Materials And Structure Property Correlations

Characterization Technique: Optical Microscopy

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OPTICAL MICROSCOPE

Introduction :

The past decade has witnessed an enormous growth in the application of optical microscopy for micron and submicronlevel investigations in a wide variety of disciplines. Rapid development of newfluorescent labels has accelerated the expansion of fluorescence microscopy in laboratory applications and research. Advances in digital imaging and analysishave also enabled microscopists to acquire quantitativemeasurements quickly and efficiently on specimens ranging from photosensitive caged compounds and synthetic ceramic superconductors to real-timefluorescence microscopy of living cells in their natural environment. Optical microscopy, with help ofdigital video, can also be used to image very thin optical sections, and confocal optical systems are now in operation at most major research institutions. Early microscopists were hampered by optical aberration, blurred images, and poor lens design, which floundered until the nineteenth century. Aberrations were partially corrected by the mid-nineteenth century with the introduction of Lister and Amici achromatic objectives that reduced chromatic aberration and raised numerical apertures to around 0.65 for dry objectives and up to 1.25 for homogeneous immersion objectives. In 1886, Ernst Abbe's work with Carl Zeiss led to the production of apochromatic objectives based for the first time on sound optical principles and lens design. These advanced objectives provided images with reduced spherical aberration and free of colour distortions (chromatic aberration) at high numerical apertures.

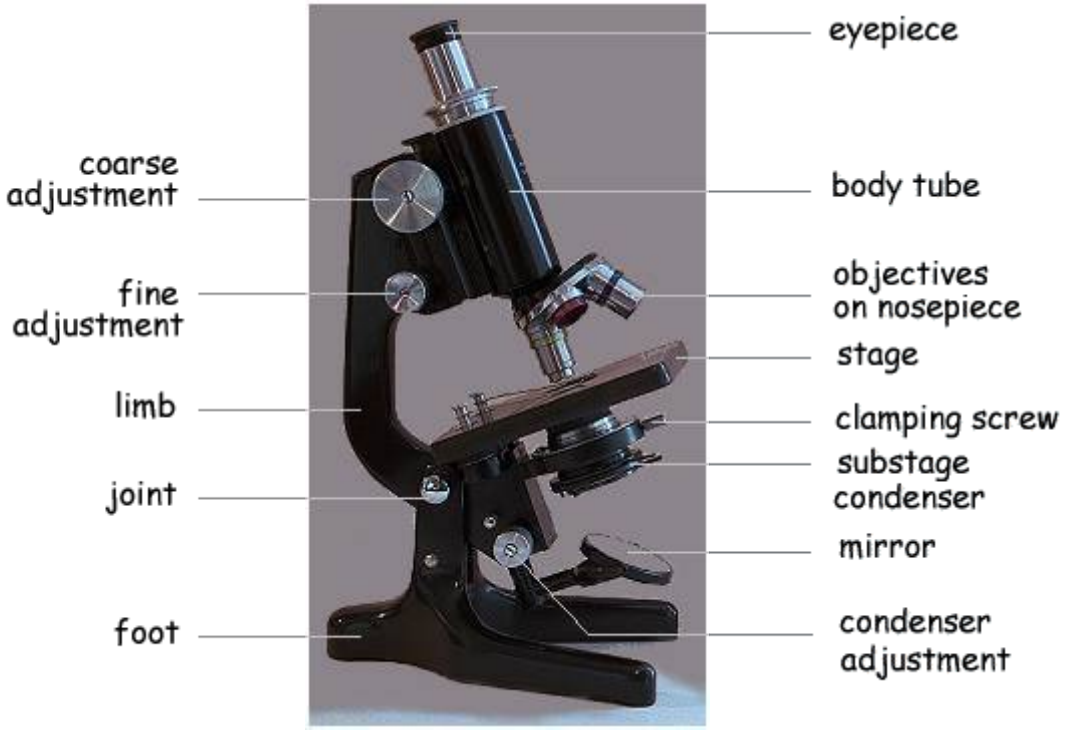
Physicist Georges Nomarski introduced improvements in Wollastonprism design for another powerful contrast-generating microscopy theory in 1955. This technique is commonly referred to as ***Nomarskiinterference*** or ***differential interferencecontrast*** (DIC) microscopy and, along with phase contrast, have allowed scientists to explore many new arenas in biology using living cells or unstained tissues. Robert Hoffman introduced another method of increasing contrast in living material by taking advantage of phase gradients near cell membranes. This technique is now termed Hoffman Modulation Contrast, and is available as optional equipment on most modern microscopes. The majority of microscopes manufactured around theworld had fixed mechanical tube lengths (ranging from160 to 210 millimeters) until the late 1980s, whenmanufacturers largely migrated to infinity-correctedoptics. Ray paths through both finite tube length andinfinity-corrected microscopes are illustrated in Figure1. The upper portion of the figure contains the essentialoptical elements and ray traces defining the optical train

The Components of the Microscope.

The Watson "Service" microscope shown here is of a general design developed in the early 1900s, and microscopes of this type are still in wide use. It is a simple, rugged, well-made instrument of a kind that, with proper care, will still be functional after hundreds of years of frequent to occasional use.

Additionally, it has a drawtube -- the collar seen between the eyepiece and the body tube -- which makes it possible to alter the mechanical and optical tube length of the instrument.

The use of this refinement will be covered in the advanced tutorials.



Component Parts of the Watson Service Microscope (c. 1950).

The optical components are those of all compound microscopes, and even though this model has no integral lamp, this discussion will begin with the lamp, and proceed in sequence via the mirror to the eye.

Microscope Lamp.

Microscopes require, especially at the highest powers,intenseillumination. The intensity of a light source depends not so much upon its absolute power as upon the amount of light emitted from a given area of the source -- lumens* per square millimetre rather than just lumens. To

achieve high intensity, various lamp designs have come and gone, but one of the commonest and most satisfactory is the low-voltage tungsten filament lamp, with the filament in the form of a tightly
woundflattened grid.

The combination of a suitable quartz-halogen bulb with a concave spherical reflector works well and is also in wide use.

Mirror.

The mirror is used only to fold the optical path of the microscope into a convenient space. It also introduces another source of potential maladjustment into the system, and another surface to collect dust. Having said this, the mirror does not need to be of the highest optical quality to do its job, nor does a small amount of dust on the mirror make much difference to the quality of the image. A slight film of fine dust (such as remains after dusting the mirror with a blower brush) can actually be useful in locating the beam of light from the lamp when setting up the instrument. Always use the flat side of the mirror in combination with a substage condenser

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Substage Condenser.

The substage condenser fitted to most microscopes is of a design originated by Ernst Abbe in the late 1800s and is usually referred to as the Abbe condenser. Whilst condensers of higher correction are available, the Abbe condenser has proven to be quite satisfactory for routine microscopy. A substage condenser of some kind is an absolute requirement for serious -- or at least satisfactory -

- microscopy. The objectives from x20 upwards require the subject to be illuminated evenly over quite a large angle, and neither a concave mirror nor (especially) a flat mirror is capable of achieving this. If no condenser is used with a high power objective, the result is an image which is dark, coarse, contrasty and lacking in detail -- described by earlier microscopists as "a rotten image". Lower power objectives however, can give acceptable, even quite pleasing images without a condenser if the mirror is directed toward a close, well frosted lightbulb or a bright white cloud.
An important point to note here is that the substage condenser diaphragm is used to control the solid angle of the light emerging from the condenser, illuminating the specimen, and filling the objective -- not for adjusting the brightness of the image. Brightness adjustment can be achieved by removing or placing a filter in the substage stop-carrier or by dimming the lightbulb, locating a brighter or a greyer cloud etc.

In practice, once the microscope has been set up, the condenser and its diaphragm setting can be largely forgotten until the objective is changed for one of higher or lower power.

Specimen

The specimen is usually supported by a slide and, essentially at the higher powers of the biological microscope, covered by a coverglass. Thus introduced into the image-forming light path, slide and coverglass become part of the optical system. The thickness of the slide is important to the correction of the substage condenser, and the thickness of the coverglass is critical to the performance of the objective, especially those of higher power (x20 and greater).

Most substage condensers are corrected to work with a slide thickness of 1.0mm, and most microscope objectives of x20 or greater power are designed to work with a coverglass thickness of 0.17mm (thickness no. 1½).

Objective.

The objective is the most important component of the optical system in terms of the quality of the final image. For over a hundred years, dating from Ernst Abbe's introduction (in the 1870's) of apochromatic corrections, the best objectives have been capable of resolving the finest detail predicted by theory. Since then, great improvements have been made in field size, field flatness and image quality toward the edges of the field. The modern microscope objective probably represents the highest degree of optical perfection and precision engineering which is manufactured in volume for public consumption. The diagram shows a construction (not to scale) typical of a x40 achromatic objective standard on most laboratory microscopes.

The screw thread of microscope objectives has been a standard across the industry since 1858, when it was first proposed by the Royal Microscopical Society. Here is a diagram of the RMS standard objective thread.
More recently, larger diameter threads have appeared to accommodate the needs of modern objective design.

Eyepiece.

The eyepiece relays to the eye an image projected by the objective into the plane of the eyepiece diaphragm, further magnifying it in the process. In older microscopes, the

eyepiece also corrected residual colour errors remaining in the objective. Modern infinity-tubelength objectives are fully corrected in themselves, but still require additional focusing optics and appropriate eyepieces to produce their image.

In short, all objectives manufactured before the arrival of infinity-correction required "compensation" of varying degrees. There was no industry-wide standard on the matter, so each manufacturer produced eyepieces which compensated the lateral colour errors of their own objectives. The degree of compensation of a compensating eyepiece can be roughly gauged by the intensity of the red fringe seen inside the eyepiece diaphragm when used on brightfield. The brighter the fringe, the greater the degree of compensation.
All of the apochromatic objectives of this (almost hundred year) period, and many of the higher power achromats, required compensating eyepieces.

Eye.

The cornea and the eye lens are the final optical components in the image forming path to the retina. In a person with normal vision, the eyelens will be relaxed as though the eye is forming an image of a very distant object, and the focusing controls on the microscope used to achieve image sharpness. The optics of the eyepiece are such that all image-forming rays pass through a circle (called the Ramsden disc) a few millimetres exterior to the eyepiece lens and just smaller than the diameter of the pupil. The eye is bought close enough to the eyepiece for the ramsden disc and the pupil to coincide, at which point the full circular field of the microscope is seen.

Learning to hold the head still in this optimum position,

especially with a binocular instrument, is one of many skills acquired by the microscopist.

Construction and Use.

The cross-section diagram (right) is of an Abbe condenser of one hundred and twenty years ago. Except for minor mechanical details, this diagram describes the condenser routinely fitted to laboratory microscopes today. Microscopes usually leave the factory with the substage condenser adjusted so that the closes approach to the specimen brings the toplens surface to within a whisker of the stage surface. With a specimen on a slide of normal thickness (c. 1.2mm) the condenser will still focus the lamp diaphragm in the plane of the specimen, but with the toplens safely below stage level. This makes it impossible to scratch the condenser toplens with the slide. In practice, most condensers achieve their focus with something less than 1mm between the toplens and the slide. It is then feasible to immerse them to the under-surface of the slide with oil (or water) to achieve a larger illumination aperture, better spherical correction and increased light throughput. The illustration is of a Watson two-lens Abbe substage condenser of c.1950, and is typical of condensers of its kind. The strongly curved top-lens can be removed to give a lower power condenser which is better suited to illuminating the field of low power objectives. The stop carrier is used for holding light filters and diffusers, and for holding and positioning darkfield stops. The use of diffusers cannot be recommended except as a last resort in an

attempt to obtain an evenly illuminated field. The penalty for using them is loss of control of the illuminated field in the specimen plane (with associated flare and contrast loss) as the lamp diaphragm cannot be seen or focused. A diffuser will also severely reduce the intensity of the illumination. If light intensity control is not possible at the lamp, it is best achieved by placing neutral density filters in this carrier.

Principle:

The Light Path through the Microscope.

Rather than attempting to illustrate all ray paths using a single optical diagram, the distribution of light in a Köhler-illuminated microscope is here represented as four light paths of practical importance to the microscopist:

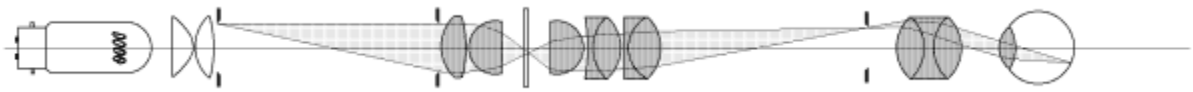
1. The path taken by light emanating from the lamp filament,
2. The conjugate image positions of the lamp field diaphragm,
3. The conjugate image positions of the substage condenser diaphragm, and
4. The image forming rays from the specimen to the eye.

...and presented as four parallel diagrams (below). The lens systems shown are not to scale, and the ray paths are representational only -- they are not the result of calculation or ray-tracing. In the real world, a differing choice of condensers, objectives and eyepieces will give slightly differing ray paths.

These diagrams (conforming to the convention that light rays should pass from left to right) extend out of the page to the right for two or three screens (depending upon your screen resolution) enabling a detailed examination of the four ray paths. It is then possible to form a complete impression of what is happening in any section of the microscope by scrolling vertically between the diagrams to compare the ray paths.

Images of a given point are formed in the optical train wherever the rays originating from that point cross over. The first image formed will be upside-down; the second will be right way up -- and so on in alternation throughout the system.

Path 4 shows that the first (inverted) image of the specimen is formed in the plane of the eyepiece, and the second (erect) on the retina. The retina normally receives inverted images of everyday objects, so the microscope image therefore appears upside down. Adjusting to this in order to follow a moving specimen is probably the first major skill required of the beginner.



The Light Microscope.Image of the Lamp Diaphragm.

The Optimized Microscope.

The term "Köhler-illuminated microscope" describes a system in which all the optical components are design-optimized to work together and are mutually focused upon one another. In this configuration, certain image planes will always coincide. The reader will have noticed that ray path 3 is contained within ray path 1, and similarly, ray path 4 is contained within 2. This demonstrates the fact that in a Köhler setup, the images of the lamp diaphragm and the specimen always coincide, as do the images of the lamp filament and the substage condensesdiaphragm.

These conditions define the principle and practice of Köhler illumination and give the microscopist control over aperture of illumination (and therefore image resolution, contrast and depth of focus) and over area of field illuminated (giving additional contrast control by reducing glare caused by extraneous light). To the extent that the setup of the microscope departs from the Köhlercondition, the above controls are less precise in their effect. If the diaphragms and their images are not correctly located, diaphragm use produces vignetting* with its attendant effects on resolution and evenness of illumination.

The next step is the hands-on business of configuring the microscope to the Köhler system.

Setting Up Köhler Illumination.

The following account assumes a laboratory grade microscope with a mirror and a separate free-standing microscope lamp with variable power supply. Users of microscopes with built-in illumination must adapt the instructions where necessary and be content with the manufacturer's setting when no means of adjustment is available.Whilst an adjustable mirror can be the cause of maladjustment, it also provides the experienced microscopist with a valuable means of making fine adjustments to the system, and is a useful tool in troubleshooting illumination problems. A separate microscope lamp and mirror also allows the easy placement of light-modifying filters and diffusers etc. in the illumination path. The instructions can be implemented without problems for dry objectives in the x10 - x60 magnification range. Some difficulties are likely to be encountered in using very low power objectives and oil-immersion objectives.

Refractive Index, Angular Aperture and N.A

Although microscopists had known for most of the 19th.century that the resolving power of a microscope objective was dependent upon its angular aperture*, this could not be used as a measure of resolution since it varied greatly depending on the refractive index of the medium through which the image-forming rays passed. It was equally clear that the information contained in the cone of image-forming rays did not change, even though the angle of the cone did. It was Ernst Abbe (1840 - 1905) who first described the relationship between image resolution, angular aperture and refractive index of medium. He called the new measure of

objective performance "Numerical Aperture" or N.A., and defined it thus: N.A. = R.I. sin Ø

where Ø is the angle the ray makes with the optical axis (half the value of the angle of the image-forming cone), and R.I. is the refractive index of the medium through which the rays pass. It is clear that Ø can never be larger than 90°, and since sine of 90° is 1, no objective can have an N.A. numerically larger than the R.I. of the medium in which it is working. This implies an acceptance angle for the frontlens of the objective of 180°. In practice, the largest angle of acceptance of glass lenses in any medium is around 140°, so the N.A. of an objective is always less than the theoretical maximum.
Consider the diagram below which represents a dry objective of N.A. = 0.95 examining a specimen in water under a coverglass. The acceptance angle of this objective is 144° in air, which becomes 77° in the coverglass, and 91° in the water containing the specimen. Even though the specimen may have detail producing first order maxima at a greater angle than shown, these rays (in blue) cannot escape the glass-air surface of the coverglass. They strike this interface at too oblique an angle for refraction, and are totally internally reflected back into the glass.

Magnification and Image Details

Of the two main design criteria which specify the performance of a microscope objective, N.A. is perhaps the most important in that it defines the limit of detail which can be resolved by the lens. The other important criterion is magnification, which determines at what degree of enlargement that detail is presented to the eye. When an operator views an image through a correctly adjusted

microscope, the eye lens is totally relaxed as though viewing a distant horizon -- the eye is focused for infinity. The focus mechanisms of the microscope bring to the relaxed eyes of its operator a sharp image of any object in the field of view. But any image arriving at the eye from infinity must be infinitely magnified. This would imply that all optical devices producing an aerial image have the same infinite magnification in spite of our experience that some images are definitely more magnified than others.

This anomaly was resolved sometime in the nineteenth century by deciding that 10 inches (250 mm) was a suitable close viewing distance for most people to see the finest detail of which the eye is capable, and that magnifications could then be calculated if it was assumed that the image seen in the eyepiece of a microscope was actually located in space at a plane ten inches from the eyes of the operator. At that distance (or any distance short of infinity) all features of the image would have calculable dimensions, and would therefore be comparable to other images measured in the same plane.

The actual magnification is determined by using a object of known size, and then measuring the size of its image when projected 250mm from the exit pupil of the instrument -- in the case of the microscope, 250mm from the Ramsden disc of the eyepiece. Conversely, the size of any feature of the object can be determined by measuring the size of that feature's image, and dividing by the magnification.

Troubleshooting Setup Errors.

There are many potential sources of error in setting up a microscope, and tracking down their precise cause is not always easy. Apart from the more obvious failures to produce an image caused by faulty lamp electrics, a knocked mirror etc, the most common problem could be described as a generally poor quality image, especially on the highest powers and oil immersion. A poor quality image obtained with dry objectives is often accompanied by a tendency for the image to shift back and forth as the fine focus adjustment is applied. In a correctly set up microscope, all points on the image should go symmetrically in and out of focus as the fine adjustment is used. The shifting effect is almost always due to one or more optical components being out of centre, or some object encroaching upon the illumination or image forming rays.

The latter possibility is best checked by removing the eyepiece and observing the objective back lens. The image of the substage diaphragm should be concentric with the objective back lens, and any obstruction to the image forming rays should be visible if present. A common cause of obstruction is a substage stop carrier which is partly obstructing the rays entering the condenser, or less commonly, some object which has fallen into the upper part of the objective.

Errors in the centration of the optical components of the microscope must be remedied by repeating the appropriate steps in the Köhler setup procedure.

Dark patches or objects which are visible with the eyepiece replaced may be located by carefully rotating each component of the optical system in turn, and noting whether the object rotates as well.Start with the eyepiece, which is the easiest component to rotate and a common location of annoyingly

obtrusive dirt particles. The objective may be carefully unscrewed and rotated, but if nothing was seen when the back lens was checked, this is less often the cause of problems. The condenser may be unclamped and rotated, though condensers can get quite grubby before they seriously degrade a bright field image.

The remaining possible location of dirt and obstructions affecting the image is the lamp. The dirt may be located on the condenser or the lamp envelope. Light modifying filters close to the lamp must also be kept quite clean. In short; the lamp, its bulb, condenser, diaphragm and any associated filters must be kept clean at all times, as any debris located there will be reimaged in the plane of the specimen (or its image) all the way through the system. Techniques for removing dirt from lenses, once located, will be dealt with in a later update of these notes. Poor image quality in oil or water immersion setups is often caused by obscuration of the objective front lens by air bubbles in the immersion medium. This can be verified by removing the eyepiece and carefully examining the objective back lens at various levels of focus using a phase telescope. This will clearly identify any bubbles if present. The remedy consists of removing the bubbles, along with the original oil or water, and reimmersing the objective.

Immersion objectives in which the frontlens is concave or somewhat recessed may require that a small drop of the immersion medium is applied to the frontlens as well as the specimen to avoid the formation of bubbles.