paths For Bright-Field and Fluorescence Microscopy) introduces the imaging and illumination light paths of the microscope and describes the principles of Köhler illumination, which is the alignment used typically for both transillumination and epi-illumination in the light microscope. Basic Protocol 1 lists steps in microscope alignment for transmitted-light Köhler illumination. Basic Protocol 2 lists steps in microscope alignment for epifluorescence Köhler illumination. Basic Protocols 3 and 4 provide brief descriptions of the principles of image formation and microscope alignment for phase-contrast and differential interference contrast (DIC) microscopy; these optical modes produce contrast of transparent specimens using transmitted-light illumination. Support Protocol 1 deals with mating cameras to the microscope and Support Protocol 2 deals with calibrating image magnification. Several procedures (see Support Protocols 3, 4, and 5) are given for testing the optical performance of the microscope; these also describe test specimens for microscope performance and their sources. Finally, Support Protocol 6 deals with the care and cleaning of microscope optics. The Commentary refers the reader to references that provide more comprehensive

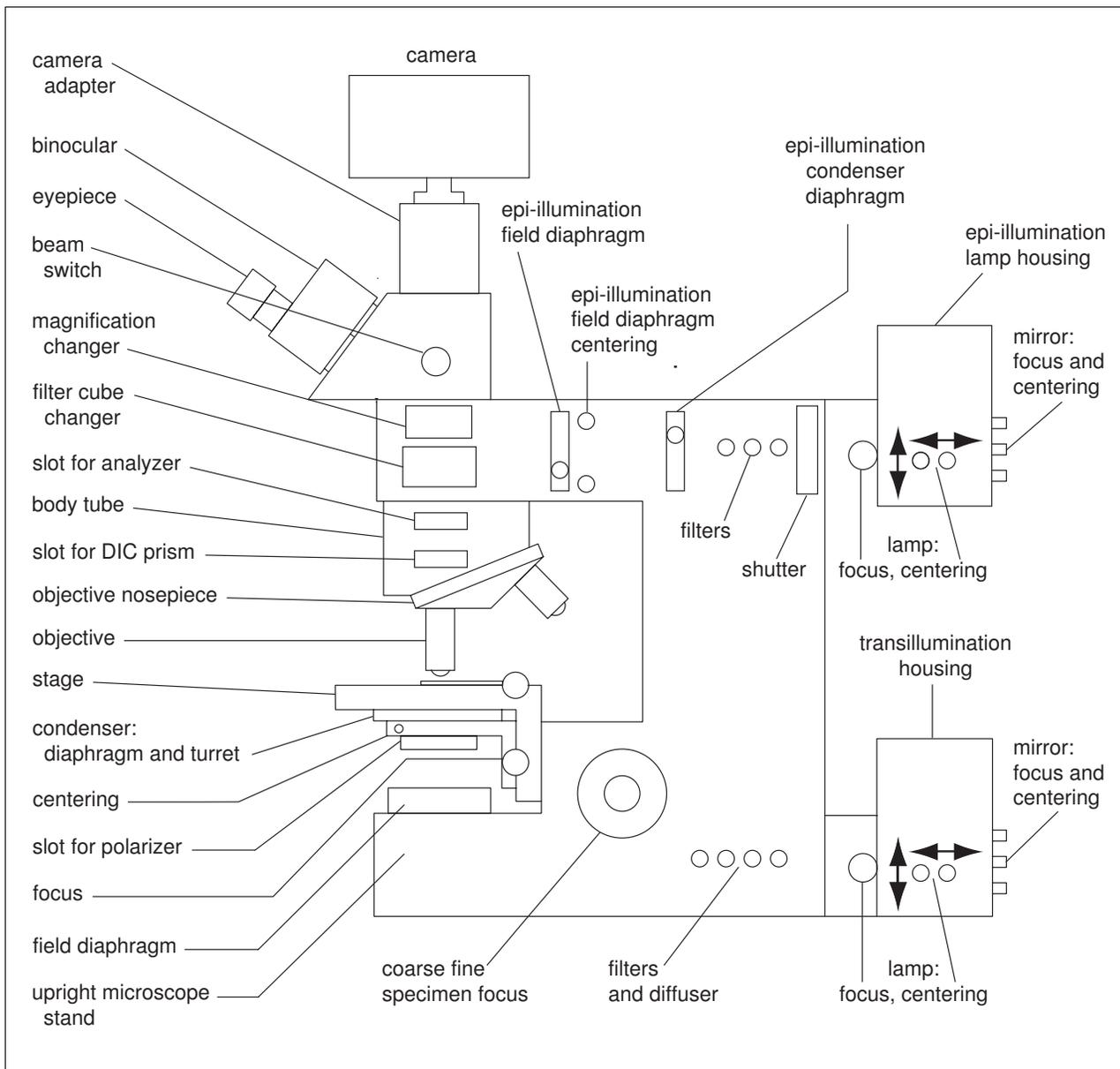


Figure 4.1.1 Diagram of the major component parts and centering screws for a research upright light microscope.

treatments of the basic modes and methods of light microscopy as well as advanced microscope imaging methods like multiwavelength, confocal, or multiphoton imaging, and electronic imaging techniques, including video and digital microscopy.

MAJOR COMPONENTS OF THE LIGHT MICROSCOPE

Familiarizing oneself with the components of the light microscope is best done using the manual for the microscope as reference. Figure 4.1.1 provides a diagram of an upright research compound light microscope equipped for both transmitted light and epifluorescence microscopy. Locate the following components on the microscope, identify their adjustment screws (if applicable) and movements (e.g., condenser focus), and ascertain that they appear correctly mounted. In this and the following sections, *z* is a direction along the microscope axis; *x* and *y* are perpendicular directions.

Image-Forming Components

Specimen stage and focus

A mechanical carrier on the stage holds the specimen slide. Knobs control movement in the x - y direction. Look for the vernier scales that mark the x and y positions. Rotatable stages are typically used for DIC microscopy. The objective is usually fixed and the specimen is focused by moving the stage along the z axis using coarse- and fine-focus knobs on the microscope body. Check the scale on the fine focus. On research microscopes, this is usually 1 $\mu\text{m}/\text{unit}$.

Objectives and revolving nosepiece

Examine the different objectives on the nosepiece. Each objective is usually labeled with the following designations: magnification (e.g., 60 \times); the degree of optical correction (Apochromat is better than Fluor which is better than an Achromat); the label Plan (if both the center and edges of the field are in focus); numerical aperture (NA, which measures the half-angle of the cone of light from the specimen accepted by the objective; Fig. 4.1.3); immersion medium to be used (e.g., air, oil, water, or glycerin between the objective front lens and the specimen); coverslip thickness; optical tube length (previously 160 mm, currently infinity); and other special features like phase contrast, DIC, or long working distance. Working distance refers to the distance from the front element of the objective to the specimen. Working distance usually decreases with increasing objective magnification and NA. Check that the objectives are mounted in series from the objective of the lowest magnification to the objective of the highest magnification. It is usually best to find the specimen or region of a specimen with a low-magnification objective, and then swing in objectives with higher magnifications and shorter working distances. The high-NA, short-working distance oil-immersion objectives often have the ability to lock up the nosepiece when changing objectives. This prevents running the front element of the objective into the mounting medium on the edges of the coverslip. Check that the nosepiece is down for imaging.

Specimen slides and coverslips

Microscope condenser lenses are usually corrected optically for 1-mm-thick glass slides. The objectives are corrected optically for coverslips with a thickness of 0.17 mm; no. 1.5 coverslips are on average this thick. The image quality decreases for thinner (e.g., no. 1) or thicker (e.g., no. 2) coverslips, particularly for non-oil immersion (“dry”) objectives with high NA. For oil-immersion objectives, this problem is not critical when the refractive index of the oil and coverslip (~ 1.515 to 1.52) match. Coverslips thinner than no. 1.5 are often used with oil immersion, to allow greater range of focus.

Body tube

In the modern research light microscope, the imaging light leaving the objective and nosepiece is unfocused and the image is projected to “infinity” (Fig. 4.1.2, left). This infinity space above the objective allows insertion of filters without changing the focal position of the image at the intermediate image plane. A positive focusing lens, the tube lens, above the infinity space, is used to bring the imaging light into focus for the eyepieces or camera.

Check for the following possible inserts in the infinity space of the microscope.

1. DIC prisms for the DIC objectives, which are usually right above the objective.
2. Analyzer; used for DIC microscopy (which should be removed from the light path for bright field, phase-contrast, and fluorescence microscopy).

3. Epi-illuminator filter-cube changer. These devices usually hold 2 to 4 filter cubes for fluorescence microscopy, each of which contains an excitation filter, an emission filter, and a dichroic mirror (Fig. 4.1.4) designed specifically for different fluorophores. Check the numbers on the filter cubes and mark the corresponding positions on the outside of the filter changer to identify the proper cube position for a given fluorophore.
4. Magnification changer and Bertrand lens. Body tube magnifications are selected from a turret, and possible values are 1.0 \times , 1.25 \times , 1.5 \times , and 2.0 \times . Often one position of the turret contains a Bertrand lens. This lens is used in combination with the eyepieces to produce a telescope view of the objective back focal plane (Fig. 4.1.2, far right). This is an important device for checking that the lamp image is centered and in focus during alignment for Köhler illumination, and for adjustment of the condenser diaphragm (see discussion of Transillumination Components).

Beam switch

This device switches the light between the binocular and the camera port. Check for the percentage of light in each direction. For fluorescence microscopy, it is important to be able to send 100% of the imaging light either to the eye or to the camera.

Eyepieces

Eyepiece magnification to the eye is marked on the barrel (e.g., 10 \times). Note that there are two and maybe three adjustments. The interpupillary distance for the eyes is adjusted by grasping the bases of the eyepiece tubes and moving them closer or further apart. At least one of the eyepieces is adjustable so that the eyes are parfocal.

Camera adapters

Check the type of camera adapter and whether it matches the detector. There are several different types of camera adapters. One type uses an eyepiece tube (not the binocular tube) and a projection eyepiece combined with a camera lens to project an image onto the camera detector. This method is common in photographic film cameras (which use ≥ 35 -mm film) and for some video cameras with large-size detectors (e.g., 1-in., equivalent to 2.54-cm). The recent video and cooled slow and progressive scan charge-coupled device (CCD) cameras have small detectors ($\leq \frac{2}{3}$ in., equivalent to 1.7 cm). These cameras require smaller projection magnifications or none—in the latter case the detector is mounted at the intermediate image plane, the focal plane of the objective (Fig. 4.1.2), and no eyepiece or projection lens is used.

Transillumination Components

Lamp and housing

The lamp is typically a low-voltage 100-W quartz halogen bulb with a tungsten-filament light source and variable control. Some lamp housings have no adjustments for centering the bulb; the socket is prefixed. Most lamp housings have x - y adjustments for the bulb socket, while an advanced lamp housing also has a mirror in the back for reflecting an image of the lamp back along the microscope axis. This mirror usually has adjustments for x , y , and z positions of the mirror image of the lamp. There is usually a knob on the side of the lamp housing for moving the collector lens back and forth along the z axis to focus the tungsten-filament image onto the condenser diaphragm plane.

Diffuser and filters

A diffuser and other filters are often inserted in slots in the base of the microscope. The diffuser helps spread the image of the source at the condenser diaphragm plane in order to uniformly fill the condenser aperture. This is important for achieving high resolution.

A heat-reflecting filter (e.g., BG 58) blocks infrared light. Video cameras, but not the eye, are often sensitive to this wavelength of light. A green filter is frequently preferred for illuminating living cells. High-efficiency interference filters with a 40-nm bandwidth around 540 nm are often best. Various neutral-density (ND) filters, which are not wavelength selective, are useful for attenuating light to cameras.

Field diaphragm

This is usually located just above or beneath the mirror that deflects the light up to the condenser lens. It controls the specimen area illuminated by the condenser (Fig. 4.1.2, left).

Polarizer

This is inserted below the condenser diaphragm for polarization and DIC microscopy. Otherwise, it is removed from the light path.

Condenser focus knob

This translator moves the condenser along the microscope z axis to focus an image of the field diaphragm on the specimen.

Condenser centering screws

There are usually two screws on the condenser carrier which move the condenser in an x - y plane. These screws are used to center the image of the field diaphragm on the z axis.

Condenser diaphragm

The condenser diaphragm is located on the bottom of the condenser. It controls the angle (NA) of the condenser cone of illumination of the specimen (Fig. 4.1.3).

Condenser turret

Condensers have turrets with inserts for special image-contrast techniques such as phase-contrast and DIC microscopy. Each insert matches certain objectives. In phase-contrast microscopy, the inserts are annuli of different diameters designed to match the phase ring in phase-contrast objectives (Fig. 4.1.5). For DIC, the inserts are typically DIC prisms designed for certain objectives (Fig. 4.1.7). To see these inserts, as well as the opening and closing of the condenser diaphragm, remove the condenser from the condenser carrier by loosening the locking screen and turning it upside down.

Condenser lens

The condenser's main function is to provide bright, uniform illumination of both the specimen field (Fig. 4.1.2, right) and objective aperture (Fig. 4.1.3) for objectives with different NAs. Check the markings on the condenser to see if it is designed for air (dry) or oil immersion with the glass slide. Dry (no oil-immersion) condensers have NA values of ≤ 0.9 and should not be used with immersion oil. Oil-immersion condensers usually have NA values of 1.0 to 1.4 when using immersion oil.

Epi-illumination Components

Lamp and housing

The light source for the epifluorescence illumination is typically an HBO 50-W or HBO 100-W mercury arc lamp or a xenon lamp of similar wattage. These lamps need to be handled with care because they can blow up if mistreated. Their glass envelopes should be carefully cleaned with 70% ethanol before installation to prevent fingerprints or other materials from inducing hot points that may result in fracturing of the bulbs. Look for the adjustment screws to adjust the x - y position of the lamp. Look also for the rear mirror

and its x , y , and z adjustment screws. Identify the focusing knob for the lamp collector lens. Do not turn the lamp on until the bulb has been installed according to the manufacturer's instructions, the collector lens has been inserted properly, and the lamp housing has been installed on the back of the microscope. These lamps produce intense light, and the mercury bulbs have large peaks in the UV range. Therefore, UV-protective glasses should always be worn when handling and installing these lamps. The light intensity decreases and the probability of explosion increases with the number of hours of operation and lamp starts. Check the timer on the lamp power supply to see that the recommended limits are not exceeded.

Shutter

Photobleaching is a big problem in fluorescence microscopy. A shutter is used to block the light from the specimen when not taking exposures on a camera or viewing by eye.

Filters

A heat-reflecting filter is used to prevent infrared illumination of the specimen and to keep infrared scattered light from reaching the camera. Neutral-density (ND) filters are used to reduce the light intensity of fluorescence illumination by the amount indicated on each filter.

Epi-illumination condenser diaphragm

This diaphragm provides variable adjustment of the illumination intensity. Some microscopes lack this diaphragm.

Epi-illumination field diaphragm

This diaphragm controls the area of the specimen illuminated. Look for the centering screws that control the x - y position of the field diaphragm.

Filter cubes

See discussion of Image-Forming Components.

BASIC IMAGING AND KÖHLER ILLUMINATION LIGHT PATHS FOR BRIGHT-FIELD AND FLUORESCENCE MICROSCOPY

The second major step in learning proper alignment of the light microscope is to understand the basic image-forming and Köhler illumination light paths and the functions of the key optical components and diaphragms. These are outlined in Figure 4.1.2, Figure 4.1.3, and Figure 4.1.4.

Imaging-Ray Paths

The upper left section of Figure 4.1.2 shows ray paths for the image-forming light from the objective. The objective, in combination with the tube lens, produces a real, magnified image of the specimen at the intermediate image plane. The eyepiece provides a second stage of magnification to the eye. The magnification to the eye is the product of objective magnification and eyepiece magnification multiplied by any magnification provided by additional lenses in the body tube of the microscope between the objective and the eyepieces.

When a camera detector—film, video, or charge coupled device (CCD)—is mounted at the intermediate image plane, the magnification to the camera is the magnification produced by the objective and body tube. When a camera is mounted above an eyepiece, then the unfocused light leaving the eyepiece must be focused onto the camera detector by a camera lens, which modifies the total magnification to the detector. Special adapters (see Image-Forming Components, Camera adapters) are also available for projecting the

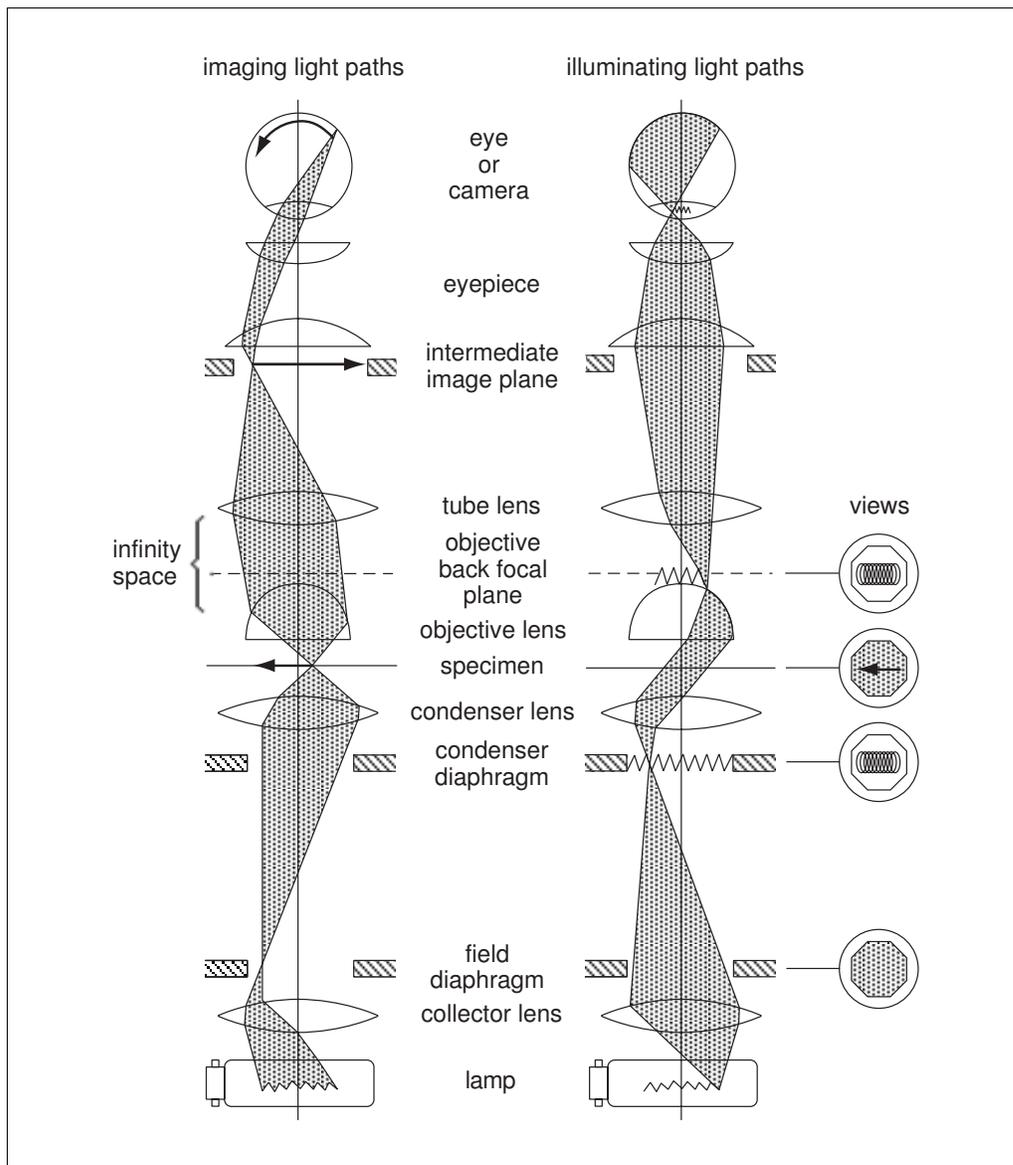


Figure 4.1.2 The imaging and illuminating light paths for a bright-field microscope aligned for transmitted light Köhler illumination. Modified from Keller (1998).

objective image onto a camera detector with magnification, but without the need for an eyepiece.

Transillumination Ray Paths

In the illustration of the standard Köhler method (Fig. 4.1.2), trace the illumination ray paths for a bright-field microscope aligned for Köhler transillumination (right side). A point on the light source is focused by the lamp collector lens onto the front focal plane of the condenser lens, where the condenser diaphragm is located. This light is projected, out of focus, through the specimen by the condenser lens, producing uniform illumination of the specimen. The objective collects the unfocused illumination light and brings an image of the light source into focus at its back focal plane, which is at the back aperture of the objective (Fig. 4.1.3). The light source is again out of focus at the intermediate image plane and at the retina of the eye or the detector of the camera. In between these two points, the light source is in focus at the exit pupil of the eyepiece, at a position ~15

mm above the eyepiece. This position is also called the eyepoint, the position of eye placement above the eyepiece (Fig. 4.1.2, right).

The condenser diaphragm controls the NA (cone angle) of specimen illumination by the condenser lens (Fig. 4.1.3). Opening the diaphragm increases the aperture of illumination, which increases both the light intensity and resolution in bright-field light microscopy (see Support Protocol 3). Note on the right side of Figure 4.1.2 that the image of the condenser diaphragm is in focus where the lamp image is in focus along the microscope axis, at the objective back focal plane and the exit pupil of the eyepiece.

Trace the imaging light rays from the field diaphragm through the microscope (Fig 4.1.2, left). Note that the field diaphragm controls the specimen area illuminated by the condenser. Note also that the field diaphragm is placed between the lamp collector lens and the condenser lens in a plane where the lamp image is out of focus (compare Fig. 4.1.2, left and right). When the condenser lens has focused the image of the field diaphragm onto the specimen, then the field diaphragm will be in focus with the specimen at the intermediate image plane and at the eye or detector.

Epi-illumination Ray Paths

Figure 4.1.4 shows the optical alignment for an epi-illuminator and Köhler illumination through the objective. As in transillumination (Fig. 4.1.2), the light source is focused by a collector lens onto the condenser diaphragm of a condenser lens in the light path. The condenser lens illuminates a field diaphragm. Another lens collects this light and projects it off a reflective mirror into the objective. The image of the light source is focused at the back focal plane of the objective so that it is out of focus at the specimen plane. In epifluorescence microscopy, filter cubes containing dichroic mirrors are used in combination with excitation and emission filters to efficiently reflect the excitation light into the objective and transmit to the eyepiece or camera only the longer-wavelength fluorescent light emitted from the specimen (see *UNIT 4.2* and Taylor and Salmon, 1989).

BASIC PROTOCOL 1

ALIGNMENT FOR KÖHLER ILLUMINATION IN BRIGHT-FIELD, TRANSMITTED LIGHT MICROSCOPY

The following procedures assume that the positions of the quartz-halogen tungsten filament and collector lens are adjustable. Inexpensive, non-research compound microscopes may have the illuminator built into the stand, with no adjustment for the lamp. Such microscopes depend on a ground-glass filter for even illumination. For these microscope stands, skip steps 2, 3, 4, and 8.

Focus the light source

1. If possible, remove the diffusion filter in the transillumination pathway during alignment so that a crisp image of the light source can be viewed at the condenser diaphragm plane and at the objective back focal plane. When alignment is complete, reinsert the diffusion filter.
2. Center and focus the lamp filament near the condenser diaphragm plane. Remove the condenser lens. Place a sheet of lens paper on the microscope stage. Close down the field diaphragm and adjust the intensity of the lamp so that the lens paper is moderately illuminated. Use the lamp-focusing knob to move the condenser lens (or lamp) along the z axis until an image of the filament is in focus on the lens paper. Roughly center the lamp on the microscope axis with the adjustment screws on the lamp housing, then adjust the mirror image (if there is a mirror) using its adjustment screws on the lamp housing.

Table 4.1.1 Suppliers for Items Used In Light Microscopy

Test specimen	Supplier
Hematoxylin/eosin-stained skeletal muscle	Carolina Biological Supply
Diatom test plate	Carolina Biological Supply
Squamous cheek cells	Freshly prepared (see Support Protocol 4)
Stage micrometer	Fisher
Red, green, and blue fluorescent tissue culture cells	Molecular Probes

- Place the lens paper on the condenser carrier and raise the condenser carrier until it is ~20 mm from the top of the stage.

This is the approximate position of the condenser diaphragm when a condenser is installed and in focus.

- Refocus the lamp and mirror images at this position of the lens paper.

If there is no mirror image, center the lamp image. If there is a mirror image, then position the images so that they sit side by side to fill the condenser aperture. Remove the lens paper and replace the condenser.

Focus for low-power viewing

- Obtain an in-focus image of a specimen with the low-power objective (10× or 16×) by placing a test specimen that absorbs light on the stage (e.g., stained muscle section; see Table 4.1.1).

The focus position can be estimated from the working distance of the objective lens. For a standard 10× lens, this is ~4 mm. So, use the coarse focus to bring the specimen to ~16 mm from the coverslip by viewing the objective position from the side of the microscope.

- Move the condenser lens up close to the lower surface of the slide and open the field and condenser diaphragms all the way. Look down the eyepieces, and use the coarse- and fine-focus knobs to bring the specimen into sharp focus.
- Focus the image of the field diaphragm centered on the specimen by adjusting the condenser *x*-*y* screws and the condenser focus. Initially close down the field diaphragm until an edge of the image can be focused by the condenser, then close the field diaphragm further as the image is centered, using the *x*-*y* translation screws.

Usually, the field diaphragm is opened just enough to match the field of view in the eyepiece or the camera. However, the best image contrast is obtained if the field diaphragm is opened just enough to illuminate the region of interest. This eliminates the presence of scattered light from outside the region of interest.

- Center an in-focus image of the lamp and mirror images at the objective back focal plane using the focus and adjustment screws on the lamp housing. View the objective back aperture (the position of the back focal plane; Fig. 4.1.3) by using the Bertrand lens in the magnification changer in combination with the eyepieces, by replacing one eyepiece with a telescope, or by simply removing one eyepiece and peering down the body tube. Open the condenser diaphragm all the way. Readjust the collector lens and the mirror so that the lamp images are in focus and sit side by side to fill the objective aperture.

This is a rough adjustment with the low-power objective and will need to be fine-tuned using the objective with the highest NA.

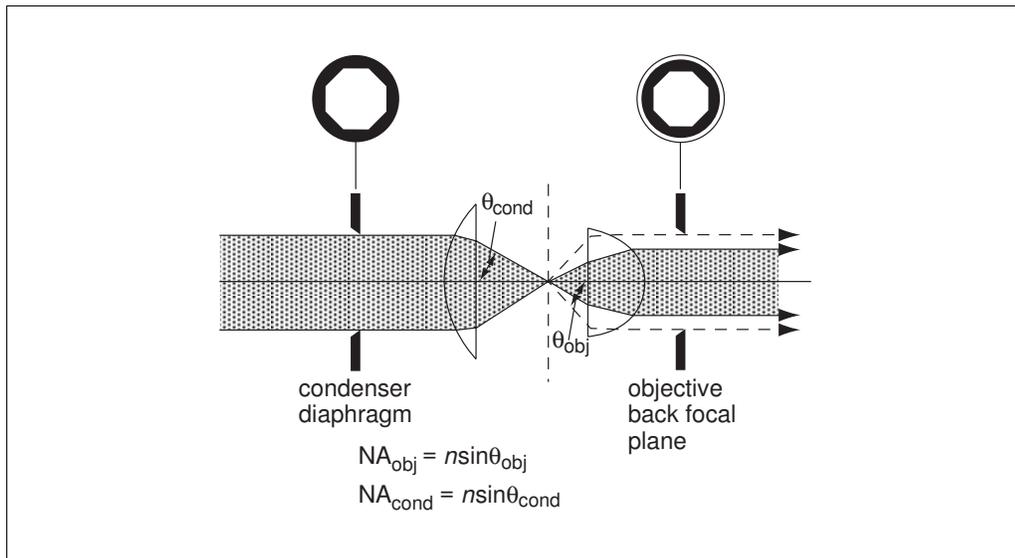


Figure 4.1.3 Numerical aperture (NA) of objective light collection and condenser illumination. The objective numerical aperture (NA_{obj}) depends on the angle of the cone of light from the specimen, which is accepted by the objective aperture while the numerical aperture of condenser illumination (NA_{cond}) is controlled by the condenser diaphragm and limited by the maximum NA of the condenser when the condenser diaphragm is wide open.

9. Adjust the opening of the condenser diaphragm so that the diameter of its image at the objective back focal plane (see far right of Fig. 4.1.2; also see Fig. 4.1.3) is slightly less than the diameter of the objective back aperture.

Some research condensers also have centering adjustments for the condenser diaphragms. The image of the condenser diaphragm should be centered in the objective back aperture.

10. Adjust the light intensity for comfortable viewing of the specimen by adjustment of the light-source intensity, either using the power-supply rheostat or by inserting neutral-density filters in the illumination light path.

The microscope is now adjusted properly for the low-power objective.

Adjust for 40× viewing

11. Switch the turret to the 40× high-dry objective.

Note that because of parfocal objective design the specimen is almost in focus. Focus on the specimen with the fine focus.

12. Focus and center the field diaphragm with the condenser focus and the condenser-carrier *x*-*y* adjustment screws.

13. View the objective back aperture (Fig. 4.1.3).

Notice that the image of the condenser diaphragm adjusted for the low-power objective is only about half the diameter of the 40× back aperture. The ratio of the diameter of this image to the diameter of the objective back aperture is equal to the ratio of the NA of the condenser illumination to the NA of the objective (Fig. 4.1.3). When the condenser illumination NA equals the objective NA, the aperture of the objective is filled with light and maximum resolution will be achieved for the objective NA (see Commentary).

14. Adjust the condenser diaphragm so that the condenser illumination NA just about matches the objective NA.

Each time objectives are changed, these procedures should be repeated.

Adjust for high-power viewing

15. Rotate the nosepiece so that the high power (60× to 100×, NA 1.25 to 1.4) oil-immersion lens is coming into place, but stop before it clicks into place. Place a small drop (~2 mm diameter) of immersion oil directly onto the coverslip above the point upon which the condenser beam is focused.

This oil drop must be free of air bubbles and dirt. The refractive index of the immersion oil should be close to that of the glass coverslip.

16. Complete the rotation of the nosepiece so that the oil-immersion objective clicks into place. Lower the objective nosepiece if appropriate.

The space between its front lens and the coverslip is now filled with immersion oil.

IMPORTANT NOTE: *Use only the fine adjustment knob when working with the oil-immersion lenses.*

17. Remove the eyepiece (or use the telescope or Bertrand lens) and inspect the back aperture of the objective. Open the condenser diaphragm as much as possible to try and match its image with the objective aperture (Fig. 4.1.3).

This will be impossible if the condenser is dry and not designed for oil immersion, since dry condensers have $NA \leq 0.9$ and the relative size of the image of the condenser aperture or condenser diaphragm seen in the objective back focal plane is given by the ratio of NA_{cond}/NA_{obj} (Fig. 4.1.3).

18. View the objective back aperture and tune up the focus and position of the lamp images so that they fill the objective aperture with light as uniformly as possible.

19. Replace the eyepiece (or remove the Bertrand lens) and examine the specimen. Adjust the field diaphragm until its margins just match the field of view.

With inexpensive condensers, the image of the edge of the field diaphragm will not be in good focus, even at the optimum position. If a condenser designed for oil immersion is being used, enormous improvement in the field iris image can be achieved by oil immersion.

20. Oil the condenser (if possible) to achieve the highest resolution and image quality. To oil a condenser, rotate the objective out of the way, remove the slide, oil the condenser (it takes much more air-bubble-free oil than the objective), then replace the slide and refocus the objective.

Before oiling the condenser, make sure it is not designed to be a “dry” condenser by checking that it is marked with an $NA > 0.9$.

The image of the field diaphragm should now be much sharper when in focus and centered.

21. Look at the objective back aperture. Again, tune up the focus and position of the lamp images so that they fill the objective aperture with light as uniformly as possible.

After oiling, the aperture should be filled by the condenser illumination when the condenser diaphragm is wide open.

Notice also that viewing the objective back aperture is the best way to see if air bubbles have become trapped in the oil. If so, carefully wipe off the oil with lens paper and repeat steps 15 through 21.

22. Reinsert the diffusion glass in the illumination light path.

This will reduce light intensity, but make the illumination of the objective aperture more uniform.

23. When finished for the day, use lens paper to remove excess oil from the objective and condenser lens surfaces to prevent dripping on specimens.

It is not necessary, however, to completely clean oil from the optics after every use (see Support Protocol 6).

ALIGNMENT OF THE EYEPIECES

The binocular usually has adjustments for the inter-eyepiece distance and visual acuity in each eye. The goal is for the image to be in focus for each eye without any eyestrain or discomfort. Only the left eyepiece tube or eyepiece is adjustable on a typical microscope. However, if the microscope has a target reticle in the microscope stand that can be rotated into view, then usually both eyepieces are adjustable.

1. With a low-power objective (10× or 16×), focus on a stained specimen placed on the stage of the microscope (e.g., stained muscle section, see Table 4.1.1) and align the microscope for Köhler illumination.
2. Move the bases of the eyepiece tubes together or apart to set the proper interpupillary distance for your eyes.
- 3a. *If the microscope has a target reticle:* Rotate the reticle into the field of view. Close the left eye and use the diopter-adjustment ring on the right eyepiece until the target is in sharp focus for the right eye. Then close the right eye and use the diopter-adjustment ring on the left eyepiece until the target is in sharp focus for the left eye. Remove the target to view the specimen.
- 3b. *If the microscope has no target reticle:* Set the right eyepiece tube to the inter-eyepiece setting (if possible). Close the left eye and bring into sharp focus a structural detail near the center of the field of view with the right eye. Close the right eye and use the diopter-adjustment ring on the left eyepiece tube to bring the specimen detail into sharp focus for the left eye. With both eyes open, fine tune the focus of the left eyepiece tube.

ALIGNMENT FOR KÖHLER ILLUMINATION IN EPIFLUORESCENCE MICROSCOPY

The eye is most sensitive to green light. Hence, the following protocol is best done using a filter cube that produces green excitation light (e.g., a filter cube for rhodamine).

Focus the lamp

1. Remove one objective, and rotate the nosepiece so the open position is centered on the microscope axis. Place a white card on the microscope stage, on top of supports that position the card at ~2 to 3 cm from the nosepiece.

This is approximately the position of the objective back focal plane, where the images of the light source should be in focus and centered after alignment (Fig. 4.1.4).

Some microscopes have a special device for lamp alignment. It screws into the objective nosepiece and projects an image of the epi-illumination arc and electrodes onto a small diffusion screen within the barrel of the device.

2. Install the mercury bulb in the lamp housing and attach the lamp housing on the back of the microscope according to the manufacturer's instructions.
3. Turn on the power source and ignite the lamp.

It takes ~10 min for the arc to brighten.

CAUTION: It is usually a good idea to make sure computers in the vicinity are turned off before starting the lamp because the high voltage pulse used to ignite the arc plasma may damage the electronics. Laboratory personnel should protect their eyes against UV light from the lamp.

4. Close down the field diaphragm and open the condenser diaphragm (if there is one) all the way.

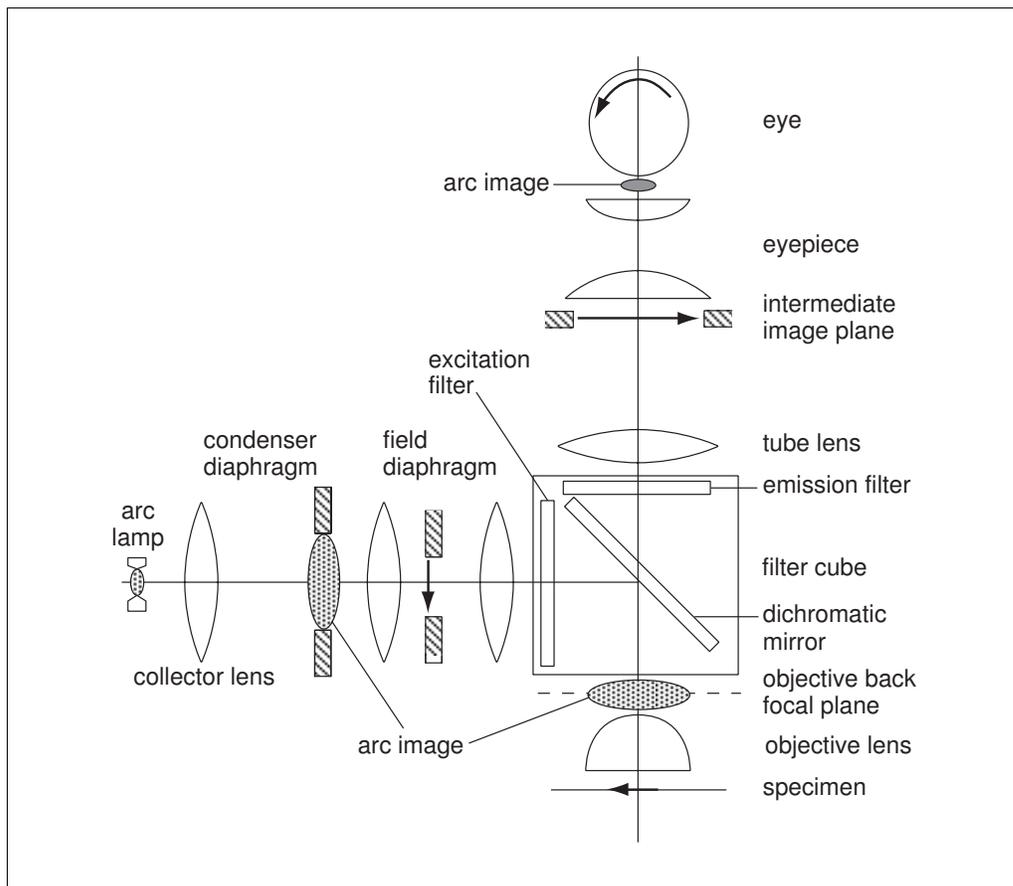


Figure 4.1.4 Microscope alignment for epifluorescence Köhler illumination.

5. On the diffusion glass screen or on the white card on the stage, the image of the arc between the tips of the two electrodes should be visible. Use the lamp focusing knob to obtain an in-focus image of the lamp arc and electrodes. Use the lamp x - y adjustment screws to approximately center the image of the arc and electrode tips (see Figure 4.1.2).
6. If there is a lamp mirror, use its adjustment screws to focus the mirror image of the arc and electrode tips, centered on the target.

Often the primary image and the mirror image of the arc are adjusted side-by-side and slightly overlapping, but centered on the microscope axis.

Focus the field diaphragm

7. Place a test fluorescent specimen (see Table 4.1.1) on the stage and rotate the objective nosepiece to a low-power objective. Select the appropriate filter cube for the fluorescent specimen. Open up the epi-illuminator field diaphragm all the way. Open the shutter and view the specimen.
8. Focus the specimen, then close down the field diaphragm until an edge comes into the field of view. Use the x - y adjustment screws for the field diaphragm to center the field diaphragm image within the field of view.
9. Close down the field diaphragm until only the region of interest is illuminated.

This prevents photobleaching of areas outside the region of interest and reduces the amount of scattered fluorescent light in the image from outside the region of interest.

**BASIC
PROTOCOL 4**

10. Switch to a high-power objective. For an oil-immersion objective, place a small drop (~2 mm diameter) of immersion oil directly onto the coverslip above the region of interest. Carefully swing in the objective. Open the shutter and focus on the specimen. Readjust the size and centration of the field diaphragm.

This oil drop must be free of air bubbles and dirt.

11. To prevent photobleaching, be sure to close the epi-illumination shutter when not viewing or taking camera exposures.

ALIGNMENT FOR PHASE-CONTRAST MICROSCOPY

Phase-contrast microscopy is often used to produce contrast for transparent, non-light-absorbing, biological specimens. The technique was discovered by Zernike, in 1942, who received the Nobel prize for his achievement (Zernike, 1942, 1955, 1958). The last of these references describes the principles of phase contrast and provides an excellent introduction to the wave optics of image formation, resolution, and contrast in the microscope.

The phase-contrast microscope is a bright-field light microscope with the addition of special phase-contrast objectives (Fig. 4.1.5) containing a phase plate or ring and a condenser annulus instead of a diaphragm; the annulus is usually located on a condenser turret because it has to be selected for different objectives. The microscope optics are usually aligned for bright-field specimen illumination by the standard Köhler method. However, there is no condenser diaphragm to adjust. Instead, the phase annulus must be selected and adjusted properly. Modern phase-contrast objectives have a phase plate containing a ring in the back focal plane within the barrel of the objective. This ring

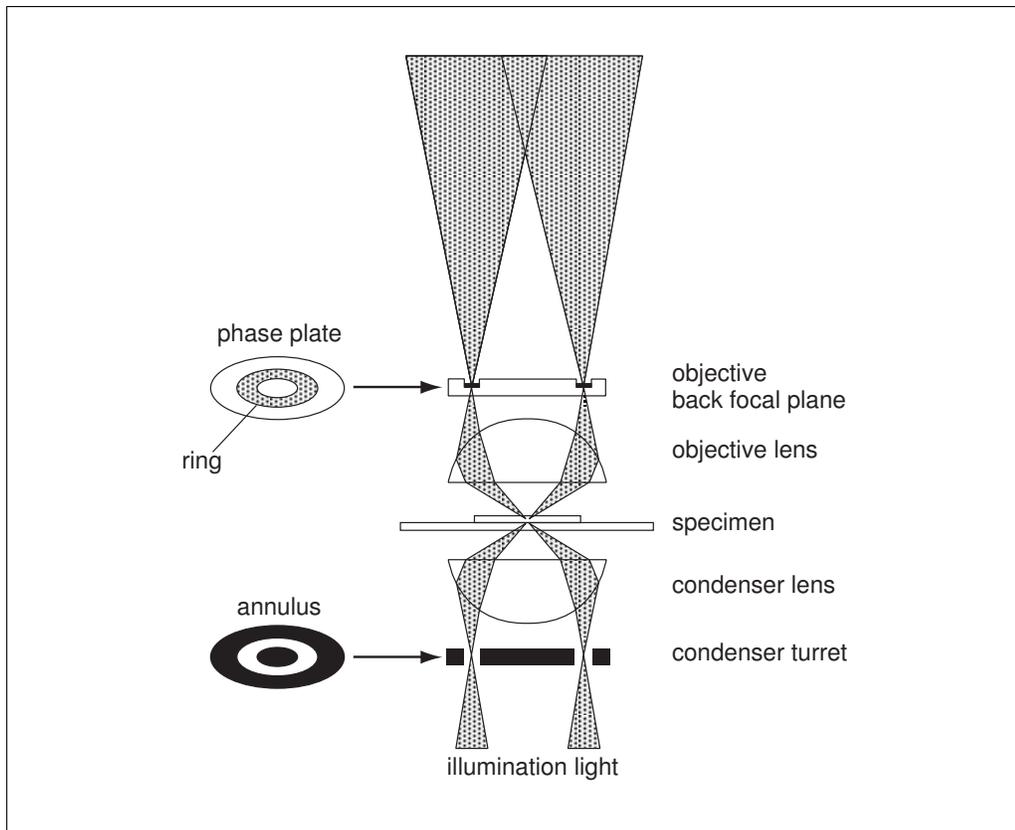


Figure 4.1.5 Illumination light path through the condenser annulus and objective phase ring in a microscope aligned for phase-contrast microscopy.

absorbs and advances the phase of the light passing through it by $\frac{1}{4}$ wavelength in comparison to light passing through the rest of the objective aperture. For each phase objective, there is a corresponding annulus in the condenser turret that has about the same relative size as the phase ring in the objective. Light passing through this annulus passes through the phase ring in the objective when the annulus is selected and aligned properly. Light scattered by the specimen mainly passes through the objective aperture outside the phase ring. Light scattered from a thin transparent specimen is $\sim\frac{1}{4}$ wavelength retarded from the unscattered illumination light. The additional $\frac{1}{4}$ wavelength retardation between the scattered and illumination light produced by the objective phase ring makes the scattered and illumination light 180° out of phase. They destructively interfere with each other at the image plane to produce the “dark” contrast of structural detail typical of phase contrast images of biological specimens.

Because the phase annulus and the phase ring reduce the intensity of the background light, a bright illuminator—e.g., a 100-W quartz-halogen illuminator—is necessary at high magnifications (because image intensity decreases as $1/\text{magnification}^2$). For living cells, heat reflection and green illumination filters should be used.

To examine the objective phase ring and matching condenser phase annulus, remove the low- and medium-power phase objectives and the phase condenser from the microscope. View the phase ring (phase plate) within the objective by looking in the back end. The phase ring is located at the position of the objective back focal plane; its diameter is usually $\sim\frac{2}{3}$ that of the objective aperture. Note that it is situated within the objective body and is visible because it absorbs light. Locate the annulus for each objective in the condenser turret. The annulus is located at the condenser diaphragm plane, which is situated at the condenser front focal plane. Notice that as the objective NA increases, the diameter of the corresponding annulus in the condenser turret increases. The NA of condenser illumination from an annulus is designed to match that of the phase ring in the corresponding objective.

Replace the condenser on the microscope and illuminate with white light. Hold a piece of tissue paper near the top of the condenser and examine the illumination cone. Notice that it is an annular cone of illumination. Change the annulus from the setting for the low-power objective to the $40\times$ setting. Note that the NA or angle of illumination increases. Points of illumination further from the central axis in the condenser diaphragm plane produce higher-aperture rays passing through the specimen.

Perform alignment using the following steps.

1. Align the microscope for bright-field Köhler illumination using the low-power phase objective ($10\times$ or $16\times$) and a cheek cell preparation for the specimen (see Support Protocol 4).

The cheek cell specimen is an excellent transparent test specimen for alignment of the microscope for phase contrast and DIC. The cheek cells are transparent and only barely visible by bright-field illumination. To find the plane of focus in bright field, initially close down the condenser diaphragm and look for the edges of air bubbles in the preparation. These edges scatter much light and appear dark in the image.

2. Align the condenser annulus with the phase ring by rotating the condenser turret to the position where an annulus matches the phase ring in the objective; use the telescope, your eye, or the Bertrand lens to view the objective back aperture.

As the condenser turret is rotated, notice in the objective back focal plane the images of the different-diameter annuli designed for the different numerical aperture objectives. The correct annulus may not be properly aligned with the objective phase ring.

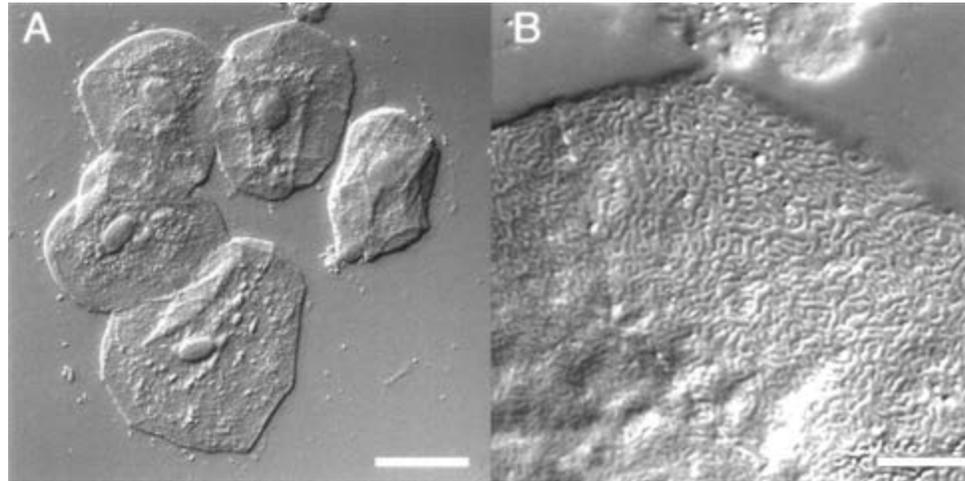


Figure 4.1.6 DIC images of a human cheek cell test specimen. **(A)** Low magnification of cheek cell preparation with a 20× objective. Bar = 20 μm. **(B)** High-resolution image of the surface of the cell at the top of (A) using a 60×/(NA = 1.4) Plan Apochromat objective and matching condenser illumination. The ridges on the cell surface are often diffraction limited in width. Bar = 5 μm. From Salmon and Tran (1998), reprinted with permission from Academic Press.

- Use the adjustment screws in the condenser (and the special tools if necessary) to move the annulus in the x - y plane to achieve alignment with the phase ring in the objective.

Note that the phase ring is slightly wider than the image of the correct annulus. It is critical that the image of the annulus be within the phase ring, but it is not critical if it is very slightly off center.

- When the objective phase ring and condenser annulus are aligned, view the specimen and properly adjust the focus and centration of the field diaphragm.

Notice that if the phase ring and annulus are slightly misaligned (rotate the turret slightly), the background light intensity goes up. This is because the phase ring is designed to absorb much of the illumination light. The unscattered illumination light becomes closer to the intensity of the light scattered by the specimen, which passes through the objective aperture outside of the phase ring. Minimizing the background light intensity while viewing the specimen image can also be used to align the annulus with its phase ring or to touch up the alignment done by viewing down the body tube.

- Switch to the 40× phase objective and then the high-power 60× or 100× phase objective (NA 1.25 to 1.4) and repeat steps 1 to 4.

It is necessary to increase light intensity at the higher magnifications. It should be possible to see the ridges on the cell surface (see Fig. 4.1.6 for a DIC image of the ridges). Notice the “phase halos” at discrete edges, a problem that limits conventional phase contrast in high-resolution imaging.

BASIC PROTOCOL 5

Proper Alignment and Adjustment of the Light Microscope

4.1.16

ALIGNMENT FOR DIC MICROSCOPY

Since its introduction in the late 1960s (e.g., Allen et al., 1969), DIC microscopy has been popular in biomedical research because it highlights edges of specimen structural detail, provides high-resolution optical sections of thick specimens—including tissue cells, eggs, and embryos—and does not suffer from the “phase halos” typical of phase-contrast images. See Salmon and Tran (1998) for details of image formation and video-contrast enhancement.

The DIC microscope is a bright-field light microscope with the addition of the following elements (Fig. 4.1.7, middle): a polarizer beneath the condenser; a DIC beam-splitting

prism (Nomarski or Wollaston) in the condenser turret; a DIC beam-combining prism (Nomarski) just above the objective; an analyzer above the objective prism in the infinity body tube space; a compensator after the polarizer or before the analyzer in some microscopes (not shown in Fig. 4.1.7); and a rotatable stage. The microscope optics are usually aligned for bright-field specimen illumination by the standard Köhler method.

The polarizer, which produces plane-polarized light (Fig. 4.1.7, left), is typically oriented with its transmission azimuth in an east-west direction facing the front of the microscope. Polarizers with high transmission efficiency are preferred. Polarizers are usually high-quality polaroid material held between thin optical glass flats. Another polarizer is used as an analyzer. The transmission azimuth of the analyzer is oriented north-south at 90° with respect to the polarizer azimuth to produce extinction of the illumination light in the absence of the DIC prisms.

The specimen is held on a rotating stage. Contrast depends on orientation, and frequently specimens must be reoriented to achieve maximum contrast of the structures of interest. Either the stage or the objective and condenser must be centerable.

For the highest sensitivity, the objective and condenser lenses should be selected free of birefringence (Inoué and Spring, 1997). Rectifiers can be used to correct for the rotation of the plane of polarization of light which occurs at the periphery of lens surfaces (Inoué and Spring, 1997).

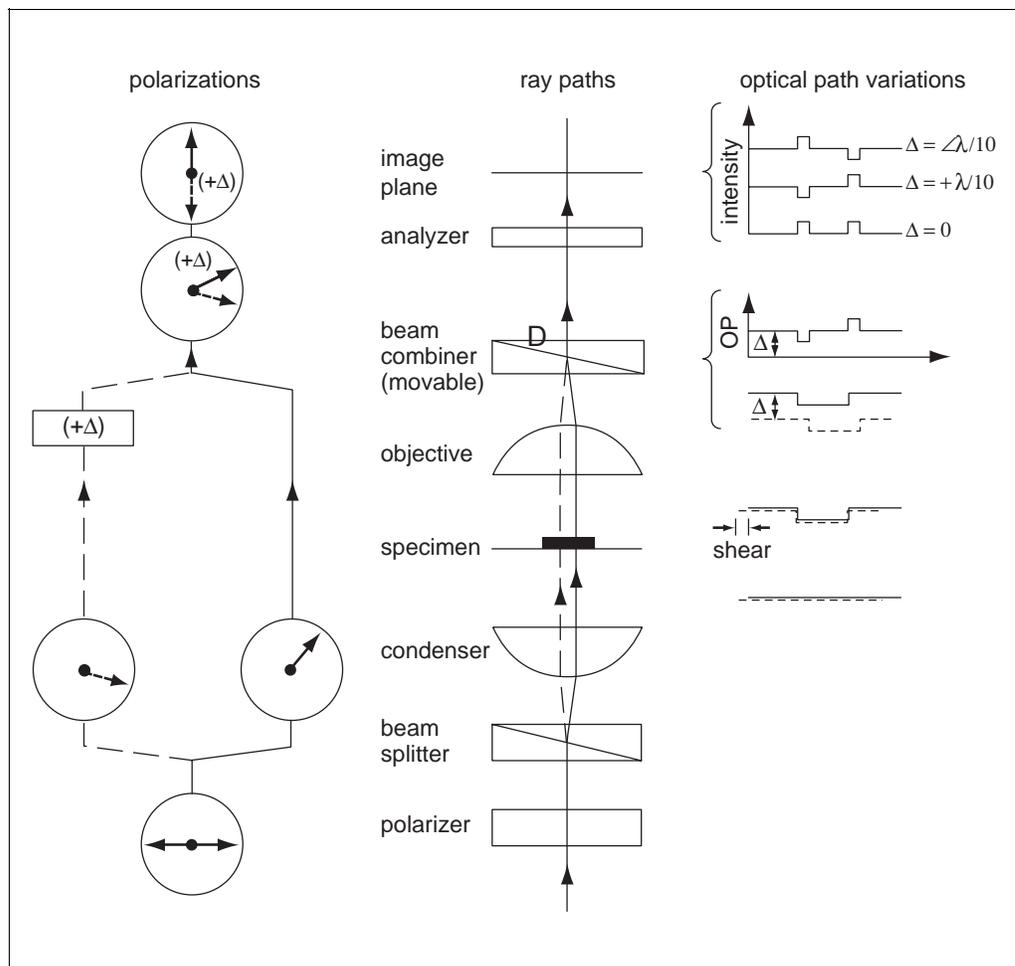


Figure 4.1.7 The optical system for DIC microscopy. From Salmon and Tran (1998), reprinted with permission from Academic Press.

Figure 4.1.7 outlines the principles of DIC image formation and contrast. The DIC beam-splitting prisms are located at the condenser diaphragm plane, just above the polarizer in the condenser turret. Objectives of different magnification and/or NA usually require condenser prisms of different optical characteristics.

The condenser prism splits the light coming from the polarizer into divergent polarized light wavefronts whose planes of polarization are orthogonal to each other and at 45° with respect to the initial plane of polarization (Fig. 4.1.7, left). The divergent beams are converted by the condenser into two wavefronts, which pass through the specimen separated laterally from each other in the direction of the prism wedge (the shear direction, Fig. 4.1.7, middle) by a tiny amount that is usually less than the resolution limit of the condenser-objective lens combination (Fig. 4.1.7, right). These two wavefronts are recombined just above the objective by a beam-combining prism. Often, each objective has its own prism so that it accurately matches the action of the condenser prism. In some microscopes, there is one beam-combining prism for all objectives and a different condenser prism for each objective. Check which is the case for your microscope.

DIC image contrast depends on the “compensation” or “bias retardation” (Δ) between the two wavefronts along the microscope axis (Fig. 4.1.7, right). When the objective beam-combining prism is perfectly aligned with the condenser beam-splitting prism and there is no compensation ($\Delta = 0$), the background light is extinguished and the edges of objects are bright against a black background (Fig. 4.1.7, upper right). When one wavefront is retarded relative to the other by Δ , this increases the optical path (OP) between the wavefronts (Fig. 4.1.7, middle right) and brightens the background light. One edge of an object becomes brighter than the background while the opposite edge becomes darker (Fig. 4.1.7, upper right). This produces the “shadow cast” appearance of DIC images. Reversing the sign of retardation, reverses the contrast of the specimen edges.

In some microscopes, the objective beam-combining prism (Fig. 4.1.7, middle) is used as a compensator by translating the prism in the direction of prism wedge away from the position of background light extinction. One direction produces positive while the other produces negative retardation (Δ). In other microscopes, both the objective and condenser prisms are fixed at positions that produce background light extinction, and typically a deSenarmont compensator (a birefringent quarter-wave retarder in combination with a rotatable polarizer or analyzer; Inoué and Spring, 1997) is inserted just above the polarizer or beneath the analyzer.

Bright illumination sources are required for high magnification because of the crossed polarizer and analyzer; at least the 100-W quartz-halogen illuminator is usually needed.

To examine the DIC prisms, remove the objective prisms and the DIC condenser from the microscope. Examine the objective prism. Notice that it is very thin and wide enough to cover the objective aperture. Also notice if there is a screw that can be used to translate the prism back and forth in one direction across the objective aperture. Examine the prisms in the condenser turret. Notice that there are different prisms for low- and high-NA objectives. The highest-resolution (NA) objectives often have special prisms designed only for the optical properties of that objective. In aligning the microscope for DIC, be careful to use the condenser Wollaston prism that matches the objective in use. Reinstall the condenser.

Steps 1 to 6 in the following procedure are for the initial alignment of the microscope. Once this is done, then begin at step 7 for routine use.

Perform initial alignment

1. Align the microscope initially for Köhler illumination using the low-power objective and the cheek cell preparation. Again look for the bright air-bubbles in the preparation to find the specimen plane.
2. Check that the polarizer is oriented with its transmission azimuth in an east-west direction as determined facing the front of the microscope (look for the line or double-headed arrow on the polarizer mount). Rotate the deSenarmount compensator (if there is one) so that it is at its zero position (no compensation).
3. Remove the objective prism and rotate the condenser turret to an open position. Make sure that the analyzer transmission azimuth is crossed to the polarizer by checking that the background light is at extinction.

This exercise is best done with the brightest light position of a 100-W quartz-halogen illuminator.

4. Insert the objective DIC prism (observe caution as the image will be very bright). Observe the extinction fringe in the middle of the prism (you must use the telescope, remove an eyepiece, or insert the Bertrand lens to focus on the objective focal plane). Make a drawing of your view of the fringe in the back aperture.

The fringe should be at 45° with respect to the analyzer-polarizer transmission azimuths.

5. Remove the objective DIC prism and rotate into place the condenser prism that matches the objective. Observe the objective back focal plane.

The orientation of the fringe should match the orientation of the extinction fringe for the objective prism in your drawing.

6. Insert the objective prism and observe the objective back focal plane. Rotate in the other condenser prisms and notice that the extinction fringe is no longer spread across the objective aperture. Rotate in the correct condenser prism.

If the condenser and objective prisms are properly matched and oriented in the same direction, the fringe should become spread across the objective aperture (a dark cross will still occur in high-power, unrectified objectives; Inoué and Spring, 1997).

Align for specimen viewing

7. View the specimen with the correct objective and condenser prisms in place. Translate the objective prism (or rotate the deSenarmount compensator if the objective prism is fixed) to induce a retardation (Δ) between the two wavefronts to brighten the background light and make the edges of the cell appear shaded. If possible, rotate the specimen and observe that contrast is directional—45° with respect to the analyzer-polarizer orientations.

As the upper prism is translated (or the deSenarmount compensator is rotated) to compensation of opposite sign, the initially bright edges become darker and the initially dark edges become brighter than the background.

8. Adjust for proper bright-field, transmitted-light Köhler illumination.

The quality of the DIC image depends critically on the field diaphragm being sharply focused on the specimen (Fig. 4.1.2, left), since this puts the condenser prism in the proper place. The highest possible resolution is achieved when the condenser diaphragm is adjusted to match the diameter of the objective aperture (view the objective back focal plane during this adjustment).

Optimum edge contrast is produced when the retardation between the wavefronts is adjusted to extinguish the light coming from one set of edges in the specimen. Further retardation increases light intensity, but not contrast to the eye. When using video cameras,

it is often important to view the specimen by eye and adjust the compensation for best contrast, then change illumination intensity to provide the camera with enough light for good image quality.

- Repeat steps 2 to 8 for the other objectives.

Once you are sure of the correct prisms and their orientation, then only steps 7 and 8 are necessary for each objective.

SUPPORT PROTOCOL 1

MATCHING MICROSCOPE MAGNIFICATION TO DETECTOR RESOLUTION

This procedure uses the diatom test slide (see Table 4.1.1 for supplier information). For more details on matching a camera to the microscope see Hinsch (1998) or Inoué and Spring (1997).

- Look down the eyepieces and obtain an in-focus image of the frustule pores of the diatom *Pleurosigma angulatum* (Fig. 4.1.8, number 6 in panel A) using 40 \times /(NA \sim 0.7) phase-contrast or DIC and proper Köhler illumination.

The pores should be clearly visible by eye.

- Project the diatom image onto the faceplate of a video or CCD camera.

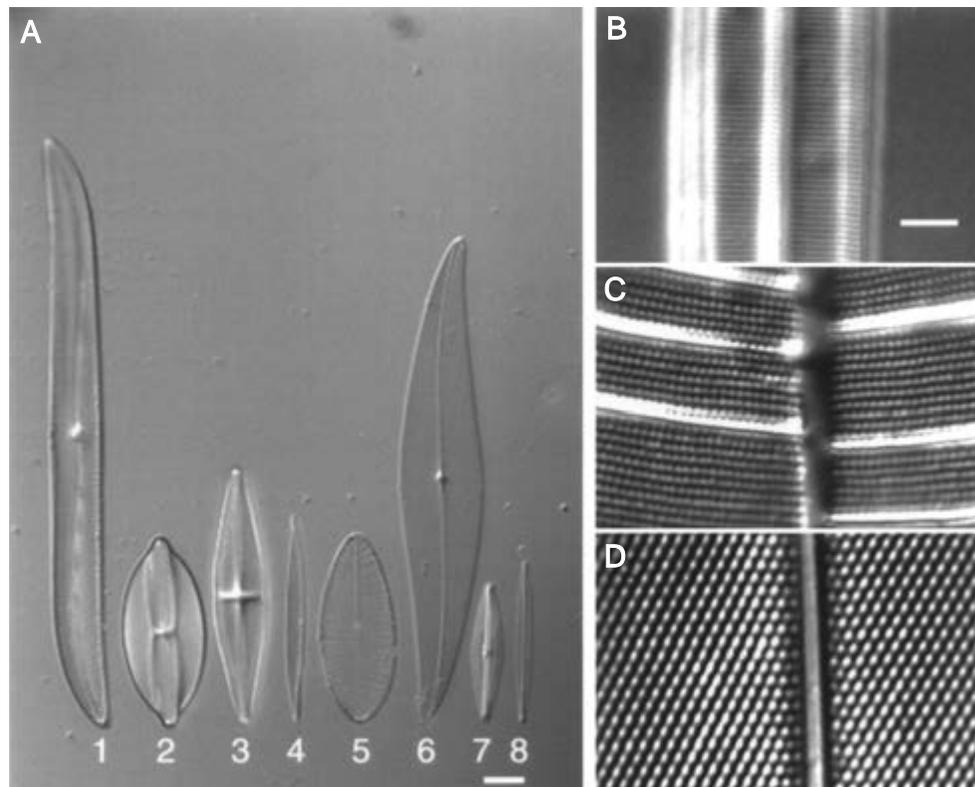


Figure 4.1.8 (A) The diatom test plate. The rows of pores are spaced in the silica shell by $\sim 0.25 \mu\text{m}$ in *Amphipleura pellucida* (panel B; also number 8 on panel A), $0.41 \mu\text{m}$ in *Surrella gemma* (panel C; also number 5 on panel A), and $0.62 \mu\text{m}$ in *Pleurasigma angulatum* (panel D; also number 6 on panel A). Bar: panel A, $10 \mu\text{m}$; panels B, C, and D, $2.5 \mu\text{m}$. From Salmon and Tran (1998), reprinted with permission from Academic Press.

3. Adjust the gain and contrast camera controls for optimum image brightness and contrast.

As you increase contrast, you will need either more camera gain or brighter illumination. Usually, a better signal-to-noise ratio is achieved by leaving the gain control in the middle of its adjustment and increasing illumination intensity.

If the projection magnification to the detector is too small, the frustrule pore lattice will be invisible in the video image, although it will be clearly visible when viewing the image by eye.

4. Increase the magnification to the camera as much as possible.

As magnification increases, resolution in the image becomes less limited by the resolution of the camera. However, the size of the field of view decreases inversely with magnification. In addition, the intensity of light in the image decreases as $1/\text{magnification}^2$. The image will become noisy at low light intensities. It will be necessary to increase illumination intensity as much as possible at high magnification or increase the integration period for the camera exposure.

CALIBRATING IMAGE MAGNIFICATION WITH A STAGE MICROMETER

1. Insert the stage micrometer (see Table 4.1.1) on the microscope stage.
2. With the low-power objective, find the region of the micrometer with 10- μm scale intervals.

The larger intervals are 100 μm .

3. Use the 100- or 10- μm scales to calibrate distance in images taken with your objectives.

The magnifications on the objective barrel and projection lenses are only approximations; accurate measurements of distances in images require a calibration scale.

4. Acquire images of the scale in both the horizontal and vertical directions to check if the camera has square pixels.

TESTS FOR THE OPTICAL PERFORMANCE OF THE MICROSCOPE

Test slides are used to evaluate the performance of the microscope under different conditions.

Testing Phase-Contrast and DIC Using Diatom Testing Slide

Diatoms have silica shells shaped like pillboxes. There are pores in the shell arranged in a lattice pattern specific for each diatom species. Figure 4.1.8 shows a low-magnification view of the eight diatoms on the test slide (panel A) plus higher-magnification views of the lattices of three diatoms most useful in testing the resolution performance of microscope optics using phase-contrast or DIC. Number 6 in panel A of Figure 4.1.8, *Pleurosigma angulatum*, has a triangular pore lattice with spacing of $\sim 0.61 \mu\text{m}$ between rows (illustrated in Fig. 4.1.8, panel D). Number 5, *Surrella gemma*, has rows of pores where the rows are separated by $\sim 0.41 \mu\text{m}$ (illustrated in Fig. 4.1.8, panel C). Number 8 is *Amphipleura pellucida*, which has horizontal rows of pores separated by $\sim 0.25 \mu\text{m}$ (illustrated in Fig. 4.1.8, panel B). In transmitted light, the diffraction limit to lateral resolution, r , is given by (Inoué, 1989):

$$r = \lambda / (\text{NA}_{\text{obj}} + \text{NA}_{\text{cond}})$$

where λ is the wavelength of light, NA_{obj} is the objective NA, and NA_{cond} is the condenser NA (see Fig. 4.1.3). The lateral resolution, r , is equal to $0.195 \mu\text{m}$ for the highest objective

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PROTOCOL 2**

**SUPPORT
PROTOCOL 3**

Microscopy

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NA, which is equal to 1.4, with $NA_{\text{cond}} = NA_{\text{obj}}$ and 546 nm green light. As seen in Figure 4.1.8B, this objective is capable of resolving the rows of pores in the shell of *Amphipleura*, but not the individual pores, which are slightly $<0.19 \mu\text{m}$ apart.

Use the above equation to calculate the diffraction limit of resolution for your other objectives and use the diatoms to test whether they achieve their theoretical limits.

**SUPPORT
PROTOCOL 4**

Testing Phase-Contrast and DIC Using Squamous Cheek Cell Test Slide

Cheek cells are a convenient specimen for testing the performance of phase-contrast or DIC microscopes. As seen in the low-magnification view (Fig. 4.1.6A) they are large and flat, $\leq 3 \mu\text{m}$ thick except near the cell center which contains the nucleus. The upper and lower surfaces have fine ridges which swirl around much like fingerprints. Many of the ridges are $<0.2 \mu\text{m}$ in width and separated by <0.5 to $1.0 \mu\text{m}$.

To prepare the cheek cells, gently scrape the inside of your mouth with the tip of a plastic pipet or similar soft tool and spread the cells and saliva on the center of a clean no. 1.5 coverslip. Invert the coverslip quickly onto a clean slide and press down to spread the cell preparation into a thin layer. Seal the edges with a thin layer of nail polish.

**SUPPORT
PROTOCOL 5**

Testing Fluorescence Using Red, Green, and Blue Fluorescent Tissue Culture Cell Test Slide

The cells in Figure 4.1.9 are triple labeled: DAPI stained nuclei and chromosomes (blue fluorescence; Fig. 4.1.9A); Alexa 488-phalloidin labeled actin filaments (green fluorescence; Fig 4.1.9B); and X-Rhodamine immunofluorescently labeled microtubules (red fluorescence; Fig. 4.1.9C). The microtubules and fine actin filamentous arrays are $<100 \text{ nm}$ in width, but they should appear sharp and in high contrast in the microscope. There should be no “bleed-through” of fluorescence from one fluorescence channel to another if the filters are chosen properly (Taylor and Salmon, 1989).

In fluorescence microscopy, the diffraction-limited lateral resolution is given by (Inoué 1989; Inoué and Spring, 1997):

$$r = 0.61\lambda/NA_{\text{obj}}$$

and the intensity of the image, I_{image} , is given approximately by:

$$I_{\text{image}} \approx I_{\text{ex}} NA_{\text{obj}}^4 / M_{\text{p}}^2$$

where I_{ex} is the excitation light intensity entering the objective and M_{p} is the projection magnification from the objective to the eye or camera. The rate of photobleaching, R_{p} , of a specimen depends on:

$$R_{\text{p}} \approx I_{\text{ex}} NA_{\text{obj}}^2 M_{\text{obj}}^2$$

where M_{obj}^2 is the magnification of the objective.

Note from these equations that resolution depends inversely on NA_{obj} ($r = 0.24 \mu\text{m}$ for $NA_{\text{obj}} = 1.4$ and 546 nm green light), image intensity depends on the fourth power of NA_{obj} , while loss of intensity by photobleaching of the fluorophore increases with the square of objective NA (a measure of the excitation light collected by the objective) and magnification (which concentrates the excitation light on the specimen).

Verify these equations by imaging the fluorescent specimen (the microtubules in the spread cells are a good choice) for objectives with different magnifications and numerical apertures. It will quickly be seen why in fluorescence one wants to use the minimum total

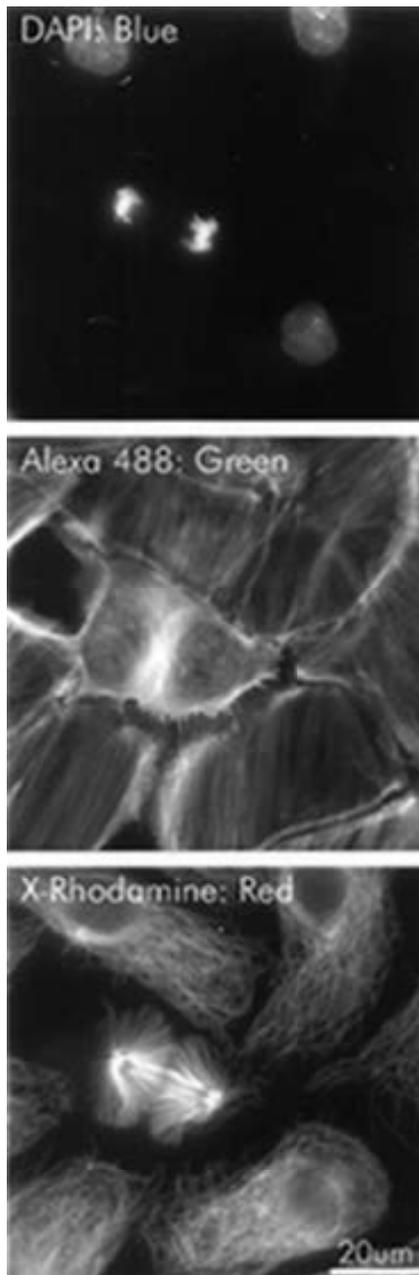


Figure 4.1.9 Epifluorescent images of fixed tissue culture cells stained with (A) DAPI, making DNA fluorescent blue; (B) Alexa 488 bound to phalloidin to label actin filaments fluorescent green; and (C) X-rhodamine labeled antibodies against tubulin to label microtubules fluorescent red. Bar = 20 μm . Images recorded with a 40 \times (NA = 1.4) Plan Fluor objective, 1.5 magnification, to a cooled CCD camera and the multi-modem multiwavelength microscope described by Salmon et al. (1998).

magnification necessary to resolve the structures of interest in the detector, as well as use the maximum NA_{obj} in order to maximize light intensity for the least amount of excitation light (and the least amount of photobleaching).

CARE AND CLEANING OF MICROSCOPE OPTICS

Keeping the microscope optics clean is important for high-quality imaging. Dust, fingerprints, excess immersion oil, or mounting medium on or in a microscope causes reduction in contrast and resolution. DIC is especially sensitive to contamination and scratches on the lens surfaces. Below are steps for keeping the microscope clean.

1. Always keep microscopes covered when not in use.
2. Make sure that all ports, tubes, and unoccupied positions on the lens turrets are plugged.

Plastic plugs are usually supplied with the microscope.

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Microscopy

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3. Store objectives in screw-top containers when not on the microscope. Keep accessories—e.g., condensers and compensators—in plastic bags or boxes. Keep slides and coverslips covered.
4. Be careful with salt water, corrosive liquids, and all solvents.
5. When cleaning lens surfaces, avoid touching the lens surface with anything (even lens paper if possible).

IMPORTANT NOTE: Never use Kimwipes or commercial facial tissue, because they may contain a filler that is part diatomaceous material (glass). One pass of a Kleenex could ruin an objective.

6. Remove dust by gently brushing with an oil-free (ether-washed) camel's hair brush or by using a low-velocity stream of purified air.
7. Remove water-soluble contamination using distilled water with a small amount of detergent, such as Kodak Photoflow solution.

Much (modern) immersion oil can also be removed by washing with detergent.

8. Remove most immersion oil by passing a high-quality lens tissue over the objective or condenser front element.

IMPORTANT NOTE: Do not rub. No area on the tissue should come in contact with the lens twice. This prevents dust and dirt removed from the lens from coming back and possibly scratching it. This is easily accomplished by passing the tissue over the lens in a "Z" pattern or by making parallel passes.

9. Clean objective lenses by holding a piece of doubled lens paper over the objective and placing a few drops of solvent on the paper. Draw the paper across the lens surface so that the solvent flows rapidly in a circular pattern over the recessed lens surface (see Inoué and Spring, 1997). Finish the stroke with a dry portion of the paper. Repeat as necessary.

In this way, the solvent contacts the lens but the paper does not, because the lens is recessed. For solvent, first use a 1% solution of Kodak Photoflow in distilled water to remove much of the oil and water-soluble material. Then use a small amount of oil solvent like ethyl ether or xylene to clean all the oil from the surface. Avoid soaking a lens with solvent, to prevent damage to lens cements.

10. To clean recessed front elements of dry objective lenses or to remove stubborn dirt, use a cotton-tipped applicator that has been soaked in cleaning solution and then shaken to remove excess fluid. Rotate the cotton tip over the lens surface to clean. Again, first use a 1% solution of Kodak Photoflow in distilled water to remove much of the oil and water-soluble material. Then use a small amount of oil solvent like ethyl ether or xylene to clean all the oil from the surface.

11. Use a detergent solution or ethanol to clean the surfaces of the eyepiece lenses.

Do not use xylene as it may solubilize enamel surfaces.

COMMENTARY

The development of lasers, electronic cameras, digital image analysis, and specific fluorescent molecular probes have recently made light microscopy an enormously powerful tool in the biomedical sciences. There are a number of excellent books and review articles about these applications, as well as the optical principles of light microscope design and image

formation. Listing of all these references is beyond the scope of this unit; only a few of these books and reviews are listed, and these can be used to find other useful references.

Spencer (1982), Zernike (1958), and Keller (1995, 1997, 1998) provide excellent introductions to the principles of image formation and contrast in the light microscope for beginners,

while Pluta (1988, 1989) provides a more comprehensive treatment. Inoué and Oldenbourg (1995) also review the basic concepts and principles of microscope design and different methods of image formation. The abovementioned references describe the many modes of transmitted-light imaging in the microscope, including bright-field, phase-contrast, and DIC, which are described in this unit, as well as dark-field, polarization, interference contrast, Hoffman modulation, and reflection interference contrast methods. Taylor and Salmon (1989) and *UNIT 4.2* give clear introductions to fluorescence microscopy. The recent edition of *Video Microscopy* by Inoué and Spring (1997) is a comprehensive introduction to the principles and practical aspects of light microscopy, video, and digital imaging. The *Video Microscopy* volume of *Methods in Cell Biology* edited by Sluder and Wolf (1998) also has contributions that cover many basic concepts and practical aspects of light microscopy in the biomedical sciences; it also includes chapters on multiwavelength, multimode digital imaging methods, camera selection, ion ratio imaging, and specimen chambers. Salmon and Tran (1998) review the principles of video-enhanced DIC methods used to image macromolecular complexes invisible in the microscope when viewed by eye. Taylor et al. (1992), Salmon et al. (1998), and Rizzuto et al. (1998) review microscope design for multiwavelength, multimode digital imaging of fluorescent specimens, and four-dimensional microscopy. Advanced quantitative fluorescence methods like ratio imaging, photobleaching, photoactivation, resonance energy transfer, chromophore-assisted laser ablation, and fluorescence lifetime imaging are also reviewed in the *Methods in Cell Biology* volumes edited by Taylor and Wang (1989), and Sluder and Wolf (1998) as well as in Herman and Jacobson (1990) and Inoué and Spring (1997). Reviews on imaging cells containing expressed protein coupled to green fluorescent protein (GFP) include Heim and Tsien (1996), and Sullivan and Kay (1998). Agard et al. (1989), Carrington et al. (1995), and Rizzuto et al. (1998) review how deconvolution of three-dimensional image stacks can produce super resolution in the light microscope. The recent edition of *Handbook of Biological Confocal Microscopy* edited by Pawley (1995) is an excellent reference on this important method for imaging fluorescent structures in thick specimens (Smith, 1997). The new technique of multiphoton laser scanning microscopy is described by Denk et al. (1994),

while biological applications of optical traps to manipulate organelles and measure molecular forces is reviewed by Svoboda and Block (1994).

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