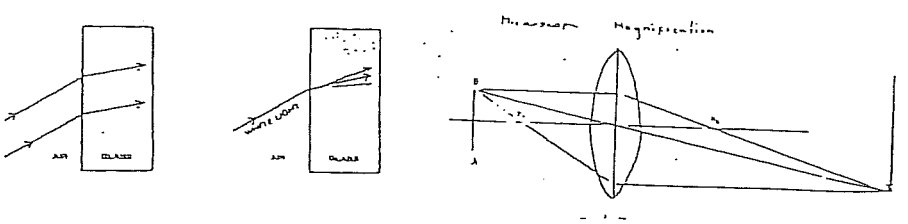
Theory of The Microscope

I will first discuss the microscope as a system of magnification. Second I will discuss it as a resolving system and then combine the two. In order to produce the phenomenon of magnification; that is, increasing the apparent size of an object, we use the property of light called refraction. Light travels 300,000 meters/seconds in a vacuum. In transparent materials light will travel at a slower speed. We can define the change in speed that light undergoes in a particular material as the refractive index of that material. The refractive index is the velocity in a vacuum divided by the velocity in the medium. Anything but a vacuum has a refractive index greater that one. Air has refractive index of 1.00029. It does not slow light very much. Glass on the other hand has a refractive index of about 1.5.

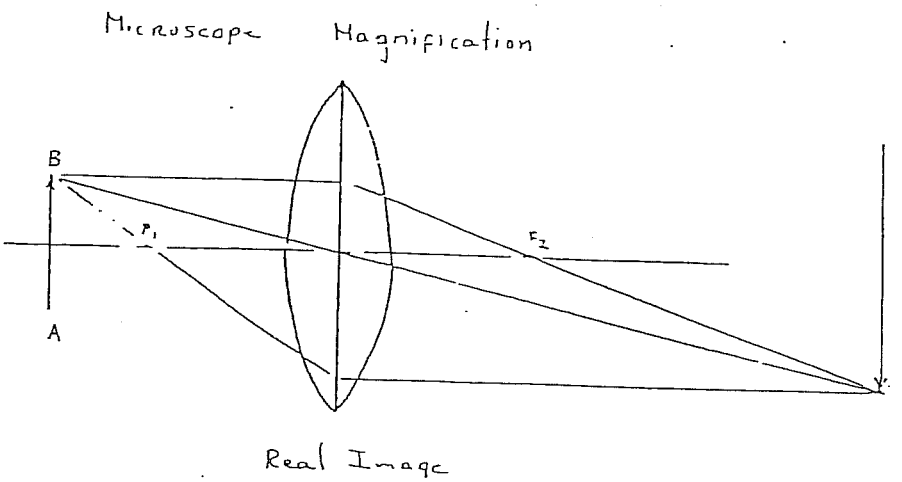
If light passes from one medium to another and the boundary of the two media are perpendicular to the path of the light, the light will enter the second medium and slow down. When it comes out the other side it will return to its original velocity. Light passing from one medium to another at an angle other than 90^0 will be bent (refracted) as it passes the boundary between one boundary and another. The angle of refraction will depend on the refractive index of the material and the angle of incidence of the light. With a curved surface, we can bend (refract) the light in a manner to cause magnification. I will describe concave lenses whose surfaces are part of a sphere. A parallel group of light rays that enter a concave lens will be focused at a point on the other side of the lens called the focal point illustrated here. In this case we are not forming an image, but focusing a group of parallel rays of light.

Figure 1



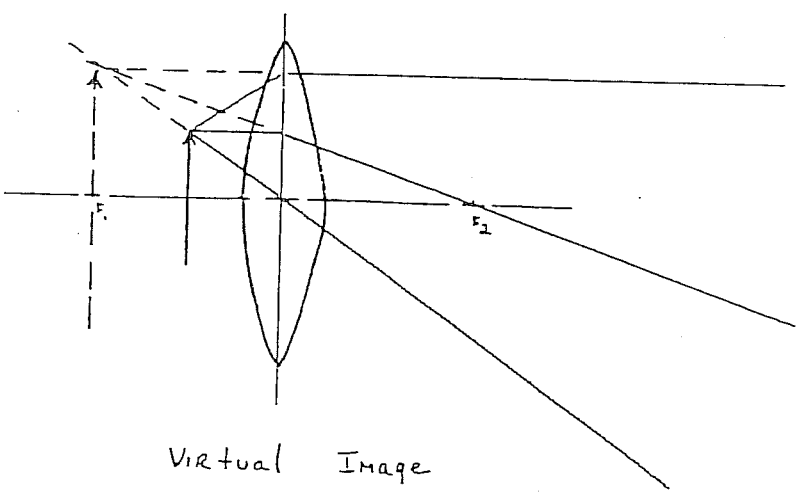
Now let's consider image format and magnification by lenses. Consider a lens as it is shown in the illustration with the same curvature on both sides. On each side of the lens we can define a focal point F1 and F2, at which parallel light rays will be focused. Now consider an object, in this case an arrow with the point labeled O. We will concern ourselves with two rays of light, because they will define the image. First let's follow the point of light leaving point O, parallel to the axis of the lens. This light will be defracted by the lens through the focal point of the lens. Second we will follow a ray of light which goes to the optical axis of the lens. This will pass from point O through the lens out of the other side. It will intersect the first ray of light we have drawn. We can do the same for 2 rays leaving the other end of the arrow. They will define a point on the other side of the lens. In an actual lens system this intersection of light rays will produce a real image. A real image can be focused on a screen behind the lens. Note that it is inverted, that it is upside down and backwards. The closer the object is to the focal point of the lens, the larger will be the magnification on the other side of the lens. Greater magnification will depend on the closeness of the object to the focal point and the focal length of the lens. The shorter the focal length of the lens, the greater its magnifying power.

Figure 2



We may arrange the lens and object in a different manner and produce a vertical image. Place the object between the lens and its focal point, and you see a different situation. Let us follow two rays from point O of the arrow. First the ray of light that enters parallel to the axis of the lens. It will go through the focal point F2. A second ray, which passes from the point of the arrow through the optical center of the lens is not refracted. In this condition where the object is not between the focal point and the lens, the rays will diverge and not come together at any point. There is no place on other side of the lens where an image can be formed. However, if we put our eye behind the lens at the appropriate place, these light rays leaving the lens will strike the eye and appear to form an image(I') behind the lens. Note if we extend these lines back behind the object they will meet behind the lens. Our eye interprets these rays as forming a point behind the lens. This image is erect, that is not upside down and backwards, and it is also magnified. This is virtual image.

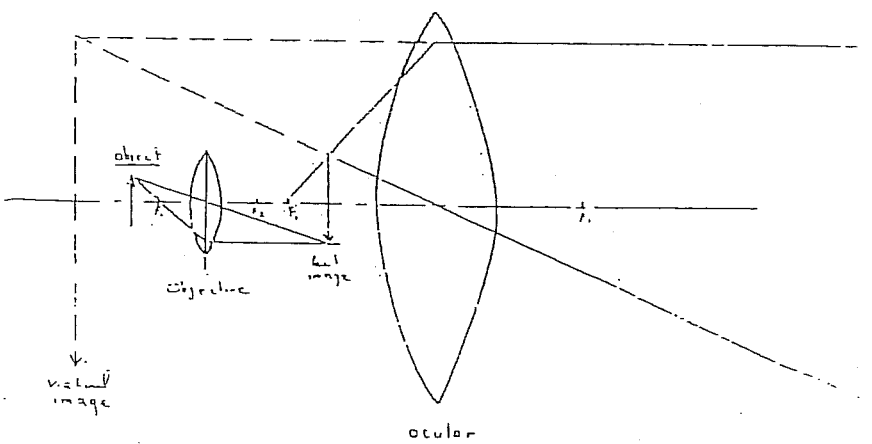
Figure 3



We can put these two types of image forming system together to form a compound microscope. Compound meaning the microscope uses two lenses to form two images. A simple microscope, also known as a magnifying glass, makes use of a single

lens. We construct a compound microscope such that the first lens, which is closest to the object, produces a real image, and then put a second lens behind the objective lens that takes the rays from the real image and refracts them again, forming a virtual image. We will then have a compound microscope. When we view something through a compound microscope the object will appear to be upside down and backwards and magnified. The magnification will depend on the placement of the object with respect to the focal point of the objective (first) lens, the focal length of the first lens and the placement of the ocular (second) lens with respect to the image produced by the first.

Figure 4



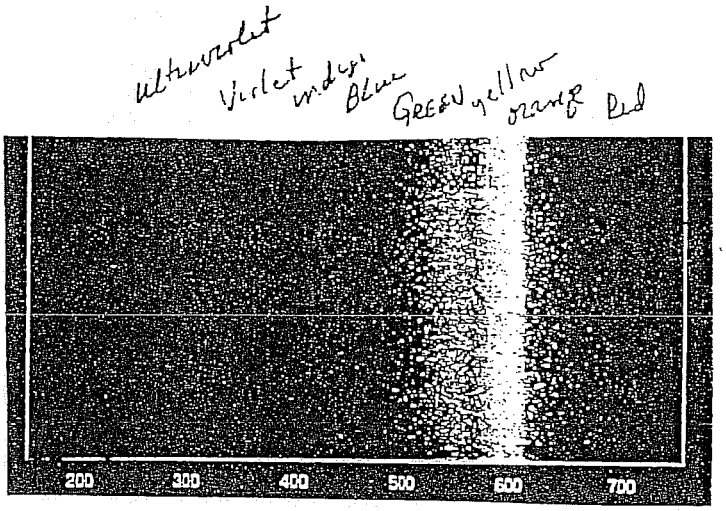
Compound Microscope

Modern compound microscopes generally have a variety of objective lenses ranging from 4x to 100x. The oculars are usually 10x or 12.5x magnification.

The lenses that I have described; that is, concave spherical lenses do not form perfect images. The distortions are created by the lens. The first type of distortion is called chromatic aberration. Light that appears to us as white light has a variety of wavelengths. Each different wavelength of light produces the sensation of a color. If we had a beam of light with all one wave length, it would appear as one particular color. Shorter

wavelengths are violet and blue, longer wavelengths are green, then yellow, then orange. The longest are red. Wave lengths of electro magnification beyond the red are known as infared. Wave lengths shorter that violet are called ultraviolet. We do not see these but they can be very destructive to tissue. Shorter wavelength light will travel more slowly in glass that longer wavelength light and so that each wave length of light be focused at a different point.

Figure 5

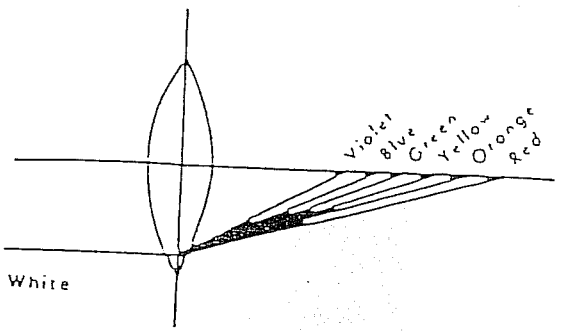


If we then look very carefully at the spot of light that is focused on a spherical lens, we see that it consists of different colors. This is chromatic aberration. In an uncorrected lens chromatic aberration will result in a ring of colors around the image. We correct for chromatic aberration by making an objective lens of a number of pieces of glass using different types of glass to bring light of various colors to focus at the same point. The achromatic lens used in most microscopes will focus red and green at the same point, thus reducing chromatic aberration. The apochromatic lens will focus red, green, and blue at the same point.

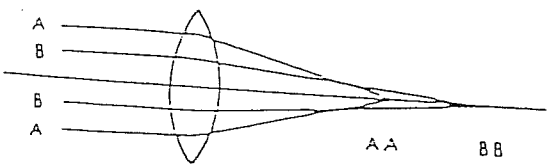
A second type of aberration is called spherical aberration. Light entering one point on the lens far from the optical axis will be focused at a different point from the

light entering the lens close to its optical axis. Instead of a fine point of light at focus there will be a blurred spot. Spherical aberration can be corrected by building a multiple glass lens. A number of lenses together produce a corrected final image.

Figure 6



Chromatic aberration. For an explanation, see text.



Spherical aberration. For a full explanation, see text.

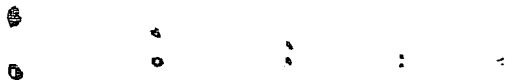
In the manufacturing of lenses, we may create aberrations. If a lens is not ground uniformally over the whole surface, but has different focal points in different planes of the lens, we say the lens has astigmatism. The light from a point entering the lens on a different axis will be focused at the focal point as a cross rather than a round point. Illustrated on the right another typed is called coma. Here a spot of light on the focal point looks like a comet rather than a single point of light. These types of aberrations can all be corrected in modern microscopes. Until these aberrations were corrected, microscopes produced images that were hard to interpret.

Resolution:

The second theoretical aspect of microscope functioning I will discuss is resolution. Resolution is the ability to distinguish two individual objects that are close together as separate objects.. Here is an example showing pairs of dots. The closer

together the pairs of dots are the more difficult it is to resolve into the separate objects. If you step back slightly from the paper you will see that the small pair of dots will become unresolved and will appear to you as a single spot.

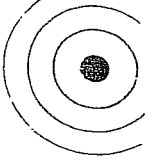
Figure 7



The unaided human eye will resolve objects between 100 and 200 micrometers apart at a distance 25 centimeters. Objects closer together than 100 or 200 microns will appear single. The function of the microscope is to increase resolution and the size of image so that it can be resolved by the human eye.

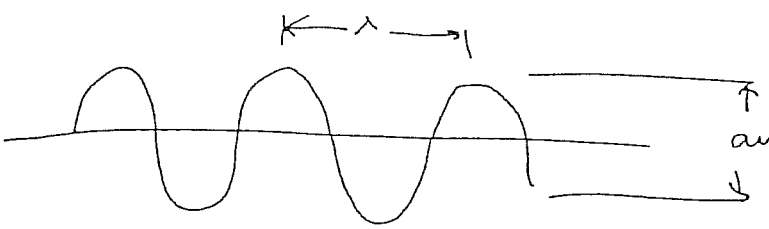
During the later part of the 19th century a German physicist named Ernst Abbe worked out a formula for determining the resolving power of a lens system. This formula allowed microscope manufacturers to produce lenses of known resolving power and to match the resolving power and magnification as it is now done in modern microscopes. Let's look at Abbe's formula and discuss it in some detail. $d = \frac{0.61\,\lambda}{n \sin \alpha}$

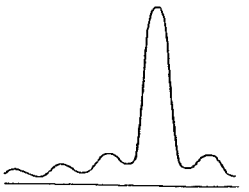
The formula for resolution is d=0.61/n sine. Let's look at each part of this equation. "d" is the distance between two points to be resolved. The smaller the number "d", the greater the resolution of the microscope because the closer together the two objects can be and still be resolved. Lambda (λ) is the wave length of light that is used in a system. As I said earlier, light is electromagnetic radiation with many different wave

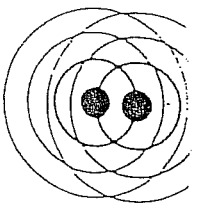


lengths. Each wave length is a different color. The amplitude represents the light intensity.

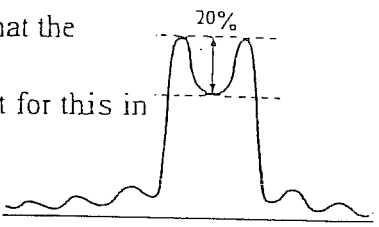
Figure 8





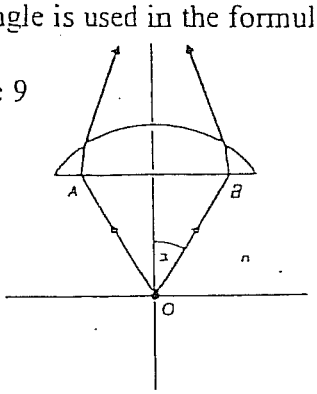


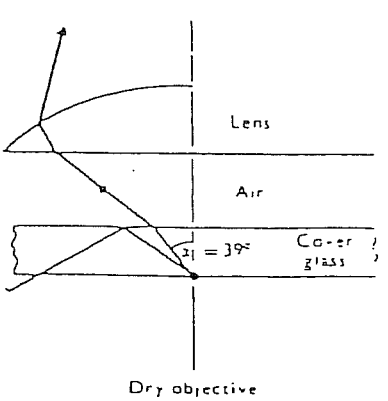
The number 0.61 is a constant related to the resolution of the human eye. Small points produce defraction rings around them as is shown here. In order for the human eye to resolve two spots as separate, the spots must be far enough apart so that the intensity between the 2 spots is 20% less than the spots. In order to account for this in the equation, we insert the 0.61 as a constant.

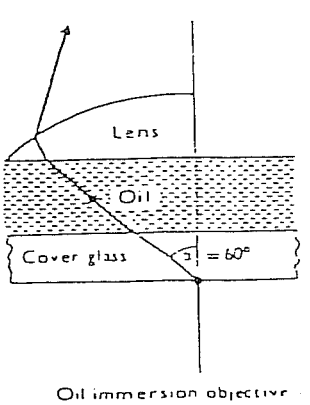


Looking at the numerator of this equation, "n" is the refraction index of the medium through which the light is passing to reach the objective lens. We use the minimum refractive index between the object and the last part of the objective lens system. This is air between the objective lens system. This is air between the objective lens and the object in a dry microscope, or in an oil immersion system, the oil between the lens and the object. Sin is the sin of ½ the angular aperture of the lens. A lens of given physical size and focal length will accept a certain cone of light. This cone of light is defined in this system by the angle AOB. This is the angular aperture or the acceptance angle of this lens. The larger the lens and the closer it is to the object the greater the angular aperture. One half the angular aperture would be sin α The sin of this angle is used in the formula.

Figure 9







Numerical aperture is a number derived from the formula n sin and is abbreviated N.A. You will find that if you look at an objective lens on a microscope, on the barrel is N.A. that stands for numerical aperture followed by a number. In some cases N.A. is left off and there is simply a number (the other numbers are 40, the magnification, the optimal cover glass thickness, and the working distance of the of the lens is the small number at the bottom. If you look at the different lenses of a microscope you will find that the lower the magnification in general, the lower will be the numerical aperture. The higher the magnification, the greater its numerical aperture. Non-oil immersion lenses are limited to numerical aperture below 1.0. I will illustrate. If we could make a lens that would accept 180°, its numerical aperture would be the refractive index times the sin of 180/2 or (the sin of 90°) which is 1. The refractive index of air is essentially 1. Therefore, the numerical aperture of the lens would be 1. The refractive index is essentially 1. Therefore, the numerical aperture of the lens would be 1. It is impossible to make lens that will accept more than 180° and so dry lenses are always restricted to numerical aperture less than 1.0. A lens must be some type of immersion lens to eliminate the air between the lens and the object. For example, a very good oil immersion lens might have an angular aperture of approximately 120°. The numerical aperture of this would be 120° divided by 2 or the sin of 60°, which is 0.87. Immersion oil, which is the most commonly used, has the refractive index of 1.5. The numerical aperture of the lens is then 1.5 X 0.87 = 1.30. This is a very good lens. Most oil immersion lenses on student microscopes have a numerical aperture of 1.25.

Now return to the formula D=0.61 lambda divided by n sin alpha. We can rewrite this equation and make it more simple. First we can substitute numerical aperture (NA)

for n sin α, since the numerical aperture will be given on the lens. Second, taking a little liberty with the formula we can round 0.61to 0.5. This makes calculation a little easier but it does not effect the results much. Then we can rewrite this to be resolution to equal to the wavelength of the illuminating light divided by twice the numerical aperture.

Figure 10

$$d = \frac{0.5\,\lambda}{N.A} = \frac{\lambda}{2N.A.}$$

We can see the conditions that we need for the maximum resolution. To maximize resolution, we can minimize λ. The smaller the wave length of light, the better the resolution, or we can maximize the numerical aperture. The larger the numerical aperture, the smaller the "d". There are some limits. First of all we have to work with a system using visible light. Therefore, we are restricted to 400-700 nm for a wave length of light. In most cases, a microscope uses a variety of wave lengths of light although often we use a blue or green filter. Second, we can only manufacture lenses of a certain numerical aperture. The practical maximum is a numerical aperture of 1.4. Most student microscopes will have a maximum numerical aperture of 1.25 and most research microscopes will have a numerical aperture of 1.3. Let us then look at maximum resolution using a microscope with numerical aperture of 1.3. If we use the given light of λ of 520 nm., the limit of resolution is approximately 2/10ths of a micron. This is essentially the limit of resolution of a light microscope. Here is a chart illustrating the limits of resolution. There have been special microscopes made that use ultra-violet

light. This allows a limit of resolution of 0.1um but requires special quartz lens and imaging. The electron microscope uses a different illuminating system. The wave length electrons are very short compared to lightless than .05nm. Therefore we can achieve 0.02nm resolution in an electron microscope.

Now let's go back to some information that I gave you at the beginning. The Human eye can resolve between 200 and 100 microns. We will use 200 microns as the limit of resolution for the human eye. The question then becomes how much do we need to magnify an object to bring it up to the resolution of the eye. If we are considering the maximum resolution of the microscope, 0.2 um, we need to magnify the 0.2 micron to 200 microns. In order to do that, we need to magnify the image 1000 times. If you look at most microscopes you will find that they have a magnification of about 1000 times. Sometimes microscopes will have an objective of up to 100x and an ocular of 12,5x which produces a total magnification of 1250x for ease of viewing. Magnification greater that this will not be necessary because the limit of the resolution of the instrument which is 0.2 microns, and above this it would produce what is know as empty magnification . The micrograph on the left was made with a low N.A. objective and magnified photographically to match the one on the right, which was made with a high N.A. objective. Figure 11

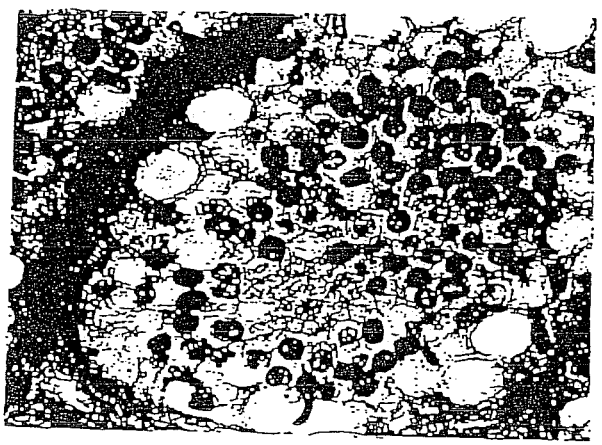


Figure 34C

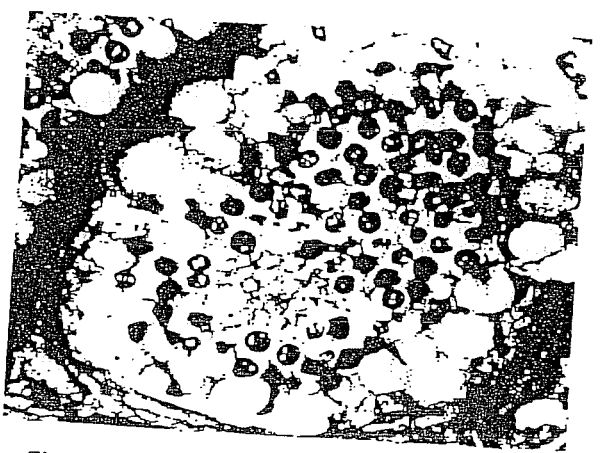


Figure 34A

Empty Magnification does not always mean that nothing is seen between two resolvable spots. We may see defraction patterns or apparent images that do not exist. This was a problem with early microscopes that produced very high magnifications with low resolution. The results were optical patterns that were interpreted as structures, but in fact did not exist. A good modern microscope has a magnification of 1000 or 1250 and no more.

We have only discussed microscope system considering the objective lens and the ocular lens. But you are probably familiar enough with a microscope to know there is another lens system called the condenser. What is the role of the condenser? Basically the condenser produces a cone of light focused upon the object. We take a beam of parallel light rays and focus it on the object. The light rays pass through the object, into the lens of the objective and are magnified by the microscope system. The condenser must have a numerical aperture equal to that of the objective in order to gain maximum resolution of the objective. If the numerical aperture is below that of the objective then the microscope will have less resolving power.

An approximation of the resolving power of the microscope considering the objective-condenser optical system is called the working aperture. It is the average of the two numerical apertures. For example if you have an objective lens with a 1.30 numerical aperture, and a condenser with a 1.00 numerical aperture, the result will be a working aperture for the microscope of 1.15. How then can we get a functional working aperture of 1.3. You must oil the condenser to the slide as well as using an oil immersion lens. In practice this is usually not necessary. However, when you are looking at very small objects near the limit of resolution or photographing , and using an oil immersion

lens you must oil the condenser to the slide as well as using an oil immersion lens to gain maximum resolution.

You will note that mounted below the condenser there is a diaphram and when the diaphram is closed down it reduces the numerical aperture of the condenser. Closing the condenser diaphram decreases the resolution of the system. However it also increases the contrast and increases the depth of the focus, that is the thickness of the area in focus. In some cases this may be desirable. Unstained specimens or thicker specimens can be viewed with a condenser that has its numerical aperture reduced. Below is a photomicrograph of a diatom made with the condenser aperture open. Next to it is the same diatom photographed with the aperture closed to increase contrast. You must keep in mind that when you close the condenser diaphragm you will reduce the numerical aperture of the system. Therefore it will reduce the resolution of the iris diagram in the condenser is not there to reduce the amount of light; it is present to control the numerical aperture. The amount of light is controlled by either controlling the voltage of the light source or by introducing neutral density filters to change the intensity. The condenser must be properly focused in order to produce maximum resolution.

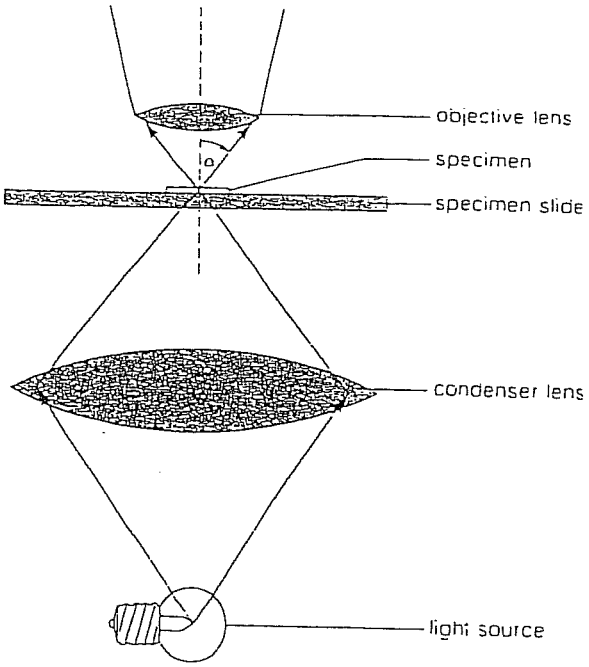


Figure A-2 Light path from the condenser to the objective lens. For maximum resolution, the half-angle (α) of the cone of light entering the objective lens should take on as large a value as possible.

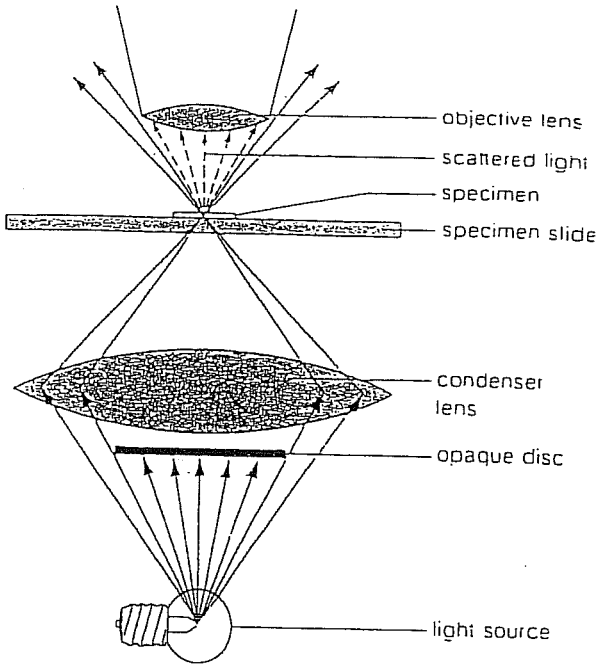


Figure A-3 Light path from the condenser to the objective lens i dark field illumination. An opaque disc blocks the central region of the condenser so that no rays from the light source directly en the objective lens. Objects in the specimen scattering light into the objective lens appear bright against a dark background (see text).

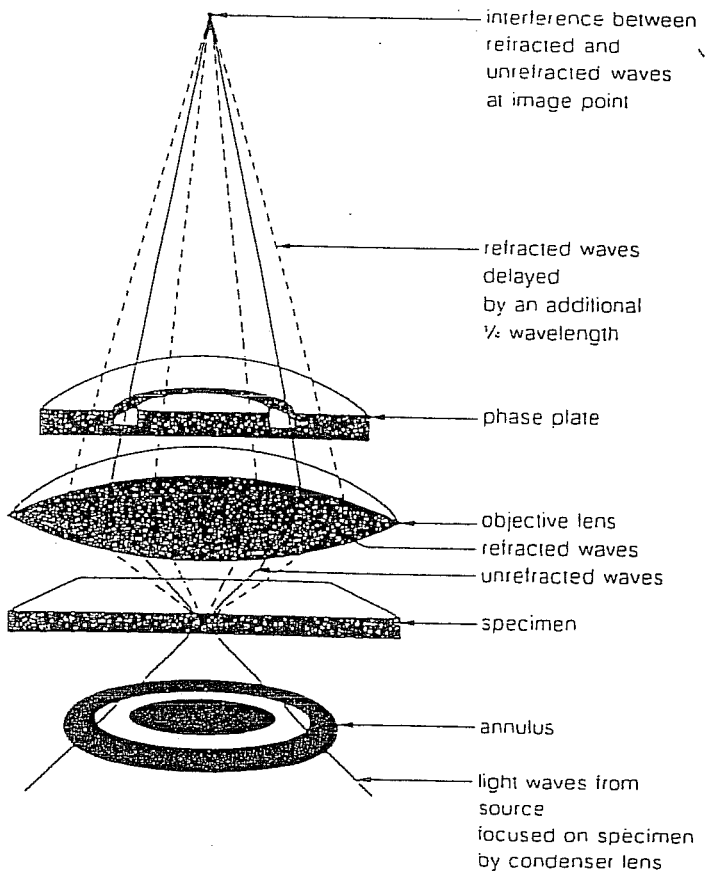


Figure A-4 The delay of refracted light waves by a phase plate in a phase contrast light microscope (see text).

INTRODUCTION TO USE OF THE COMPOUND MICROSCOPE

1) Getting started:
   a) Place a good slide on the stage.
   b) Rotate the 10X objective in viewing position.
   c) Focus on the slide
2) You can use the microscope without using your eyeglasses if you are near or farsighted. If you have moderate to severe astigmatism you will need to use your glasses.
3) Adjusting the condenser:
   a) Close the field diaphragm (on the light source) until it is visible in the field.
   b) Check to see that it is centered. If it is not centered, move the field diaphragm ring or the condenser adjustment screws to bring the image of the field diaphragm into the center of the field.
   c) Bring the image of the diaphragm into focus using the condenser focus knob.
4) Adjusting the interocular distance to your eyes:
   a) Move the oculars closer or farther apart to accommodate you interocular distance.
   b) It will be correct when you see a single image of the slide.
5) Adjusting for left/right eye differences:
   a) Determine which eye is dominant.
      i) Using both eyes, frame a distant object in a circle made by your index finger and thumb of your preferred hand.
      ii) While viewing and without moving your head or hand, close one eye and then the other.
      iii) The eye that keeps the object framed is the dominant eye.
   b) Set each eyepiece to the 65 diopter setting or halfway between the extremes of the adjustment.
   c) Bring a specific structure on the slide into the best focus using your dominant eye only.
   d) Close your dominant eye and observe with the other eye only.
      i) Do not touch the microscope focus.
      ii) Change the diopter setting of the non-dominant eye ocular until the structure is in the best focus.
      iii) Note the diopter number for future reference.
6) Use of the Condenser diaphragm.
   a) It is not for controlling the amount of light!
   b) It is used to control contrast.
   c) As it is closed the contrast will increase, however, the resolution will decrease.
   d) The best compromise is to close the diaphragm until you first notice a reduction in light.
   e) You must readjust the diaphragm for each objective.
7) Use only enough light to resolve the detail you need!

LIGHT TO ELECTRON MICROSCOPES – form and function

I. Compound microscope images
   a. Real Image is produced when the object being imaged is outside of the focal length of the lens
   b. Virtual Image is produced when the object being imaged is between the focal point of the lens and the physical lens.
   c. A compound microscope in its simplest form consists of these two elements

II. Corrections for aberrations
   a. Spherical aberration is the result of the light entering different regions of the spherical lens and being focused at different distances from the lens
   b. Chromatic Aberrations is the result of the different wavelengths of light (color) being focused at different distances from the lens
   c. Manufacturing aberrations are primarily quality control problems
      i. Astigmatism occurs when different sectors of the lens have different curvatures
      ii. Coma occurs when the surface is asymmetrical
   d. All these can be corrected and are taken care of in a modern professional microscope

III. Magnification is what usually comes to mind when one thinks of a microscope.
   a. Magnification is approximately the product of the magnification of the objective multiplied by the magnification of the ocular
   b. If it was this simple we would have light microscopes of magnification above 1000 times.

IV. Resolution is as important or more important than magnification in any imaging system
   a. Zeiss Corporation hired Ernst Abbe in the early 1870's to work on the theory of resolution to improve their microscopes
   b. His work led to the following equation

      $d = 0.61\lambda / n \sin \alpha$

      d is the distance between two points to be resolved

      0.61 is the Rayleigh constant for the effect of diffraction in resolving two small objects as described by George Airy.
      If two holes are very close together they will have overlapping Airy discs and will make it difficult to determine the two holes or objects as separate.

      λ is the wavelength of the illuminating light

      n is the refractive index of the space between the object and the front element of the objective lens

      $\sin \alpha$ of the half angle of the cone light that can enter the lens when it is in focus

      $n \sin \alpha$ is a design characteristic of the lens and is referred to as the Numerical Aperture (NA) of the lens

      We can simplify this equation a bit for ease of use:

      $d = 0.5 \lambda / NA = \lambda / 2 NA$

      Substituting real numbers:

      Green light: $\lambda = 0.52$ μm

      NA = 1.30

      $d = 0.52 / 2 (1.3) = 0.52 / 2.6 = 0.2$ μm

V. Abbe understood that there were two parameters to change that would increase resolution
   a. Increase the Numerical Aperture of the lens
   b. Decrease the wavelength of the of the illuminating light
   c. Helmholz working independently demonstrated the relationship between resolution and wavelength

VI. Electrons and their wavelength
   a. J. J. Thompson describe negatively charged particles which later were named electrons in 1897
   b. Louis de Broglie demonstrated that electrons had wave-like characteristics that were considerably shorter than visible light in 1924
   c. H. Busch demonstrated that electrons would be focused by magnetic lenses in 1926

d. In 1931 Davisson and Germer, and independently Thompson and Reid demonstrated diffraction phenomenon with electrons. This demonstrated their wave characteristics

e. Knoll and Ernst Ruska demonstrated a functioning electron microscope in 1932
f. In 1936 Vickers EMI built the first electron microscope designed as a commercial microscope
g. In 1938 the first scanning electron microscope was built by von Ardenne
h. Seimens Corporation with von Borries and Ruska began production of a commercial electron microscope in 1939. 10nm resolution. Production was stopped until after the Second World War ended in 1945.
i. RCA Corporation made the first commercial EM in the USA. 2.4nm resolution

VII. Electromagnetic Radiation (table 6.1, page 151)
   a. All travel at the speed of light
   b. Two descriptors
      i. Wave length – the distance between two peaks in the wave
      ii. Frequency - number of oscillations per second

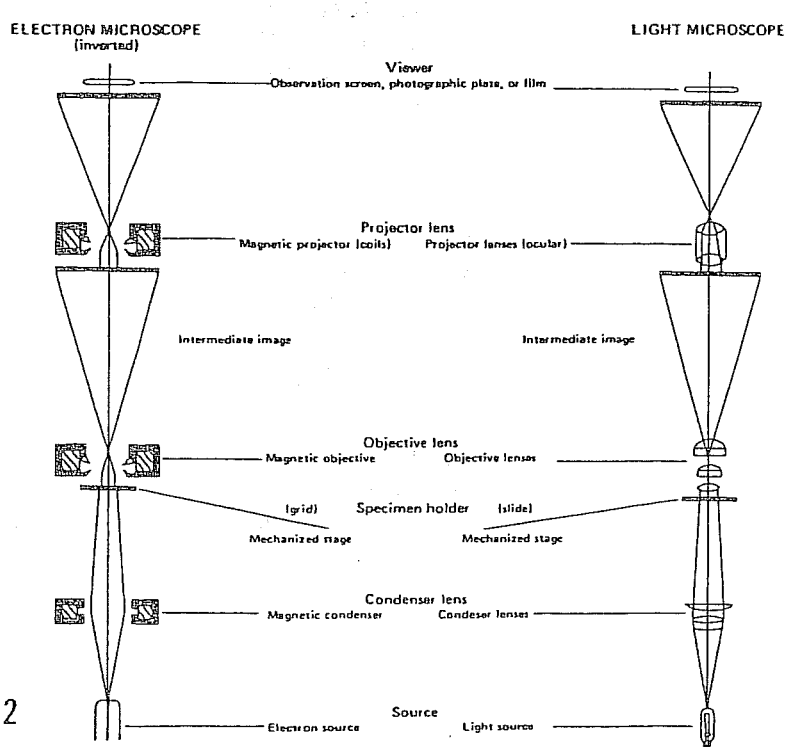
VIII. Wave length of Electrons
   a. de Broglie for the wavelength of an electron
      i. $\lambda = h/mv$
      ii. h = Planck's Constant = 6.626×10^{-23} ergs/sec
      iii. m = mass of the electron = 9.1×10^{-23} kg
      iv. v = velocity of the electron

IX. Resolution using electrons accelerated by 60KV
      i. $\lambda = 1.23\text{nm}/\sqrt{V}$
      ii. V = accelerating voltage
      iii. Example: λ at 60KV = 0.005nm

X. The Electron gun – source of illumination

XI. Electron lenses
   a. Electrons have a negative charge and are affected by magnetic fields
   b. This can be used to bend the path of a beam of electrons
   c. Make an electromagnetic lens out of a coil of wire with a direct current passing through the wire
   d. Electrons follow a helical path through the electromagnetic lens

XII. Vacuum Systems
   a. Electrons are small particles and cannot travel through air for any great distance without being deviated from their path and loosing energy.



diagramatic comparison of a light and electron microscope. e electron microscope is inverted to permit similar position- of the various lens systems. The two major differences in s comparative diagram are the type of lenses (electromag- tic vs glass) and the types of radiation (electron vs light).