

The Micrographia Tutorials.

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This tutorial is for those whose work involves day-to-day use of the light microscope, as well as those approaching the instrument for the first time.

It gives an easy to follow procedure for ensuring that the instrument is set up not a hundred miles from the maker's specification. It is in effect an instruction on how to set up brightfield Köhler illumination. (August Köhler, an employee of the Zeiss microscope company, proposed this method of setting up the microscope around a hundred years ago).

An understanding of this simple and elegant method of illumination is a prime requirement for both routine and critical microscopy, as it underlies all the commonly encountered forms of microscope illumination -- darkfield, phase contrast, interference contrast and the various types of incident illumination -- which are covered in detail by other tutorials.

The instruction is aimed at the user of the laboratory microscope. Instruments below this level may or may not have an adjustable substage condenser, and since this component is essential for satisfactory imaging, especially at the higher powers, instruments without one are not considered here (but are dealt with in the tutorial on minimal microscopy).

The intention is to pass on sufficient information that microscope users may better understand their instrument, set it up in a way that produces a satisfactory image in a comfortable setting, and proceed with their work.

Those aspiring to aficionado status in critical microscopy and to the use of the microscope as a source of pleasure in itself should proceed to the Advanced Tutorials.

Types of Microscope Stand.

Even though the outward appearance of the microscope has changed greatly over the last century or so, the purpose of the design has not. The function of the microscope stand has always been to hold the same components -- mirror, condenser, specimen, objective and eyepiece -- on a common optical axis, and to allow finely controlled axial movement between them.

The stage supports the specimen to be examined and allows specimen movement in a plane perpendicular to a fixed optical axis.



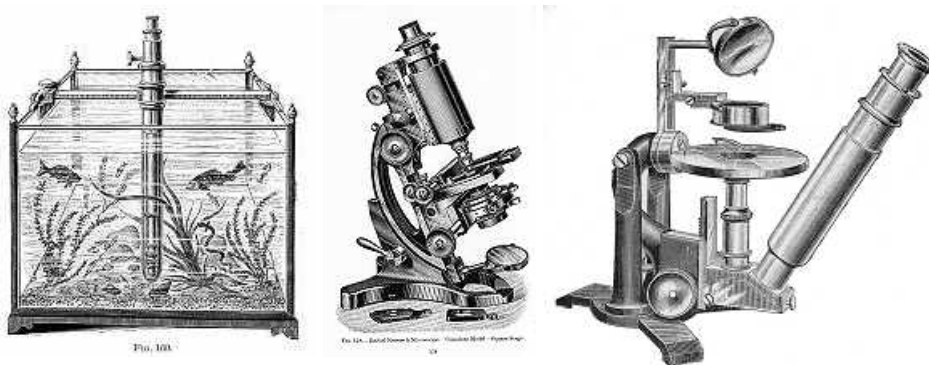
Three basic designs of microscope stand of the past 150 years.

In terms of mechanical stability, the end result of this evolutionary process is the modern design in which the limb/base is a rigid fixture and all focusing actions are applied to the stage -- seen on the Olympus microscope (right) above -- a construction much better suited to supporting heavy accessory devices such as zooms, trinoculars and photomicrographic cameras.

Recent microscopes incorporate the lamp into the base, enabling the instrument to be moved from one place to another without upsetting the relationship of the components, so the lamp can now be added to the list of components held in alignment by the stand.

The detachable lamp unit seen on the Olympus is now rare, except as an optional extra for less expensive microscopes sold originally with only a mirror.

In the four hundred years of its development, the microscope has been adapted to any number of specialized tasks. The illustrations below give some idea of the variety in design. Inverted microscopes have become standard if somewhat specialized laboratory instruments, and the aquarium microscope, whilst no longer manufactured, would be sure to have a modest following if it were offered for sale today.

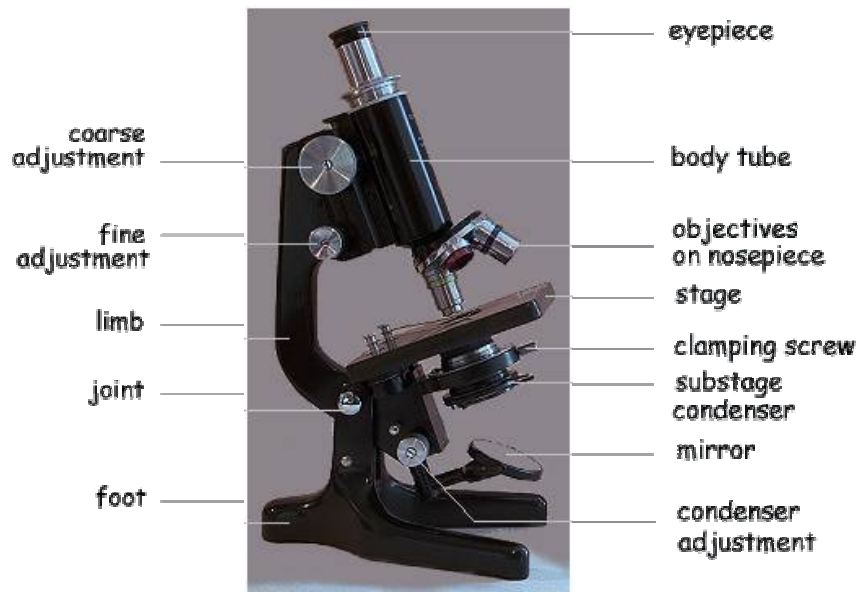


Some unusual microscope designs of the last hundred years.

The Component Parts 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.

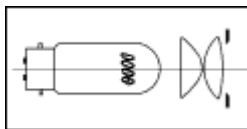
Additionally, it has a drawtube -- the collar seen between the eyepiece and the body tube -- which makes it possible to alter the mechanical and optical tubelength of the instrument. The use of this refinement will be covered in the advanced tutorials.



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.

The Microscope Lamp.



Microscopes require, especially at the highest powers, intense illumination. 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-wound flattened grid.

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

For more detail on lamp issues:

- [Closeup of lamp filament.](#)
- [Centring the lamp using the projected lamp filament image.](#)
- [Considerations in the design of a microscope lamp.](#)
- [A microscope lamp utilizing electronic flash.](#)
- [Historical background: Microscope lamps of the past.](#)

The Mirror.

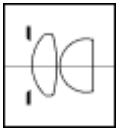
The mirror is used only to fold the optical path of the microscope into a convenient space. It also introduces another source of potential maladjustment into the system, and another surface to collect dust. Having said this, the mirror does not need to be of the highest optical quality to do its job, 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.

The 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.

More Information on Condensers.

1. [Notes on Construction and Use.](#)
2. [Dismantling and Cleaning the Condenser.](#)

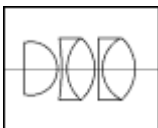
The Specimen.



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).

The Objective.



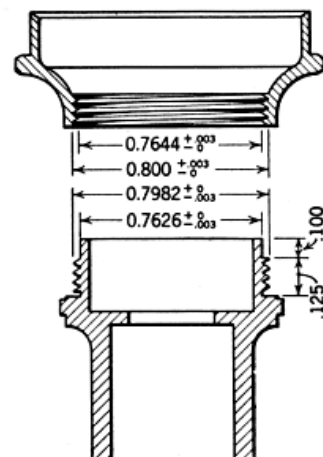
The objective is the most important component of the optical system in terms of the quality of the final image. For over a hundred years, dating from Ernst Abbe's introduction (in the 1870's) of apochromatic corrections, the best objectives have been capable of resolving the finest detail predicted by 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.

FIG. 8. This drawing, which gives the dimensions of the screw thread of the objective, in inches, as adopted by the Royal Microscopical Society, presents the information necessary for the tool or instrument maker so that he can properly fit accessory apparatus to the microscope. The form of thread is Whitworth, pitch, 36 threads per inch. This standard seems to be the only one to which microscope manufacturers consistently adhere. (By courtesy of The Royal Microscopical Society, London.)



More Information on Objectives.

A Low Power Objective.

This objective is a low power planachromat by the Czech company Meopta. It is engraved with all the information a microscopist needs for a fairly good idea of the quality of image it would produce.

Starting at the top: the figure 170 is the optical tubelength of the Meopta microscope it was designed to be fitted to. Meopta, as well as Leitz, adopted a 170mm tubelength standard, whilst most of the rest of the world went with the Zeiss standard of 160mm.

Next comes a slash and a dash. It is common practice for the tubelength figure to be separated by a slash from the figure representing the coverglass thickness for which the objective is corrected. In this case, the dash indicates that the objective's performance is independent of coverglass thickness -- you may use one or not as you please.

Next line down -- Pl indicates that the objective is a planachromat; ie, an objective of normal achromatic correction, but additionally corrected to eliminate or reduce field curvature, and therefore giving a sharply focused image of flat subjects. The figures 6.3:1 are the objective magnification expressed as a ratio to one. The final figure, 0.15, is the objective N.A.

By noting the ratio of the objective's magnification to its N.A., the experienced microscopist can predict within fairly narrow limits what the performance of the objective is likely to be. The tubelength figure is also important, as if it is intended to use the objective on a microscope stand built to the 160mm tubelength standard, all other things being equal, the objective will produce a lower magnification and have a longer working distance than if used on a Meopta (170mm) microscope.



A High Power Objective.

This is a high-power water immersion objective of Chinese manufacture and another completely specified objective.

WI of course indicates the important fact that the objective is designed to work with its frontlens immersed in water. The next line of figures are the magnification and the N.A. separated by a slash, and the bottom figures are the tubelength and coverglass correction -- much as in the Meopta objective above. The figure 0.17 is the thickness in millimetres of the coverglass for which the objective is corrected. This thickness is now standard and designated No. 1½ by most coverglass manufacturers.

In terms of predicting performance, the following considerations. The figure of 0.85 for the N.A. indicates a higher resolving power than a 40 power objective with an N.A. of 0.65. The magnification of x63, however, indicates that the image will appear less sharp than the x40, because the increase in magnification has not been matched by a corresponding increase in the N.A.



If this objective were to be used on a microscope built to the 170mm tubelength standard, the magnification would be slightly more than the engraved value, the image would show a degree of spherical over-correction, and its working distance would be less than if used on the microscope for which it was designed. There are ways of compensating these departures from specification, but they are outside the scope of the present discussion.

In the absence of any other markings, it can be correctly assumed that the objective is not flatfield, and is a normal achromat.

There are other things that the microscopist must know about an objective to obtain the best possible image from it, particularly in the choice of a suitable eyepiece, but they can be known only either by hands-on experimentation, or by familiarity with the optical practices of the company which produced it. This latter information is often only obtainable by direct contact with the technical department of the company in question.

The resolving power* of the light microscope depends upon two factors:

1. The absolute limit to resolution imposed by the wavelength of the light illuminating the specimen. No instrument which forms its image by wave interference can resolve detail which is smaller than about half the wavelength of the wave energy (light in the case of the microscope) being used to examine the specimen. This is as true of the acoustic and the (transmission) electron microscope as it is of the light

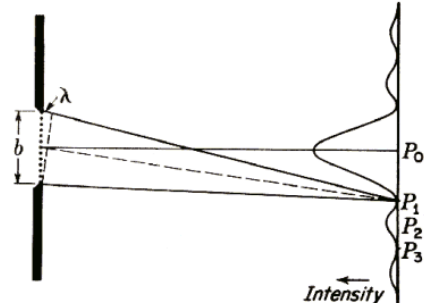
microscope.

2. The Numerical Aperture (N.A.) of the objective in use. To explain this term, it is necessary to show why the amount of detail that can be seen with the microscope depends not only on wavelength, but on the angle over which the objective is capable of receiving light from the specimen. This is in turn dependent upon the refractive indices of the media in the light path.

N.A. is the lens specification which takes these factors into account, and is effectively an index of the objective's ability to resolve fine detail.

An explanation of N.A. must necessarily deal with the optical phenomenon of diffraction.

The following account of image formation by a microscope objective is essentially a simplified version of the diffraction theory (minus the mathematics) put forward by Ernst Abbe in 1873.



Diffraction and Subject Detail.

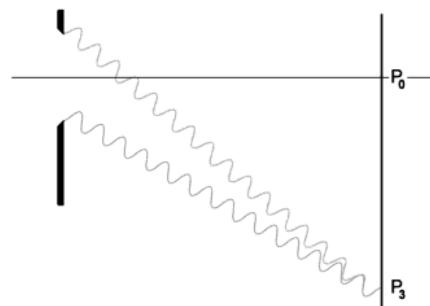
Consider a subject under a brightfield microscope which has a pattern of detail in which very small opaque objects are separated from one another by a distance equal to their own diameter. The diagram below represents the diffraction which occurs at a single narrow slit, and is used here to illustrate what happens when light passes through the space separating the opaque objects of the above example.

Given the approximation that the wavefront of light arriving at this slit from a very distant point source is planar, Huyghens's principle states that along the imaginary line b which represents the wavefront momentarily present between the edges of the slit, each point on b could itself be considered a secondary source of wavelets which radiate from that point. This provides a basis for determining the distribution of the light energy passing through the slit, which, due to interference between the rays, is neither even nor random.

The point P_1 on the screen is so situated that the light wavelet emanating from a point very close to the upper edge of the slit, and another emanating from a point very close to the lower edge of the slit have a path difference of one wavelength. Whilst these two rays interfere constructively, they are only two rays of the infinite number of ray pairs along the line b .

To determine the net effect of the interaction of ray pairs across the entire aperture, consider the ray passing very close to the upper edge of the slit, and the ray immediately adjacent to and below it. Between these two rays, the path difference at P_1 is extremely small, and the rays are very close to a condition of constructive interference -- but not quite.

As second rays emanating from points further from the uppermost ray are considered, the path difference steadily increases until the ray from the centre of the slit is reached. With this ray, the path difference is half a wavelength, and total destructive interference occurs. A condition very close to total destructive interference also occurs with rays very close to the central ray. The degree of destructive interference gradually diminishes as second rays approaching the remote edge of the slit are selected and the interference is once more constructive. The result of this at the screen P is a zone of darkness on either side of the point P_1 , with P_1 at the position of maximum darkness.



Similarly, at the point P_2 , the path difference between the upper and lower rays is $1\frac{1}{2}$ wavelengths, and P_2 marks the centre of a zone of constructive interference -- called the first order diffraction maximum. And similarly on the other side of the axis.

Second, third and higher order diffraction maxima are formed at points where the path difference is an odd number of half-wavelengths, and the intervening minima where the path difference is an even number of half-wavelengths.

The diagram on the left shows the condition for the formation of the second order diffraction minimum (P_3 above), where the path difference between the upper and lower rays is two wavelengths (four half wavelengths).

For an objective to form an image of the object detail represented by these diffracted rays, it must be capable of accepting them.

As a minimum requirement, the objective must be capable of accepting both the axial rays (centred on P0) and at least part of the first-order diffraction maximum. The clarity with which any detail is rendered depends upon the percentage capture of the diffracted rays it generates. Complete capture of the first-order diffracted rays will produce an image which is capable of revealing detail close to the diffraction limit for that aperture. Second and third order maxima, being decreasingly intense, make less contribution to image detail.

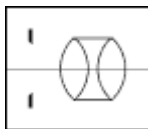
It should be clear from the diagram that the finer the detail in the specimen (the smaller the value of b), the greater the angle which must be assumed by the rays forming the various maxima in order to achieve the necessary path differences -- requiring the use of an objective of correspondingly larger angular aperture to capture them. Very fine detail will have maxima passing outside the aperture of the objective and will not be imaged.

The ability of the objective to accept diffracted rays of a given angle is however strongly dependent on the refractive indices of the media between the objective and the specimen -- usually some combination of air, water, glass and oil.

At this point the concept of Numerical Aperture becomes useful, and is dealt with in the next section.

The 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. The matching of older objectives to a suitable eyepiece is also discussed in the advanced tutorials.



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

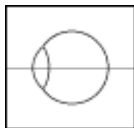
when used on brightfield. The brighter the fringe, the greater the degree of compensation.

All of the apochromatic objectives of this (almost hundred year) period, and many of the higher power achromats, required compensating eyepieces.

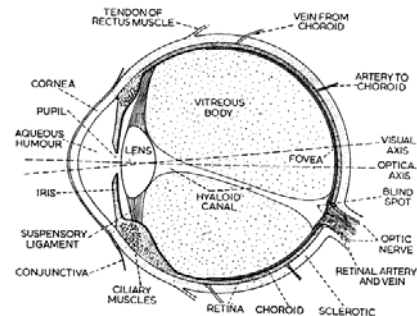
The other variety of eyepiece in common use was the Huyghenian -- best suited to achromatic objectives in general, and particularly to low power achromats which often require little or no correction. These eyepieces are distinguished by a blue fringe around their field diaphragm when the eyepiece is used in brightfield.

The 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 brought 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.

Having briefly covered the components of the microscope and their function, the next step is to refer to diagrams of the Köhler setup to see the optical relationship between the components prior to the setup procedure itself.

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.

1. Illumination Light Path.

(with notes on the major components).



The Microscope Lamp.

Light from a low-voltage high intensity bulb with a tightly wound grid filament is collected by a large aperture condenser system and focussed to produce an enlarged image of the filament in the plane of the substage condenser diaphragm. This image must be large enough to fill the substage condenser otherwise the back focal plane of the objective will not be filled with light.

The diameter of the illuminated field is controlled by means of an iris diaphragm (the lamp field diaphragm) immediately in front of the lamp field lens.

The Substage Condenser.

The condenser accepts light coming from the lamp and focuses it onto the specimen at a far greater angular aperture than the lamp alone could achieve.

It is focused to form an image of the lamp diaphragm blades in the plane of the specimen, which means that the microscope field is filled with an image of the lamp condenser lens filled with light. (See next light path).

Specimen

The Objective.

This is the most critical component in the system with regard to the quality of the image produced by the instrument. The lens system shown here is a representation of a typical X40 achromat, and in practice has a working distance of about 1 mm.

The back focal plane of the objective is shown external to the lens system. Whilst this is true for lower power objectives, the BFP of a real x40 objective is usually within the thickness of the rear elements.

The Eyepiece.

The second most critical component in the optical system. It further magnifies the image produced by the objective, and on older microscopes, also compensates for any residual chromatic difference of magnification remaining in the objective.

The additional optics of the modern infinity-corrected systems are not shown.

The Human Eye.

Along with curiosity, the reason for the existence of the microscope. The optics of the eye are simple enough -- it's how

the brain makes sense of the weirdly distorted image which falls on the spherically curved surface of the retina and processes it into the familiar images of the external world which is food for speculation.

2. Conjugate Images of the Lamp Diaphragm.



3. Conjugate Images of the Substage Condenser Diaphragm.



4. Image-forming Rays from the Specimen to the Eye.



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 condenser diaphragm.

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 Köhler condition, 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. They will be dealt with in later sections of this tutorial.

Preliminaries.

If you are thoroughly familiar with your microscope, you may not need to carry out the following steps. If the microscope is new to you, then check the following points as a minimum preparation.

1. Take the coarse adjustment through its travel to give yourself a feel for the sense and resistance and gearing of the movement. Both focusing adjustments on a microscope are highly geared, and in the case of the coarse focus, often lubricated with a stiff grease, and it is quite easy for a novice to drive an objective through the specimen slide and into the condenser toplems without being conscious of doing it.

2. Do the same with the fine adjustment and if there are marks on the stand defining the limits of its travel, set the index mark to about half way between them.

3. Focus the substage condenser upwards and, taking a line of sight across the surface of the stage, note whether the focus action stops with the condenser toplens just a fraction below the surface of the stage. A condenser toplens which can be raised above the level of the stage will not only be easily scratched by slides placed on the stage, but is capable of driving the specimen slide into the objective in the hands of the hasty or inexperienced.

A condenser which comes to a stop too far below the surface of the stage may not come to a focus when setup is attempted.

A warning. When the microscope is set up with the x40 objective, there will be about half a millimetre between the objective and the specimen slide, and a similar space between the slide and the condenser toplens. That doesn't leave much margin for operator error, and the margin is even less for the oil immersion objective.

4. Check that the nosepiece rotates and locates easily, and be careful when changing objectives if they are not parfocal, set, and/or differ greatly in length.

5. Check that at least the front lenses of the objectives are clean. No point in continuing if they're not. Notes on cleaning lenses will be included later.

6. Check that the eyepiece can be easily removed and that it is not too dirty, by holding it up to the light. The eye lens usually benefits from a careful wipe.

7. If the microscope has a mirror, check visually that the mirror pivot is on the optical axis of the condenser because sometimes a damaged mirror or its mount has been replaced with another made for a different microscope.

8. Check the motions on the mechanical stage if the instrument has one.

The users of microscopes with built-in illumination will have to trust to the manufacturers factory settings. Hopefully, they will have access to lamp focus and centration. If not, it is unlikely that the system will be so far out of adjustment that routine microscopy will be much affected (provided that the recommended lamp bulb is in use, and factory settings have not been seriously deranged).

Given the means of doing so, the first task of the process is to centre the microscope lamp.

Centreing the Microscope Lamp.

Arrange the lamp so that it throws an image of the filament onto a nearby wall or other suitable surface. It will not be a perfect image -- it will show colour fringing or surrounding haze even at the point of best focus. Adjust the lamp bulb centreing screws until the haze or fringing is symmetrically distributed around the filament image. Focus the lamp back and forth to ensure that these artifacts are concentric with the image. When this is the case, the lamp filament is centred to the optical axis of the lamp condenser.

Click to see the notes on [centreing the lamp](#) using the projected lamp filament image.

Positioning the Microscope Lamp.

Place the lamp on its stand in front of the microscope at a convenient distance (200mm or so) from the microscope. Open the lamp diaphragm fully and increase the intensity of the lamp until its beam can be seen striking the microscope mirror.

If the mirror is too clean to show the beam, breathe on it.

Move the lamp so that the beam strikes the mirror in the centre. (We shall assume at this point that the mirror is centered to the microscope optical axis). Move the mirror so that the beam now strikes the centre of the closed substage condenser diaphragm. (A small mirror lying beside the microscope or attached to the microscope base helps here). Focus the lamp until an image of the lamp filament large enough to fill the condenser backlens is projected onto the closed diaphragm blades.

Users of built-in illumination should use their lamp focus and centration adjustments to ensure that an image of the lamp filament is projected onto the closed substage condenser diaphragm at a sufficient size to fill the condenser lens.

When this is achieved, for the purposes of routine microscope use, the lamp need not be touched again.

Adjusting the Substage Condenser.

Before proceeding with this step, a specimen of some kind must be placed on the stage of the microscope to define a point on the optical axis to which the condenser focus must be brought. For this purpose, even a scratch on a plain microscope slide would serve, but most microscopists have favourite specimens which have been used over many years for setting up and testing the microscope, and this is probably the best approach, as familiarity with the specimen under varied conditions makes it much easier to spot any lighting anomalies as they occur.

The ideal test slide would have the same thickness as the slides to be used for the specimens, as well as having approximately the same coverglass and mountant thickness. A stained plant or animal tissue section is suitable.

Reduce the intensity of the lamp to a comfortable level, and using an x10 objective, focus the microscope on the specimen.

Another warning. Due to the ease with which the point of focus can be missed, especially with high-power objectives, and the damage which can be caused when this happens, the following procedure is recommended in most texts on the microscope.

Whilst looking across the stage, lower the objective until it almost touches the specimen. Then, looking through the eyepiece, carefully raise the objective using the coarse adjustment until the specimen comes into focus.

If the point of focus is missed, repeat the procedure.

Although some departure from this procedure is called for when using immersion objectives, it remains a sound general approach in routine microscopy.

Close the lamp diaphragm to a small aperture and use the substage condenser focusing adjustment to obtain a sharp image of the lamp diaphragm blades in the plane of the specimen.

Move the mirror about until any colour fringing around the image of the lamp diaphragm is concentric with the image, then use the substage condenser centring screws to move the image back into the centre of the microscope field. Rack the condenser back and forth through its focus to check that any haze or fringing is concentric with the diaphragm image, and leave it at the position of best focus. Open the lamp diaphragm until it just clears the eyepiece field.

The substage condenser is now focused and centred.

If the microscope has no substage centring screws, the lamp diaphragm image must be centred by using the mirror. This means that any centration errors of the substage condenser will have to be tolerated, but they are unlikely to be gross, and Abbe condensers, being poorly corrected themselves, are not sensitive to small errors of this kind.

Now that a just-sufficient area of illumination has been established, the next step is to adjust the substage condenser diaphragm to fill the aperture of the objective.

This is best done by removing the eyepiece and observing the objective backlens. With the substage condenser diaphragm fully open, the backlens of the objective should be seen filled with a well-focused image of the lamp filament. If the filament has a tightly wound rectangular grid structure the light fill should be fairly even. It will never be completely even with a filament lamp, but if the lamp has a spherical reflector behind the filament, now is the time to adjust the reflector so that any spaces between bars of the filament are filled with reflected light and the most even possible fill of the objective backlens achieved.

Now close the substage condenser diaphragm until its blades are seen encroaching on the illuminated backlens. If the condenser has been properly centred, the image of the substage condenser blades will be concentric with the objective backlens mount. Set the substage diaphragm to obscure about one quarter of the area of the backlens. The objective is now set up to produce an image of good contrast and definition for an average specimen.

"Critical" microscopy, as practiced in the early 1900s using the (then) best available optics, required that the illuminating cone be $\frac{7}{8}$ ths of the objective backlens. The $\frac{3}{4}$ cone trades a slight loss of resolution for a larger gain in image contrast, and is probably better for everyday use.

In either case, the purpose of restricting the aperture of the illuminating cone is to prevent the scattering and flare which results when light strikes the lens mountings inside the objective.

If difficulty is experienced observing the objective backlens, there are a number of possible solutions:

1. Use a telescope of the kind included in phase contrast outfits for adjusting the phase annulus. This is the best solution.
2. Leaving the eyepiece in place, use a x10 hand magnifier held just above the eyepiece to view a relayed image of the objective backlens. Fiddly but effective.
3. Use another eyepiece, held upside down over the microscope eyepiece as in method 2. This is the

least elegant solution, but useful in an emergency.

The microscope is now set up for Köhler illumination using the x10 objective.

Using Higher Power Objectives.

If the x20 or x40 objective is substituted for the x10, the following adjustments will be necessary:

1. Close the lamp diaphragm to just clear the reduced field now seen by the eyepiece. A slight trim of condenser focus may be needed to sharpen its image. On those stands with substage centring screws, slight adjustments of centration may also be called for, otherwise adjust the mirror.
2. Check the objective backlens again. The substage condenser diaphragm will need to be opened to fill the larger aperture of the higher power objective and duplicate the three-quarter illumination cone condition as for the x10 objective.

A Useful Tip:

When you have determined the 3/4 cone condition for each objective, make a coloured inkdot on the condenser mount opposite the substage diaphragm lever. Longer experience will enable you to gauge the condition visually, but the dots will help a lot until then.

Using Lower Power Objectives.

The main difficulty encountered in the use of low power objectives (less than x10) is that of filling their larger fields with light from the lamp. A quick and crude solution in an emergency consists of placing a diffuser in the substage stop carrier and increasing the lamp intensity, but this usually produces an illumination of reduced contrast and increased glare, since the diffuser prevents the use of the lamp diaphragm to control the illuminated field.

The best solution is to use a substage condenser of lower power, which projects a larger image of the lamp condenser. This can often be achieved by unscrewing the toplens from the a two-lens Abbe condenser (see [detailed notes](#)), giving a condenser of lower power which may well do the job for objectives down to x4 or x5. Some substage condensers have a toplens which may be flipped out of the optical path by a knob or lever, making the illumination of the lower powers much easier.

If lower powers than this are required, it is often less trouble to abandon the laboratory microscope and use a stereo microscope which is designed for low power use.

The Specimen.

Specimens for the light microscope are traditionally prepared on glass slides measuring 3 x 1 inches (25mm x 75mm), and covered with a piece of thin glass of 0.17mm thickness called a coverglass. The slides are most commonly 1.0 to 1.2mm in thickness, and thinner slides at 0.8 to 1.0mm are also available. With the specimen placed on the stage of the microscope, these two thicknesses of glass become an important part of the microscope illumination and imaging system.

The thickness of the slide goes a small way toward correcting the large spherical errors of the Abbe condenser, and the thickness of the coverglass is essential to the spherical correction of the objective, especially those of x20 and higher powers. The x40 objective in particular will give very poor images if used with an uncovered specimen. A more detailed account of spherical aberration in microscope image formation and the means of correcting it is given in the advanced tutorial.

Quite apart from optical considerations, a coverglass is essential for examining specimens in water and other liquids to reduce sensitivity to vibration and to prevent contact with liquid or condensation of vapour on the frontlens of the objective. The thickness of the slide is an important consideration also when the substage condenser is immersed in oil for use with the oil immersion objective (a thicker slide requires less oil), and when the microscope is fitted with an achromatic condenser (see advanced tutorial).

Now that the microscope is correctly set up, a slight detour into the theory of microscope image formation is necessary to clarify terms before we proceed to apply the same principles to the use of the oil immersion objective.



Page 2. 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_a, 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:

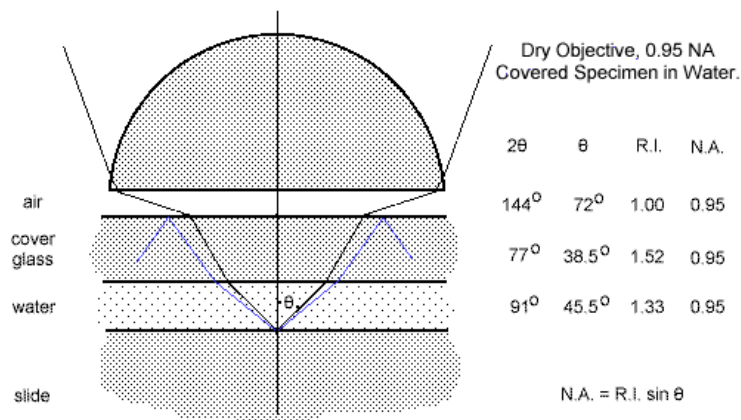
$$N.A. = R.I. \sin \theta$$

where θ 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 θ 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.

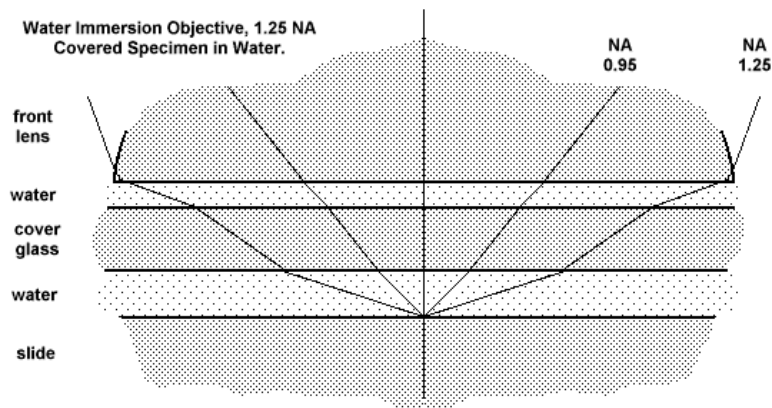
Consider the diagram below which represents a dry objective of N.A. = 0.95 examining a specimen in water under a coverglass. The acceptance angle of this objective is 144° in air, which becomes 77° in the coverglass, and 91° in the water containing the specimen. Even though the specimen may have detail 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.



Ray path from a covered specimen in water to a dry objective of 0.95 N.A.

If the dry objective of 0.95 NA were replaced with a water immersion objective of 1.25 NA, and the space between the coverglass and the frontlens filled with water, the acceptance angle of this objective in the immersion water is 143° , which, after refraction through the coverglass, is also 143° in the specimen water. This results in the gathering of a far larger solid cone of diffracted light than was possible with the dry objective, and hence an image with finer detail. The increase in detail revealed is directly reflected in the difference between the two N.A.s.

In the diagram below, the thickness of the water film, the thickness of the coverglass and the working distance of the objective have been left unchanged in order to show the increase in diameter required to produce a frontlens capable of collecting the wider aperture cone. In the real world, the dry objective would have a magnification of about x40, a longer working distance on the scale of the diagram, and hence larger diameter than shown. The water immersion objective of NA 1.25 would have a magnification of about x70, and therefore a shorter working distance.



Ray path from a covered specimen in water to a water immersion objective of 1.25 N.A.

Abbe extended this thinking to a situation in which the entire space between the specimen and the objective frontlens was filled with a medium having the same refractive and dispersive qualities as the glass of the frontlens. He found a medium of the required properties in cedarwood oil, and the oil-immersion objective began its career. For certain specimens, such as stained bacterial smears and blood films, the coverglass could be dispensed with, and it exerted no refractive effect upon the image-forming rays from a specimen mounted in balsam. Because of this continuity of optical properties in the object space, the system was also called "homogeneous immersion".

It enabled the highest resolution possible for an objective having a glass frontlens. The diffracted rays from the specimen passed undeviated into the glass of the frontlens, undergoing their first refraction at the strongly curved upper surface. An advanced tutorial deals with the way Abbe made use of this situation to solve the otherwise difficult problem of spherical aberration in such a powerful frontlens.

For the present business of image formation, it remains only to deal with the not-so-obvious relationship between magnification and image sharpness.

Page 3. Magnification and Image Detail.

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 some time 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.

This standard is still in use today in that the magnifying power of simple lenses and lens systems is determined by dividing their focal length into 250 mm. For example, a hand magnifier with a focal length of 25 mm is a ten-power magnifier.

For many years in the 1800s, English microscope manufacturers produced, in observance of this standard, microscopes having a tubelength (between objective and eyepiece) of ten inches, but these proved unwieldy in use. Until the quite recent development of infinity-corrected objectives and for most of the

twentieth century, the accepted standard for microscope objectives was an optical tubelength of 160 mm. In other words, a microscope objective produced an image of the stated magnification only when it projected its image a distance of 160 mm measured from its back focal plane. This was also the conjugate distance required for spherical correction of the magnified image -- a topic dealt with in more detail in the advanced tutorial.

According to these criteria, a microscope objective having a focal length of 16mm is a ten-power objective (160 divided by 16), a forty-power objective has a focal length of 4mm, and so on.

An objective would of course produce larger or smaller magnifications with greater or lesser projection distances, but only at an optical tubelength of 160 mm was it both corrected for spherical aberration and delivering the engraved magnification.

Such is the magnification of the image which arrives at the plane of the eyepiece diaphragm in a properly set up microscope. What magnification should this eyepiece have?

The general answer would be -- enough extra magnification for an operator of good/normal vision to see all the detail in the image. So with regard to magnification, there is a close relationship between NA (as a measure of the amount of detail in the image), and the visual acuity of the person using the microscope.

Based upon long experience, it has been said that the microscope should have a total magnification of around a thousand times the NA of the objective for a person of good vision to see all the detail the image has to offer. People of better than average vision could see the detail at lower magnification; people with less acute vision would require more.

According to this rule of thumb, a good quality x40 objective of 0.65 NA could use a x15 eyepiece and still look sharp (at x600), and a x100 oil-immersion objective of 1.30 NA would not be revealing all of its detail with x10 eyepieces (at x1000).

Given that the highest practically achievable NA is about 1.40 it follows that x1400 is the highest useful magnification obtainable before passing into what is generally called "empty magnification" -- giving enlargement of the image but revealing no more detail.

For the person of average eyesight using an expertly set up microscope of the highest correction, specimens of high contrast would probably still look good at x2000 -- which could be seen as the outer limit of useful magnification in the light microscope.

These observations apply to the visual use of the microscope. Different criteria apply to the recording of microscope images on film of various formats, and in video and other CCD cameras. These issues are dealt with in the advanced tutorials.

Using the Oil Immersion Objective.

The only reason to use an oil-immersion objective is to take advantage of the highest resolving power available to the microscopist. Unless this is a necessary requirement of the work in hand, stay with the lower power dry objectives -- they are much less trouble to use, and can provide magnifications up to x600 (using a x40, 0.65 NA dry objective with x15 eyepiece) at quite acceptable resolution.

Having said that, much of the work requiring the high powers and resolution of the oil immersion objective has adapted itself to the requirements and constraints of the immersion technique. Blood films, stained bacterial films etc. are all prepared on plain microscope slides as dry films, and the drop of immersion oil becomes the only optical medium between the specimen and the frontlens of the objective.

When the examination is over, the slide with its film may be discarded or stored without even bothering to remove the immersion oil, since more will be added if the slide is examined again. In histology, thin (10 μm or less) stained sections of tissue are mounted under a coverglass in canada balsam or other resin having the same optical properties as glass, so with the addition of immersion oil, the conditions for homogeneous immersion are met. Much thicker sections than this cannot be examined, as the working distance of these objectives is very small, and the front-lens mount is soon in contact with the coverglass. Sometimes, thinner than standard coverglasses are used to allow deeper penetration of focus into the specimen. With a covered balsam mount, the oil may be removed with no risk of damage to the specimen.

Oil Immersion: Routine Technique.

The most common oil-immersion objective in use in routine microscopy is the achromatic* objective of magnification x100 and NA of 1.25, used in combination with a dry two-lens Abbe substage condenser having a maximum aplanatic* NA of about 0.6.

It is clear that the condenser cannot fill more the half the NA of the objective, and the resulting image will

be high in contrast and showing the coarsening of detail characteristic of images formed by narrow axial cones -- but acceptable, especially for good visibility of low contrast subjects.

If you require such an image of say a balsam mounted section of stained plant or animal tissue, or a stained dried blood film on a slide, follow these steps:

1. Place the slide on the stage and locate the area of interest using the x40 objective in a Köhler setup.
2. With the object of interest focussed in the centre of the field, close the lamp diaphragm to a small aperture in the centre of the field, and using the substage condenser adjustment, focus its blades sharply in the plane of the specimen. Increase the lamp intensity a little.
3. Swing the x40 objective out of the way and place a drop of immersion oil on the coverglass or film at the spot where the subject is illuminated by the condenser.
4. Swing the oil immersion objective into position, so that (if the objectives are parfocal) the front lens is now immersed in oil. If the objectives are not parfocal, the OI objective should be carefully racked down using the coarse adjustment until contact is made with the oil drop. In either case, whilst looking through the eyepiece, continue racking the objective down, noting the increasing brightness of the image. The coarse adjustment may be used for this operation if it is sufficiently controllable, or the fine adjustment may be used if it has sufficient travel.
Either way, as the image approaches maximum brightness, either the specimen or the lamp field diaphragm or both will come into focus.
If this operation is carried out in haste, it is very easy to pass rapidly through the focus before you realize it. In this case, the sound of breaking glass will alert you to what has happened.
However, if you are able to close the lamp diaphragm to smaller than the field of the OI objective, and it has been carefully focused in the plane of the subject, the point of focus is hard to miss. Objectives with spring-loaded optics are an added insurance against disaster.
5. Once focus has been established, open the lamp diaphragm to clear the field, and trim the substage condenser diaphragm for the best image. This will probably be with the blades fully open. That's it.

When you have finished examining the specimen, rack the objective up to break oil contact and swing it forward to enable removal of the remaining oil with a piece of soft paper tissue. A fresh piece, moistened with cigarette lighter fluid will completely remove any residual oil film. The strict rule of not using alcohol for this operation is still a good one, especially for older objectives in which the more-than-hemispherical front lens is held in place with a cement which is dissolved by alcohol.

Oil Immersion at the Highest Resolutions.

If higher resolutions are required, the main problem is that of supplying the objective with a sufficiently wide cone of illumination to fill its aperture and obtain best performance. A dry two-lens Abbe condenser produces an aplanatic cone of no more than 0.6 NA, and this is hardly sufficient to exploit the higher NA of the OI objective. Immersing the condenser helps a little, but is a fiddly operation providing very little gain in image quality.

The most practically satisfactory method involves replacing the Abbe condenser with a dry achromatic/aplanatic condenser with an aperture of 0.95 which will provide a 3/4 illumination cone for an objective of 1.3 NA. This extracts an optically satisfactory performance from a better than average objective without the need to immerse the condenser.

If the highest resolutions are required, the objective will need to be of apochromatic correction and will have an NA of 1.4. The condenser required will be of achromatic/aplanatic correction, and with oil immersion, provide an aplanatic illumination cone of 1.4 NA. Additionally, the microscope stand employed will require substage centring adjustments to enable accurate centration of the condenser.

These necessarily expensive optics are capable of producing the finest possible images -- but their deployment is an exercise in critical microscopy and outside the scope of this basic tutorial.

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 brightfield 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 front lens is concave or somewhat recessed may require that a small drop of the immersion medium is applied to the front lens as well as the specimen to avoid the formation of bubbles.

Settings for Comfortable Microscopy.

Many people spend as much time each day working with a microscope as others spend working with their computers. Fortunately, more thought is now devoted to ensuring that working conditions and practices be optimized to reduce the fatigues and increase the enjoyments offered by the occupation in question.

In the case of microscopy, the bilateral symmetry of both the instrument and its operator determines many of the commonsense requirements for an optimized working space, which are largely personal and ergonomic in nature.

The physical arrangements of that working space aside, the main detractor from image quality and a major cause of eye fatigue in routine microscopy are the unwanted reflections of surrounding light sources in the many optical surfaces of the microscope -- most importantly those of the eyepieces.

If there is a window in the room, the microscope is best set up facing the opposite wall so that the eyepieces of the instrument are in the shadow of the operator's body. The microscope should be standing on a preferably dark surface to minimize reflections and glare from whatever local lighting is needed to provide working light levels. A well balanced anglepoise lamp with an easily accessible dimmer control is ideal.

Eyecups with extended side-pieces (such as bird-watchers have on their binoculars) fitted to the eyepiece/s of the instrument are very effective in enabling a reflection-free image.

A binocular viewing system is itself an aid to comfort as both eyes share their intensive task with much less strain than is often the case with a monocular microscope. Users of monocular microscopes should cultivate the habit of using each eye in turn, and of keeping both eyes open at all times. This is difficult at first, but the brain soon learns to ignore the image from the unoccupied eye, and the task is made much easier if the ambient lighting level is lower than the microscope illumination.

The very worst situation would be a monocular microscope set up on a shiny white bench in a brightly lit lab with no lighting controls, and an operator who keeps one eye closed whilst the other does all the work.

The best situations begin with the ownership of your own microscope. Apart from anything else, one's home is usually warmer and more comfortable than most laboratories, and the grease-lubricated adjustments of a microscope run more smoothly in a warm setting. A comfortable working temperature also reduces the inconvenience of eyepiece misting and breath condensation on colder parts of the instrument.

In conclusion, it can be said that the possession of a good quality, well equipped light microscope, set up in a situation optimised for personal convenience so that any specimen which suggests itself may be examined quickly and easily, is one of the most rewarding luxuries a technological world can offer the intelligently curious person.