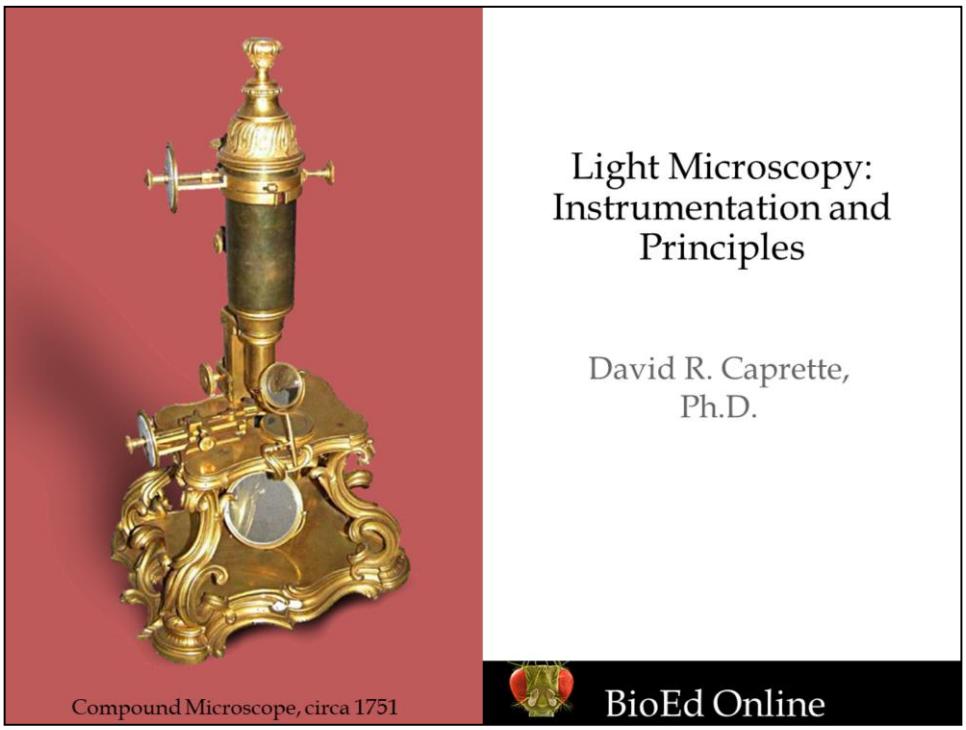


Light Microscopy: Instrumentation and Principles

A light microscope is so named because it uses visible light to produce a magnified image. Compound light microscopes are indispensable to almost any teaching laboratory in biological science, yet many of us have a difficult time using them. Part of the problem is that with any light microscope, a user must select the right magnification, contrast and resolution, position, and focal plane, all at the same time. A second complication stems from the fact that most teaching lab microscopes are designed for bright field viewing only. A good bright field microscope can produce excellent high resolution images. However, many light microscopes are equipped with specialized optics that enhance contrast so that any specimen, living or preserved, can be imaged.

For satisfactory contrast and resolution, some specimens are best examined using phase contrast or dark field optics. Polarized light provides the basis for differential interference contrast (D.I.C.), which produces three dimensional images. Specialized optics are usually necessary for imaging very small unstained living organisms, such as bacteria or the smallest protists. To maximize their capabilities, most research microscopes are equipped with some combination bright field and specialized optics.

Here, we will explore the features of compound microscopes, principles of imaging, magnification, contrast, and resolution. We also will look at the components of compound light microscopes and their functions.

References:

Alberts, B., et al. (2002). *Molecular biology of the cell* (4th ed.). New York: Garland Science.

Caprette, D. (2005). Light microscopy. Retrieved 09-12-2005 from http://www.ruf.rice.edu/~bioslabs/methods/microscopy/microscopy.html

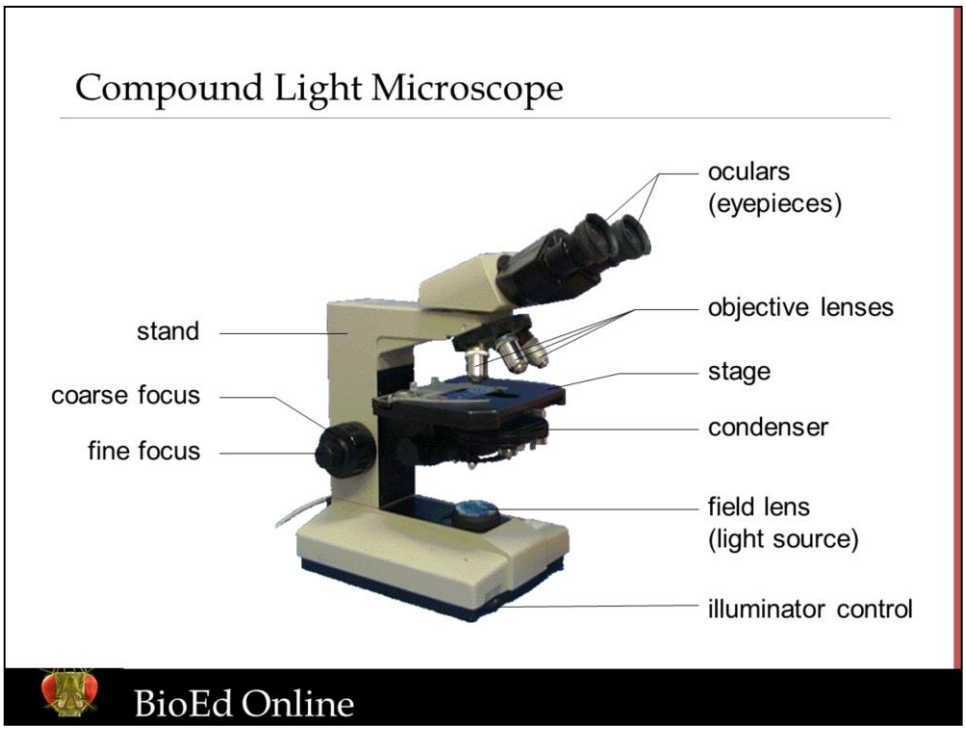
Lodish, H.,et al. (2000). *Molecular cell biology* (4th ed.). New York: W.H. Freeman and Co.

Nave, C.R. (2005). Hyperphysics (light and vision). Retrieved 09-12-2005 from

http://hyperphysics.phy-astr.gsu.edu/hbase/hframe.html
Wolfe, S.L. (1993). *Molecular and cellular biology*. Belmont, CA: Wadsworth Publishing Company.

Image Reference:

Compound Microscope, circa 1751. Roby 11 janvier 2005. Retrieved 09-15-2005 from http://commons.wikimedia.org/wiki/Image:Microscope1751.jpg.



Compound Light Microscope

A compound microscope employs multiple lenses that combine to produce a high quality, well resolved, magnified image. A component called a condenser collects light from an external or internal source and projects it toward a specimen. A condenser is essential because it modifies the light beam to match the properties of the objective lens. The user can control and optimize contrast and resolution by adjusting the condenser. If specialized optics are available, their use requires changing a condenser position or exchanging condensers.

Light from a specimen passes into an objective lens that magnifies the image. A good quality objective lens (often simply called an objective) is composed of multiple individual elements, producing a much better resolved and corrected image than one could obtain using a simple lens. The user should have a choice of lenses, arranged on a turret in order of increasing magnification. Specialized objectives that are required for phase contrast or D.I.C. microscopy usually can produce bright field images as well.

An eyepiece lens (called an ocular) magnifies the image from the back lens of the objective. Final magnification is the product of the objective magnification and ocular magnification. For example, a 20 power (20x) objective magnifies the image of a specimen twenty times. A 10x ocular magnifies the magnified image 10 times further. In this example the final magnification is 20 x 10 = 200x.

Well designed modern microscopes are equipped with a binocular eyepiece tube (i.e., two oculars) so that one can view with both eyes. One's acuity is much better using both eyes in the natural fashion than when squinting with one eye down a monocular eyepiece tube. A measuring device, called a reticule, may be placed in an ocular to aid in counting or measuring dimensions of objects.

These features are common to all types of light microscopes, including bright field, dark field, phase contrast, polarizing, and fluorescence microscopes, as well as instruments that combine

two or more optical systems. The least expensive option when equipping a teaching lab is to purchase dedicated bright field microscopes with no other special features. Of all of the other options, dark field is probably the most versatile and least expensive upgrade, but it is highly underused. A combined bright field/dark field microscope permits a user to see virtually any biological specimen at the cellular or tissue level, living or dead, stained or unstained.

References:

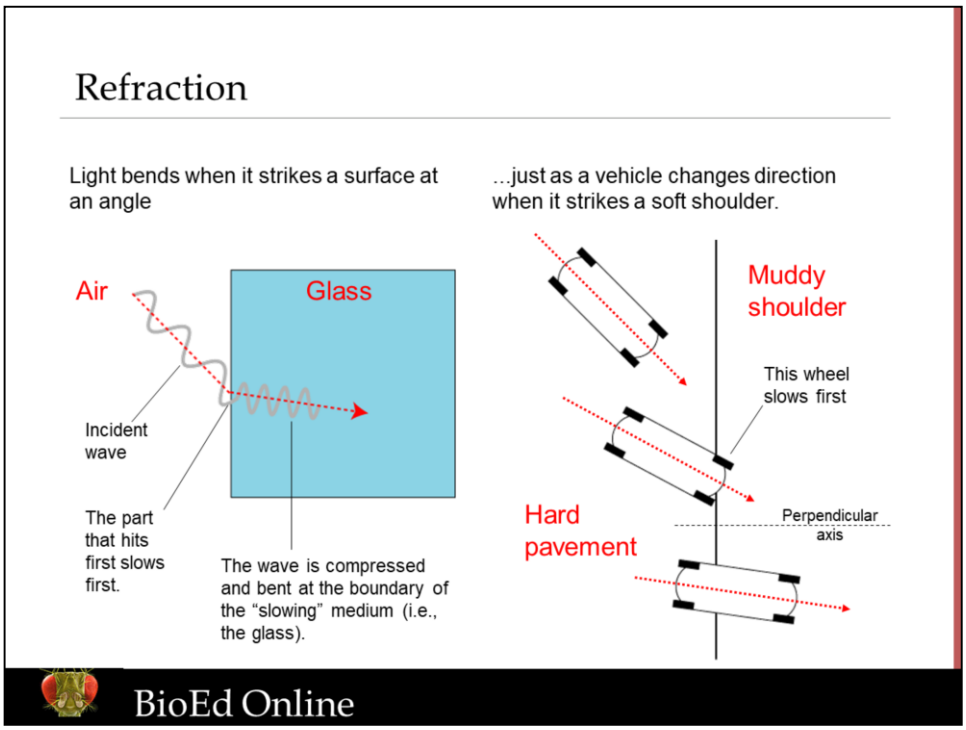
Alberts, B., et al. (2002). *Molecular biology of the cell* (4th ed.). New York: Garland Science.

Caprette, D. (2005). Light microscopy. Retrieved 09-12-2005 from http://www.ruf.rice.edu/~bioslabs/methods/microscopy/microscopy.html

Lodish, H.,et al. (2000). *Molecular cell biology* (4th ed.). New York: W.H. Freeman and Co.

Nave, C.R. (2005). Hyperphysics (light and vision). Retrieved 09-12-2005 from http://hyperphysics.phy-astr.gsu.edu/hbase/hframe.html

Wolfe, S.L. (1993). *Molecular and cellular biology*. Belmont, CA: Wadsworth Publishing Company.



Refraction

The lenses used in a compound microscope collectively magnify images by taking advantage of the refraction of light. When light enters optical glass from the air, its speed is reduced by one-third and its wavelength is shortened proportionally. When it strikes a glass surface at an angle, it not only slows but also changes direction, a property that we call refraction. The light bends because the part of the wave that strikes the glass first slows down first.

To understand the refraction of light, you might picture an automobile leaving a paved surface onto a soft shoulder. If the vehicle drives straight off the road so that both wheels hit at the same time, it slows down but does not change direction. If it hits at an angle, the first wheel to hit the soft ground slows down before the opposite wheel slows. The vehicle changes direction toward an axis that is perpendicular to the edge of the road. One side of the vehicle travels faster until both front wheels are in the mud.

When light strikes a surface of a slower transmitting medium at an angle, it changes direction toward the perpendicular axis in the same way a vehicle changes direction toward the perpendicular when it goes off the road. The opposite effect takes place when light exits a slow transmitting medium into a faster transmitting medium, such as from glass to air. In that case, the direction changes away from the perpendicular. That is, the angle of exit becomes greater. To visualize the direction change, picture what happens when an automobile drives from a muddy shoulder back onto the road surface at an angle. The first wheel to hit the road gains a better grip and encounters less resistance, pushing the vehicle more toward the same direction.

References:

Alberts, B., et al. (2002). *Molecular biology of the cell* (4th ed.). New York: Garland Science.

Caprette, D. (2005). Light microscopy. Retrieved 09-12-2005 from http://www.ruf.rice.edu/~bioslabs/methods/microscopy/microscopy.html

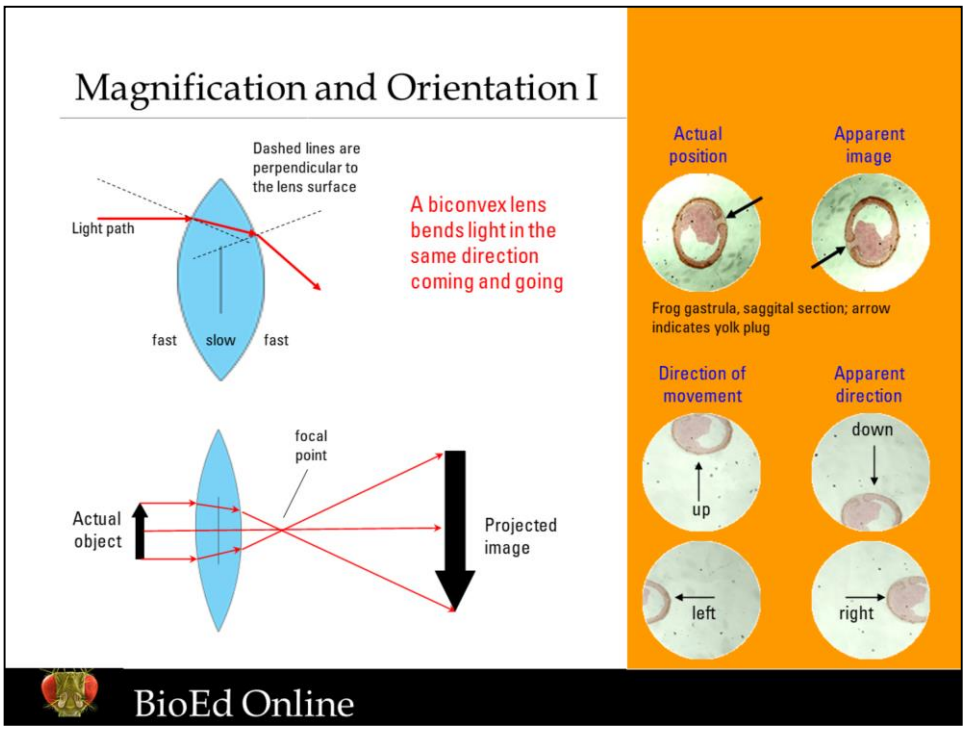
Lodish, H.,et al. (2000). *Molecular cell biology* (4th ed.). New York: W.H. Freeman and Co.

Nave, C.R. (2005). Hyperphysics (light and vision). Retrieved 09-12-2005 from http://hyperphysics.phy-astr.gsu.edu/hbase/hframe.html

Wolfe, S.L. (1993). *Molecular and cellular biology*. Belmont, CA: Wadsworth Publishing Company.

Image Reference:

Dave Caprette. (2005).



Magnification and Orientation I

Suppose that a beam of light transmitted in air strikes a curved glass surface. It will slow down and change direction toward an axis that is perpendicular to the surface at the point of impact, then continue through the glass in the same direction. Suppose that the beam exits a glass lens through a curved surface into air. Because the light now travels from a slow transmitting medium to a faster transmitting medium it will now bend away from the perpendicular.

A biconvex lens is a circular piece of optical glass that is ground so that it bulges in both directions from a central plane. Picture parallel rays of light striking the surface of the lens perpendicular to the central plane. All such light rays will be bent toward the middle when they strike the convex surface, regardless of where they hit. When the same light rays exit the lens they again bend toward the central axis because the surface is curved in the opposite direction. If the surfaces are curved just right, not only does the light bend toward the central axis, but it also converges on the same spot, called the focal point.

After the light rays cross each other at the focal point the projected image of an object is upside down and reversed. Thus we see an image as upside down and reversed when looking through a microscope. For the same reason, if you move an object while looking into a microscope its apparent motion, either left/right or forward/back, will be opposite to its actual direction of movement.

References:

Alberts, B., et al. (2002). *Molecular biology of the cell* (4th ed.). New York: Garland Science.

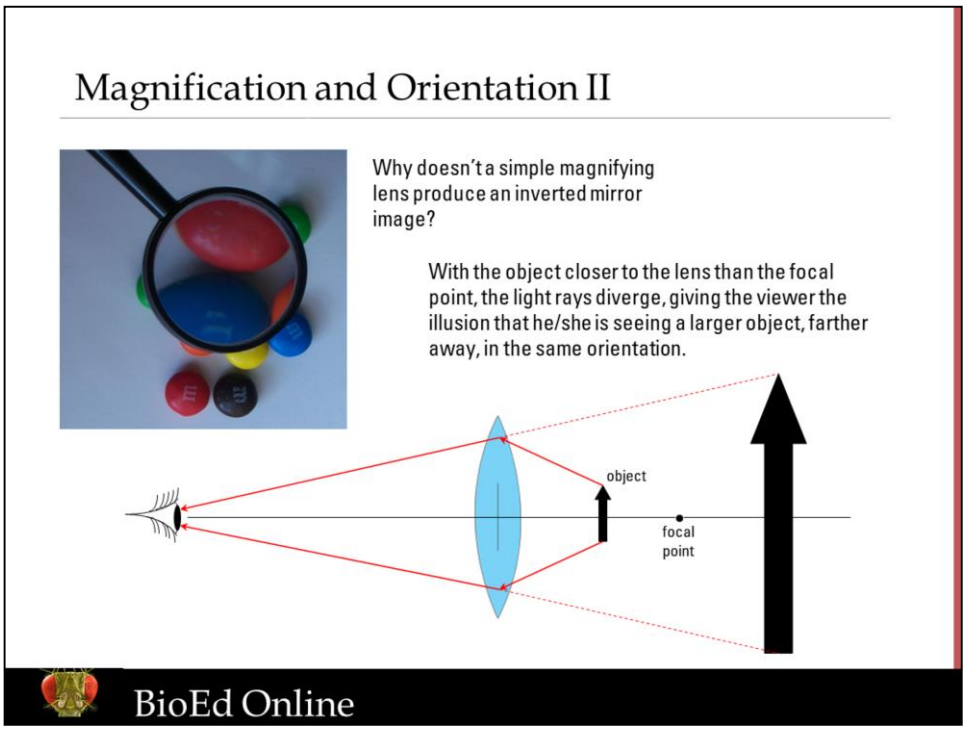
Caprette, D. (2005). Light microscopy. Retrieved 09-12-2005 from http://www.ruf.rice.edu/~bioslabs/methods/microscopy/microscopy.html

Lodish, H.,et al. (2000). *Molecular cell biology* (4th ed.). New York: W.H. Freeman and Co.

Nave, C.R. (2005). Hyperphysics (light and vision). Retrieved 09-12-2005 from

http://hyperphysics.phy-astr.gsu.edu/hbase/hframe.html
Wolfe, S.L. (1993). *Molecular and cellular biology*. Belmont, CA: Wadsworth Publishing Company.

Image Reference:
Dave Caprette. (2005).



Magnification and Orientation II

You may be wondering why the image isn't inverted and reversed when one looks through a simple magnifier. An ocular lens, or eyepiece, is a simple magnifying lens. If it inverted the image as an objective lens does, it would change the image orientation back to where it was. That isn't what happens in a compound microscope, though.

A biconvex lens projects a magnified inverted image when an object is placed in its focal plane, as we saw previously. If an object is placed inside the focal plane, that is, if the lens is placed too close to the object, then light rays from the object never converge on the opposite side of the lens. If you look through the lens at the object, though, it will look exactly as if the light rays came from a plane beyond the object. The object will appear to be farther away than it really is, and will appear magnified. The image from an objective lens is projected to a plane closer to the eyepiece than its focal plane, so that the image is simply magnified and not inverted and reversed.

You might be wondering, "Doesn't the magnification depend on how close the object is to the lens?" In fact it does. The light rays diverge more the closer the object is to the focal plane. Thus, the magnified image appears proportionally larger. The rating of a magnifier (3x, 5x, etc.) is based upon its maximum practical magnification.

So, can you use an eyepiece as you would a magnifying glass? Yes, but chances are the focal plane is so close to the lens that the object would have to be placed inside the barrel of the eyepiece to work the same as an ordinary magnifying glass. Can you use a simple magnifier to view a microscope image? Yes, but why would you want to? Eyepieces are designed to fit in the light path just right so that you can see the entire field without distortion. You might be wondering how the image reaches the eyepiece in the first place. The light path from the objective lens usually reaches a mirror or prism that projects the magnified image to a plane within the focal plane of the eyepiece, where it is then magnified.

References:

Alberts, B., et al. (2002). *Molecular biology of the cell* (4th ed.). New York: Garland Science.

Caprette, D. (2005). Light microscopy. Retrieved 09-12-2005 from http://www.ruf.rice.edu/~bioslabs/methods/microscopy/microscopy.html

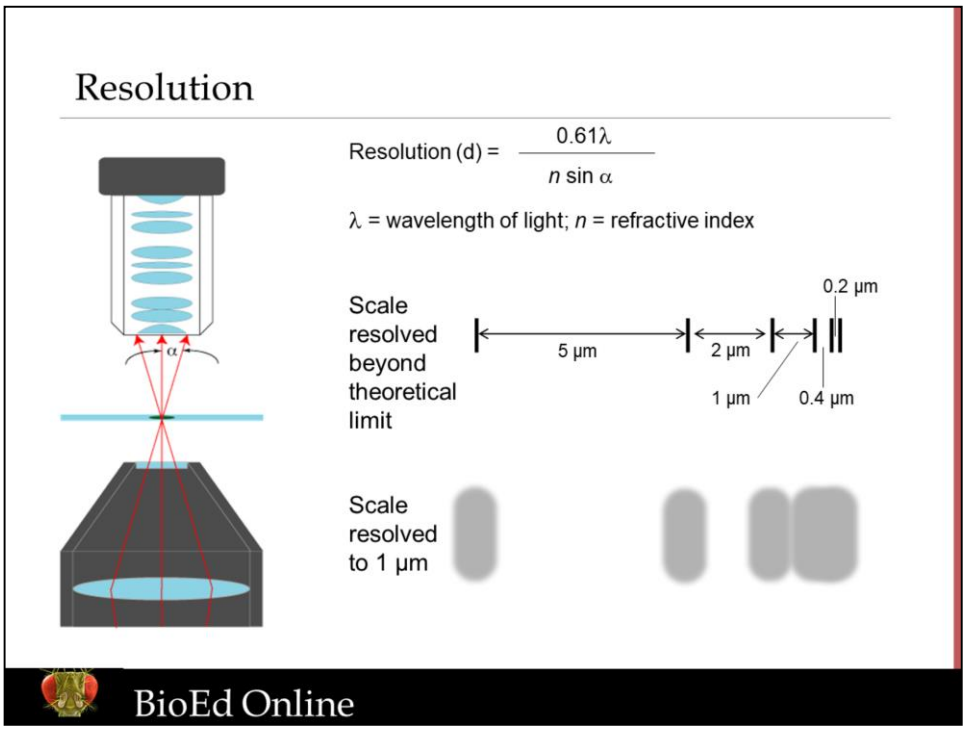
Lodish, H.,et al. (2000). *Molecular cell biology* (4th ed.). New York: W.H. Freeman and Co.

Nave, C.R. (2005). Hyperphysics (light and vision). Retrieved 09-12-2005 from http://hyperphysics.phy-astr.gsu.edu/hbase/hframe.html

Wolfe, S.L. (1993). *Molecular and cellular biology*. Belmont, CA: Wadsworth Publishing Company.

Image Reference:

Dave Caprette, photographer. (2005).



Resolution

Resolution (d) is the degree to which a microscope can distinguish fine details. The number represents the minimum distance that must separate two points for them to be distinguished by the human eye. The lenses on a compound light microscope are composed of multiple elements placed close together. A very good objective lens may contain as many as eight to ten individual lenses, while an ocular may contain two or three. The result is a single very well corrected lens that can magnify many times without blurring or distorting an image.

The resolving power of an objective lens should approach the theoretical limit of about 0.2 µm. A good 40x objective lens not only magnifies an image 40x, but also provides a resolution such that when the image is further magnified 10x by an ocular lens, the eye can distinguish objects that are separated by as little as two or three micrometers. Above 40x, an objective lens produces empty magnification without the use of immersion oil, a method that will be described later. Resolving power using a 100x oil immersion lens to achieve 1,000x final magnification can be brought very close to the theoretical limit. At resolution 0.2 µm, one can distinguish larger organelles, such as chloroplasts, mitochondria, and cell nuclei. Raising magnification beyond 1,000x using a visible light microscope produces only empty magnification.

Good quality objective lenses are expensive, ranging in price from hundreds to thousands of dollars. Quality and cost typically increase with magnification. Low magnification lenses, often called “scanning” lenses, are the least expensive and typically magnify from 3.5x to 4x. Typical lenses of intermediate magnification are 10x or 20x. “High dry” lenses are high magnification objectives that are used without immersion oil. The most common magnification is 40x, although you might find objectives, especially older ones, ranging from 35x to 40x. Oil immersion lenses typically magnify from 95x to 100x.

To preserve their function, objective lenses must be handled with extreme care. Organic solvents can dissolve adhesives or remove a lens coating. Distilled water or dilute acetic acid (e.g., vinegar) can be used along with good quality lens tissue to clean a dirty lens. It also is

safe to use a cotton-tipped applicator stick, provided the tip is 100% natural cotton. The “rules” for cleaning lenses apply to any optical surface, including ocular and condenser lenses, filters, and even eyeglasses.

References:

Alberts, B., et al. (2002). *Molecular biology of the cell* (4th ed.). New York: Garland Science.

Caprette, D. (2005). Light microscopy. Retrieved 09-12-2005 from http://www.ruf.rice.edu/~bioslabs/methods/microscopy/microscopy.html

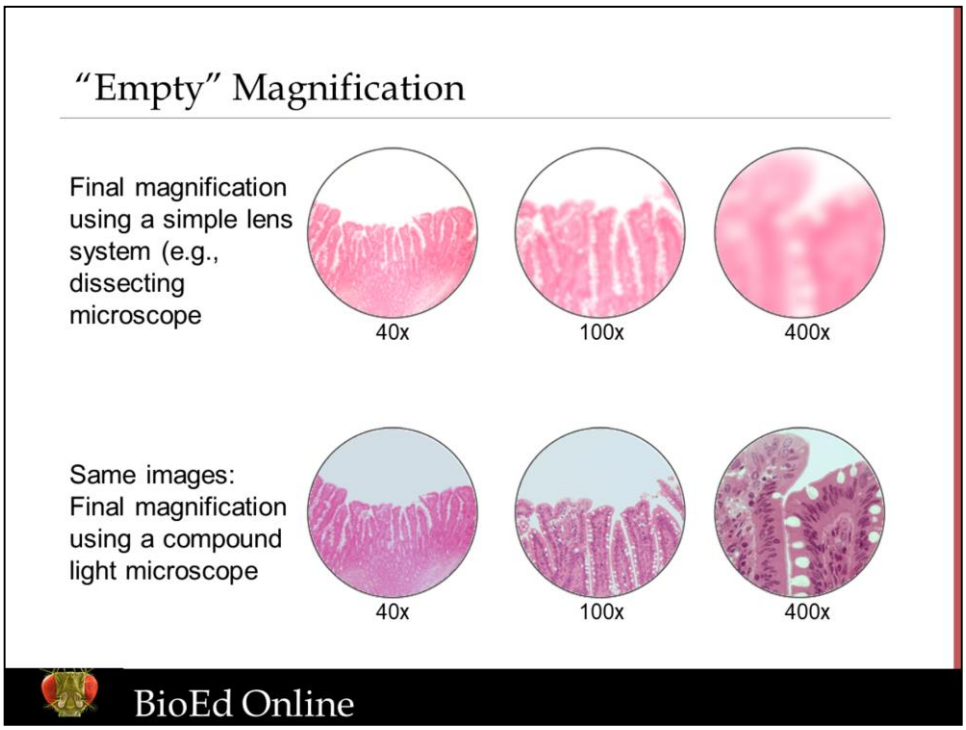
Lodish, H.,et al. (2000). *Molecular cell biology* (4th ed.). New York: W.H. Freeman and Co.

Nave, C.R. (2005). Hyperphysics (light and vision). Retrieved 09-12-2005 from http://hyperphysics.phy-astr.gsu.edu/hbase/hframe.html

Wolfe, S.L. (1993). *Molecular and cellular biology*. Belmont, CA: Wadsworth Publishing Company.

Image Reference:

Dave Caprette. (2005).



"Empty" Magnification

The best quality (and very expensive) magnifying glasses may produce reasonably good quality images with up to about 25x magnification. We can obtain magnifications up to 40x or maybe a bit more using what is called a stereomicroscope or dissecting microscope. Dissecting microscopes are designed to provide room to work on a specimen while looking at a magnified image. The objective lens may bring the image into focus when it is three or four inches above the specimen.

Why can't a dissecting microscope be used in place of a compound light microscope? Suppose your dissecting microscope can produce up to 40x total magnification when used with 10x eyepieces. Suppose that you find an eyepiece rated as magnifying 20 times. You will make the apparent image twice as large, but you won't see any more detail. This kind of magnification, in which we try to enlarge images beyond a practical limit, is called "empty" magnification. There is no benefit to making a blurry image larger.

We encounter a similar situation when we enlarge a digital image. We produce empty magnification once we reach the point at which the eye can distinguish individual pixels (somewhat less than 200 pixels per inch). Resolution is determined by the number of pixels that define the image, regardless of the physical size of the image. Enlarging pixels themselves does not improve resolution. In a microscope, resolution is limited by the quality and design of the lenses, by the medium in which light travels, and ultimately by the wave nature of light itself.

References:

Alberts, B., et al. (2002). *Molecular biology of the cell* (4th ed.). New York: Garland Science.

Caprette, D. (2005). Light microscopy. Retrieved 09-12-2005 from http://www.ruf.rice.edu/~bioslabs/methods/microscopy/microscopy.html

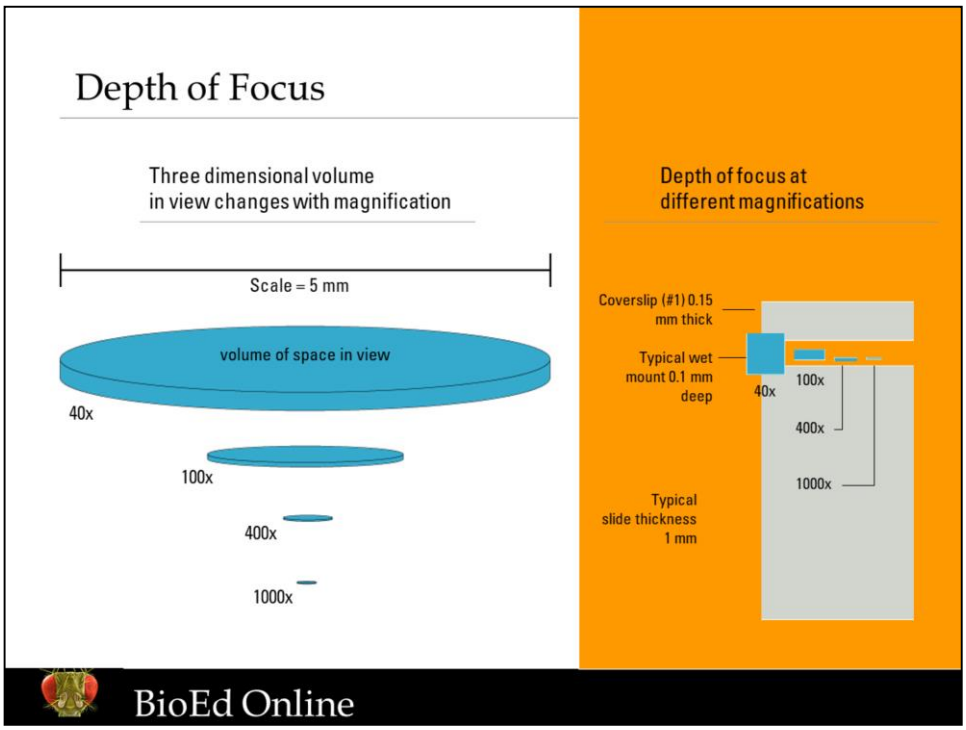
Lodish, H.,et al. (2000). *Molecular cell biology* (4th ed.). New York: W.H. Freeman and Co.

Nave, C.R. (2005). Hyperphysics (light and vision). Retrieved 09-12-2005 from http://hyperphysics.phy-astr.gsu.edu/hbase/hframe.html

Wolfe, S.L. (1993). *Molecular and cellular biology*. Belmont, CA: Wadsworth Publishing Company.

Image Reference:

Dave Caprette, photographer. (2005). Peyer's patches in monkey small intestine, h.-e. stained.



Depth of Focus

Some specimens, such as stained animal or plant tissue, will be obvious to the naked eye and not hard to find at all. Others require some searching, especially if they are very small, sparsely distributed, and/or very light in color. Students often have trouble finding Gram negative bacteria, for example, because they are typically less than a micrometer in diameter and because they stain a very light pink. For hard-to-find objects, it is important to remember that to locate your target, you must bring it into focus, or at least close.

When we look for a particular object, we are looking within a volume of space. The volume of space in which an object will show up depends on the object itself and the choice of objective. Many objects, such as stained bacteria, become invisible when they are far out of focus. Let's define depth of focus as the vertical range over which a very small specimen, such as stained bacteria, remains recognizable.

My microscope has four objectives of 4x, 10x, 40x, and 100x magnification, and oculars that magnify 10x. With the 4x lens in place, the total magnification is 40x and the depth of focus is 160 µm, or 0.16 mm. It is only necessary to position the 4x objective so that the surface of the slide, and thus the specimen, is more or less in focus. The area in view at any one time is that of a circle of 2.5 mm radius. The volume visible with the 4x lens, then, is pi times 2.5 mm squared, times 0.16 mm, or 3 cubic mm. With such a large field of view, specimens should be easy to locate, provided that they have sufficient contrast and that they are recognizable at 40x.

At 100x final magnification, the depth of focus is reduced to 40 µm and the area in view now has radius 0.6 mm. The visible volume of space is now 0.05 cubic mm. At 400x and 1,000x, the depth of focus is 12 and 5 µm respectively, and the areas in view are correspondingly smaller. At 1,000x, you are looking at a volume of space of less than 0.0002 cubic millimeter, which is less than 1 ten thousandth the volume in view at 40x.

Experienced microscopists start at low magnification and work up. Trying to find a tiny object

at high magnification without systematically working up from low magnification is not at all unlike looking for a needle in a haystack.

References:

Alberts, B., et al. (2002). *Molecular biology of the cell* (4th ed.). New York: Garland Science.

Caprette, D. (2005). Light microscopy. Retrieved 09-12-2005 from http://www.ruf.rice.edu/~bioslabs/methods/microscopy/microscopy.html

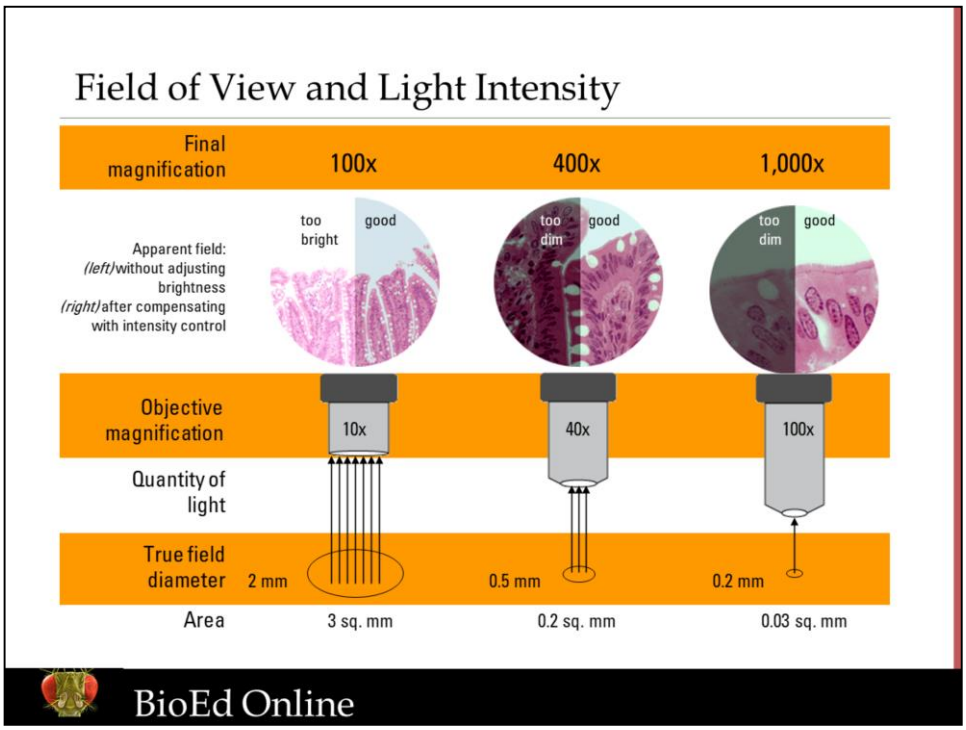
Lodish, H.,et al. (2000). *Molecular cell biology* (4th ed.). New York: W.H. Freeman and Co.

Nave, C.R. (2005). Hyperphysics (light and vision). Retrieved 09-12-2005 from http://hyperphysics.phy-astr.gsu.edu/hbase/hframe.html

Wolfe, S.L. (1993). *Molecular and cellular biology*. Belmont, CA: Wadsworth Publishing Company.

Image Reference:

Dave Caprette. (2005).



Field of View and Light Intensity

When you look into a microscope, the true field of view is the actual circular area of specimen magnified by an objective lens. With increased magnification, the area diminishes, and so does the amount of light entering an objective. An ocular lens magnifies an image so that the view we see has the same apparent diameter, regardless of magnification. It follows, then, that each time we raise magnification the image becomes dimmer.

The loss of light at high magnifications is dramatic. The area of the true field and amount of light it transmits to an objective are proportional to the square of its radius. When we increase magnification by a factor of ten, we reduce the radius of the true field of view tenfold. The amount of light entering the objective lens and eventually reaching the eye is reduced a hundredfold.

With a sufficiently intense light source, it is necessary to reduce the light intensity at low magnifications to avoid hurting one's eyes. Enough light should be available to view a specimen at the highest available magnification without having to look at a dim image. With source intensity at maximum, if an image is still too dim it may be time to change the bulb or re-align the light path.

Only the source intensity control should be used for adjusting image brightness. The aperture diaphragm in the condenser also will affect brightness, but it should be used strictly for adjusting contrast and resolution.

References:

Alberts, B., et al. (2002). *Molecular biology of the cell* (4th ed.). New York: Garland Science.

Caprette, D. (2005). Light microscopy. Retrieved 09-12-2005 from http://www.ruf.rice.edu/~bioslabs/methods/microscopy/microscopy.html

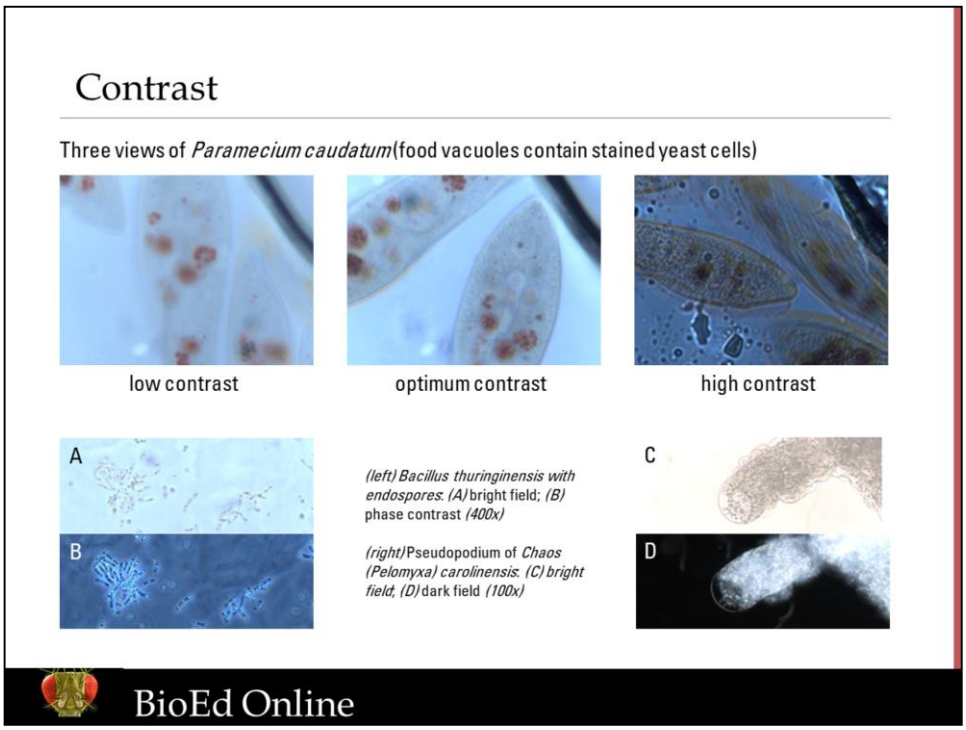
Lodish, H.,et al. (2000). *Molecular cell biology* (4th ed.). New York: W.H. Freeman and Co.

Nave, C.R. (2005). Hyperphysics (light and vision). Retrieved 09-12-2005 from http://hyperphysics.phy-astr.gsu.edu/hbase/hframe.html

Wolfe, S.L. (1993). *Molecular and cellular biology*. Belmont, CA: Wadsworth Publishing Company.

Image Reference:

Dave Caprette, photographer. (2005). Peyer's patches in monkey small intestine, h.-e. stained; 1,000x image shows brush border of epithelial cells.



Contrast

To be viewed successfully in bright field, a specimen must be thin enough to transmit light yet thick enough to absorb or scatter enough light to provide contrast. Very small, thin, and/or colorless specimens may be nearly invisible. In addition, the thickness of a specimen limits the available resolution. That is because any objective lens has a measurable depth of focus. Within a vertical distance, all parts of a specimen are in focus or close to focused, so that if structures overlap it is difficult to distinguish fine detail. Very thick specimens block too much light, resulting in an image that is nothing more than a silhouette.

The condenser is used to adjust both contrast and resolution. Bright field condensers, for example, are fitted with an aperture diaphragm that should be adjusted for optimum contrast when viewing specimens. If the aperture is wide open, one obtains a washed out image with little detail. When the aperture is stopped down there may be some dimming of the light, but more important, details in the specimen begin to appear, details that were not apparent with the aperture all the way open. When the aperture is stopped down fully, the image is often distorted. Boundaries are exaggerated and fine details are actually obscured.

With bright field microscopes, do not expect to see nearly transparent structures, such as cilia and flagella, subtle differences in regions of pseudopodia, or very thin and colorless cells, such as living bacteria. Other relatively colorless specimens may be visible, but the view will be poor compared to the view in a phase contrast or dark field microscope. On the other hand, bright field microscopes are ideal for stained and/or pigmented specimens, and they can give very satisfactory views of larger specimens at low magnification.

References:

Alberts, B., et al. (2002). *Molecular biology of the cell* (4th ed.). New York: Garland Science.

Caprette, D. (2005). Light microscopy. Retrieved 09-12-2005 from http://www.ruf.rice.edu/~bioslabs/methods/microscopy/microscopy.html

Lodish, H.,et al. (2000). *Molecular cell biology* (4th ed.). New York: W.H. Freeman and Co.

Nave, C.R. (2005). Hyperphysics (light and vision). Retrieved 09-12-2005 from http://hyperphysics.phy-astr.gsu.edu/hbase/hframe.html

Wolfe, S.L. (1993). *Molecular and cellular biology*. Belmont, CA: Wadsworth Publishing Company.

Image Reference:

Dave Caprette, photographer. (2005). *Paramecium caudatum*, *Bacillus thuringinensis,* Pseudopodium of *Chaos (Pelomyxa) carolinensis*