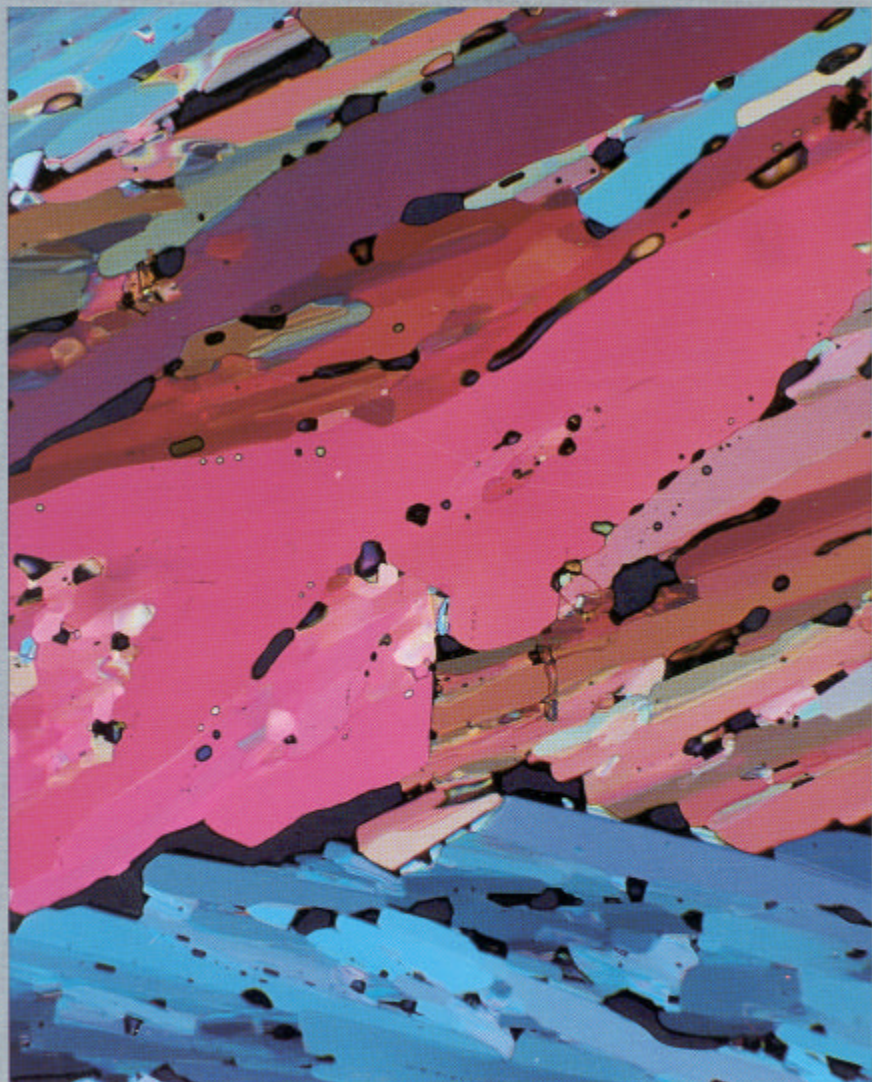


# Microscope Basics and Beyond

by Mortimer Abramowitz  
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For Olympus Corporation

Volume 1



# Microscope

## Basics and Beyond

*Published by Olympus America Inc., Precision Instrument Division,  
Two Corporate Center Drive, Melville, NY 11747-3157.  
516-844-5000, Fax: 516-844-5112*

*Cover photo: Salol Crystals (Polarized Light).  
All photos by the author.*

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Figures 2, 3, 6, 12, 22, 24, 25 and 26*

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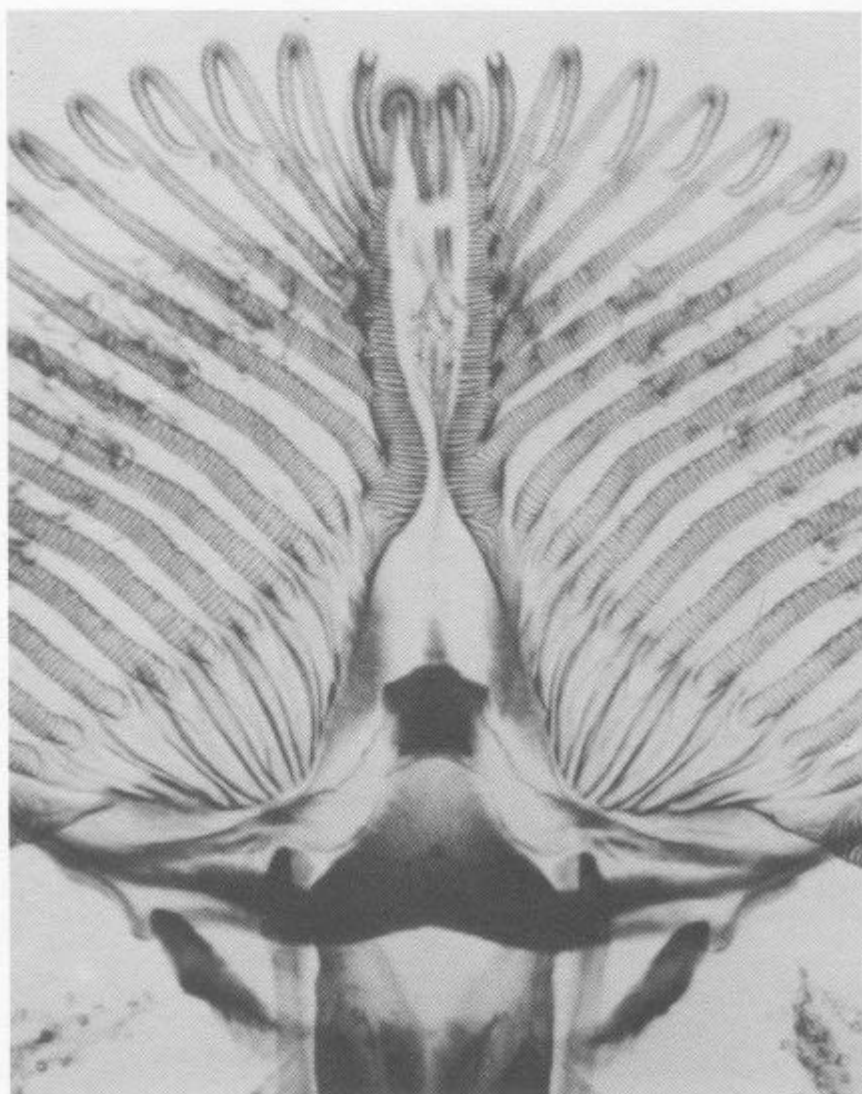
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**PART  
ONE**

# An Introduction to the Compound Microscope



*Photo: Mouth Parts  
of the Blowfly*

## The Basic Concepts of the Compound Microscope

What is a microscope? How does it work?

This booklet, "Microscope Basics and Beyond," is designed to answer these questions with a minimum of technical and mathematical explanation. The knowledge of basics is fundamental to the skilled use of the microscope; the information in this booklet will be a guide in the selection of equipment suited to various purposes; the fundamentals will clarify the simple but elegant ideas of microscope design.

First, an overview of the basic concepts; then the parts and their functions; lastly the significant details.

Microscopes are instruments designed to produce magnified visual or photographic images of small objects. An easy way to understand the microscope is by means of a comparison with a slide projector, a device familiar to most of us.

Visualize a slide projector turned on its end with the lamp housing resting on a table. The light from the bulb passes through a condensing lens, and then through the transparency, and then through the projection lens onto a screen placed at right angles to the beam of light at a given distance from the projection lens. The *real image* on this screen emerges inverted (upside down and reversed) and magnified. If we were to take away the screen and instead use a magnifying glass to examine this real image in space, we could further enlarge the image, thus producing another or second-stage magnification.

The above is essentially what a transmitted light microscope does. Light from a lamp passes through a substage condenser; then passes through the transparent specimen placed over the opening in the stage; then through an objective lens (similar to the projection lens of the projector), and forms a real, inverted, magnified image inside the body tube of the microscope. This real image is further magnified by the eyepiece (similar to the magnifying lens in Fig 1) and is seen by the observer's eye as if this twice enlarged image were at a distance of approximately 10 inches from the eye.

Fig 1. Comparison of slide projector and microscope.

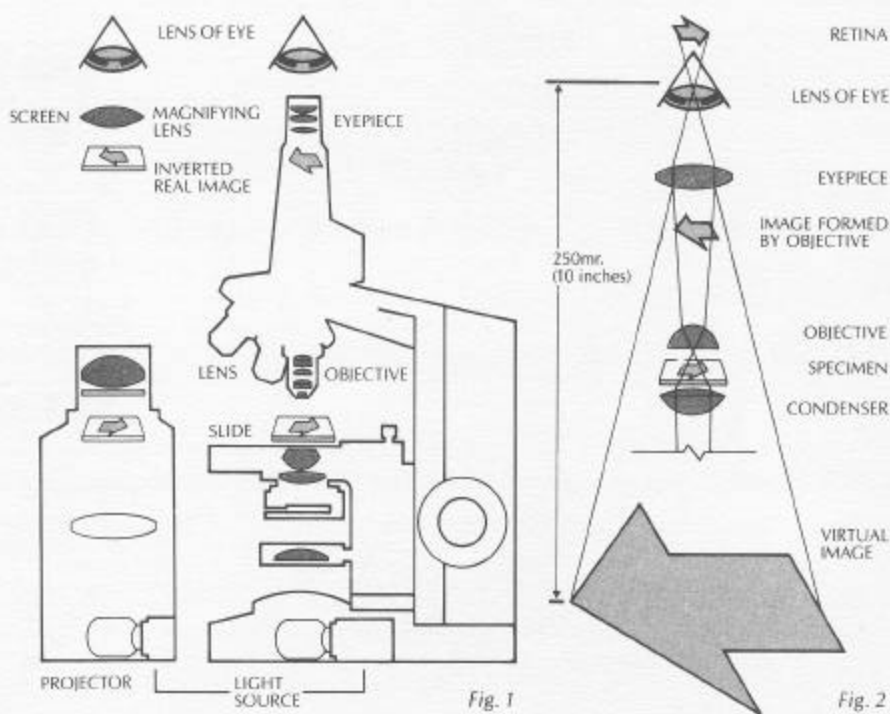


Fig. 2. Compound Magnifier. In the compound microscope, the intermediate image formed by the objective is enlarged by the eyepiece.

These basic principles underlie the operation and construction of the compound microscope—so-called because, unlike a magnifying glass or simple microscope, the compound microscope employs a group of lenses aligned in series. The elaboration of these principles has led to the development, over the past several hundred years, of today's sophisticated instruments. Today's microscopes are often modular with interchangeable parts for different purposes; such microscopes are capable of producing images from low to high magnification with remarkable clarity and contrast.

**The Parts  
and Their  
Functions,  
Illumination  
and Light**

**THE MAGNIFYING LENSES**

A simple microscope or magnifying glass produces an image of an object. The lens is convex, that is thicker at the center than it is at the periphery. The image is perceived by the eye as if it were at a distance of 10 inches or 25 centimeters. Since the image appears to be on the same side of the lens as the object, it cannot be projected onto a screen; it is called a virtual image; the image is upright, not inverted.

Fig. 3. Simple Magnifier. A simple magnifier uses a single lens system to enlarge the object in one step.

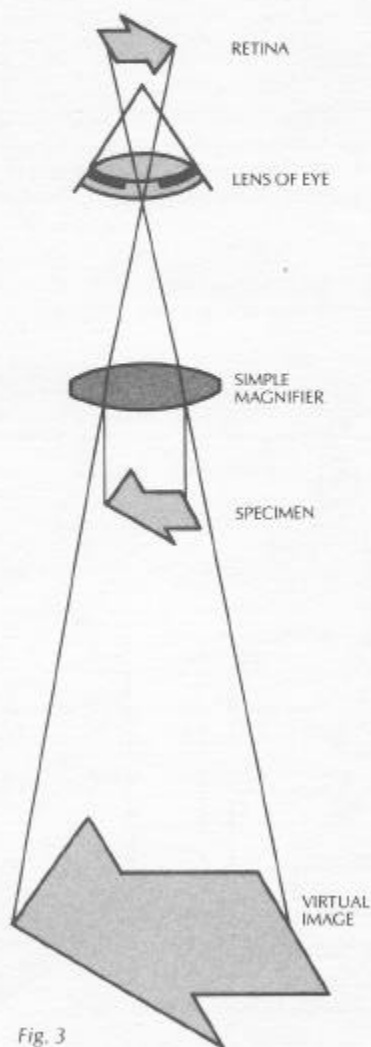


Fig. 3

Fig. 4. The relationship between optical tube length and mechanical tube length.

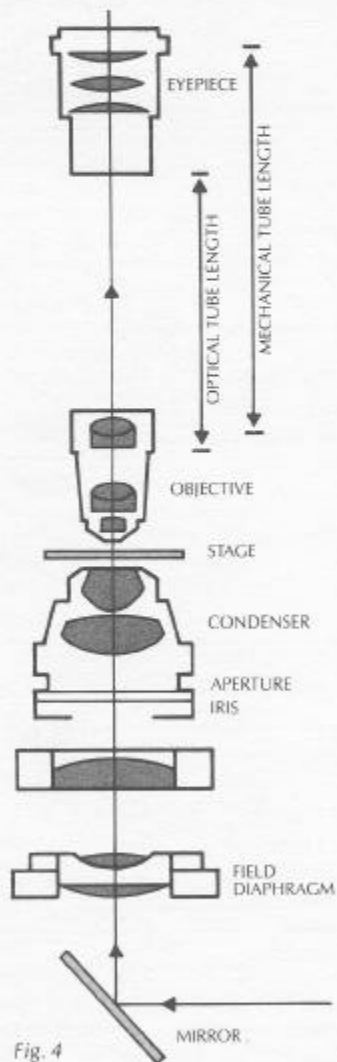


Fig. 4

In the compound microscope, there are two kinds of magnifying lenses:

- a. An objective lens at the lower end of the body tube which is focused quite close to the object and which projects a magnified real image of the specimen or object into the body tube of the microscope. This image is inverted and projected at a fixed distance up into the tube (known as the optical tube length). If one were to place a piece of ground glass or a small screen down inside the microscope tube, this real image could be seen. The objective is the most important lens of the microscope for producing a clear image of high resolution. If the objective is of poor quality and uncorrected for errors (aberrations), the image will be of poor quality.

The objective has several major functions:

1. The objective must gather the light coming from each of the various parts or points of the specimen.
2. The objective must have the capacity to reconstitute the light coming from the various points of the specimen into the various corresponding points in the image. (Sometimes called anti-points.)

Fig. 5. The image of a point, formed by a lens, is never a point, but a circular disc of definite diameter, known as the anti-point.

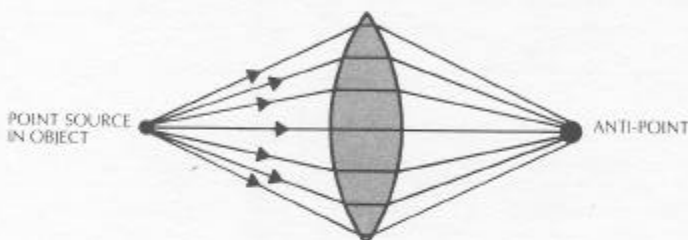


Fig. 5

3. The objective must be constructed so that it will be focused close enough to the specimen so that it will project a magnified, real image up into the body tube.
- b. The eyepiece or ocular which fits into the body tube at the upper end, farthest from the specimen. The eyepiece is held in place by a shoulder which keeps it from falling into the tube. The placement of the eyepiece is such that its eye (upper) lens further magnifies the real image projected by the objective. The eye of the observer sees this secondarily magnified image as if it were at a distance of 10 inches from the eye; hence this virtual image appears as if it were near the base of the microscope. The distance from the top of the microscope tube to the shoulder of the objective (where it fits into the nosepiece) is usually 160 mm. This is known as the mechanical tube length.

The eyepiece has several major functions:

1. The eyepiece serves to further magnify the real image projected by the objective.
2. In visual observation, the eyepiece produces a secondarily enlarged virtual image.
3. In photomicrography, it produces a secondarily enlarged real image of the image projected by the objective. This augmented real image can be projected on the photographic film in a camera or upon a screen held above the eyepiece.
4. The eyepiece can be fitted with scales or markers or crosshairs in such a way that the images of these inserts are superimposed on the image of the specimen.

Thus the image is enlarged in two stages; first by the objective, then by the eyepiece. This tandem arrangement produces a visual magnification which is the product of the magnification of the objective and that of the ocular; e.g. a 20x objective and a 10x ocular yield a visual magnification of 200x. Higher or lower magnifications would result from other combinations of objectives and eyepieces.



Fig. 6. Image formation from specimen to film.  
 A. Specimen.  
 B. Objective.  
 D. The image plane within a Huygen eyepiece lies between the field-lens C and eye lens E, in the plane of the diaphragm.  
 F. Camera.  
 G. Image of A recorded at G, the focal plane.

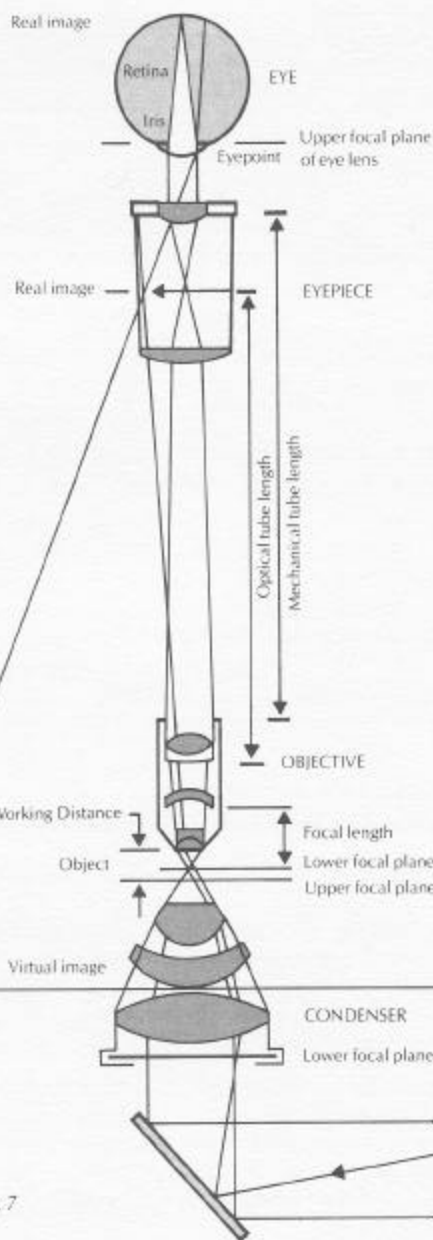


Fig. 6

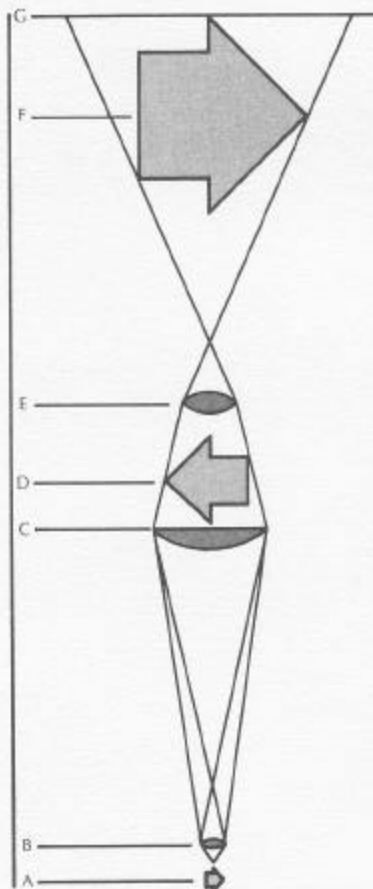


Fig. 7. The optical system of the microscope.

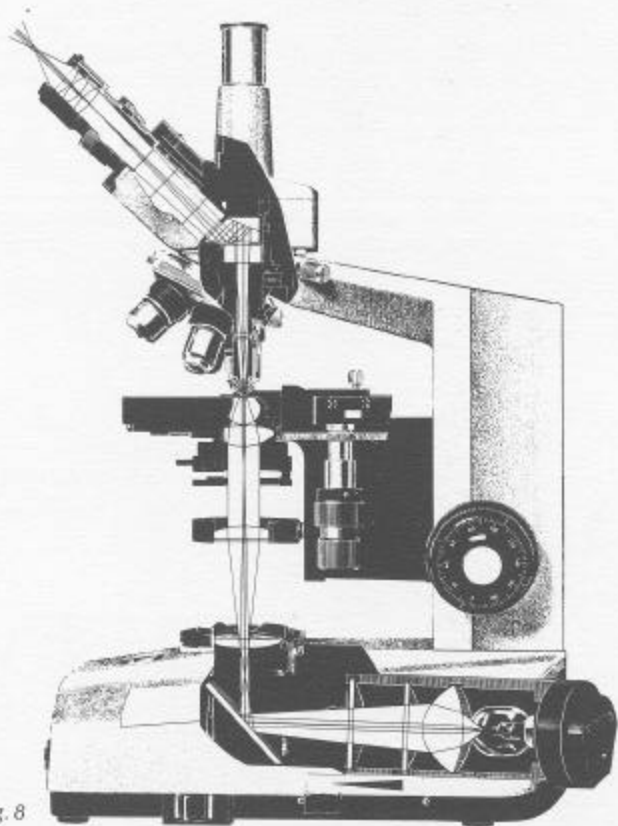
Fig. 7

## THE MICROSCOPE STAND

The microscope stand or frame carries the objectives at the end of the body tube nearest the specimen. Usually there is a nosepiece to accommodate several objectives, each of which can, by means of rotating the nosepiece, be lined up with the body tube opening directly over the specimen in the optical axis of the microscope. The end of the body tube farthest from the specimen holds the eyepiece. In a straight monocular microscope, there is but one eyepiece at the upper end. In a binocular microscope, by means of prisms, the image projected by the objective is "split" in two and "sent on" to each of the two eyepieces of the binocular. In a trinocular tube, the observer can, by means of a movable prism, divert the image to the eyes or to the straight photo-tube or sometimes to both the eyes and the straight tube simultaneously.

In order to minimize vibration, the microscope is constructed with a heavy and rigid base. The microscope tube is attached to the frame. On both sides of the microscope tube or the frame, there are two sets of adjustment knobs: the coarse adjustment knobs for larger increment focusing movements; the fine adjustment knobs for small increment focusing movements. The adjustment knobs serve to bring the objective and the specimen closer together or farther apart. In most microscopes the adjustment knobs raise or lower the stage; in some the knobs raise or lower the microscope tube or the nosepiece.

The specimen rests on a stage, rectangular or circular, with an opening in the center to allow light to pass from the lamp through the specimen in order to enter the objective. Often the stage is equipped with a mechanical device which holds the specimen slide in place and can smoothly move the slide back and forth as well as from side to side.



*Fig. 8. Longitudinal section of a microscope.*

*Fig. 8*

Below the stage is the substage which holds the condenser. The lenses in the condenser serve to concentrate the light from the lamp onto the specimen. The condenser is held in place on a mount which can be moved up and down by means of a focusing knob. The condenser is usually centerable so that all lenses of the microscope above and below the stage can be kept in alignment. As part of the condenser, or part of the substage, there is an iris diaphragm called aperture diaphragm which can be opened or closed by a knurled ring or a lever. The aperture iris diaphragm and the condenser are of critical importance in securing good illumination.

Below the condenser, a mirror is fitted which serves to deflect the light from the light source into the condenser and further through the specimen. The microscope may have a built-in light source and right angle mirror or prism to do this—or the microscope may have a movable mirror which has to be used with an outside light source.

The microscope stand has the following functions:

1. To insure stability and rigidity of the microscope.
2. To provide the frame for holding the objective and eyepiece at the opposite ends of a body tube.
3. To make it possible by means of adjustment knobs to focus the microscope objective on the specimen.
4. To hold the specimen on a stage and enable the specimen to be readily moved on this flat surface.
5. To carry the substage condenser and mirror which will deflect the light from a lamp up through the specimen.

## ILLUMINATION

Since specimens rarely generate their own light, illumination is usually furnished by means of a built-in lamp. The light beam passes through the substage condenser after deflection from a built-in mirror or right angle prism. The light from the condenser then passes through the specimen on the stage, into the objective, thus illuminating the specimen. If the lamp is of high intensity, its brightness is controlled by a built-in or separate transformer. In microscopes without a built-in lamp, a separate lamp is used. The light beam from such a lamp is deflected into the substage condenser by means of a flat mirror fitted to the base of the microscope tilted to send the beam up into the condenser.

## LIGHT

One explanation of the nature of light is that it is made up of very small waves vibrating at right angles to the direction of the beam's path. Light travels in straight lines; its path can be deflected or reflected by means of flat mirrors or right angle prisms. Light can be "bent" or refracted by means of glass lenses that are thicker or thinner at the center than at the periphery.

Fig. 9. Variation of wavelength with light velocity.

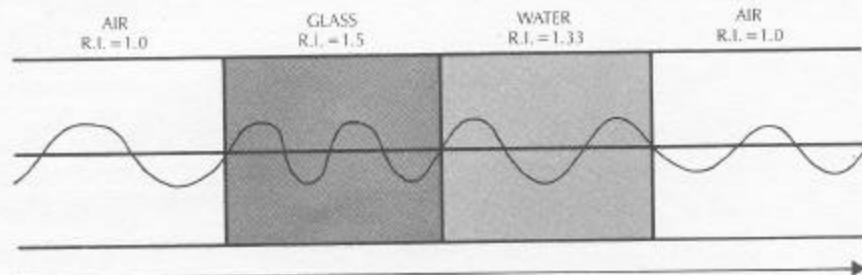


Fig. 9

Light travels at different speeds in air and glass (faster in air which is taken approximately as the standard of 1). Light is slowed and "bent" or refracted when it passes through air and enters a convex lens. Thus light is refracted when it enters a convex lens from air; refracted when it leaves the convex lens and reenters air; refracted when it passes from air through oil; or from oil through air. Oil has a refractive index of 1.515 as does glass. The refractive index of air is 1.

Fig. 10A. Snell's Law. Refraction of a light ray at a glass surface.

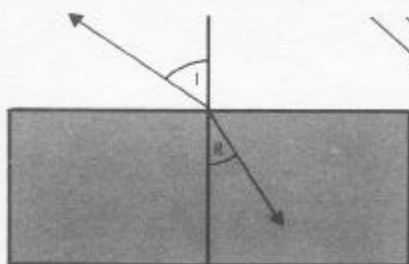


Fig. 10A

Fig. 10B. A light ray is laterally deflected by a sheet of glass unless it passes perpendicularly through it.

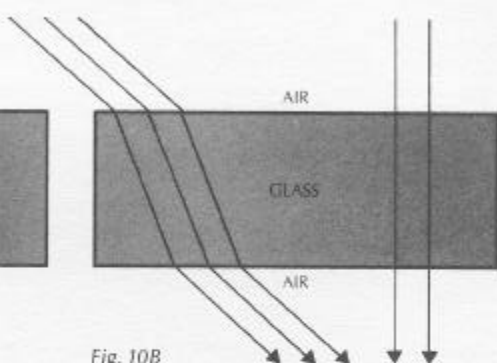


Fig. 10B

When light at an angle (other than  $90^\circ$ ) passes from a less dense medium (e.g. air) to a more dense medium (e.g. glass), the rays are bent toward the perpendicular. When light at an angle (other than  $90^\circ$ ) passes from a more dense (e.g. glass) medium into a less dense medium, (e.g. air), the rays are bent away from the perpendicular. When light passes from glass into air, if the angle is too great (critical angle) the rays do not emerge but are totally reflected. When light passes from glass (refractive index 1.515) into immersion oil (refractive index 1.515), the rays are not refracted or reflected since the refractive indices are identical.

Light is composed of both invisible and visible waves. The visible light frequencies represent the familiar spectrum or rainbow from violet to red. The violet-blue end of the spectrum is made up of the shorter wave lengths; the red end of the spectrum is made up of longer wave lengths.

Invisible (to the eye) parts of the spectrum that may be of use in microscopy are the ultra-violet (shorter than violet) and infra-red (longer than red).

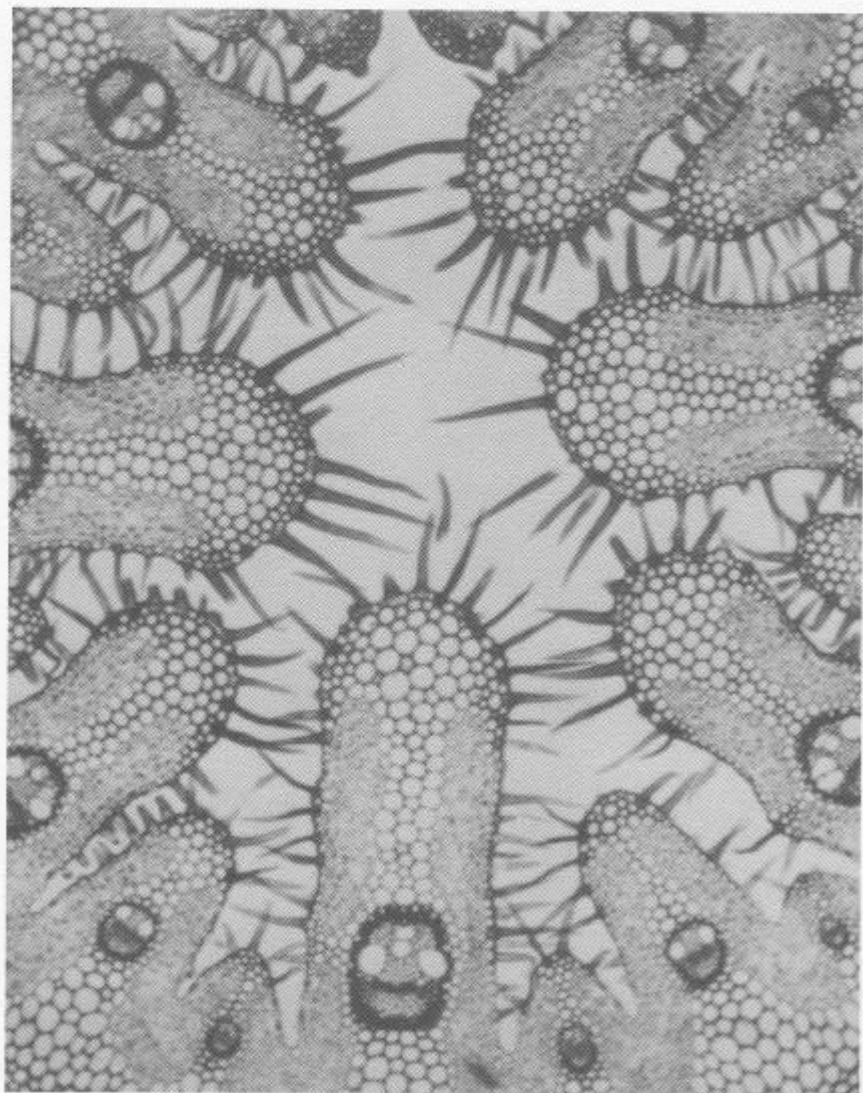
It will be seen later in this booklet how the microscope utilizes the fundamentals of illumination and light in making possible excellent image rendition of the observed specimen. Remember, it is the enlarged image of the specimen, not the specimen itself, that is seen or photographed.



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PART  
TWO

The  
Significant  
Details



*Photo: Cross  
section of Beach  
Grass Leaf*

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## The Microscope Stand

Just a few additional details on the microscope stand:

The sensitivity of the fine adjustment knob is calculated by the size of the interval of motion as the knob is turned clockwise or counter-clockwise. In good instruments, this interval is usually .002 mm or 2  $\mu$ ; in the finest research instruments the sensitivity of the fine adjustment may be as low as .001 mm or 1  $\mu$  (micron).

The microscope stage may be rectangular or circular. It is more useful if the stage can be rotated so that the specimen can be oriented either for viewing or photomicrography.

The microscope tube may be monocular, binocular or trinocular. For comfortable viewing, a binocular tube produces less eye strain. For photomicrography, a trinocular tube (one of its tubes is upright for transmission of light to a photomicrographic attachment camera) offers the greatest convenience since a simple flip of a lever redirects the light or a portion of the light to the camera. Thus in a trinocular microscope, it may be possible to simultaneously view the specimen and take the picture. In most microscopes, these tubes are interchangeable.

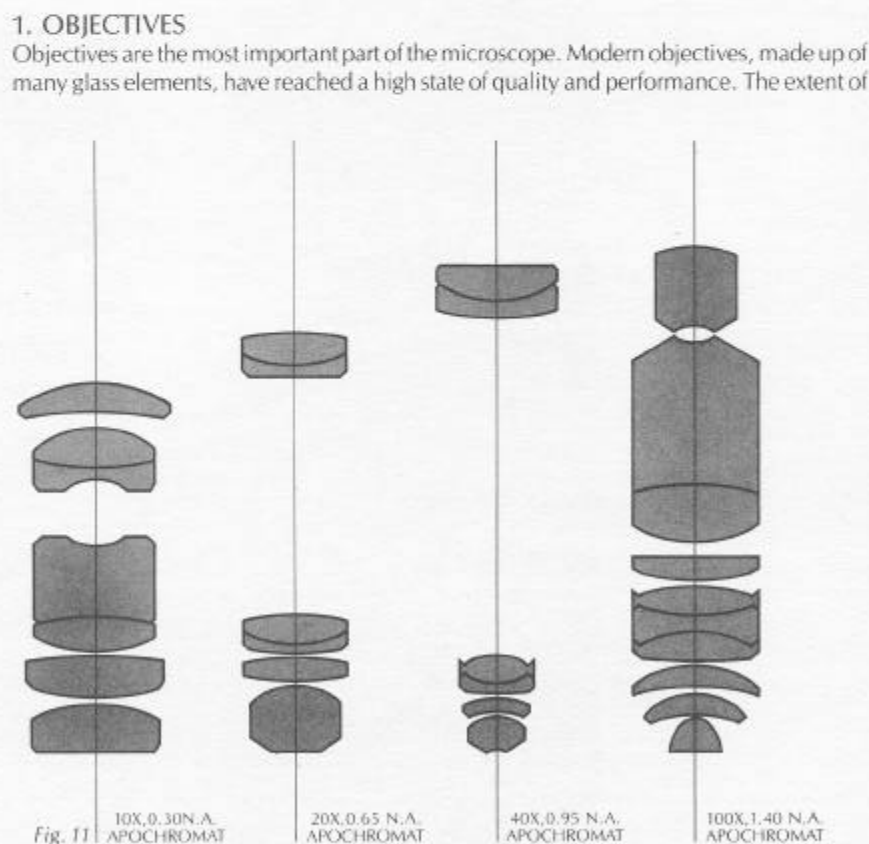
Present-day binocular tubes are constructed at an angle (preferably 30°) for viewing ease. The horizontal distance between the eyepiece sleeves is adjustable to fit the interpupillary distance of the eyes yet still automatically maintain the standard 160 mm. mechanical tube length. There may also be a rotatable knurled ring on one of the sleeves to allow adjustments for individual eye acuity.

Modern microscopes have low-positioned coarse and fine adjustment knobs. These are usually coaxial so that the user's hands can readily move from coarse to fine adjustment or vice versa.

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## Objectives, Eyepieces, Condensers

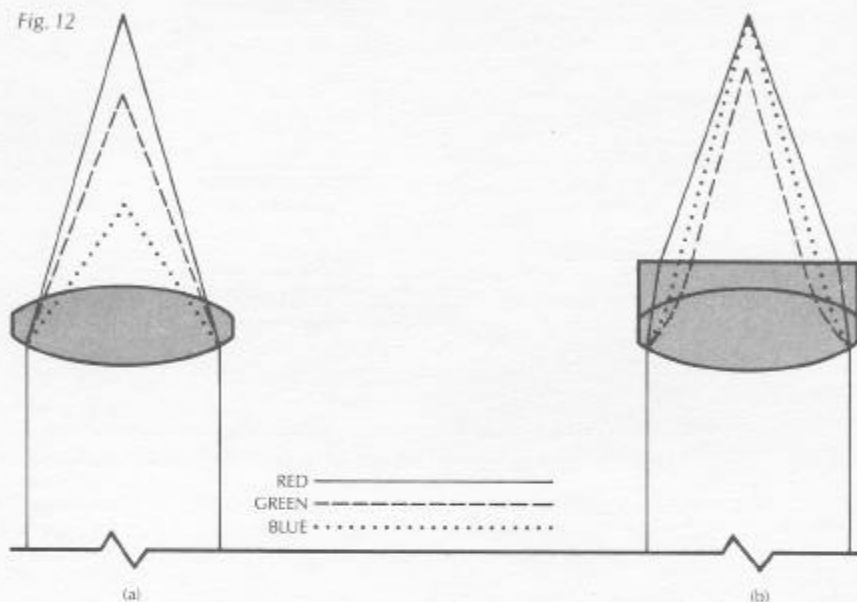
Fig. 11. A series of  
Apochromatic  
Objectives.



corrections for aberrations and flatness of field determines the usefulness and cost of an objective. (The booklet explains aberrations later.)

The least expensive objectives are the achromatic objectives. These are corrected chromatically to bring red and blue light to the same focus. Further, they are corrected for spherical aberration for the color green. Thus achromatic objectives yield their best results with light passed through a green filter and, when employed for photomicrography, with black and white film.

Fig. 12. Chromatic aberration. Failure of a simple lens to bring light of different wavelengths to a common focus (a) can be compensated in part by use of an achromatic lens (b).



The next higher level of correction and cost is found in objectives called fluorites or semi-apochromats. These objectives too are corrected chromatically for red and blue light. However, the fluorites are corrected spherically for two colors. Hence fluorite objectives (their lens elements contain some natural or synthetic fluorite) are better suited than achromats for color photomicrography in white light.

The highest level of corrections (and expense) is found in apochromatic objectives. These objectives are corrected chromatically for three colors: red, blue, and green; they are corrected spherically for two colors. Apochromatic objectives are the best for color photomicrography in white light. Because of their high level of correction, such objectives usually have, for a given magnification, higher numerical apertures (see the importance of this characteristic later in the booklet) than do achromats or fluorites.

All three types of objectives project images that are curved rather than flat. To overcome this inherent condition, lens designers have produced flat-field objectives which yield flat images. Such lenses are called plan-achromats, or plan-fluorites, or plan-apochromats. Such correction, although expensive, is valuable in photomicrography.

Each objective has inscribed on it the magnification (e.g. 20x or 10x etc.); the tube length for which the objective was designed to give its finest images (usually 160 mm.); the thickness of cover glass, covering the specimen, which was assumed by the designer in correcting for spherical aberration (usually .17 mm.). If the objective is designed to operate with a drop of oil between it and the specimen, the objective will be engraved OIL or OEL or HI (homogeneous immersion). If these latter designations are not engraved on the objective, the objective is meant to be "used dry," that is with air between the lowest part of the objective and the specimen.



Objectives also always carry the engraving for N.A. (numerical aperture). This may vary from .04 for low power objectives to 1.3 or 1.4 for high power apochromatic objectives.

Fig. 13. Objective marks.



Some objectives, usually the higher power, "dry" objectives of 40x-60x magnification are fitted with a correction collar. Since these objectives are particularly sensitive to incorrect thickness of the cover glass covering the specimen, the rotation of the collar can compensate for cover glasses thicker or thinner than .17 mm.

If the objective carries no designation of higher correction, one can usually assume it is an achromatic objective. More highly corrected objectives have inscriptions such as apochromat or apo., plan-, fl. etc.

Fig. 14A. A 4mm objective of 0.95 N.A. with correction collar marked in terms of cover-slip thickness.

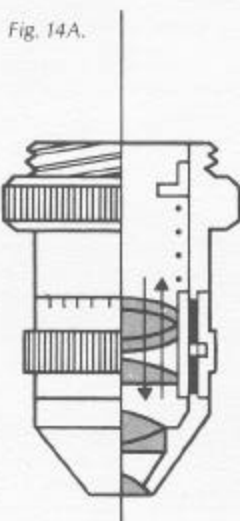
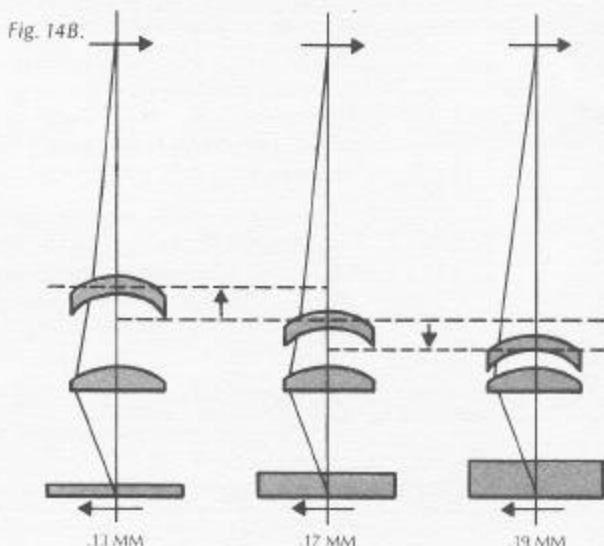


Fig. 14B. Action of a correction collar (diagrammatic).



When a manufacturer's set of similar objectives, e.g. all achromatic objectives of various magnifications, are mounted on the nosepiece, they are usually designed to project an image to approximately the same plane in the body tube. Thus changing objectives by rotating the nosepiece usually requires only minimal use of the fine adjustment knob. Such a set of objectives is described as being parfocal—a useful convenience and safety feature.

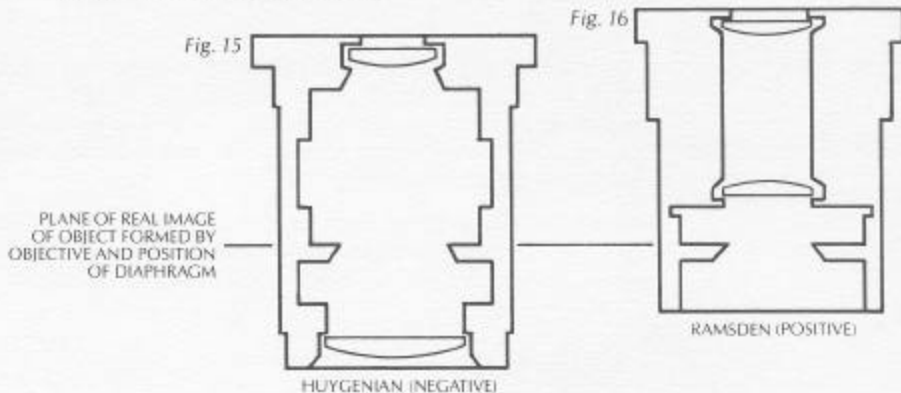
Sets of objectives are also designed to be parcentric, that is, a specimen which is centered in the field of view for one objective remains centered when the nosepiece is rotated to bring another objective into use.

## 2. EYEPIECES

Best results require that objectives be used in combination with eyepieces that are appropriate to the correction and type of objective. There are several main kinds of eyepieces: negative and positive.

Fig. 15. Huygenian Eyepiece, in Cross-section

Fig. 16. Ramsden Eyepiece, in Cross-section

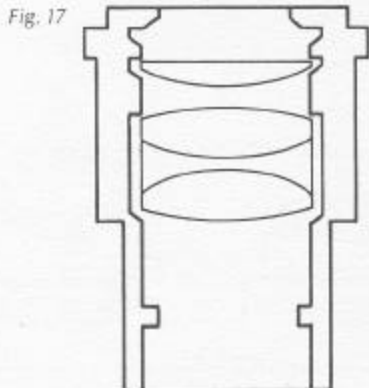


In negative eyepieces, there are two lenses: the upper or eye-lens and the lower or field lens. In their simplest form, both lenses are plano-convex, with convex sides "facing" the specimen. Approximately mid-way between these lenses there is a fixed circular opening or diaphragm which, by its size, defines the circular field of view that is observed in looking into the microscope. The simplest kind of negative eyepiece, or Huygenian eyepiece, is found on most routine microscopes fitted with achromatic objectives. Although the Huygenian eye and field lenses are not well corrected, their aberrations tend to cancel each other out. More highly corrected negative eyepieces have two or three lens elements cemented and combined together to make the eye lens.

The simple Huygenian eyepiece shows a blue fringe at its periphery. If the eyepiece carries only the magnification inscribed on it, it is most likely to be a Huygenian eyepiece, best suited for use with achromatic objectives of 5x-40x magnification.

The other main kind of eyepiece is the positive eyepiece or Ramsden eyepiece. Its eye lens and field lens may be cemented together and better corrected than the simple Huygenian type. In the positive eyepiece, the circular, fixed diaphragm is below the field lens.

Fig. 17. Positive eyepiece, corrected lens.



Micrometer discs, pointers, grids, etc. are placed on the diaphragm of either type of eyepiece. Since the image is projected by the objective at the plane of the eyepiece diaphragm, such discs and grids will appear as if superimposed on the focused specimen.

Compensating eyepieces may be either of the positive or negative type. Compensating eyepieces must be used at all magnifications with fluorite, apochromatic and all kinds of plan objectives (and also can be used to advantage with achromatic objectives of 40x and higher). Compensating eyepieces play a crucial role in compensating for residual chromatic aberrations inherent in the design of highly corrected objectives. Hence it is preferable that the microscopist use the compensating eyepieces designed by the maker to accompany that maker's higher-corrected objectives.

Compensating eyepieces are readily identified by the yellowish fringe of light appearing at their periphery of view. They are inscribed with K or C or comp. as well as their magnification. Those eyepieces used with flat-field objectives are sometimes labeled Plan-comp. Eyepieces that are especially designed to give a particularly wide diameter field of view are inscribed WF or S.W.

Some manufacturers make objectives (CF) that contain all of the color correction in the objectives themselves and thus cannot be used with compensating eyepieces. Special eyepieces can be designed without showing color in the periphery of the field of view.

### 3. CONDENSERS

The substage condenser is fitted below the stage of the microscope, between the illuminating lamp and the specimen. Condensers are manufactured according to different levels of correction needed.

The simplest and least corrected (and least expensive) condenser is the Abbe condenser, numerical aperture up to 1.4. While the Abbe condenser is capable of passing bright light, it is not corrected chromatically or spherically. As a result, the Abbe condenser is suited mainly for routine observation with objectives of modest numerical aperture and magnification.

The next higher level of correction is found in the aplanatic condenser which is well-corrected for spherical aberration but not for chromatic aberration. This condenser can be used for photomicrography, particularly for photomicrography in black and white through a green filter.

The highest level of correction is incorporated in the aplanatic-achromatic condenser. Such a condenser is well-corrected for both chromatic aberration and spherical aberration. It is the condenser of choice for use in color photomicrography done in white light.

Fig. 18. Condenser systems.

Fig. 18

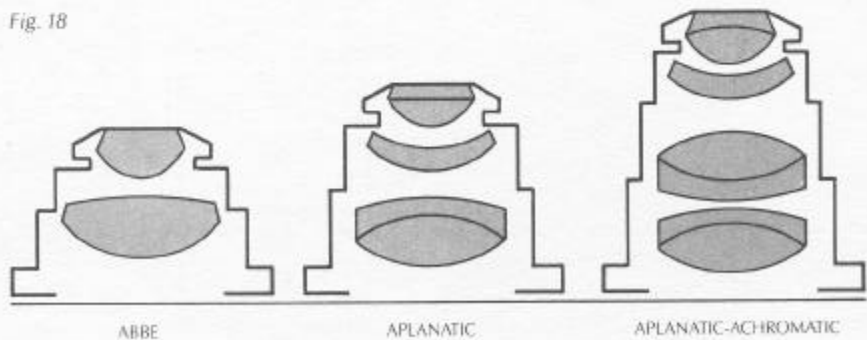
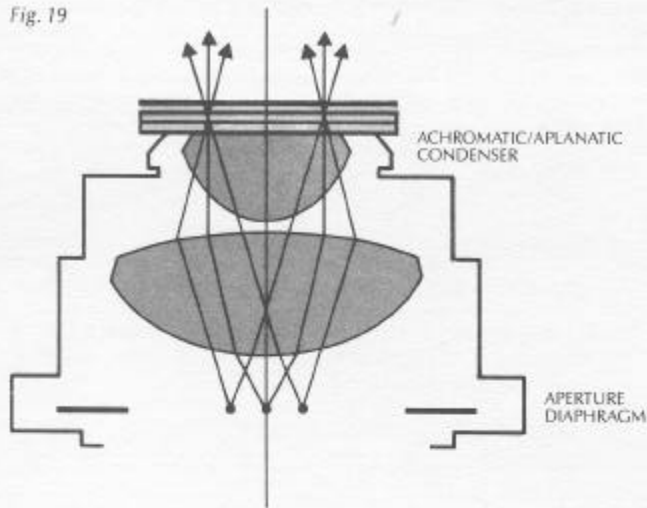


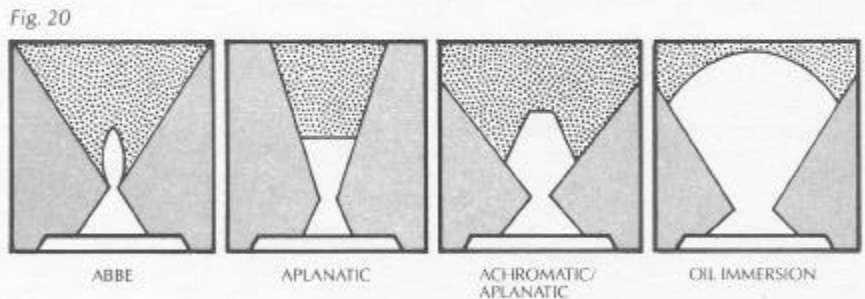
Fig. 19.  
Achromatic/  
Aplanatic  
Condenser N.A.  
1.4



The engraving on the condenser includes its numerical aperture and its designation, if aplanatic or aplanatic-achromatic. Condensers with numerical apertures above 1.0 perform best when a drop of oil is applied to their upper lens in contact with the *undersurface* of the specimen slide.

The condenser aperture and the proper focusing of the condenser are of critical importance in realizing the full potential of the objective in use. Likewise, the appropriate use of the adjustable aperture iris diaphragm (incorporated in the condenser or just below it) is also most important in securing proper illumination and contrast. The opening and closing of this aperture iris diaphragm controls the angle of illuminating rays (and thus the aperture) which pass through the condenser, through the specimen and then into the objective.

Fig. 20. Brightfield  
condensers. Cones  
of light transmitted.



For low power objectives (below 10x), it may be necessary to unscrew the top lens of the condenser in order to fill the field of view with light. Some condensers are produced with a flip-top upper lens to accomplish this more readily. Some manufacturers produce a condenser which flips over completely when used with low power objectives. Other companies may incorporate auxiliary correction lenses in the light path for securing proper illumination with objectives less than 10x. When a condenser is used without its top lens, the aperture iris diaphragm is opened wide (compare with Koehler Illumination).

The height of the condenser is regulated by a condenser knob which lowers or raises the condenser. Thus, the condenser is capable of focusing light from the lamp onto the specimen (more details on this will appear under Koehler Illumination later in this booklet).

## Light and Illumination

Fig. 21. Chromatic aberration of white light

- A. Uncorrected lens. The light is dispersed along the axis in the order of the colors of the spectrum.
- B. Achromatic lens. Green is brought to the shortest focus. The color error is much reduced.
- C. Semi-apochromatic lens. The color error is similar to (B) but still further reduced.
- D. Apochromatic lens. For all practical purposes chromatic aberration may be considered as eliminated.

W: white light  
G: green  
O: orange  
R: red  
B: blue  
M: magenta  
(“minus green”)

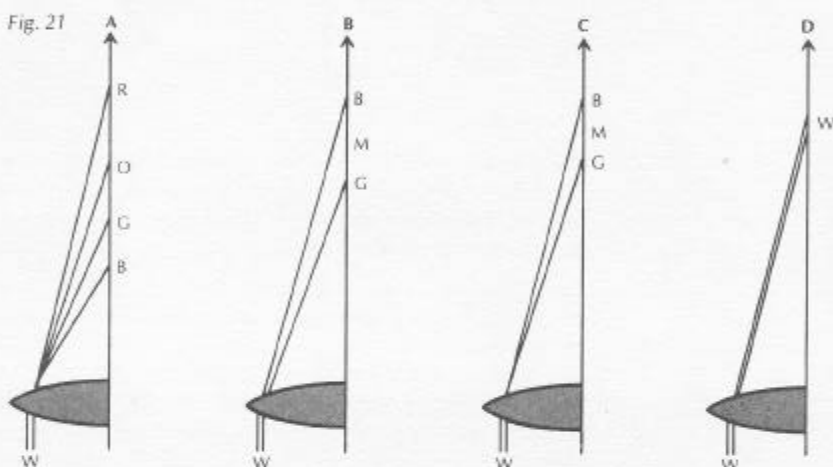
Fig. 22. Spherical aberration. Failure of the lens system to image central and peripheral rays at the same focal point arises with spherical lenses. Optical correction is possible, but care must be taken not to introduce additional spherical aberration when setting up the microscope.

## LIGHT

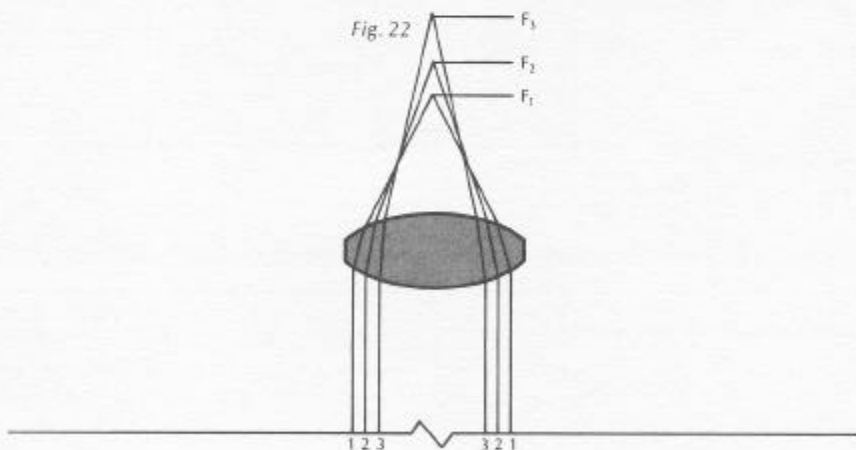
Knowledge of the behavior of light and the effects resulting when light passes from air through a glass convex lens and out into air again is fundamental to the understanding of image formation.

When light passes from air through a convex lens, the speed of light is slowed. The various colors, differing in wave length, are slowed at different rates. Thus the bending (refraction) effect differs for the various colors. Those rays which strike the central area of the lens at a perpendicular emerge unrefracted. Light passing through other parts of the convex lens is refracted or “bent.”

When white light passes through the convex lenses of objectives, eyepieces, or condensers, two main kinds of aberrations may occur. These aberrations can be corrected in the design of the lenses.



a) Chromatic Aberration—The various color frequencies of white light pass through a convex lens and, instead of being brought to the same common focus, come to different foci. The lens designer strives, by combining various kinds of glass and several lens elements, to bring the main colors of red, green, and blue into common focus.



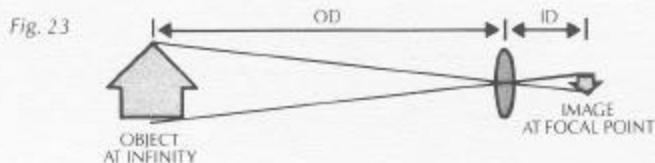
b) Spherical Aberration—Light passing through a convex lens will be brought to different foci depending upon whether the light passes through near the center of the

lens or closer to the periphery. Lens designers strive to correct this kind of zonal aberration to bring peripheral and near-central rays to a common focus.

In image formation, light from all of the illuminated points of the specimen passes through the objective which then reconstitutes the rays into an image. The finer and more accurate this reconstitution, the clearer the image will be.

The following describes in simple form the geometric optical laws involved. For clarity of the basic principles, we'll assume that the object or "specimen" is a self-illuminated arrow.

Fig. 23. Relationship between Image size and Object size. ( $I : O :: ID : OD$ )

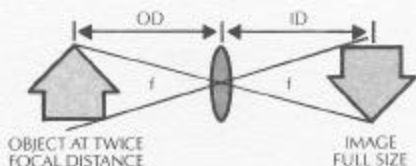


Case A—The arrow is very far from the convex lens.

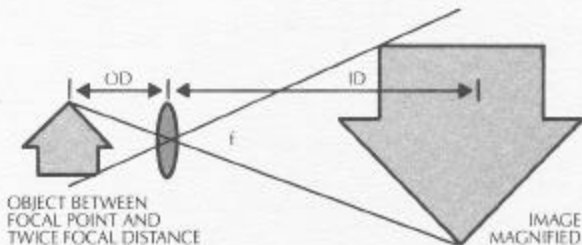
In this case the light from the arrow passes through all parts of the convex lens (assume it is well-corrected) and comes to a focus at a distance which is characteristic for that particular lens. The distance from the lens center to this focal point is called the focal length of the lens. The image produced is real and inverted and smaller in size than the arrow. This image could be projected on a screen placed at the focal distance.



Case B—The arrow is brought closer to the lens but is still at a distance of more than twice the focal length of the lens. Here again, the image is real and inverted but now is bigger than in Case A but not quite the size of the arrow. The image is now further from the lens.



Case C—The arrow (remember, this is our "specimen") is brought to a distance in front of the lens that is equal to twice the focal length. The real image, still inverted, is exactly the size of the arrow. The image now is at twice the focal length behind the lens.



Case D—The "specimen" is brought nearer to the convex lens at a distance which is between twice the focal length and the focal length (between 2F and F as lens designers say). The image is real, inverted, but now is larger (magnified) than the

"specimen." This is the case for microscope objectives. The specimen on the microscope stage is focused by an objective at a distance between  $2F$  and  $F$ . This results in the projection of a magnified real image up into the microscope tube coming to a focus in the upper part of the tube.

*Case E*—The "specimen" (or real image of the specimen) is brought even closer to the lens at a distance smaller than the focal length. Again, the image is magnified but it is not inverted; it is a *virtual image* that appears to be on the *same side of the lens as the specimen*, at a distance of approximately 10 inches. This is the case for eyepieces in visual observation. The image projected by the objective comes to a focus at or just within the focal distance of the eyepieces' eye lens. The eyepieces' eye lens, together with the lens of the human eye, produce a further magnified, virtual image of the magnified image projected by the objective. (See Fig. 3, page 3.)

The various lenses of the microscope function according to the cases governed by the geometric optical laws of image formation described above, especially Case D and Case E.

## ILLUMINATION

All too frequently, sophisticated and well-equipped microscopes fail to yield excellent images because of incorrect use of the light source. Excellent illumination of the specimen should be bright, glare-free, and evenly dispersed in the field of view. Since most modern microscopes achieve such excellence by use of Koehler illumination (so-called after its discoverer, August Koehler), this description will deal with the method to achieve Koehler illumination.

There are several physical-mechanical requirements. The substage condenser must be capable of being focused up and down, preferably by a knob operating on a rack and pinion. The substage condenser must be fitted with an aperture iris diaphragm that can be opened and closed by a lever or knob. The lamp must be fitted with a condensing lens, a collector, and a field iris diaphragm that can be opened and closed. It is also desirable that the lamp filaments or bulb be centerable or pre-centered. To repeat, there are two important adjustable iris diaphragms; the *aperture* diaphragm at the substage condenser and the *field* diaphragm nearer to the lamp. The *aperture* iris diaphragm controls the angular aperture of the cone of light from the condenser. The *field* iris diaphragm controls the area of the circle of light illuminating the specimen.

Fig. 24.  
Microscope illuminator. The essential elements of the illuminator are the lamp, a condenser lens, and a lamp, or field diaphragm. The diaphragm is adjustable.

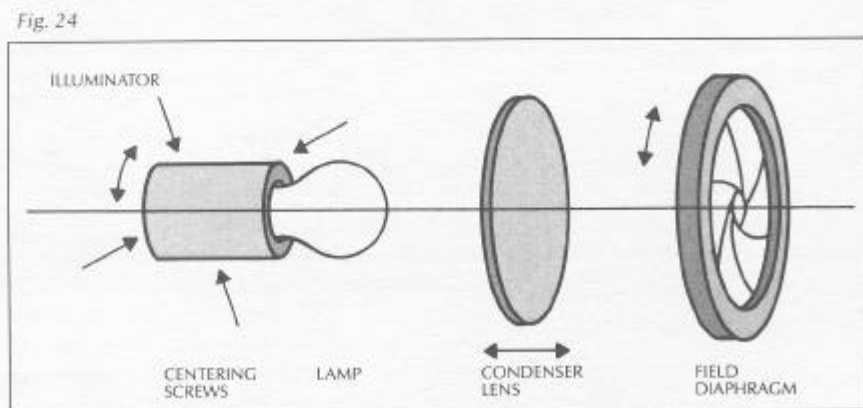
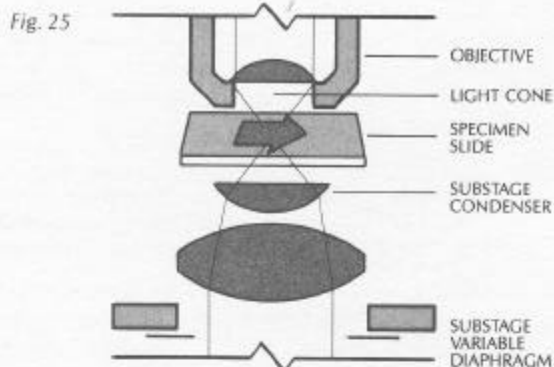


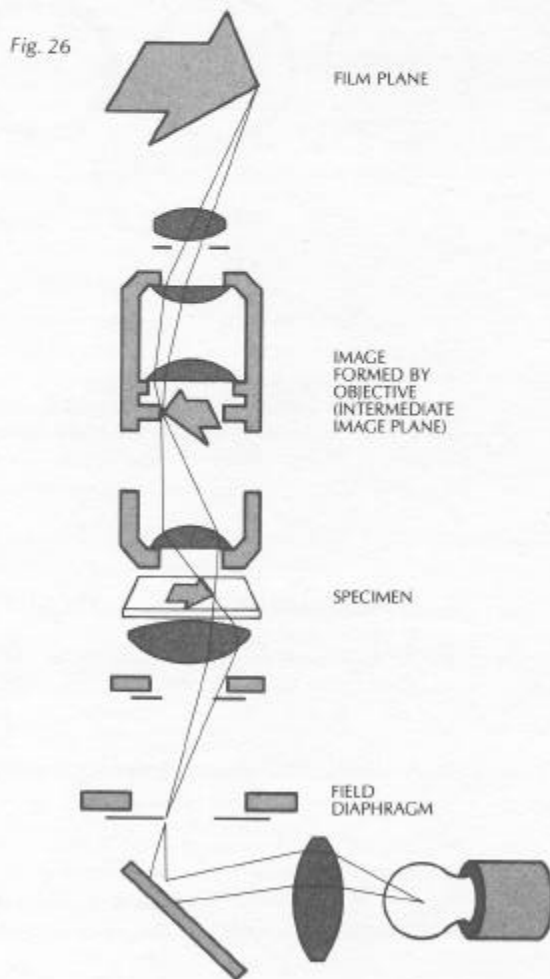
Fig. 25. Cone of illumination. The substage condenser must be focused and the diaphragm adjusted so that the cone of illumination completely fills the aperture of the microscope objective.



## KOEHLER ILLUMINATION

Step 1. Open the *aperture* iris diaphragm wide and also open the *field* iris diaphragm wide. Turn on the lamp. Using a low power objective (10x or so) and a 10x eyepiece, slowly focus the specimen that has been placed on the microscope stage over the stage opening.

Fig. 26. Image-forming ray paths are traced from two ends of lamp filament. Conjugate foci are field diaphragm, specimen plane, intermediate image plane (entrance pupil of eyepiece), and, with camera in place, the film plane. See text for Koehler illumination.





Step 2. Close the *field* iris diaphragm most of the way. Using the rack and pinion knob of the condenser, raise the condenser until the edges of the partly closed *field* iris diaphragm appear superimposed on the already-focused specimen. The edges of the *field* iris diaphragm should appear sharply focused. The substage condenser is usually close to its highest position.

Step 3. If the *field* diaphragm does not appear centered in the field of view, use the substage condenser centering screws to center the *field* diaphragm. Then slowly open the *field* iris diaphragm until it just disappears from view. This step must be repeated each time a different objective is turned into place on the nosepiece.

Step 4. Now lift the eyepiece out of the body tube and look down the tube at the back lens of the fully-lighted objective. (This is best accomplished by the use of a pinhole eyepiece—an eyepiece with a tiny hole but no lenses—or a focusing telescope such as is provided for use in phase-contrast microscopy.) While looking down the microscope tube, slowly open and close the substage condenser *aperture* iris diaphragm. It will be seen that closing the *aperture* iris diaphragm “cuts into” the periphery of the back lens of the objective. For excellent illumination and contrast, approximately  $\frac{1}{4}$ - $\frac{1}{3}$  of the back lens should be occluded, thus leaving  $\frac{3}{4}$ - $\frac{3}{4}$  of the back lens illuminated. Then replace the eyepiece in the tube. This step too must be repeated each time a different objective is turned into place on the nosepiece.

Fig. 27.  
Appearance of  
light at the back  
lens of the  
objective.



Fig. 27

The *aperture* iris diaphragm may have a calibrated scale (sometimes the calibration refers directly to numerical aperture) which can be used to make the iris adjustment more accurate as well as readily repeatable.

It will now be found that the specimen is well-illuminated with even, glare-free light, giving good image contrast. The intensity of the lamp can be adjusted by proper use of the transformer or by neutral density filters, *not* by raising or lowering the condenser, *not* by closing the *aperture* iris diaphragm. Increasing the voltage of the transformer controlling the lamp increases the color temperature of the light. Such color temperature must be properly adjusted in color photomicrography because different color films are balanced for different color temperatures of the light source.

## NUMERICAL APERTURE AND RESOLUTION

If the geometric optics described earlier were the sole consideration in image formation, it would be possible to secure clear magnifications of many thousands of times larger than the specimen itself. However, it was discovered by the optics experts of the 19th century—Abbe, Rayleigh, Airy et alia—that other factors operate to limit useful magnification. (Additional magnification which yields no additional detail of the specimen is called “empty magnification.”)

These experts recognized that, when light from the various points of a specimen passes through the objective and is reconstituted as the image, the various points of the specimen appear in the image as small discs (not points) known as Airy Discs. This phenomenon is caused by diffraction or scattering of the light as it passes through the

Fig. 28. Decrease in size of the anti-point with increase in the numerical aperture of the lens.

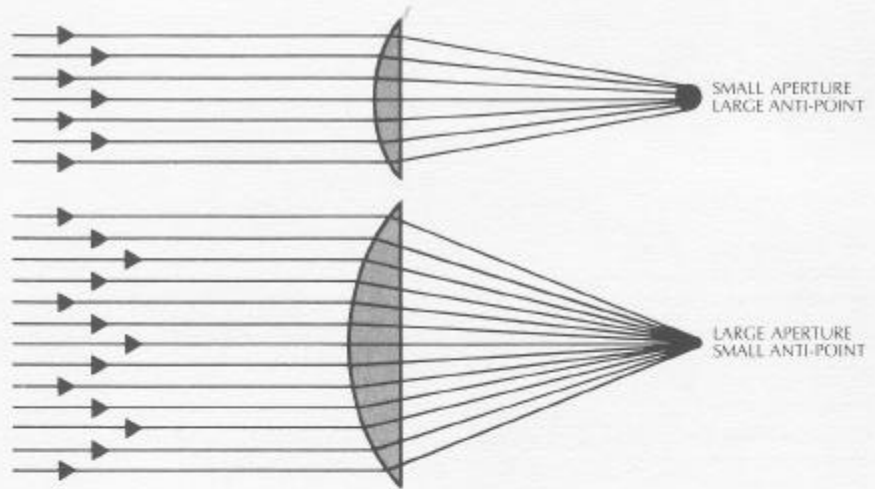


Fig. 28

minute parts and spaces in the specimen. These "Airy Discs" consist of small, concentric light and dark circles. The smaller the "Airy Discs" projected by an objective in forming the image, the more detail of the specimen is discernible. Objectives of better correction produce smaller "Airy Discs" than do objectives of lesser correction. Objectives of higher numerical aperture (more on this to be explained) are capable of producing smaller "Airy Discs." For these reasons, objectives of high numerical aperture (N.A.) and better correction can distinguish finer detail in the specimen. The ability to distinguish (separate) clearly minute details lying close together in the specimen is known as resolving power.

Fig. 29. Decrease in size of airy discs accompanying an increase of numerical aperture.

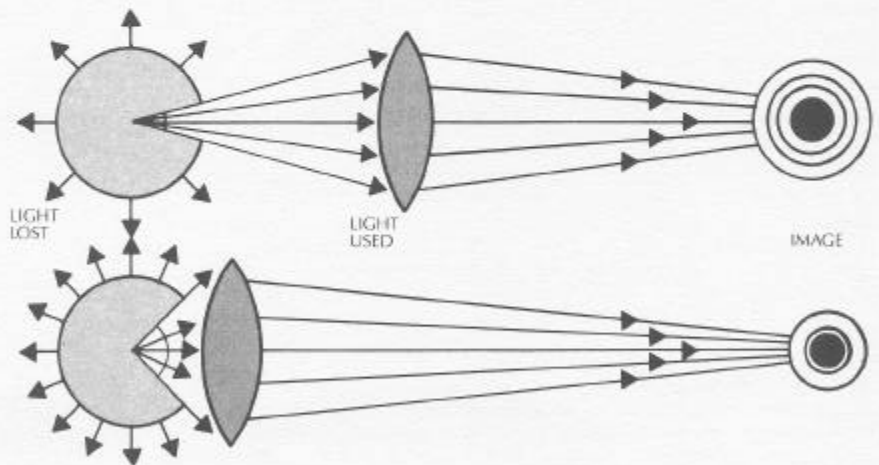


Fig. 29

The phenomenon of diffraction and the limiting effect of the size of light waves dictate that the useful magnification of an objective is 500-1000x the numerical aperture (e.g. upper limit of 250x for an objective with numerical aperture of .25; upper limit of 1300x magnification for an objective of 1.3 numerical aperture).

In achieving a desired magnification, it is generally good practice to use objectives of higher magnification accompanied by eyepieces of lower magnification (e.g. for a magnification of 200x, use an objective of 20x and an eyepiece of 10x rather than an objective of 10x and an eyepiece of 20x).

Fig. 30. Angular apertures of objectives compared. The 15° narrow angle of a low power objective compared with the 110° wide angle of the high power oil immersion lens.

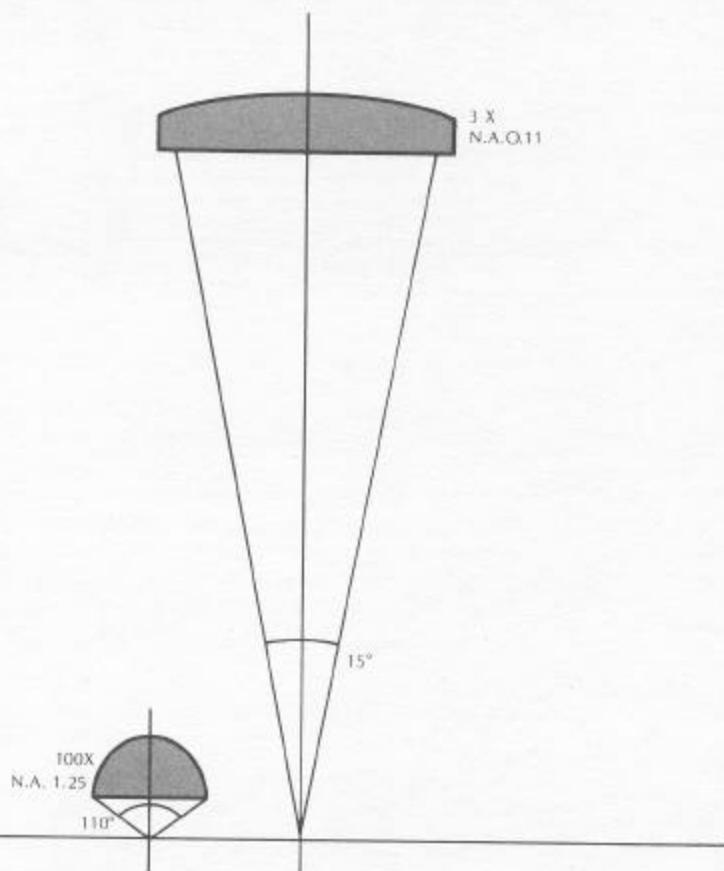


Fig. 30

Fig. 31. The principle of oil immersion. In A, five rays are shown passing from the point P in the object through the coverslip into the air space between the latter and the lens. Only rays 1 and 2 can enter the objective. Rays 4 and 5 are totally reflected. In B, the air space is replaced by oil of the same refractive index as glass. The rays now pass straight through without deviation so that rays 1, 2, 3 and 4 can enter the objective. The N.A. is thus increased by the factor  $n$ , the refractive index of oil.

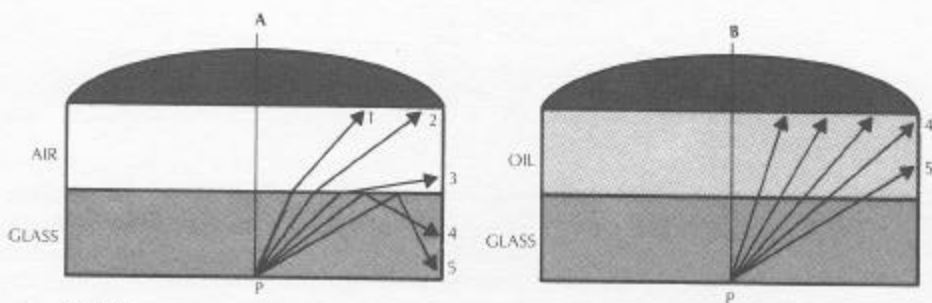


Fig. 31

Now for the explanation of the term numerical aperture, referred to as N.A.

The ability of a microscope objective to include or "grasp" the various rays coming from each illuminated part of the specimen is directly related to the angular aperture of the objective. Objectives with lower angular aperture can include only a narrower cone of light as compared to objectives with higher angular aperture.

The equation for determining numerical aperture (N.A.) is:  $N.A. = n \sin u$ .

In this equation N.A. is the numerical aperture;  $\sin u$  is the sine of  $\frac{1}{2}$  the angular aperture of the objective;  $n$  is the index of refraction of the material in the object space, that is the space between the specimen and the lowest lens of the objective. (The refractive index of air is 1; of immersion oil it is 1.515).

Fig. 32.  
Comparison of a  
dry with an oil  
immersion  
objective

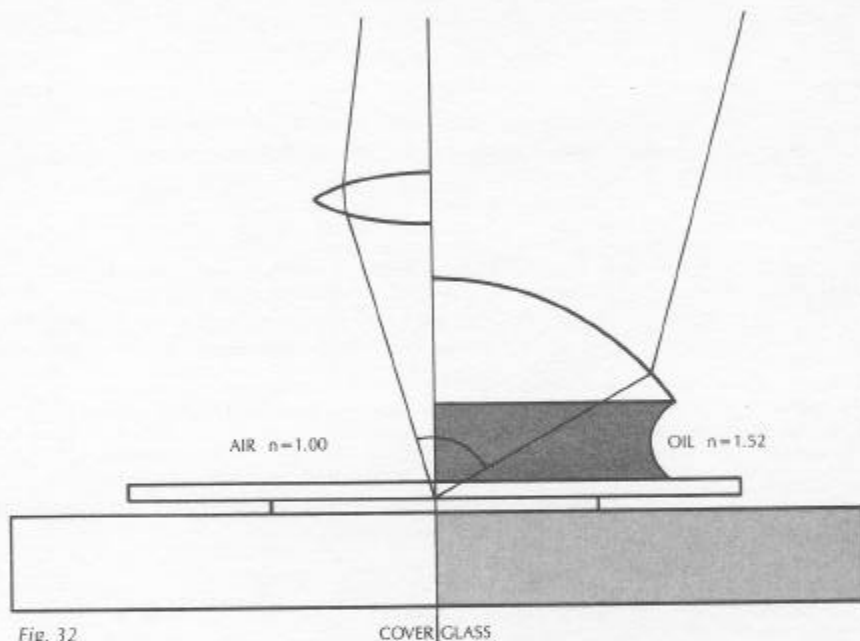


Fig. 32

COVER GLASS

Study of the above equation will yield the following inferences:

1. For a given angular aperture, oil immersion lenses can have higher numerical aperture since  $n = 1.515$  for oil.
2. Since  $u$  cannot exceed  $90^\circ$ , the sine of  $u$  must be 1 or less. Since a "dry" objective is used with air in the object space ( $n$  or refractive index = 1), the maximum theoretical N.A. of a dry objective is 1 (in practice, not more than .95).
3. Increasing the angular aperture of an objective increases  $u$  and thus increases sine  $u$  and thus increases the numerical aperture.
4. Since oil has a refractive index of 1.515 it is possible to utilize oil immersion objectives which can yield an N.A. of up to 1.515. (In practice, N.A. 1.4 is usually the upper limit of apochromats; more usually 1.3 N.A.)

And now, the important relationship between N.A. and resolution.

Resolution has been defined as the ability of an objective to separate clearly two points or details lying close together in the specimen.

The equation for resolution is generally agreed to be:

$$R = \frac{.61\lambda}{\text{N.A.}}$$
 (according to Lord Rayleigh)

In this equation R is the size of the distance between two minute points lying close together in the specimen;  $\lambda$  is the wave length of the light being used; N.A. is the numerical aperture.

An analysis of this equation will lead to the following inferences:

1. As N.A. increases R becomes smaller; the size of the distance between the adjacent points is smaller; hence the resolution is better.
2. If shorter wave lengths of light are used (violet-blue end of the spectrum), the resolvable distance is smaller; resolution is better. Longer wave lengths (e.g. red) yield poorer resolution.
3. Resolution varies inversely with numerical aperture. High aperture objectives operating in violet-blue light are capable of yielding the best resolution in visual microscopy.

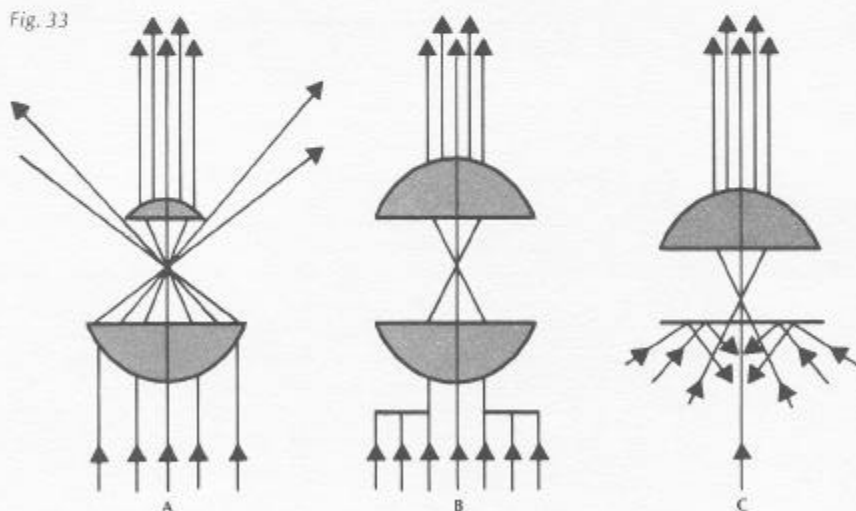
The numerical aperture of the entire *microscope system* depends on the apertures of the substage condenser and the objective working together.

$$\text{N.A. of the system} = \frac{\text{N.A. of objective} + \text{N.A. of condenser}}{2}$$

This equation demonstrates that, for full realization of the aperture of the objective, it should be matched or exceeded by the numerical aperture of the condenser. In practice, the partial closing of the iris aperture diaphragm of the condenser reduces the working aperture of the microscope system in order to improve the contrast in the image.

The N.A. system equation also shows that in order to realize the full aperture of the system, any condenser with a numerical aperture of more than 1.0 should have oil placed between its top lens and the *underside* of the slide. Highly corrected oil immersion objectives of N.A. greater than 1 should be used with oiled condensers of N.A. greater than 1.

Fig. 33. Limitation of working N.A. by  
A) Restriction of objective diameter.  
B) Restriction of illuminating cone.  
C) Lack of immersion contact with specimen.



In many uses of the microscope, it is not necessary to use objectives of high N.A. because the specimen is readily resolved with use of lower N.A. objectives. This is

particularly important because high N.A. and high magnification are accompanied by the disadvantages of very shallow depth of field and short working distance. Thus, in specimens where resolution is less critical and magnifications can be lower, it is better to use lower magnification objectives of modest N.A. in order to yield images with more working distance and more depth of field. (Depth of field refers to good focus in the area just below or just above the area being examined.)

Fig. 34. The diminution of field depth by increased N.A.

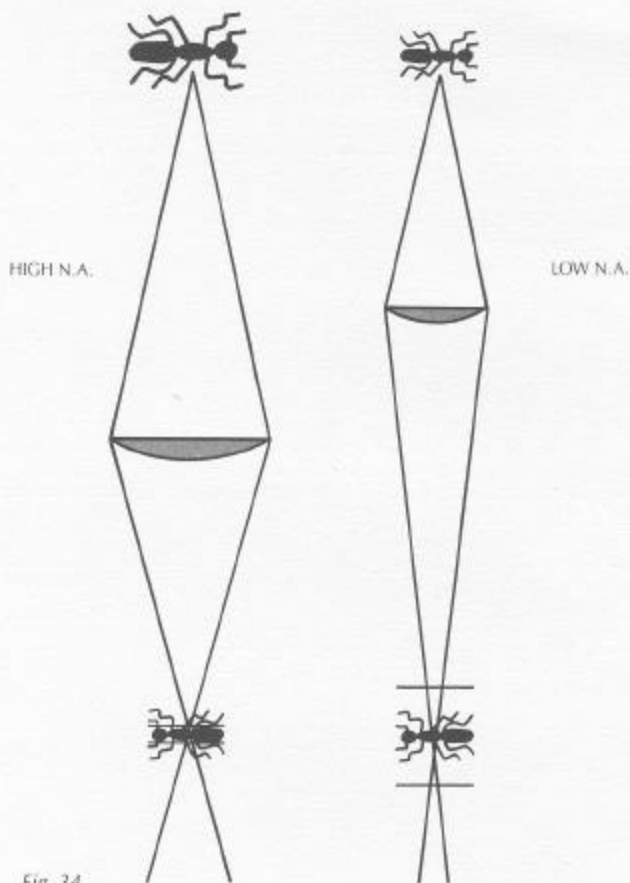


Fig. 34

This booklet has described the "basics" of microscopy. It was severely condensed in order to provide the most important ideas. Our hope is that you, the reader, will find it helpful.

