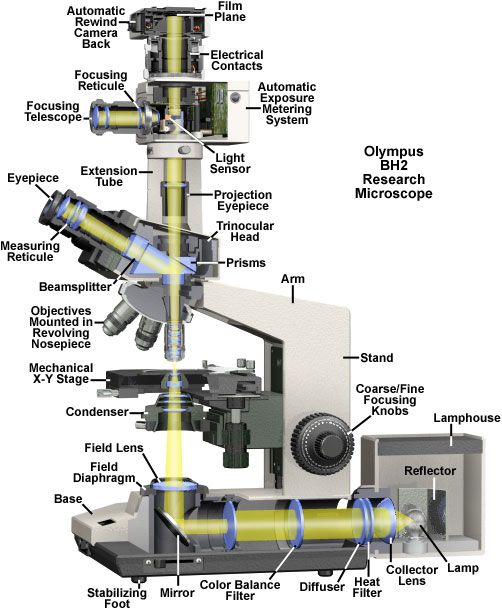
**OPTICAL MICROSCOPY : A Brief Tour**

J. Fong

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Microcrystalline habit

**OPTICAL MICROSCOPY : A Brief Tour**

**1. INTRODUCTION**

The microscope has been part of the technical workbench ever since 1685 when Anton Von Leewonhoek ,

a Dutch janitor, built the first compound microscope, examined the scrapings of his teeth under it and saw `animucules’ .





Anton Leeuwonhoek circa 1685 First compound microscope

Now, even middle school science labs have at least a half-dozen student `scopes little different in basic principles from Leewonhoek’s, and, it is the rare professional lab that does not sport at least one or two medium-priced (often polarizing) light microscopes.

Because of the microscope’s long history, popularity and availability it is therefore easy to forget the complicated set of image transformations undergone by any object being examined under a microscope.

Often, one’s introduction to a microscope amounts to the single instruction, “Look down the eyepiece and focus until you see something.” Indeed, the complete absence of instruction on microscopic methods in current college chemistry curricula points to a distressing lack of respect for this venerable technique. Yet, in any chemical problem involving solid material of unknown character, the chemist’s first instinct is to `have a look at it under the microscope.’

At Alfred State, the microscope is recognized as integral to any investigation of a solid material’s properties. With little more than a few glass slides, intelligence and a basic notion of how the scope works, it is often possible to home in on a material’s identity and character far faster than by any other (superficially) more `high tech’ methodology. To do so, however, requires more than just the

simplistic assumption that all you have to do is focus and look.

**2. GOALS OF THIS MODULE**

In this module your goals are:

1) to understand the basic optical properties giving rise to microscope magnification

2) to become familiar with various practical terms associated with modern microscopes

(NA, objective types and rating, image types)

3) to acquire a hands-on set of skills allowing you to align and take pictures through

a microscope

4) to gain some experience with chemical microscopy methods connected with

qualitative chemical analysis (methods of Chamot and Mason, taken from

their “Handbook of Chemical Microscopy,” volume II, 2nd edition, John Wiley and Sons, Inc., 1938)

**3. BASIC CONCEPTS UNDERLYING MICROSCOPY**

In microscopy, the sample you `see’ through the eyepiece is not what is actually there. This perhaps startling assertion reflects a constant fact of life in measurement science, to wit:  **all instrumentation, irrespective of its simplicity or sophistication provides at best, an imperfect picture of a sample’s `real’ nature.** The microscope is no exception. The light from a sample that arrives at your eye from a microscope is the result of a large number of `transformations’ undergone by the light as it passes from the sample through one medium (e.g. air or glass) into another medium (e.g. glass or air.) Key to these transformations is the phenomenon of refraction.

**3.1 Refraction**

Suppose a beam of light strikes a glass surface at right angles (90o) , as shown in Figure 1. What happens ? Experience teaches that if the light is not absorbed or reflected, it passes from air to glass `undeviated’ e.g., a beam incident at 90o remains at 90o on either side of the interface. However, since the glass is denser than air, it is natural to expect that the glass offers greater `optical resistance’ to the passage of light. By extension, this means the light `slows down’ traveling in the glass relative to the air. Conversely, if the light moves from glass back into air, it `speeds back up’. In both cases we case we say the light is ***refracted.***

The medium’s power or ability to `refract’ light is measured in terms of **n**, the refractive index (often simply referred to as the material’s index.) A material with high index slows light more than a material with low index. More precisely, if **c** is the speed of light in vacuum and **v** is the speed of light in any other medium, then the index of the medium is:

**1 n = c/v**

As you might expect, minimum refractive index occurs in vacuum, e.g. light moves `fastest’ in vacuum. This is logical. In vacuum nothing is present to `resist’ the passage of light. Hence, all matter must by definition have **n** values greater than 1.0000.

Typical values of **n** at a wavelength of 589.3 nm (Sodium D line[[1]](#footnote-1)) are listed below. Consistent with intuition they correlate with the material’s density.[[2]](#footnote-2)

**TABLE 1: Refractive Indexes and Densities** **FIGURE 1: Refraction**

**of Some Typical Materials at 25o C Effect at 90o** **Incidence**

**material n=refractive index at 25oC density (g/cm3**)

vacuum 1.0000 0.0000 ***air*** `fast’

air 1.0003 0.0013 light

distilled water 1.3330 0.9997

window glass 1.510 2.5 ***glass*** ‘slow’

lead glass 1.615 2.9 light

What happens if the angle of incidence is **not** 90o ? This circumstance is sketched in Figure 2 where the incident beams of light strike a piece of glass obliquely at angles of A1 or A2 relative to the perpendicular ( ) line.

**FIGURE 2: Refraction Effect at Angles Other Than 90o**

**air medium**

***incident light beam*** A1 (n~1.00) A2

***refracted light beam*** **glass medium**

**perpendicular** B1  (n~1.51) B2

As seen, when the incident beam (or ray) moves from air to glass, the refracted beam within the glass tends to bend **towards** the perpendicular formed at the air-glass interface. This can be generalized, e.g:.

**When light moves from lower into higher n=> light bends towards perpendicular;**

**When light moves from higher into lower n=> light bends away from perpendicular.**

Furthermore, Figure 2 illustrates that the amount of bending or ‘ refraction,’ as measured by the refracted angle B **increases** as the impinging beam’s angle A deviates more and more from the perpendicular. That is:

**The larger the angle of incidence (A), the larger the angle of refraction (B).**

The combination of the qualitative refraction properties in **bold** forms the foundation of lens properties.

These two properties take mathematical form in Snell’s Law, e.g.:

**2**  **sin A/sin B = nrefracted/nincident**

**3.2. Lenses and Real Image Formation**

The foregoing refraction properties lead directly to the operative basis of lenses. By curving and polishing glass into the `double convex lens’ shape as shown in Figure 3, a set of parallel light rays can be brought `into focus’. This effect arises directly from the simple refraction behavior of light just described. Follow for example the **bolded** ray in Figure 3.

**perpendiculars to interfaces**

At both the in-going and outgoing air-glass interface, **tangents defining**

the ray bends (`refracts’) in accordance with the rule **interface at point**

that it must move **towards the perpendicular to the**  **of ray entry and exit**

**inteface** **as the light moves from low** **to**

**high index** (the in-going ray does this), ***in-going rays out going rays***

and **away from the perpendicular**

**to the interface as the light**

**ray moves from high to low index.**

The overall effect is to bend the light twice, **f**

and in the same direction, i.,e. towards the center line

defined by the middle of the lens. [[3]](#footnote-3)The distance from **FIGURE 3:** **Parallel Ray Diagram**

the center of the lens at which the focus is attained **for** **Double** **Convex Lens**

is called the focal length **(f)** of the lens.

Note that at the extreme edges of the lens, the refraction angle should be the greatest since an in-coming parallel ray deviates most from the perpendicular to the lens surface. That is, the outermost rays are bent in the most, while those traversing the center of the lens are not bent at all. (See Figure 4 below.)[[4]](#footnote-4)

**FIGURE 4: incoming rays**

**Variation of Refracted**

**Ray Angle in Double Convex**

**Lens For Parallel, Incoming**

**Rays**

Now let us consider the case where the incoming light is **not** parallel. This is what is normally expected.

The light emanating from any object spews off in all directions and at all angles. It is reasonable to wonder then how we can `see’ anything clearly through a lens since the rays from any point on the object we wish to examine are arriving at the lens `higgly-piggly.’. The key is connected to another feature of the concave lense, e.g. its ability to create a `real’ image .

Examine Figure 5. The object (**O**), which is placed **outside** of the focal length, f , is shown emanating 2 rays from its top. The first ray is parallel, the second passes through the front focal point of the lense. As already shown, a parallel ray refracts to the back focal point of the lens. Conversely, when a ray appears to emanate from the front focal point, it is refracted to become parallel . (Think of these two rays as being `inverse’ to one another in their paths.) Both converge to a single point **behind** the back focal point, to form the `real’ image of the object. This `real’ image (**R**) is what in fact forms on the back of your eyeball once the light has passed through your cornea (the eyes’ lens). [[5]](#footnote-5)

**O**

**f**  **q**

**FIGURE 5:**

**Real Image Formation by**

**A Double Convex Lens**

**R**

Now at this point, we have a `real’ image formed behind the back focal plane of the lens. How big is it

relative to the original object ? Common sense tells us that the further away the real image is from the

lense, the greater the magnification. (Think of what happens when you pull a slide projector away from the projection screen, e.g. the image enlarges.) Relatedly, the closer the object is to the lens, the greater the magnification. (Think of what happens as you bring you hand closer and closer to your eyes. It appears

to grow in size until it blots out all other objects in your field of view, e.g. it enlarges.) Using the distance

symbols in Figure 5, simple or `real image’ magnification **Mr** is therefore:

**3 Real Image Magnification = Mr = q/f = real image size/object size**

The value of **Mr** is determined by how the lens has been ground and shaped.

**3.3. Simple Magnifiers: Virtual Images**

It is re-emphasized that the real image just described represents a physical reproduction of the object at some distance from the object and lens, using light as the effective `paint’ . The real image actually exists in space and it is what forms on the retina of our eyeballs when we peer through a magnifying glass or a microscope. What we actually `see’ however, when we look through a convex lens such as in Figure 5, is **not** the real image. To explain this, we need to have some background regarding the human eye.

You are probably aware that the human eye is capable of varying its focal length by means of stretching or compressing the cornea (the eye’s lens) through muscle action. This allows us to focus on objects both near and far away. However,for reasons likely related to some evolutionary quirk in human development, the human eye is most comfortable and able to `see’ objects held at about 10 inches (25 cm) from the face. Indeed, this is the `default’ value of focus for the eye. (It explains why we hold books about 10 inches away while we read.)

For objects held at distances much below 10 inches, our eye becomes increasingly unable to `focus’.

(This is easily shown by simply moving your hand or a pencil tip closer and closer to your face.

Eventually, at distances below around 25 cm or 10 inches, the hand or tip goes blurry.)

Imagine now, that the real image formed by the glass lens in Figure 5 is `painted’ onto the back of

your eyeball, where the retina is located. (See also, Figure 6) The retina contains the signal `transducers’ (rods and cones) that convert light into electrical pulses which make their way to the brain and are somehow `seen’ by the human mind. ***But at what apparent distance is the ‘real image’ being `seen’ ?***

Unlike looking directly at the object, there are no reference points for the mind to sort out and judge distance. Now the reader might initially disagree with this observation noting that the lens system itself can serve as `reference’. However, the lense is, practically speaking, well within the 10 inch limit of our eyes and out of focus. Hence, the only `focused’ and sharp image the brain records is the **real image**. Left with no other reference or guide to `how far away’ it is, the confused eye therefore automatically assumes the default value of 10 inches.

The gist of this process is shown in Figure 6. As seen, the human mind `assumes’ that the rays coming to it from the real image are at 10 inches from the front of the eye. It then `follows’ those rays backwards to a plane 10 inches (25 cm) away from the face, where the so called `virtual image’ of the object lies. The effect of the mind `projecting’ the image at this distance is like that achieved by increasing the distance from screen to slide projector e.g., magnification. In a sense, therefore, the main magnifying effect of

a lense is an optical quirk of our own eyes.

**FIGURE 6: Ray Diagram for Simple Magnifying Glass**

***Eyeball***

***virtual*** ***image***

***“seen” by eyeball***

**O** **R**

**f**

***10 inches (25 cm) projection distance assumed by human brain***

The magnification of the object by a simple magnifying glass can be deduced from Figure 6 (with some geometry and a few minor assumptions) to be **Mo** (if f is measured in centimeters):

**4**  **Mo = Magnifying Glass Magnification = 25/f + 1**

**3.4. Compound Magnification: The Microscope**

The foregoing simple magnifier is practically limited to magnifications of about 20X [[6]](#footnote-6). In order to achieve substantially higher magnifications appropriate for seeing objects at say, the cellular level

(e.g 10-100 u sized specks), 100-400X magnifications are necessary. This is achieved as shown in Figure 7 through use of a compound lens system, that is, using a microscope.

The basic idea of the microscope is simple: take the `real’ image formed by the objective (essentially the simple magnifier lens) and further magnify that lense’s `real’ image, before it enters the eye. This second magnification is accomplished by a convex eyepiece with magnifications of 4-25X. Compound magnification, Mtotal , is then computed as the product of the objective’s magnification, Mo and the eyepiece magnification, Me.

**5** **Mtotal  = Mo x Me**

A simple computation assuming a 20X objective and 5X eyepiece leads to a 100X compound magnification, e.g. an original object of 10 u=0.01 mm will appear about 1 mm in size. Because two magnifications have occurred, the image is additionally inverted (e.g., when you move the object right, it appears to go left under the microscope eyepiece.) Note, however, that nothing has really been changed compared to the simple magnifier except for an additional amplification . The projected distance of the virtual image remains at about 10 inches from the eye. Not surprisingly, the approximate height of typical microscopes lies around 11-12 inches from eyepiece to sample stage.

So why not continue this process ? That is, why not simply place many pairs of objectives and eyepieces

in a series and gain seemingly unlimited magnification ? To answer this question, we must consider

some practical aspects of getting the object’s light up into the optical system.

**3.5. PRACTICAL LIMITATIONS ON MICROSCOPES**

**3.5.1 Lens Abberations**

The first and most basic of problems with a glass lens is that if it is polished with a purely spherical

curvature (which is the easiest to do, physically), the focus of light from the extreme edges of the lens does not coincide with that attained by light passing closer to the center of the lens. (See Figure 8 below.) The effect is termed ***spherical abberation,*** e.g., the real image is defocused because the lense curvature is not exactly right.

**FIGURE 8: Spherical Abberation**

***monochromatic light***

A second abberation is termed **chromatic abberation**. It is related to the fact that the amount of bending by the light depends on the **color of** **light.** That is, the degree of refraction (or index) is wavelength dependent. This effect, often referred to as dispersion, is present even if spherical aberration is eliminated.

The dispersive effect on a double convex lens is shown in Figure 9. (Note that dispersion also forms the basis of how older models of uv-vis spectrophotometers use a prism to disperse a light source into individual wavelengths suitable for spectral analysis.) The shorter the wavelength, the closer in the focal length for that wavelength. Thus, the notion that we can `stack indefinitely’ lens pairs on top of one another to achieve unlimited magnifications is stymied by manufacturing practicalities and physical limitations of the lens materials. This is not to say, though, that these shortcomings can’t be addressed

to a reasonable degree.

**FIGURE 9: Chromatic Abberation**  Both kinds of abberations are correctable. Spherical abberation is fixed by reshaping the lens

through grinding and must in principle be done

white light red light (650 nm) so that all wavelengths of light are focused

to a common spot, irrespective of incident

beam position. In practice, this is done

only for one or two wavelengths.

blue light (450 nm)

Chromatic abberation can also be fixed, albeit only at selected wavelengths so that at least those wavelengths end up coincident on the back focal plane of the lens. This is done by by combining double convex lenses and/or concave lenses , as shown in Figure 10.

**R + B R + B**

**R** **R**

**B**  **B**

**FIGURE 10: Various Methods of Correcting Chromatic Abberation**

Objectives which have had this done to them are termed ***`Achro’*** or ***`Apo’*** depending on whether the lens matches, respectively, one or two other wavelengths (normally blue at ~450 nm and /or red at ~660 nm) to a reference wavelength (normally the sodium D line at 589.3 nm.) That is, an `achromatic’ lens is corrected for two wavelengths including the sodium D line; an `apochromatic’ lens is corrected for three

wavelengths including the sodium D line. It also common that the `apochromatic’ lens is spherically

corrected at the sodium D and at either the red or blue wavelength. An achromatic lens, however, is spherically corrected only at the sodium D line.

In addition to spherical and chromatic abberation, a third problem arises when considering portions of the real image at reasonable distances from the center line of the lens. As shown in Figure 11, when a curved

lens surface is used, the real image is actually in focus on a curved, **not** planar surface.

**FIGURE 11:**

**Field Curvature** **Effect**

***real image***

***object***

***focal plane focal plane of***

***of arrow ends arrow center***

This problem can be particularly bothersome if you need to perform photomicrography . It is corrected by flattening the lens shape in various ways by grinding. Such corrective measures are designated by the prefix **’plan’** (indicating lens correction which makes the real image fall on a `plane’ )

Finally the quality of glass in the lenses is of concern. The best objectives (used in polarized light microscopy) are strain-free and have no glass fines (small defects created by incomplete glass mixing and melting.) The ratings scale for optical glass is (in order of increasing quality): **E, D, S.**

Thus, when you hear an objective described as a ***S-plan-apochromat*** you may assume it is composed of ‘good glass’ corrected for field curvature, spherical abberations at 2 wavelengths (including the sodium D line) and chromatic abberations at 3 wavelengths (including the sodium D line.) This is a `good’ objective. On the other hand, an ***E-achromat*** lens is composed of lousy glass, spherically corrected only at the sodium D line and chromatically corrected at only 1 wavelength in common with the sodium D line.(...a real piece of junk) . These descriptors are often on the objective housing.

**3.5.2. Resolution and Contrast**

Ultimately, the value of any microscopic image comes down to a single question: ” how detailed a picture

of the object am I getting ?” This is not tantamount to asking, however, how much magnification can I get. An image magnified to 1000X often yields ill-defined, fuzzy blobs in the eyepiece, which are useless for characterizing the object. However, at 50X, sharp, detailed images can be had which are useful-even if they represent features some 20 times larger than those at 1000X. Information content, not `empty magnification’ is the bedrock of good microscopy.

The two basic quantities governing `how detailed a picture you can get’ are: ***Contrast*** and ***Resolution***

***(or Resolving Power)***.

**Resolution**

Resolution (also called resolving power) measures the ability of the microscope to distinguish two adjacent points as being discrete and different. A simple numerical measure of resolution, ***R***, is often taken to be the minimum distance between two spots that still allows us to distinguish them as two spots.

(See Figure 12) . So what factors in microscope design control **R** ? The initially obtuse answer is provided in the equation below:

**6** **R= Resolution=Resolving Power= 0.61\*λ**

**NA**

*where NA = numerical aperture for the objective*

**FIGURE 12: Resolution (R)**

***R***

resolved unresolved***.***

According to the above equation, ***R*** (which we want as small as we can get) varies with the wavelength **λ**

of light illuminating the sample. This is sensible. The smaller the object, the smaller the wavelength of light needed to `probe’ that object. Having too large a wavelength (e.g. a 600 nm wave to look at

a 200 nm object) is like trying to measure a dust mote diameter with a yardstick.

The inverse relationship with numerical aperture needs a bit more explanation. First you need to know what numerical aperture (**NA)** is. The physical sense of it is illustrated below for two different objectives.

**FIGURE 13: Numerical Aperture (NA)**

***low power objective high power objective*  NA=light gathering power of lens *=* n sin θ**

***5X*** low NA (~0.10) ***20X*** high NA (~0.61)

sample θ θ

Incoming light

(***Expanded view of objective)***

Generally, we want as large an **NA** as we can get. Qualitatively, **NA** measures the light gathering power of the microscope’s objective. That is, the larger the value of **NA**, the bigger the fraction of emanated light per unit sample area captured by the objective lens. This is desirable since more light means more information.

As a general rule, higher magnification objectives are correlated with higher **NA**. This is because the 20X objective has a shorter focal length and must be be focused much closer to the sample. As a result, the 20X objective captures a higher fraction of the light per unit area of the sample, i.e., the rays have little room to diverge before entering the objective lens. In contrast, the 5X objective is focused considerably further away from the sample, so that the light diverges substantially before reaching the front objective plane.[[7]](#footnote-7)

The mathematical expression of **NA** is:

**7** **NA =n sin θ,**

where **θ** is defined in the expanded view of the objective shown in Figure 10**. The maximum value of NA is NAmax = n=refractive index** of the medium in immediate contact with the lens. This is normally taken to be ~1.00 since air is the usual medium. [[8]](#footnote-8) All microscope objectives will have their NA in air stamped someplace on the body of the objective.

The reason that NA enters into the expression for ***R*** is interesting, if somewhat complicated by the need

to discuss diffraction . We will side step this issue since this is not a physics course. Instead we can reason intuitively by viewing the microscope objective as if it were a ham radio antenna receiving only a partial, intermittent signal from a distant sender (the sample). Obviously, the more information transmitted into the antenna , e.g, the higher the NA, the clearer and more intelligible the final message (the sample’s image) will be. That is, with more information, we should be able to resolve more clearly what is being `said’ by the sender.[[9]](#footnote-9)

With the foregoing as a background, we can now proceed to compute the typical resolving power for a common microscope objective, e.g., a 20X objective using the 589.1 nm sodium D line for illumination.

Assuming NA for a 20X objective is around 0.75, and recalling from **6** that:

**8** ***R*= 0.61\*λ nm**

**NA**

We obtain for the conditions just described:

***R* = 0.61 \* 589.1 nm = 269.5 nm = 0.00002695 cm ~ 0.27 m**

**0.75**

This is excellent resolution, and one rarely achieved in practice. A second computation for a 5X objective (**NA**= 0.10) using the same light illustrates the range in variation of R created by varying the objective magnification.

**R= 0.61 \* 589.1 = 3593.5 nm = 0.0003593 cm ~ 3.6 m**

**0.10**

The reader should note that **R** is related to magnification, but does not scale precisely with it. The ratio of magnifications in the two examples above, (20/5=4) is not identical to their computed ratio of 1/***R*** (3.6/0.27~11). Indeed, objectives with identical magnification factors, can have quite different **NA**.

**Contrast**

You are likely to have visited a local science museum and witnessed the optical illusion sketched below. By apparent magic, when a solid glass rod is immersed in a clear oil, it `disappears’ from sight.

**FIGURE 14: Disappearing Glass Rod Trick**

solid glass rod

oil

The secret is that both glass and oil have identical refractive indices. In order to see the rod, it must be ‘different’ (e.g. have different index) from the medium it is immersed in. This simple insight holds true under the microscope. If the sample and immersion medium are identical in index, you will not be able to see the sample.

Thus, even with a perfect lens and extraordinary resolution, an object is invisible to your roving

microscopic eye unless there is **Contrast.**  An object can provide contrast by being : 1) an amplitude

object and/ or 2) a phase object. The human eye is sensitive, however, only to amplitude objects.

An **amplitude object** generally speaking has an index different than the medium and therefore

**can be seen in `bright field’**, e.g. by transmitting light through the object. Think of an amplitude

object as one which `absorbs the light differently’ than the medium.[[10]](#footnote-10)

A **phase object,** generally speaking has an index nearly the same as the medium and therefore **cannot be (easily) seen in bright field**. That is, it does not produce a difference in ‘absorption’ that contrasts with the medium. This unhappy circumstance is often the case for biologists. Biological cells contain a variety of sub-structures separated by a thin boundary of material virtually the same in composition (optically) as the inside and outside of the structure, e.g.: cells are substantially phase objects.

For this reason, it is common to `stain’ biological samples using various colorants. Provided that the colorant uptake is different on different cellular components, staining artificially enhances the amplitude contrast between these components. The most familiar stain is the `Gram’ stain , which is actually a pair of stains: one blue {+} and one yellow {-}. The Gram stain forms the basis for classifying bacteria into two major types, e.g. Gram {+} or Gram {-}.

But what if you can’t stain your sample ? This might occur if the object won’t hold a stain, or if staining is disallowed because your sample needs to be kept unaltered. (The latter situation is especially true in forensics applications where evidence must be kept as much as possible, unaltered.)

One clever solution to this dilemma comes through the Nobel prize-winning work of F. Zernike , a Dutch physicist. Suppose as shown in Figure 15, that three separate points on an object (**o** **O** *o)* are composed of different materials and/or morphological characteristics, yet all three points have the same refractive index as the medium. Then as already discussed, the three points will be indistinguishable from the background in the real image. That is, the same amount (amplitude) of light appears to exist at all three real image points.

Zernike observed that our inability to distinguish these points arose because in a sense **too much light** emanated from the real image spots, e.g., the spots are effectively so bright that the human eye cannot distinguish the spots from the background illumination. The problem is analogous to trying to deduce the presence and size of stars during the day time. Obviously, during the day, light from the sun effectively masks the stars. In similar fashion, the source lamp of the microscope masks phase objects. But since we necessarily need light to see our microscopic objects, we cannot just turn the source lamp off. Zernike thus reasoned that if we could **reduce the brightness of the real image spots**, we could see them as dark spots

against the bright light background of the source lamp. It is analogous to somehow turning off

the intensity of the stars in the sky. In this unlikely circumstance, we might see black pinholes

in an otherwise sunny sky. To produce such an effect under a microscope Zernike observed that the light in the real image spots is the sum of two parts:

**real image spot = `undeviated’ + `deviated’ light = `amplitude’**

The undeviated light is essentially the light emanating at near 90o angles from the object spots relative to the lense’s back focal plane. It is `undeviated’ since such light does not change angle when penetrating

a surface (recall Figure 1). The deviated light is the collective light emanating from the object spot at

obtuse or acute angles. When entering the lense, these beams will be substantially bent or `deviated.’

(recall Figure 2).

back focal plane of lense

**FIGURE 15:** ***Real image of Object points + medium***

**Phase Object** (no contrast)

**Ray Diagram**

deviated light

medium undeviated light

with similar NA

to object points **o**  **O**  *o*  ***Object points***

deviated+ undeviated

amplitude sum

Now since the two kinds of light are both waves, the **brightness of the real image spots must arise**

**because the deviated and undeviated light constructively add at the point of focus** on the back focal plane of the lense. If one could somehow **‘slow down’ or ‘speed up’ the undeviated light,** however**,**

it would be possible to **create a destructive addition** at the back focal plane. That is, we could create a dark spot that contrasts with neighboring bright spots or the medium. The reverse can also be created, e.g., we make a `bright’ spot (in contrast to neighboring spots or medium) by forcing `**constructive’ interference** between deviated and undeviated rays. In practice, it is the former (dark or positive phase) case that is most often encountered.

Zernicke attained this **phase contrast** by placing two new components in the light path. The first is a **phase** **annulus**  placed before the objective but after the light condenser beneath the microscope stage. This has the effect of shifting the undeviated and deviated light contributions apart by about 1/4 wavelength in phase from each other. The second component is a **phase plate** placed at the back focal plane of the objective. The phase plate is essentially a disk of glass whose center is either thinner or thicker than its outside. This further phase shifts the light so that cumulatively, the deviated and undeviated light from the object are now about 1/2 wavelengths apart. This allows the requisite interference to occur. Figure 16 illustrates these several additions to the light path. In general, the use of dark, positive contrast is preferred, so the phase plate will be thinner in the center. This creates a speeding’ up of the un-deviated beam and destructive interference.

With such an arrangement, it now becomes possible to distinguish objects in a medium with identical refractive index **since only the objects will exhibit both** **deviated and undeviated light.**

The medium, in contrast, `emanates’ only undeviated light and hence will undergo no phase

contrast enhancement . The result of this distinction is illustrated in Figure 17.

**FIGURE 16:** **Diagram of Light Path with Phase Plate and Phase Annulus**

**PHASE PLATE**

thicker=>`**slows’** light compared to outside

part of phase shifter ring

**back focal plane** ---------------------------- ----------------------------

**of objective** *thinner*

**undeviated ray**

deviated ray

***objective***

**object** **o** **O**  *o*

***substage condenser***

**PHASE ANNULUS**

**FIGURE 17: Effect of Phase Contrast Attachments**

**View down eyepiece w/o phase rings View down eyepiece with w/ phase rings**

**SUMMARY OF BASIC CONCEPTS IN OPTICAL MICROSCOPY**

**1. The physical basis of optical magnification is the differential bending**

**of light in media of different refractive index (n). (section 3.1)**

**2. Simple magnification Mr occurs to form a *real* image when light from an object**

**passes through two index changes (low-high, then high-low) across a double convex-shaped lense. (section 3.2)**

**3. Virtual magnification from the foregoing `simple’ magnifier, occurs because**

**the mind and eye forms a `*virtual’* image of the real image. This is essentially**

**a `quirk’ of the human optical sensory system. A maximum magnification**

**of about 20X is attained in this fashion. (section 3.3)**

**4. Compound magnification, e.g., the use of two lenses (objective + eyepiece)**

**constitute the basic structure of a microscope. The magnification of the**

**microscope is simply the product of the `virtual’ magnifications of each**

**lense taken separately: e.g. compound magnification = Mobjective x Meyepiece**

**A maximum useful magnification of about 2000X is attained in this fashion.**

**(section 3.4.)**

**5. The microscope’s key performance characteristics are reflected in NA,**

**the numerical aperture of the objective, and the degree of correction**

**for various `facts of life’ about lenses e.g.: chromatic aberration, spherical**

**aberration, field curvature. (section 3.5)**

**6. The usefulness of a microscope ultimately turns on the twin issues of resolution**

**and contrast. Both are tied mathematically to NA and wavelength. Generally,**

**resolution is enhanced by lower wavelength and higher NA. Contrast is**

**enhanced either by sharp differences in the refractive index of the medium**

**versus objects, or, through use of various `tricks’ such as staining or**

**phase contrast. (section 3.6)**

**MENTALLY FOCUSSING QUESTIONS ABOUT MICROSCOPY**

**1) Based on what you have read herein, which refracts more when moving from lower to higher n: red or blue light ?**

**2) Is it possible to have the light that penetrates from glass to air end up moving parallel to the surface of the two media ? Explain why or why not.**

**3) Which way does the cornea `flex’ (thicker or thinner) when it tries to focus**

**on an object which is moving closer ? Explain your answer.**

**4) You have two objectives. Objective #1 is an S-plan apochromatic 10X (NA=0.4) Objective #2 is an E-plan-achromatic 25X (NA=0.5). Your eyepiece is a 5X**

**power lens and you have the good fortune to have a single wavelength source**

**(green light, =565 nm). For both objectives:**

**a) compute the minimum diameter object (assume it is a circle) you can expect to see, given that your eye can detect things down to about 0.2 mm in diameter.**

**b) compute the mimimum distance between two objects that still allows resolution.**

**5) You want to see a monocococcal bacteria suspected of causing a new kind of intestinal illness. It’s diameter is about 5 The bacteria is virtually the same in refractive index as water and doesn’t hold stain of any sort. List some of the steps and some of the equipment you would take to maximize your chances of seeing this little booger under the `scope.**

**6) Optical fiber is composed of two glasses in a `core and clad’ arrangement as shown**

**below. One critical `trick’ with this design is to pick refractive indexes so that the**

**light traversing the core never `leaks’ out into the clad. Suppose we assume the**

**core has a refractive index of 1.50, a diameter of 2\*10-3** **cm and that our fiber optic**

**light transmission system looks so:**

**source**

**clad**

**core 2\*10-3** **cm**

**10 cm**

**Estimate the cladding index necessary to operate our optical fiber transmission**

**system at `zero’ loss, e.g, so that no light penetrates into the cladding**

1. The sodium D line, which is the strong, orange-yellow flame produced when table salt is burned, is traditionally selected as the reference

   wavelength in many optical characterizations. [↑](#footnote-ref-1)
2. Note that n is unitless. Furthermore; 1/n = the fractional amount light is slowed in a medium with an index of n relative to vacuum. [↑](#footnote-ref-2)
3. a single convex lens (curved on one side, flat on the other) bends the light just once but otherwise is the same in function as adouble convex lens. [↑](#footnote-ref-3)
4. The astute student may be wondering why the lens must also be thicker at the center than at the edges. As described thus far, just curving the glass surface can produce refraction to a focus. That is, why not just bend a a piece of flat glass into a curve for a lens ? You can !

   However, we would like to have all the light rays arrive at the focus simultaneously. The outermost rays travel the greatest distance, hence would arrive `late’ compared to light traversing the center of the proposed planar glass lens. Adding thickness to the center, however, `slows’ the latter light down, thus allowing a better coinicidence of individual rays as they come to focus.) [↑](#footnote-ref-4)
5. Note that the object must lie outside the focal length of the lens. If it lies within focal length, there is no way to construct the object’s image since the ray that must pass through the front focal plane point is by definition not available. Put simply, if the lens is `too close’ the object goes out of focus. [↑](#footnote-ref-5)
6. ...although it has been reported that simple magnifiers of 300X power can be used...Svihla, G. **Microscope**  **15** 298-300 (1967). [↑](#footnote-ref-6)
7. It cannot be surmised, though, that we should always work at the highest magnification since NA is larger there. The size of the 20X lens is sharply reduced compared to a 5X. So while more light per unit lens area is had at 20X, there is also less total lens area, hence less net light at the higher magnification. You will observe this if you switch from 5X to 20X; the latter always results in a darker field . [↑](#footnote-ref-7)
8. While we will generally look at samples with a `dry’ lens, e.g., air is in immediate contact with the objective, the definition of NA implies that immersing sample and lens in a high index oil can substantially improve NA, and thus ***R.*** As a result of this observation, `oil immersion’ objectives are commonly available from microscope makers. [↑](#footnote-ref-8)
9. The interested reader is referred to Chamot and Mason, pp 9-11 for a more detailed explanation of NA and resolution based on Abbe’s theory of resolution. [↑](#footnote-ref-9)
10. More precisely, if less obviously, the amplitude object creates an image because the light emanating out of the sample and through the medium either constructively or destructively adds to make bands of light or dark interference patterns we collectively call the `real image’. [↑](#footnote-ref-10)