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Lenses are the microscope's jewels. Understanding their properties is critical in understanding the microscope. Objective, condenser, and eyepiece lenses have information about their properties inscribed on their housings. Table 7.1 is an example from objective lenses. This chapter will unfold the meaning and significance of these symbols. The three main categories of information are Numerical Aperture, Magnification / Tube Length, and Aberration Corrections.

| Zeiss | Leitz | | |
|---|-------------------------------------|--|--|
| Plan-Apochromat 63X/1.40 Oil ∞/0.17 | 170/0.17 Pl 40 / 0.65 | | |
| Nikon | Olympus | | |
| Fluor 20 Ph3 0.75 160/0.17 | ULWD CDPlan 40PL 0.50 160/0-2 | | |

Table 7.1 – Common Objective Lens Inscriptions

Numerical Aperture & Resolution

Inscribed on every objective lens and most condenser lenses is a number that indicates the lenses resolving power – its numerical aperture or NA. For the Zeiss lens in table 7.1 it is 1.40. The larger the NA the better the resolving power. Ernst Abbe invented the concept of numerical aperture in 1873. However, prior to Abbe's quantitative formulation of resolving power other people, such as Charles Spencer, intuitively understood the underlying principles and had used them to produce superior lenses.

Spencer and Angular Aperture

So, here is a bit about Charles Spencer. In the mid 1850's microscopists debated the relationship of **angular aperture** to resolution. Angular aperture is illustrated in



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figure 7.1. The 3 X lens has an angular aperture of 14° compared to 110° for the 95 X

lens. Some people thought that magnification was the sole contributor to resolution. Charles Spencer (figure 7.2) - a lens maker in New York - defended the idea that angular aperture was equally important. The fact that larger angular aperture improved resolution was empirical. The finer details of diatoms were visible when using lenses of larger aperture but comparable magnification.

Abbe and Numerical Aperture

In 1873, Ernst Abbe, working with Carl Zeiss in Germany, devised a method for determining the resolving power of lenses. Abbe took into account the refractive index of the



medium between the objective and the cover glass (**n**), the natural sine of one half the angular aperture (α) and the wavelength of light (λ). The first two he multiplied together to produce a value he called **numerical aperture**: **NA** = **n** sine α . Abbe's formula is: Resolving Power (D) = $\lambda/2$ NA. This applies when the NA of the condenser lens is equal to or greater than the objective's NA and when the illumination consists of nearly parallel rays formed into a cone of light whose angle <u>matches</u> the objective lenses angular aperture. All of these criteria are fulfilled in Köhler illumination. Today, the accepted formula for resolution is D = $1.22\lambda/NA$ condenser + NAobjective. The constant 1.22 comes from the work of Lord Rayleigh in 1898. For a more complete explanation of how Abbe made this discovery and how Rayleigh extended it, see the footnote at the end of this chapter.

Typical numerical apertures for dry objectives range from 0.04 for a 1 X objective to 0.95 for a 60 X objective.

(The largest possible NA for a dry objective is around 0.95. Here is why based on Abbe's formula. The refractive index of air is 1. The largest possible angular aperture is 180 degrees. This would put the lens on the specimen so a <u>slightly smaller</u> angular aperture is assumed. Half the largest angular aperture is 90 degrees. The sine of 90 degrees is 1 and NA = n sine α so 1 X almost 1 = almost 1 (i.e., ~ 0.95).)

Now, here is where refractive index (**n**) comes in. The largest possible NA for liquid immersion objectives depends on the refractive index of the immersion medium. From Abbe's formula you can see that as **n** gets bigger so does NA. Table 7.2 lists the refractive index for several common optical materials. Numerical apertures may be as high as 1.40 or 1.50 for a 60 X or a 100 X oil immersion lens.

| Optical Material | RI - Sodium D Line |
|-------------------|--------------------|
| Air | 1.00029 |
| Fused Quartz | 1.45845 |
| Crown Glass | 1.52300 |
| Dense Flint Glass | 1.67050 |
| Cover Glass | 1.52500 |
| Immersion Oil | 1.51500 |
| Water | 1.33300 |

Table 7.2 - Refractive Index of Common Optical Materials

Table 7.3 shows resolution versus NA based on Abbe's formula assuming a wavelength of 0.5 μ m (blue-green) and assuming the condenser fully illuminates the objective's aperture.

| Numerical Aperture | Resolution In μ m | Typical |
|--------------------|-----------------------|---------------|
| | X - Y | Magnification |
| 0.04 | 7.62 | 1 X |
| 0.08 | 3.81 | 2 X |
| 0.20 | 1.52 | 4 X |
| 0.45 | 0.67 | 10 X |
| 0.75 | 0.40 | 20 X |
| 0.95 | 0.32 | 40 X |
| 1.30 | 0.23 | 40 X |
| 1.40 | 0.21 | 60 X |
| 1.50 | 0.20 | 100 X |

Table 7.3 - Numerical Aperture and Resolution

Index of Refraction

Numerical aperture also takes into account **Refractive Index (RI)**. RI is extremely important in light microscopy so let's take a closer look at it.

Light bends from its normally straight path when it passes from a medium of one density into a different density medium. This is called **refraction**. A common example is the apparent bend in a pencil inserted into a glass of water. The pencil looks bent because light travels a different path in the water than in the air. Lenses work by refraction. As illustrated in figure 7.3, when going from a less dense to a more dense medium light bends toward a line drawn normal to the surface of the medium at the point of incidence. When going from a more dense to a less dense medium, light bends away from the





normal. In 960 AD, the Muslim scholar Al Hazan attempted to derive a mathematical relationship between a light ray's angle of incidence and its angle of refraction. He was unsuccessful, as was everyone else for the next 600 or so years. The correct law of

refraction was derived in 1621 by Willibrord Snell of Holland and is known as **Snell's law of sins**.

Snell's law of sins states: when a ray of light passes from one transparent medium into another, its direction will change by an amount that depends on the **refractive index** of the new medium. The refractive index of a substance is equal to the sine of the angle of incidence divided by the sine of the angle of refraction.

The refractive index of a substance will vary for different wavelengths of light. Shorter wavelengths are

refracted more strongly than longer wavelengths. This is what causes chromatic aberrations in lenses. Unless otherwise stated, a refractive index is based on the yellow sodium D line of 589 nm.

A practical example of the effect of index of refraction can be seen in the use of oil versus air immersion lenses. Figure 7.4 is a classic illustration of this principle. An American working in New York, Robert B. Tolles (student of Charles Spencer) described the **homogeneous immersion** principal and made immersion lenses in 1874 (figure 7.5). This method connects together the condenser, the slide with its specimen, and the objective lens using a medium of refractive index close to that of glass. Tolles' immersion medium was Canada Balsam (RI 1.535). In 1878 Ernst Abbe created the type of homogeneous immersion objective used today. His immersion medium was cedar wood oil (RI 1.515).





Critical Angle



There is a case in which refraction cannot occur. This is shown in figure 7.6. When light is traveling from a denser to a rarer medium and strikes the interface at an angle greater than the **critical angle**, the light is totally reflected back into the denser medium. This is called total internal reflection. The critical angle is the angle of incidence for which the angle of refraction is 90°. The critical angle for glass to air is 41.3°, and for glass to water it is 61.7°. There is no critical angle for glass to oil. The angle 67.5° in figure 7.6 is equivalent to a numerical aperture of 1.40. Dark field condensers use total internal reflection, as do some polarizing prisms such as the Nicol prism.

Total internal reflection produces an **evanescent wave** along the reflective interface that penetrates about Pathology 464 Light Microscopy 4 100 nm into the lower refractive index medium. It can be used to reveal features attached to the surface of the interface such as adhesion plaques of motile cells. The technique known as TIRF (total internal reflection fluorescence) can be utilized to <u>visualize</u> structures far below the resolution limit of the microscope.

Diffraction

This discussion of numerical aperture began with the historical observation that larger angular apertures produce better resolution. Why should this be so? The answer has to do with **diffraction**.

When a beam of parallel light strikes the sharp edge of an object the light's path tends to bend a little toward the edge. This bending of the light path is called diffraction. Suppose the object is a narrow slit rather than a single edge. The angle at which the light is diffracted at the slit becomes greater as the slit becomes narrower. Figure 7.7 illustrates parallel light passing through increasingly narrow slits (bottom to top) and the resulting diffraction.

According to Ernst Abbe, a microscopy specimen, in effect, consists of many slits of varying widths - the slits being regions of the sample where refractive index changes abruptly, as at membranes. Diffracted light spreads out from the slits and the narrower the slit the greater the angle of spread. In other words, the finer the specimen detail, the greater the angle at which it diffracts light. It is important to collect as much of this light as possible from the specimen to resolve fine details. A lens of large angular aperture will collect more-highly diffracted light than one



of small angular aperture and will therefore have better resolution. Also, the shorter the wavelength of light, the smaller its angle of diffraction for a given slit width. Thus shorter wavelengths give better resolution, and this is taken into account in Abbe's formula for resolution.

Resolution

Based on his work with diffraction from a line grating, Abbe devised an equation for the resolving power of the microscope:

D = lambda / 2 NA

where lambda is the wavelength of light and NA is numerical aperture. Why "2" NA? If a specimen is illuminated with a parallel beam of light (Fig. 7.*), Abbe's equation would be D = lambda / NA. But Abbe realized that by tilting the illumination to match the acceptance angle of the objective (that is to match the objective's NA) you could double the resolving power (Fig. 7.*).





Figure 7.* Tilted Illumination

Years later, Lord Raleigh, working with diffraction points known as Airy Disks rather than diffraction lines, showed that resolution was:

D = 0.61 lambda / NA objective

Or

D = 1.22 lambda / NA of objective + NA of condenser.

This was based on the fact that two diffraction spots had to be separated by about 60% of the diameter of the first dark ring to be clearly seen as two spots by a human (Fig. 7.*).

Numerical Aperture of the Condenser

Numerical aperture of the condenser is extremely important. In transmitted light, the numerical aperture of the entire microscope system can be no larger than that of the condenser regardless of the NA of the objective lens. In air immersion (dry) systems, the largest numerical aperture possible is about 0.95. However, even if the objective's NA is 0.95, if the condenser's NA is not at least this then the entire aperture of the objective cannot be illuminated using the Köhler system. Also, even if the condenser's NA is equal to or greater than the objective's, the condenser iris may be used to limit the condenser's aperture and therefore the NA of the entire system. In oil immersion systems, unless the condenser is immersed to the specimen slide with oil, the numerical aperture of the system can be no greater than 0.95.

Magnification and Tube Length

There are two inscriptions on an objective lens that relate its magnification. The first is the **magnification** indication – e.g., 10X. The second is the **tube length** at which this lens produces this magnification – e.g., 160. This lens produces a magnification of 10 X at a distance of 160 mm behind the lens.

Linear or Axial Magnification

There are several ways of defining magnification. Perhaps the most general definition is the ratio between the size of an image and the size of the object it represents. If a lens forms a real image of an object you could measure the size of the image and the object. If the size of the image is 100 times bigger than the size of the object, the magnification is 100X.

(Human vision uses a different definition of 1X. The closest point at which a normal adult can comfortably focus is about 250 mm. By definition this is taken as the **near point** of human vision. An object viewed at this distance is said to be viewed at 1 X. If you could focus on the object at 125 mm, it would be viewed at 2X. The magnifications written on objectives and eyepieces do not consider this human definition of 1X. However, the optical system of the microscope is designed to produce virtual images that are seen at 250 mm.)

The Lens Equation

Magnification can also be defined in terms related to geometric optics. This is worth considering because it involves the simplest and most famous equation in all of optics: the **lens equation**. The lens equation is illustrated in figure 7.9. When a converging lens forms a real image, the distance from the object to the center of the lens is called **u** and the distance from the image to the center of the lens is called **v**. If the focal length of the lens is called **f**, the lens equation is

$$1/u - 1/v = 1/f$$

Magnification can be defined as the ratio of the **image distance** (v) to the **object distance** (u):

$$M = v/u$$
.

This is a purely mathematical definition of magnification. Let's apply this to a real microscope.

On older microscopes, the distance between the nosepiece and the top of the eyepiece tube is a fixed distance called the **mechanical tube length** - which is 160 mm in many instruments. The image distance v is fixed at 160 mm. If we let the object distance u be at the lenses focal length f then we can calculate the lenses magnification as 160 mm / f in mm.

(In the early days, before the advent of standardized tube lengths, a lens did not have its magnification written on it. Instead, an objective lens had its focal length in inches inscribed on its barrel. To calculate the magnification one had to divide this value into whatever the tube length of the microscope happened to be (and you could vary the tube length on some instruments). For example a lens may have had the value 1/4 inscribed on it. If you had set your tube length to 5.0 inches your objective's magnification would be 5.0 / 0.25 = 20X.)



Figure 7.9

Total Magnification

When more than one magnifier is involved, the total magnification is the product of all the individual magnifications. Thus if a 10 X objective lens is used with a 10 X eyepiece, the total magnification is 100X as seen by the observer. If a micrograph is made, magnification of all lenses between the specimen and the film or CCD must be known and multiplied together to determine the final magnification. A simple way to keep track of magnification in a digital micrograph is to photograph a **stage micrometer** and include a magnification bar, based on the micrometer, in the specimen image. Even if a print is made at some unknown enlargement or reduction factor in a publication, the size bar will remain an accurate indicator of total magnification.

Tip: Use a combination of higher objective and lower ocular magnification to achieve the final magnification. For example use a 20X objective and a 10X ocular instead of a 10X objective and 20X ocular to achieve a total magnification of 200X. This takes advantage of the higher NA of the 20X objective.

Empty Magnification

There is no theoretical limit to the amount that an object can be magnified, but there are practical limits. Normal human vision can clearly resolve about 1/4 mm at 250 mm. To see a small object, all that is required is to enlarge its image to 1/4 mm. Based on the idea of numerical aperture, there is a limit to the size of structure that a lens can resolve. With the highest NA objectives, this size is about 1/4 μ m. The magnification required to enlarge an object of 1/4 μ m to 1/4 mm is 1000X. Ernst Abbe proposed the following rule regarding magnification and numerical aperture: Useful magnification will be between 500 and 1000 times the objective's NA. Magnification above 1000 times the numerical aperture will be empty of further detail and is referred to as **empty magnification**.

Magnification Color Codes

An international color code has been devised to make it easy to identify the magnification of an objective lens. A colored stripe that corresponds to this code encircles modern objective lenses. The code is black= 1X, brown= 2X, red= 4X, yellow= 10X, green= 20X, light blue= 40X, dark blue= 63X, and white= 100X.

Why 63X?

Many microscope manufacturers produce a 63X lens rather than a 60X lens. The reason is to comply with an international standard geometric series of magnifications for light microscopes called the R_a -10 standard series of the ISO. The standard tries to make each succeeding step in magnification about 25% different from its neighbors and the product of any two values is a standard value. The values in the series that we are most familiar with are: 10, 12.5, 16, 20, 25, 32, 40, 50, 63, 80 and 100.

Tube Length

An objective lens can produce real images of different magnifications just as a slide projector can project images at different magnifications by moving the projection screen closer to or father away from the projector and adjusting the focus. The screen in the slide projector set up corresponds to the intermediate image plane inside a microscope. Unlike a projector, a microscope usually has a fixed distance between the objective lens and the eyepiece. This distance is called the **mechanical tube length**. There are three common microscope tube lengths: 160 mm, 170 mm, and infinity (∞).

How can tube length be infinite? A converging lens focuses at infinity when the object is at one focal length from the lens. Infinity corrected microscopes use an **auxiliary lens** near the eyepiece to create the intermediate image. Infinity corrected optics allow the tube length to be changed, without changing the magnification. This is often useful if optical accessories, such as an illuminated pointer or a confocal pinhole, are to be inserted into the optical path.

You should not use objectives of one tube length on a stand of a different tube length even though the objectives may focus the specimen. The primary reason for not doing so relates to the objective's aberration corrections that are optimal only at the indicated tube length. A secondary reason is that the indicated magnifications will be incorrect for 160 mm and 170 mm tubes.

Depth of Field and Depth of Focus

For a given lens system, the distance along the optical axis on the image side, over which the specimen appears to be in focus, is termed the depth of focus. The distance along the optical axis, on the object side that will be in focus on the image side is termed the depth of field. Both the depth of focus and depth of field have an inverse relationship to the numerical aperture: higher NA produces smaller depth of focus and depth of field. (The equations are Depth of focus = magnification squared X resolving power / NA; Depth of field = wavelength / NA squared). So, higher NA objective lenses

have a thinner depth of field. This is why you have to focus up and down with higher NA lenses to get an idea of what a specimen is like throughout its depth. You can close the aperture iris in the condenser to increase the depth of field in a specimen at the expense of lowering the NA of the system and therefore worsening the resolution. Depth of Field and Focus are illustrated below.



Aberration Corrections

Lens aberration corrections can be made in objective lenses, condenser lenses, and eyepiece lenses. The types and degree of correction vary for each of these and they will be discussed in this section. The general appearance of the important aberrations when observing a normal specimen will be discussed. Binocular tubes can make diopter corrections for the microscopist. The correct use of different binocular tube types is described in Chapter 1. This section ends with a simple test for aberrations that you can apply to your objective lenses. The optical explanation of these aberrations and the way in which they are corrected is interesting and is presented in Appendix A.

Some discussion of the effect of these aberrations upon the appearance of a specimen will help clarify your choice of one type of objective over the other.

Spherical Aberration

When a lens suffers from spherical aberration, the image of a well prepared, stained section will always seem a little hazy. No matter how carefully you focus, the image cannot be made completely sharp.

Chromatic Aberration

Chromatic aberration is not noticeable in stained sections, but in unstained preparations faint color fringes around objects may be noticeable with achromats.

Field Curvature

Field curvature causes an inability to focus the entire image simultaneously. When the center of the field is in focus the periphery is not and when the periphery is in focus the center is not. This may not be too troublesome for observational purposes, but it is a real problem for photomicrography. Lenses that are labeled "**Plan**" have been corrected for field curvature.

Cover Glass Correction

Use of a cover glass over a specimen introduces additional spherical aberration. Figure 7.10 illustrates how this happens. For all lenses, this effect is compensated in the lens design. The thickness of cover glass for which a lens is designed is inscribed on the lens housing. Most modern lenses are designed for a 0.17-mm thick cover glass. This is a number 1.5 cover glass. Some older lenses may be designed for a 0.18-mm cover glass. Some special lenses are designed for use with no cover glass and are labeled NCG. High magnification dry lenses are susceptible to differences of 0.01 mm in cover glass thickness. These lenses are usually provided with a cover glass correction collar. Turning this collar adjusts the position of an internal rear lens element thus allowing the lens to be corrected for slight variations in cover glass thickness. Proper use of the cover glass correction collar is covered in Chapter 8.

Figure 7.10 A shows a cover glass of correct thickness. The lens has been designed so an in focus specimen appears to be just at the lower surface of the

Figure 7.10 A B C

cover glass. Light refracted by the cover glass just fills the lens aperture. Figure 7.10 B shows the effect of a cover glass that is too thick. The cover glass moves the specimen image closer than the lenses optimum correction for spherical aberration. The lenses full aperture is not filled. Figure 7.10 C shows the effect of a cover glass that is too thin. The image is father away than optimum. Part of the refracted light misses the lens.

Off Axis Corrections

Three other aberrations are corrected in objective lenses. These are **astigmatism**, **coma**, and **distortion**. Modern objective lenses are completely corrected for these. See appendix A to learn more about them.

Objective Lens Types

Objective lenses are named based on how well they are corrected for the common lens aberrations. Table 7.4 lists the lens types and their respective combinations of corrections. Different microscope manufacturers use different inscriptions on their objectives to indicate these levels of correction (Table 7.1). Regardless of the terminology on the lens, there will be a direct correlation to one of these four lens types.

| Aberrations / | Spherical | Chromatic - | Chromatic - | Field |
|---------------|-------------|--------------|---------------|-----------|
| Lens Types | | Longitudinal | Lateral | Curvature |
| Achromat | green | red & blue | red & blue in | if plan |
| | | | eyepiece | |
| Fluorite | red & blue | red & blue | red & blue in | if plan |
| | | | eyepiece | |
| Apochromat | red & blue | red, blue & | red & blue in | if plan |
| | | green | eyepiece | |
| CF | red, blue & | red, blue & | red & blue | if plan |
| | green | green | | |

Table 7.4 - Corrections of Different Lens Types

Practical Considerations in Choosing Objective Lenses

There are some practical points about usage of the different lens types.

Achromat lenses are the least corrected and least expensive. These are fine for general observation.

Fluorite lenses are better corrected than achromats and are the best choice for fluorescence microscopy.

Apochromats are the best-corrected lenses and they are the most expensive. They should be used when the highest resolution possible is required. Most older apochromats pass very little UV light and thus are not desirable for UV excited fluorescence. Modern apochromats are much better.

CF lenses are as well corrected as apochromats with all corrections being made within the objective lens itself – they do not require compensating eyepieces. They cost as much or more as apochromats. CF stands for chromatic aberration free and is a Nikon designation. Other manufacturers have their own designations for this type of lens. It is common to use the terms Fluorite and Apochromat even for CF type lenses.

Tip: The final correction for lateral chromatic aberration is accomplished in the eyepiece for all but the CF lens types. See compensating eyepieces below. Be sure that your eyepieces and objective lenses match.

Condenser Lenses

Condenser lenses are generally not as well corrected as objective lenses and the terminology associated with them is different from that of objective lenses.

There are three types of bright field condensers:

- 1 The Abbe type has no chromatic or spherical correction.
- 2 The **aplanat** is only spherically corrected.
- 3 The **aplanat-achromat** is corrected for both spherical and red and blue longitudinal chromatic aberrations.

Tip: Use the aplanat-achromat for bright field color photomicrography. The Abbe type is acceptable for observational work, even with the highest NA best corrected objectives.

Eyepiece Lenses

The information written on eyepiece housings tends to be scanty and cryptic. Even without documentation you can learn a little by examining the upper and lower lenses of the eyepiece. Figure 7.11 illustrates the difference among three common types. The blue lines in this illustration indicate the position of the intermediate image. The following is a description of eyepieces with a historical perspective.

Huygenian Eyepiece

In 1684 Christian Huygens invented an eyepiece for the telescope that had two lenses. The lens nearest the eye was called the **eye lens** and the second lens was called the **field lens**. In the late 1600's, Robert Hooke used this type of eyepiece on his microscope. This type of eyepiece has good chromatic aberration correction but its **exit pupil** is very close to the eye lens.

Ramsden Eyepiece

In 1782 J. Ramsden designed an eyepiece that had an exit pupil father from the eye lens and was better corrected for field curvature.

Compensating Eyepiece

Ernst Abbe developed the compensating eyepiece in the late





1800's. This eyepiece is intentionally designed with sufficient lateral chromatic aberration (chromatic difference of magnification) to just balance that of the objective. Modern microscope manufacturers still use this technique. Thus eyepieces and objectives are produced as a matched set.

CF Eyepieces

Nikon, in the 1980's, developed several special types of glasses. These glasses have refractive indices and dispersion characteristics that enabled correction of lateral chromatic aberration without the need for compensation. Thus Nikon's Chromatic Aberration Free (CF) optics use **CF eyepieces** rather than compensating eyepieces. Other manufacturers are now also producing chromatic aberration free optics.

Telling One Eyepiece Form Another

You can tell something about the level of correction of your eyepieces by looking through them at a white light. In an uncorrected eyepiece you will see a blue fringe around the edge; a compensating eyepiece will have a yellowish fringe and a CF eyepiece will show no color.

Photo Eyepieces

A microscope equipped for photomicrography will have an eyepiece (or some other optical adapter) between the objective lens and the camera. This eyepiece is designed to produce a real image at its exit pupil whereas the other eyepieces produce a virtual image. The **photographic eyepiece** is referred to as a **projection eyepiece**. This eyepiece may have a different magnification from the viewing eyepieces; thus the magnification on a micrograph can be different from that observed in the microscope.

Eyepiece Markings

Eyepieces are marked with their magnification (e.g., 10X), and perhaps other information such as **K** or **C** or **comp** to indicate that they are compensating eyepieces; an indicator of the **field width** such as **W** for wide field or a number indicating the **field number** (diameter of the field of view in mm); an indication of aberration corrections such as Plan for flat field; and an indication if the ocular has an exit pupil far enough away to allow the use of eyeglasses.

The Star Test for Detecting Objective Lens Aberrations

The following excerpt from Needham describes a simple way to check an objective for aberrations. Figure 7.12 (adapted from Spencer) illustrates the appearance of spherical aberration, coma and astigmatism using this method. I have added comments in parentheses.



Figure 7.12 - Appearance of the Airy Disk for Various Aberrations

THE STAR TEST

The Star Test is a valuable and delicate method of testing for spherical and chromatic aberrations of the objective. Also for determining the tube length which gives the minimum spherical errors and for testing the setting of the correction collar of high dry objectives. (These days we cannot adjust the tube length at will.)

For this test an extremely thin film of silver or aluminum is deposited on a 0.172 mm. thick 15 mm. diameter cover glass, and then the film is polished with chamois moistened with rouge until minute scratches and holes are apparent. The cover is then mounted on a 3" x 1" slip with clarite or balsam. Such a test slide can be prepared by the microscopist or one can be purchased from W. Watson & Sons of London. (We make our own by evaporating aluminum onto #1.5 coverglasses in a vacuum evaporator then rubbing the coated surface with a bit of cotton and mounting them, aluminum side down, on a slide with some DPX. One could also simply mount some sub-resolution polystyrine beads in an antifade medium under a 1.5 coverglass.)

The lamp and condenser necessary are similar to those required for the Abbe test plate. A wide axial cone of light is used for each objective. A 15x-compensating eyepiece will be found best. Check to see that the tube length is 160 mm. or 170 mm. (Leitz). (Align the microscope for Köhler illumination.)

Spherical Aberration. Search for a minute, *circular*, *brilliant* spot of light in the film which shows several diffraction rings when in focus. Change to above and below the focus with the fine adjustment. With an ideal objective *free* from spherical errors, the following optical phenomena would occur when this change is made:

1. Exact duplication of concentric rings.

2. The rings would be perfect concentric circles with equal spacing and brilliance.

When in focus, the minute circle of light would be surrounded by perfect diffraction rings.

As every objective has more or less spherical aberration, the closer a lens approaches to the above ideal, the better the objective. Should the rings downward be plainer than the rings upward, the tube length of the microscope should be increased in 5 mm. steps until the rings are as nearly balanced as possible in and out of the focus. With most objectives, one set of rings seems to be more brilliant than the other set. Should the rings upward be more pronounced than the rings downward, shorten the tube length until compensation takes place.

Two high dry achromatic objectives tested as above required an increase of 7 and 23 mm. from the standard tube length of 160 mm. to give the minimum spherical aberration.

Two apochromatic objectives of 40x and 60x required a correction of -0.02 and -0.01 mm., respectively, to the correction collar reading set at the 17.5 mark when tested spherically as given above. This is a very severe test for the correction collar setting and few high dry apochromats or fluorites will meet this test.

Chromatic Aberration. Search for a fairly large break in the film and note the fringe of the film above and below the focus. The following effects will occur, according to the type of objective:

Achromat. Pronounced color fringes. Above the focus, yellow-green, below the focus, red or purple. The wider the fringes, the poorer the lens is corrected for color.

Fluorite. Narrow color fringes of green and red. The narrower the width of the color fringes, the better the lens.

Apochromat. No color fringes in and out of the focus.

(For further information on the Star Test, see Coles, A.C.: The Star Test for Microscopic Objectives. *Watson's Microscope Record*, 7:16-19, January, 1926.)

Exercises

NOTE: A prepared diatom slide can be purchased from Carolina Biological Supply Co.

1) Observe the effect of the thickness of the glass slide and coverglass on microscope alignment: Using a standard 1.2 mm thick slide with no specimen, mount a #1.5

coverglass that is about 20 X 40 mm using standard mounting media (I used DPX). Leave an area of the slide uncovered. Mount another #1.5 coverglass that is about 20 X 20 mm square on top of the first coverglass. Use just enough mounting media to just fill the coverglasses. Let the slide dry. You might put a mark on the slide in the area where there will be only one cover glass before you begin to give something to focus on for alignment. Using this test slide, align the microscope for Köhler illumination using the part of the slide with just one coverglass. Bring the field iris into view and remove the slide from the image path. What happens to the image of the field iris? Replace the slide and observe what happens to the image of the field iris as you move from slide-only to slide plus coverglass to slide plus two covergalsses. Adjust the condenser hight each time to focus the field iris and note the direction in which you have to move the condenser. Compare this to figure 7.10.

2) Chromatic aberrations are rarely seen in modern optics. Still, it is worthwhile for the student to have some appreciation of the appearance of chromatic aberration. For this exercise you will need an Abbe type condenser. The Abbe condenser has no corrections for chromatic or spherical aberrations. Align a slide for Kohler illumination. Move to an area of the slide with no specimen. Close the field iris nearly all the way. Lower the condenser then gradually raise it and observe the color shift at the edge of the field iris as the condenser moves to, through, and past focus. The color at the edge of the iris will shift from blue to red as you raise the condenser through focus. Try the same experiment with an aplanat-achromat condenser.

3) Confirm the fact that higher NA gives better resolution. For this exercise you will need:

a) A diatom Test Plate (B 25D) from Carolina Biological Supply or equivalent

b) two 10 x objective lenses of different NA (I have a 0.3 and a 0.45)

c) a blue filter.

Put in the blue filter. Align the microscope for Köhler. Focus on the diatoms and bring Navicula lyra (next to the longest one on the end) into the center of the field. (This diatom has 8 striae per 10 microns). Reduce the field iris to an area just around the diatoms. Close the aperture iris to achieve good contrast. Compare the image of the horizontal striae between the 0.3 and 0.45 lens. The difference will be obvious. Note that neither lens can resolve the striae on the next diatom.

Repeat this exercise with 20 x objectives of 0.50 and 0.75 NA on the next diatom Stauroneis phoenocenteron. (This diatom has 14 striae per 10 micrometers.)

4) Check your eyepieces; note what kind they are by looking through them at white light; see whether they match your objective lenses. (See Eyepiece Lenses – this chapter.)

Footnote

Ernst Abbe's work on the resolving power of the microscope:

(I hope Abbe will forgive me for this extremely light summary of his work.)

Abbe created a simple system for examining resolution. He started with light that was formed into as nearly parallel rays as he could make for illuminating the specimen. He knew that the objective lens would form this light into a bright spot in the center of the objective lens back focal plane (OBFP) surrounded by a dark space. He also knew that light from the bright spot would continue on, spreading out in all directions in a homogenous pattern as it moved up the microscope. Next he used a simple specimen that consisted of a set of parallel light and dark lines of known spacing. Under these conditions, when he looked at the OBFP he observed a bright spot in the center flanked, not by a dark space, but by a series of bright spots that extended into the formerly dark space. The spots were arranged at right angles to the direction of the lines in the specimen. Abbe reasoned that this was a diffraction pattern made by the affect of the specimen on the nearly coherent illumination. He then used a mask that he could place in the OBFP that blocked different parts of the diffraction pattern and noted the result on the observed image at the evepiece. When all the diffracted orders but one were blocked, the image did not resemble the specimen. In fact, he determined that at least 2 diffraction orders had to be present in the OBFP to completely resolve the specimen. Under these conditions he determined that the resolving power of the objective lens was the wavelength of the illuminating light divided by the numerical aperture of the lens (lamda / N.A.). Then, using a condenser that produced a cone of illumination whose angle exactly matched the acceptance angle of the objective lens (i.e. their N.A.s were the same), the resolving power of the lens system was the wavelength of the illumination divided by 2 times the numerical aperture (lamda / 2 N.A.). This was in 1873.

Lord Rayleigh's extension of Abbe's work:

In 1896 Lord Rayleigh investigated resolution for point objects in the microscope (Abbe had investigated lines). He knew, based on Airy's work, that a point object was not imaged as a small point in the OBFP, but rather as a small circle of light that was brighter in the center than it was at the edge – an Airy disk. He found that for two point objects to be resolved by a human observer, they had to be separated by a distance equal to the radius of the Airy disk. For example, given a 100X 1.3 N.A. objective lens and 0.55 μ m light, the radius of an Airy disk of a point object will be measured to be 0.26 micrometers. This is 1.22 λ / 2 N.A. which, for all practical purposes, is exactly what Abbe found. This is known as the Rayleigh criterion for resolution.