THE OPTICAL MICROSCOPE (DRAFT)

Introduction

The optical components of a microscope are its two imaging lenses (eyepiece and objective) and a condenser lens. The eyepiece and objective are responsible for magnifying the image of the specimen and projecting it onto the viewer's retina or onto the film plane in a camera. The job of the condenser lens is to focus a cone of incident light onto the specimen. To provide the incident light there is an illumination system which may include the source of the incident light or may direct external natural or artificial light towards the condenser lens. It can also provide means for enhancing the contrast and detail seen in the image. Finally, there is a movable stage which holds the specimen in the optical path and allows the specimen to be moved in and out of the focal plane and even left, right and rotated about the optic axis. To complete the instrument the microscope may include other attachments: cameras, a viewing screen, even hot and cold stages.

With this brief description of the anatomy of an optical microscope we can look at each part in a bit more detail. The objective here being to introduce the reader to the basic but essential working principles of a microscope so an intermittent microscopist may get the results they desire.

Theory of Microscopy

- Ernst Abbé, 1880's, found that image formation requires collecting transmitted and diffracted rays.
- Light is diffracted when it passes through narrow slits or other structural elements.
- Zero-order diffraction, undeviated rays, n-order diffraction, at some angle from the zero-order.
- Smaller slit spacings produce diffraction at higher angles
- To form an image all of these rays have to be recombined
- Finer structure involves higher diffraction angles
- Can see why objective lenses with small angle aperture will not reveal fine details
- Importance of aperture diaphragm in condenser

Optical Components

Objective Lens

- Highly corrected lenses
- Often with several glass elements (compound lens)
- Varying degree of corrections for spherical and chromatic aberrations

Optical Aberrations

Chromatic Aberration: The focal length of a lens varies with wavelength, blue rays focusing shorter than red rays. This problem was solved with the invention of apochromatic lenses.

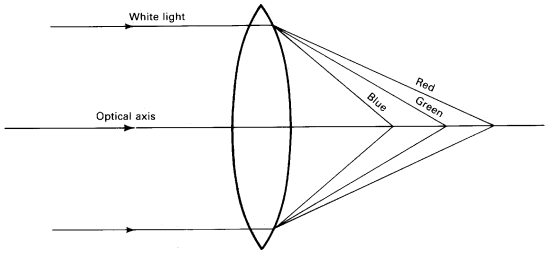


Diagram showing chromatic aberrations (3)

Spherical Aberration: Light rays passing through the center and outer parts of a lens do not focus at the same distance from the lens. This is due partly to more refraction of light at the edges and also to the wavelength dependence of refraction. Thus problem can be minimized by "stopping down" the lens, i.e., not using the edges of the lens.

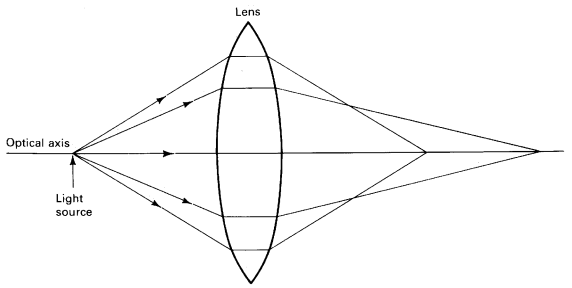


Diagram showing spherical aberrations (3)

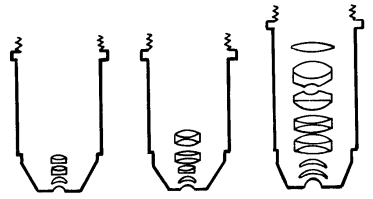
Types of Objective Lenses

Achromat

- Simplest and least expensive
- Corrections for chromatic aberrations at two points in the visible spectrum, blue and red, and spherical aberrations at one point, usually yellow-green.
- Color fringes might be seen when using white light
- Moderate numerical aperture, good working distance
- Good serviceable objective, used in some 90-95% of all microscope in use today
- Adequate for visual observations and B/W photography when green filters are used

Fluorite (semiapochromat)

- Next level of quality above achromats
- More complicated and more expensive than achromats
- Made from the mineral fluorite ($CaFl_2$), usually natural but possible synthetic
- Larger NA that achromats: brighter, sharper and more contrasty image
- More highly corrected, chromatic corrections at two points in the visible spectrum and spherical corrections at two points
- Requires matching eyepieces
- Suitable for color photography


From left to right: achromat, fluorite and apochromat lenses. (1)

Apochromat

- Highest quality lens, most complicated and most expensive
- Most highly corrected, chromatic corrections at three points in the visible spectrum and spherical corrections at two points
- Highest NA: best resolution and fewest optical defects

Specialized Objective Lenses

The following are enhancements which may be applied to the types of lenses above to make them either more useful in general or useful for specialized applications. Note: the short name given on the left for each type of lens is often engraved on the lens itself.

"Plan" lenses	Additional corrections for errors caused by different distances the on- and off-axis rays must travel, produces a flatness of field, essential for high-quality photography
Phaco	Used for phase contrast imaging, includes a special aperture and "phase ring" and specialized condenser and objective lenses

Pol	Strain-free objectives suitable for high-quality work employing polarized light
DIC	Used for differential interference work
BF/DF	Used for bright field/dark field work
Met	Used for metallurgical work, reflected light microscopy which does not employ a cover slip

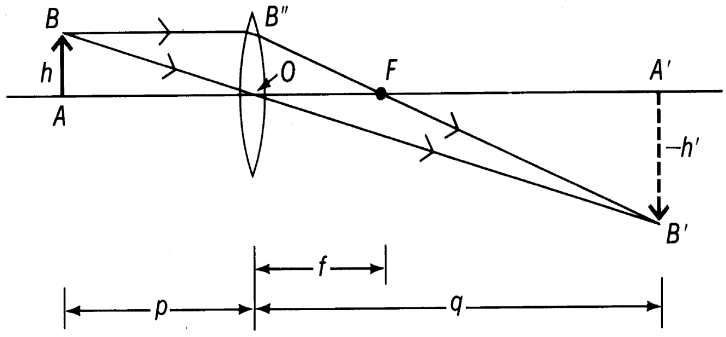
A type of specialized objective lens which does not have a common shorthand designation are those which are used on microscopes equipped with high-temperature stages. These are made of quartz (dimensionally stable and heat resistant) and provide long working distances.

Essential Specifications of Objective Lenses

Magnification

A combination of the index of refraction and the curvature of a lens deflects the light coming from the specimen in such a way that an image in front of the lens is reproduced at some distance behind it. The ratio of these distances determines the magnification of the lens.

The magnification of a series of lenses is the product of the magnifications of each lens. The maximum practical magnification of a single lens is around 3X. For compound lenses magnifications up to 100X are possible. In microscopes the overall magnification, the product of the magnification of the eyepiece and the objective lens can in principle be as high as 2500X but limitations imposed by optical quality and the wavelength of light give a practical maximum of 1500X. Magnifications above the point where no additional feature may be resolved are generally useless (hollow magnification).



$$m = \frac{h'}{h} = -\frac{q}{p}$$

Note that while the combination of a 10X eyepiece and a 25X objective lens will give the same magnification as a 25X eyepiece and a 10X objective image quality will be better in the former due to the higher numerical aperture (light gathering power) of the 25X objective.

Finally, the magnification engraved on the objective lens is nominally correct. For accurate measurements using a microscope one should either use a stage micrometer (a flat piece of glass or polished metal into which lines spaced 0.01 mm (typical) apart are cut) or refer to the manufacturer's specifications for your particular objectives.

Numerical Aperture

Loosely, the ability of a lens to collect the undeflected and diffracted light coming off of a specimen (Ernst Abbé), is defined as:

$$NA = n \sin a \quad \textbf{(1)}$$

where n is the refractive index and a is half the "angle of acceptance" of the lens. The index of refraction indicates the of degree of deflection of light when it enters and leaves a medium. For air its value is 1.0 and, since an angle of acceptance of 180° is not possible, the highest NA for a lens used in air is roughly 0.95. For oil immersion lenses the refractive index can be as high as 1.51, thus a numerical aperture of 1.4 is possible.

As a general rule the angle of acceptance increases with the lens' magnification. Therefore in general higher magnification lenses of the same type will have higher numerical apertures.

NA has significant influence on the overall performance of the lens, particularly:

Resolution
Resolution is the minimum distance between two objects which can distinguished, is given by

$$R = 0.61\frac{\lambda}{NA} \quad \textbf{(2)}$$

where 8 is the wavelength of the light and R is the resolution, also called resolving power. Resolving power increases with increasing NA which in turn increases with increasing magnification. Note that it also increases with decreasing wavelength, making the use of UV and near-UV incident light attractive. The theoretical maximum resolution in visible green light (8=550 nm, NA=1.5) is 0.22 microns. If UV light is used (8=365 nm) the resolution drops to 0.15 microns.

Depth of Field
Depth of field is defined as the distance from the nearest object plane and the farthest object plane which are in acceptable focus.

Related to object-to-lens and lens-to-image distance. In microscopy object-to-lens distance is much less than the lens-to-image distance. The depth of field is small, on the order of microns. It can be expressed mathematically as

$$d = \frac{\lambda\sqrt{n^2 - NA^2}}{NA^2} \quad \textbf{(3)}$$

for which a typical range using green light is 8.5 to 0.19 microns. Larger depth of field can be achieved by using longer wavelengths, but this would be done at the expense of resolution. In practice the aperture diaphragm is adjusted to achieve and acceptable compromise.

Note that while higher magnification provides both greater enlargement and higher resolution the depth of field decreases, making focusing, flatness of the specimens and the tilt of the specimen with respect to the optic axis more critical.

Tube Length
The mechanical tube length is defined as the distance between the point where the objective lens is screwed into the microscope tube to the top of the draw tube. A common tube length is 160 mm but 170, 200 and 210 mm tube lengths are also used.

Objective lenses are designed to work properly in a microscope of a specific tube length. Poor performance and incorrect magnification may result from using objectives of incorrect tube length. Usually the mechanical tube length is marked on the lens. Lenses marked with "∞" are corrected for infinite tube length.

Cover Glass Thickness
A cover glass introduces spherical aberrations which are corrected for in many objective lenses. This correction, however, requires that the cover glass have a specified thickness. Thicknesses typically specified are 0.17 and 0.18 mm and this is usually marked on the lens housing.

For reflected light microscopy no cover glass is used and the lens is not corrected. In this case the cover glass thickness specified is 0 mm and are marked with either a "-" or with the initials o.d. (German for ohne decke, without cover).

Immersion Lenses
As mentioned above, the numerical aperture of a lens can be increased from 0.95 when used in air to 1.4 when a droplet of oil or a similar liquid fills the gap between the lens and the object. Other liquids used are water and glycerol.

Immersion lenses will be clearly marked to indicate which liquid medium which should be used. Water immersion lenses will be marked with "WI", "wasser", "water", "water immersion", oil immersion lenses will be marked "oil", "oel" or "HI" and glycerol immersion lenses will be marked "Glyz".

While immersion lenses should be used with the appropriate liquid medium, dry lenses should always be used in air. There is the danger that the liquid will seep into the lens assembly, damaging and possible destroying it.

Eyepieces
The function of the eyepiece is to magnify (typically 8X to 25X) the image formed by the objective lens. Some are compensated to correct for residual defects in this image. Other may have a high-point for people who wear glasses and others, specified as "wide field", allow one to see a larger area of the image.

There two basic types of eyepieces, Huygen and Ramsden. Both consist of two lenses and an aperture. The differences are mainly in the location of the aperture and the orientation of the lenses.

Eyepieces may be fitted with reticles which are useful in measuring the size and shape of features and for framing the area which will photographed.

Condenser Lens

The condenser lens, often called the substage condenser, is the third optical element of the microscope. Its function is to focus a cone of light onto the specimen. It is a very important part of the microscope but is often the most ignored part (2). Proper use of the condenser lens will provide the clarity and resolution you expect from the objectives of a given numerical aperture.

The condenser lens is actually an assembly consisting of from one to several lenses, possibly corrected for chromatic and spherical aberrations. Like the objective lens the condenser has a numerical aperture.

Associated with the condenser is the aperture diaphragm. This aperture diaphragm is adjustable, often the only adjustable component in the condenser, but is very important to image quality. The size of the opening in this diaphragm defines the angular size of the cone of light which illuminates the specimen.

Illumination System

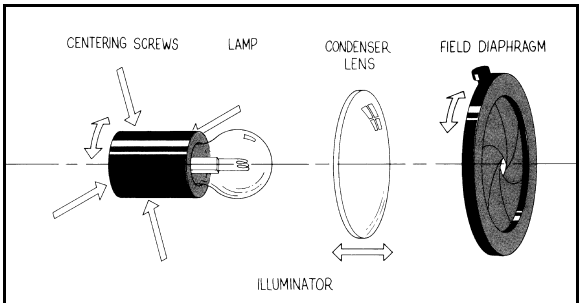
The illumination system consists of the light source and a series of lenses and apertures whose function is to focus an intense, homogeneous cone of light onto the specimen plane. It may also include filters, diffusers and polarizers.

Correct illumination is critical but is often neglected. Eighty percent of micrographs submitted for contests and exhibitions are rejected due to improper illumination and another ten percent are rejected because of improper aperture diaphragm adjustment. The same can be said of published micrographs. Only when the illumination system is correctly adjusted can the rest of the microscope perform as it should.

Parts of the Illumination System

Light Sources

The ideal light source would be intense, homogeneous and would include the desired spectral range. In general, the power of the lamp will determine overall intensity and the type of lamp used will determine the spectral content of the light. Lamp design will determine the homogeneity of the source while the rest of the illumination system is set up to make it appear as homogeneous as possible at the object plane.



A simple illumination system (2).

Types of Light Sources

- Tungsten coiled-filament: 6-12 volt, 60 to 100 watts. Color temperature varies from 2700K to 3200K as a function of electrical power, lamp design and lamp age.
- Tungsten ribbon filaments: 6 volts, 18 amps, bright homogeneous source
- Xenon arc: high intensity and daylight quality with continuous spectrum in the visible and UV ranges. Used for high resolution and fluorescence microscopy.
- Mercury vapor: excellent monochromatic light sources, 546 nm (green), 436 nm (blue) and 365 nm (UV) when proper filters are used
- Zirconium arc: point source, color temperature of 3200K.

Lamp Condenser
Also called the field condenser, collector lens and lamp collector, it should not be confused with the substage condenser. Its function is to project an image of the lamp filament.

Field Diaphragm
Controls the size of the illuminated field of view in the microscope

Filters
Filters are used to improve the quality of illumination, to reduce the intensity of illumination, to alter the spectral content of the light and to enhance specimen contrast and resolution. Common types of filters are:

Diffusers	Renders the light from the lamp more uniform
Neutral density filters	Reduces the intensity of the incident light without altering the spectral content
Green filters	Improves resolution in B/W photography
Blue filters	Compensate for the excess in yellow in the light from tungsten lamps

Filters are usually located in front of the field diaphragm. If they are located near the lamp must be able to withstand the heat. Also, they must be clean or else dust spots can be projected, partially focused, onto the object plane.

Adjusting the Illumination System
The precise details of this procedure varies with the type of illumination system but they do have several things in common. A few things to look for when adjusting your illumination system are:

1. Centering of the light source

2. Adjustment of the field diaphragm. Open it until the object stops getting brighter.

3. Alignment and focus of the condenser lens

4. Adjustment of the aperture diaphragm. Stop down this diaphragm to where it can be seen through the eyepiece and then open it until it just disappears from view.

Contrast Methods
Once a good image of the specimen has been obtained it may be necessary to take measures which make it easier to distinguish one feature from the next. This might be something as simple as adjusting the intensity of the light source, using colored filters, or another method, including possibly one of the methods described below.

Bright Field
In bright field microscopy the cone of incident light is focused onto the specimen and all undeflected and all diffracted rays are used to form the final image. Contrast is a function of specimen transparency, color and other properties.

Dark Field

In dark field microscopy a beam stop is placed in the middle of the cone of light which is focused onto the specimen by the condenser lens. This enhances the details in the image by excluding most of the undeflected beam and possibly a number of low-order diffracted beams from the final image. The background appears black and the specimen is bright.

For reflected light microscopy one way to look at dark field illumination is that the specimen is illuminated from an angle and that light hitting the edges of features are diffracted or reflected into the objective lens and are used to form the final image. Flat areas will appear dark and edges will appear bright.

Rheinberg Contrast

Instead of using a beam stop one can use a two-colored filter in which one color fills only the center portion and another color the outer portion, for example a blue center and a yellow edge. The result would be a blue background and a yellow object.

Polarized Light

For reflected light microscopy contrast enhancement using polarized light is used for certain optically anisotropic materials where conventional etching and imaging techniques fail to reveal sufficient detail. The process goes like this. When light enters the polarizer (a rotatable polarizing film on the incident light side of the specimen) only the light whose "vibrations" are parallel to the polarizing plane of the polarizer can pass through it. When this plane polarized light reflects off an optically anisotropic metal surface the light is reflected as two components which are rotated and plane-polarized at right angles to each other. The strength of each component and the degree of rotation varies depending on the crystallographic plane. Finally, when this light passes through the analyzer (a fixed polarizing film in the path of the reflected light) those elements of the light which are aligned with the analyzer may pass. All others are estinquished. If the analyzer and polarizer at set at 90 degrees to each other then only the rotated components of the reflected light will pass. In this way one can obtain varying intensities from different grains since the orientation of each grain will be different, exposing different crystallographic planes at the surface. If the specimen is rotated the brightness of each grain will change.

Phase Contrast

In phase contrast microscopy additional contrast is obtained by retarding the undeflected and diffracted rays by differing amounts before recombining them to form the final image. As usual, a cone of light is focused onto the specimen and some of this light will be diffracted and some will not. Both sets of rays then pass through the objective lens but before they form the final image the undeflected rays also pass through an annular phase ring. This ring retards these rays by one-quarter wavelength (green light). The result is a two-component light which forms an image which includes the partially destructive interference due to the one-quarter wavelength phase shift.

The specimen can also retard these rays by up to one-quarter wavelength. This can have a significant effect on the final image since the ¼ + #¼ wavelength shift can create blacked out areas due to nearly completely destructive interference. The results can be striking and this technique is not

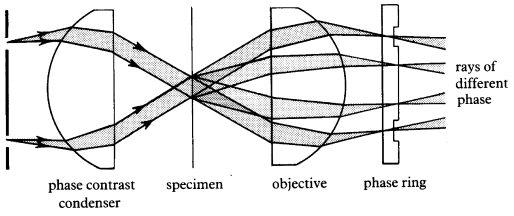


Diagram showning phase contrast optics (1)

effected by staining, allowing the microscopist to examine unaltered specimens. One disadvantage is an artifact which appears as halos around sharply delineated edges.

When used in reflected light microscopy this technique reveals slight, as small as 0.005 microns, changes in height of the specimen. But like the halo artifact seen in transparent specimens, bands of light and dark regions are often observed, for instance, along an inclined section of the specimen.

Phase contrast microscopy require special condenser and objective lenses. The are usually marked "Phaco" on the lens housing.

Differential Interference Contrast

DIC microscopy is similar to but more complicated than the phase interference system. The DIC technique begins by polarizing the incident light and then passing it through a prism which splits the light and rotates the two parts 90 degrees so that they are cross-polarized. This light now passes through or is reflected by the specimen, is recombined using a prism and is once again polarized.

The result is high-resolution images, brilliant colors and a striking three-dimensional illusion. The mechanism responsible for the contrast and color is similar to that in phase contrast method. In reflected light microscopy the contrast comes from slight differences in the distance between the prism and various spots on the surface of the specimen.

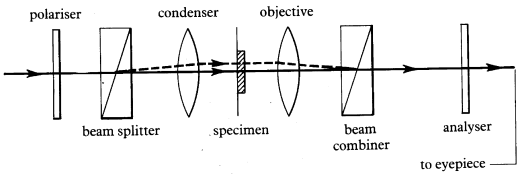


Diagram showing DIC optics (1)

In the DIC technique one polarizer, the analyzer, is fixed while the other can be rotated. This rotation can produce either negative, positive, cross and neutral positions/rotations. In the neutral position the DIC effect is minimized and the image appears flat and contrast is similar to that of bright field illumination. Positive and neutral settings generate varying levels of contrast accompanied by changing colors and the illusion of relief, negative where scratches appear as ridges and positive scratches appear as they should. In the cross polarized position the image appears almost as it would in dark field illumination.

DIC does not produce the halos seen in phase contrast microscopy, but it does produce a few of its own artifacts. The appearance of three-dimensions is an illusion. This can be demonstrated by looking at a micrograph of a fairly flat specimen (i.e., a polished and etched metal) and noting which parts appear raised and which appear lowered and then rotate the micrograph 180 degrees and notice that the relief has been reversed. A second artifact are the colors, which while they can be fabulous, are false.

References

1. J.Burgess, M.Marten and R.Taylor, Microcosmos, Cambridge University Press, Cambridge, 1987.

2. J.G.Delly, Photography Through the Microscope, Eastman Kodak Company, 1980.

3. Metallography and Microstructure, ASM Metals Handbook, Volume 9, ASM, Metals Park. Ohio, 1985.