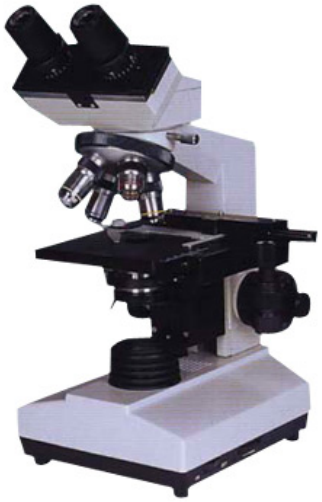

Using Microscopes

Life Science: Molecular

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.

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Image Reference:

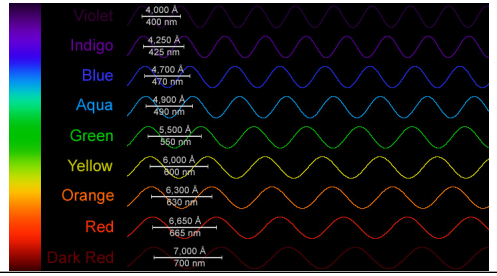
Compound Microscope, circa 1751. Roby 11 janvier 2005. Retrieved 09-15-2005 from http://commons.wikimedia.org/wiki/Image:Microscope1751.jpg.

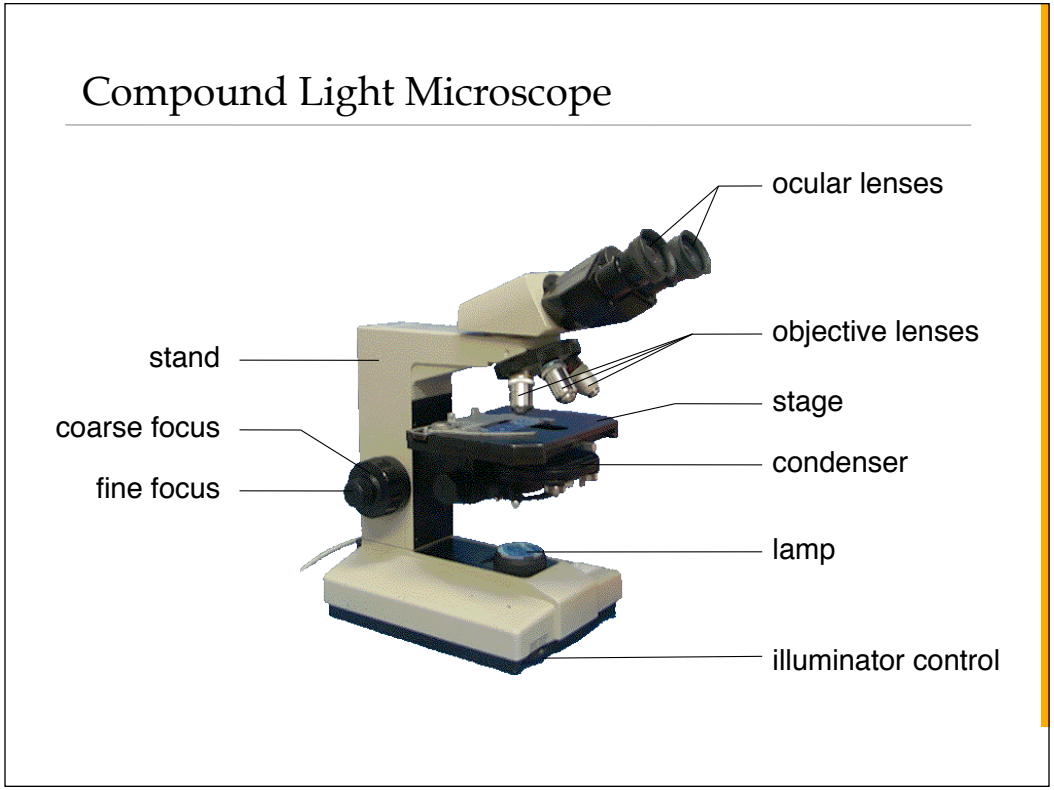
Learning Objectives

1) Name all the parts of a microscope, and describe the function of each.
2) Explain how to properly carry a microscope, and prepare and focus a slide correctly.
3) Calculate total power of magnification.
4) Estimate the size of a specimen being observed

Light Microscopes

- A **microscope** is a device that produces magnified images of structures that are too small to be seen with the unaided eye.
- Light microscope uses visible light and lenses to produce a magnified image.
- Light microscopes are limited by the wavelength of light to about 0.2 μm (micrometers)





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.

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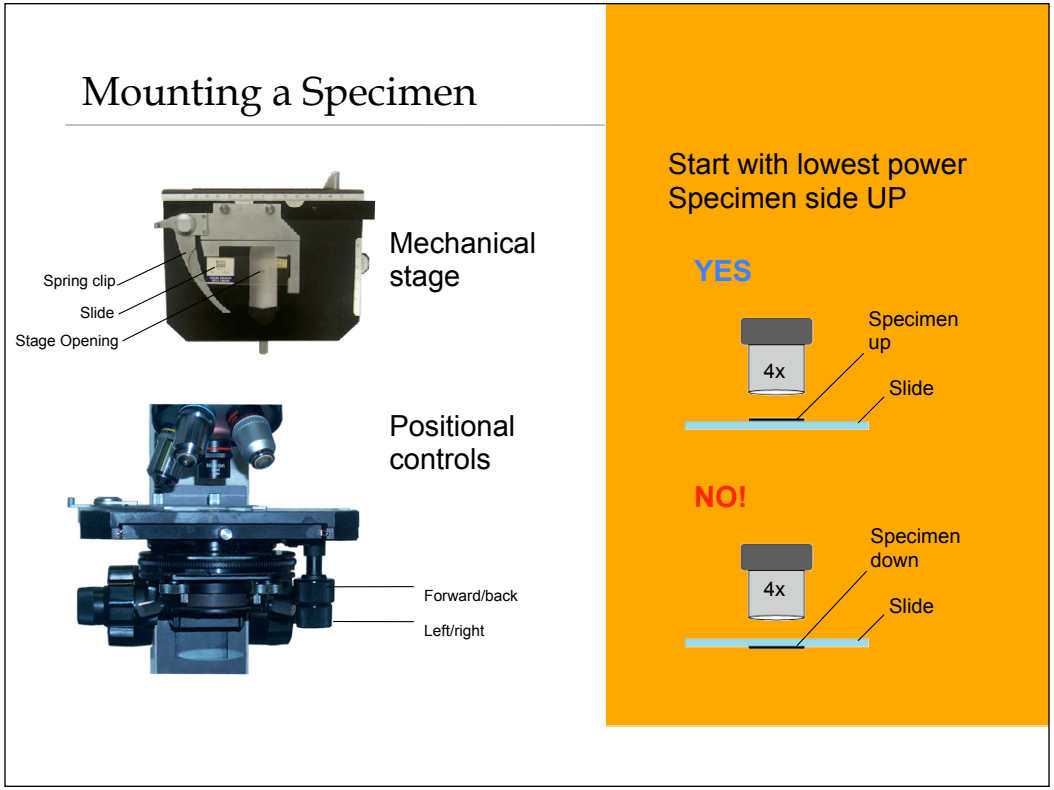

Mount a Specimen

Prior to mounting a specimen, one should put the lowest available magnification in the light path. An objective of low magnification is shorter than one of high magnification, giving one more room for placing the slide. More importantly, when we look for an object on a slide, we search for it in three dimensions, namely the x-y plane and the vertical dimension (i.e., the focal plane). At low magnification, we see a much greater area of specimen and have a much deeper focal plane than at high magnification.

A mechanical stage makes it convenient to search an area systematically for objects of interest and to collect replicate data. Using the translational controls, one can manually "chase" a fairly fast moving living organism around a microscope slide without losing it from view.

Whether you have a prepared slide, wet mount, or a smear with no coverslip, it is critical to mount the slide with the specimen toward the objective lens. Usually, that means the specimen will be facing up, although some microscopes (inverted microscopes) have the stage above the objectives.

If the slide is upside-down, you may be able to focus at low magnifications without compromising the view. You will not be able to focus at a high magnification, though. High resolution requires that the half angle at which the cone of light enters the objective (alpha in the equation for resolution) be as large as is practical. Proximity to a specimen is necessary to obtain a large enough half angle when the light comes from a very small area. It follows, then, that to obtain the necessary resolution, a high magnification objective lens must be brought very close to the specimen.

Coverslips are made of very thin glass or plastic for two reasons. One is to allow an objective to approach within a very short distance of a specimen. The other to prevent the thickness of the glass, which is not optically perfect, from significantly compromising contrast or resolution.

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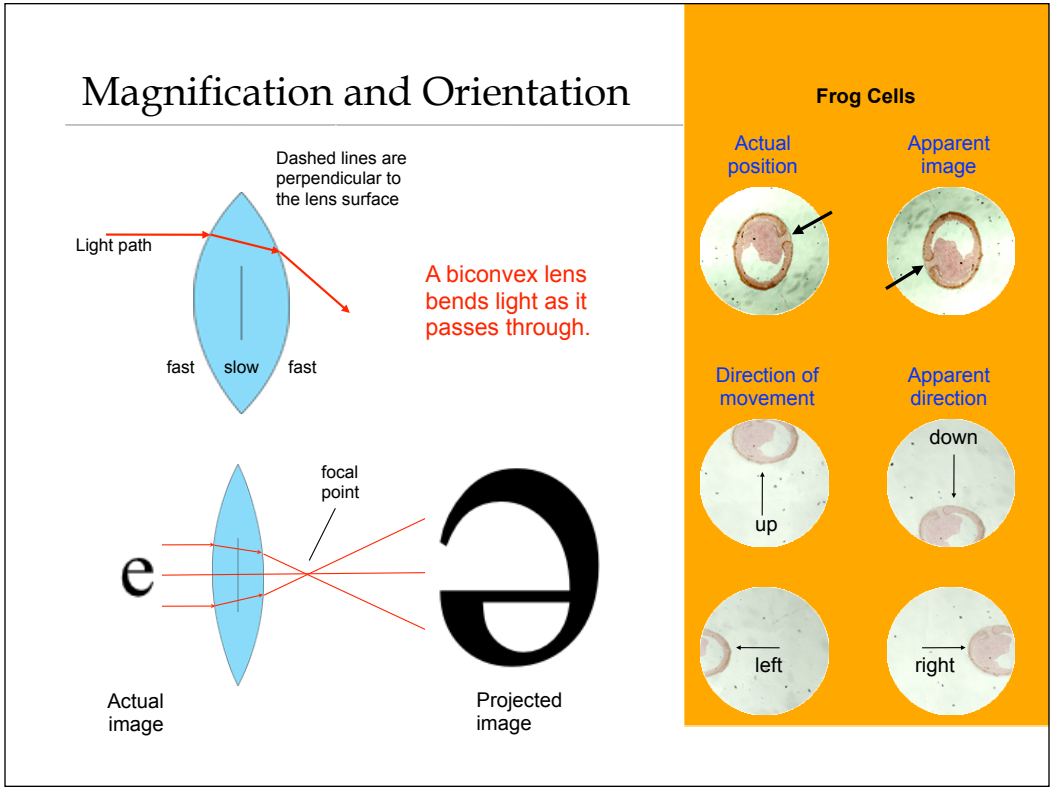

Magnification and Orientation I

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.

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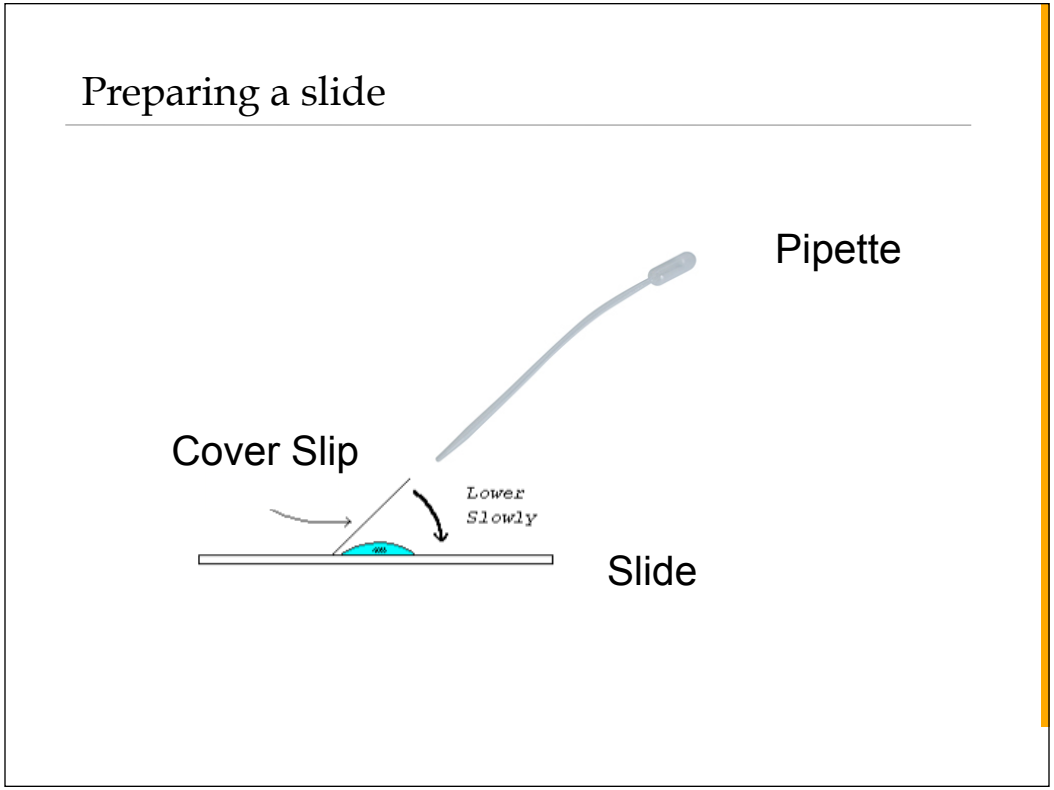

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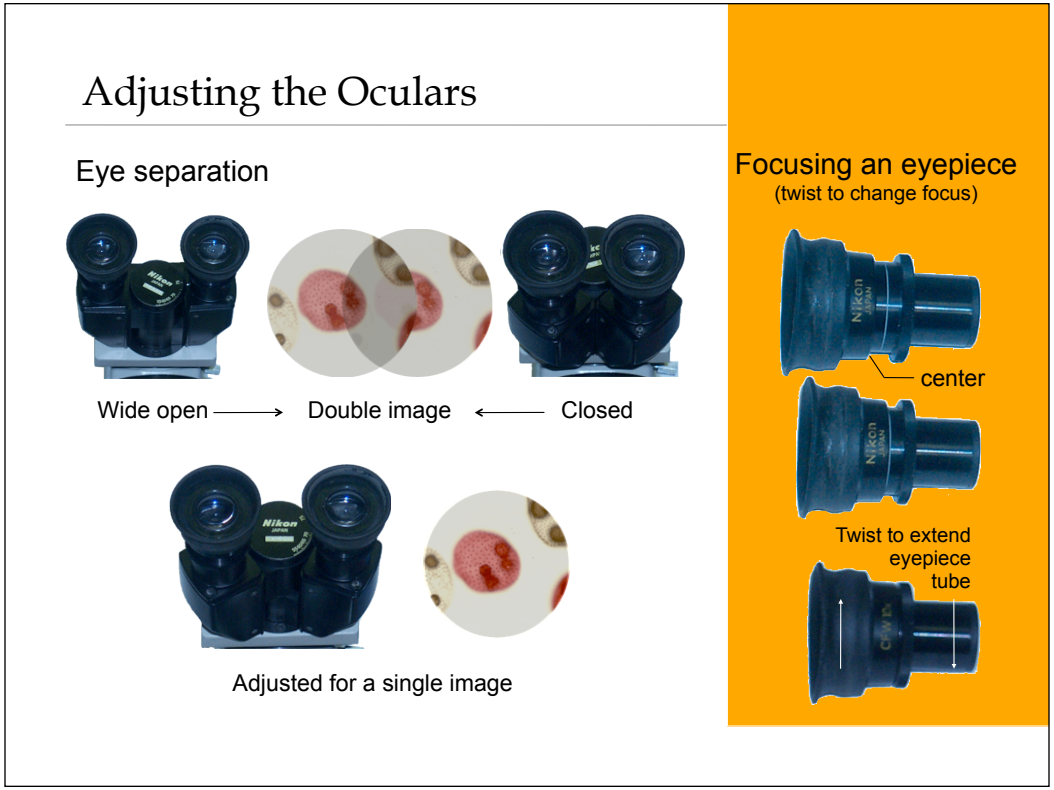

Adjusting the Oculars

You may not need your eyeglasses when using a microscope, unless they correct for astigmatism. Using a single ocular, the focus control alone can bring an image into sharp focus. If you have a binocular microscope, the eyepieces should be adjusted to compensate for eye differences.

Anyone who has used binoculars should find it easy to adjust the oculars on a binocular microscope. Before even focusing on a specimen, you should be able to adjust for eye separation so you will see a single field of view. When the oculars are separated to match your eyes, you should be able to look into them with both eyes relaxed, just as if you are looking across a room. If you have trouble with binocular vision, you could be among the minority of users with eyes set close together, making such viewing difficult. It is more likely, though, that the individual oculars are simply out of adjustment, which prevents you from bringing the image into focus for both eyes at the same time.

Your microscope may be equipped with one fixed and one adjustable eyepiece, or with both eyepieces adjustable. Either way, the first step is to place each adjustable eyepiece in the center of its range of travel, giving you the most latitude for adjustment either way. The next step is to obtain an image at high enough magnification so that you can see fine details. Step three is to observe with the fixed eyepiece only (or one of the two adjustable eyepieces) with the appropriate eye, and focus the microscope on the image. Recalling one or two specific details from the image, observe with the other eye only, and this time, adjust only the eyepiece until the details come into focus. From this point on, when you focus the microscope, you should be able to look comfortably using both eyes.

If you had trouble seeing a single image when adjusting for eye separation, it may be worth trying again once the oculars are adjusted to match your eyes.

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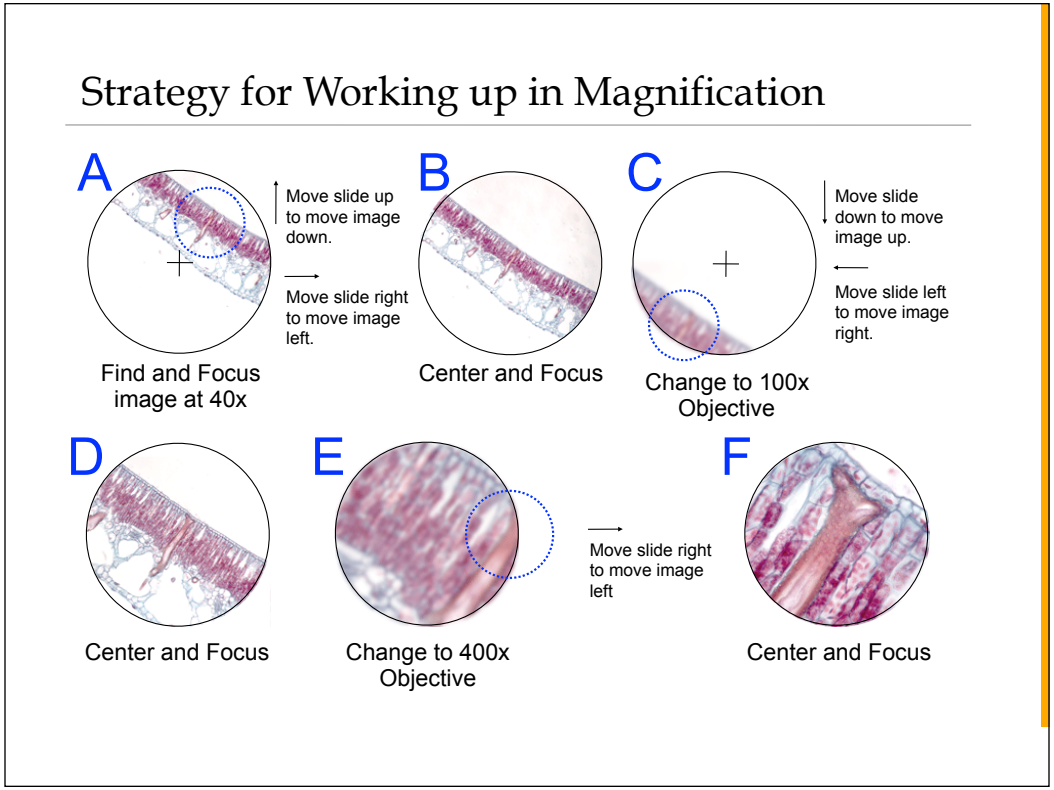

Strategy for Working up in Magnification

Unless you are so familiar with a type of specimen that you can go straight to an appropriate magnification and find your target immediately, it is best to take the same approach to finding specimens each time you observe. The most consistently effective strategy is to start at low magnification, find the target, adjust illumination, resolution and contrast, focus and center the object, and then raise magnification. Most sets of objective lenses are parfocal, meaning that the objectives are matched, so that if a specimen is in focus using one objective, it will be very nearly in focus when you raise the magnification using the next objective lens. Thus, if you re-focus, using only the fine focus control, and center the target each time you change magnification, you should have no trouble obtaining the image you seek at the desired final magnification.

After reaching 100x magnification, it is a good to re-adjust the microscope for binocular viewing, if you have a binocular eyepiece tube. You can see more detail now, and the better the oculars are adjusted to match your eyes, the more satisfactory the viewing.

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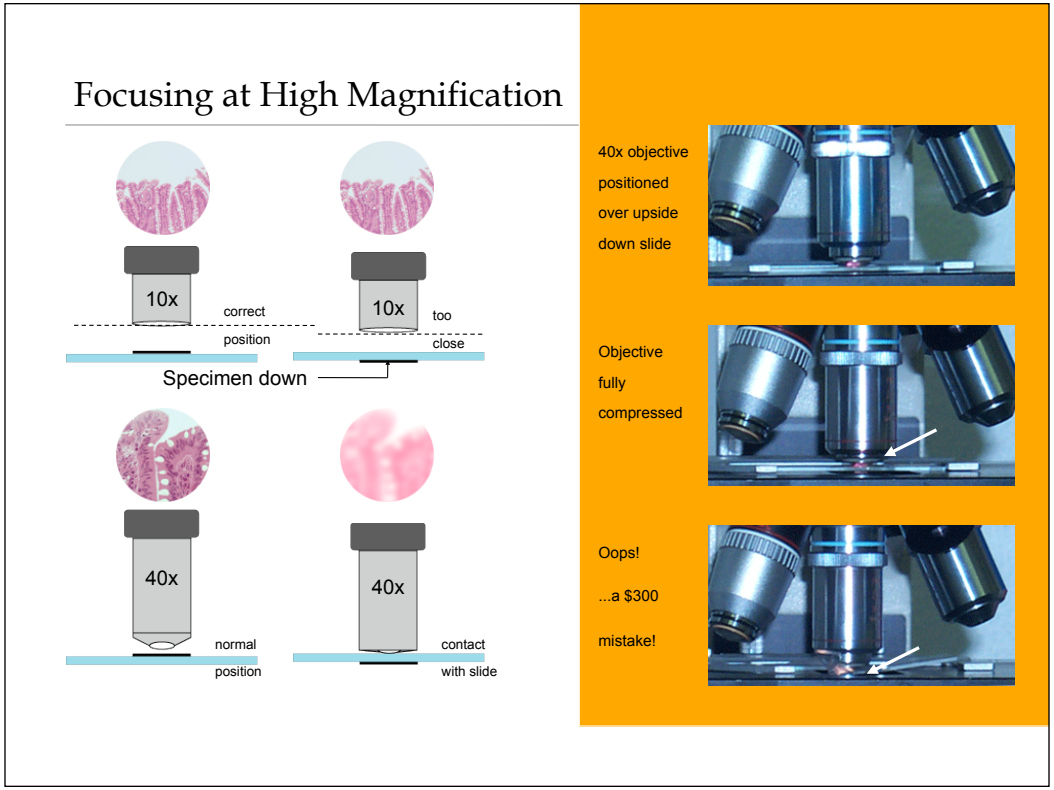

Focusing at High Magnification

At low magnifications (up to 100x or so total magnification), you should use the coarse focus control. Not only does it take too long to move a distance with the fine control, but the limit of travel with the fine focus may be less than with the coarse. Trying to focus past the limit of travel can damage a focusing mechanism.

When you bring in a high dry objective (a high power lens which is used without oil, usually a 35x or 40x lens) with the specimen in focus, the end of the objective will approach the specimen closely. It is unwise to use the coarse objective with such a lens, because it is too easy to ram the lens into the slide. In this case, use the fine control only.

Suppose you mount your slide upside-down. You will be able to focus at 40x total magnification, and again when you go to 100x magnification by swinging in the 10x objective. However, the thickness of the slide may exceed the depth of focus with the high dry objective (35x or 40x). If so, you won't be able to focus at all. If you don't pay attention, you probably will bump the slide with the end of the objective. Good high power lenses will telescope so as to buffer such shocks, but if you reach the limit, further movement will damage the slide and also may scratch the objective, and even the exit lens of the condenser. Such damage cannot be repaired.

Because high magnification lenses come so close to the specimen, to reduce the risk of a disaster, you might want to take your eyes from the eyepieces and instead watch the lens as you rotate it carefully into place. Until you are used to your microscope, you should check the position of the lens frequently while focusing, or (better) have someone else watch the objective and warn you if it contacts the slide.

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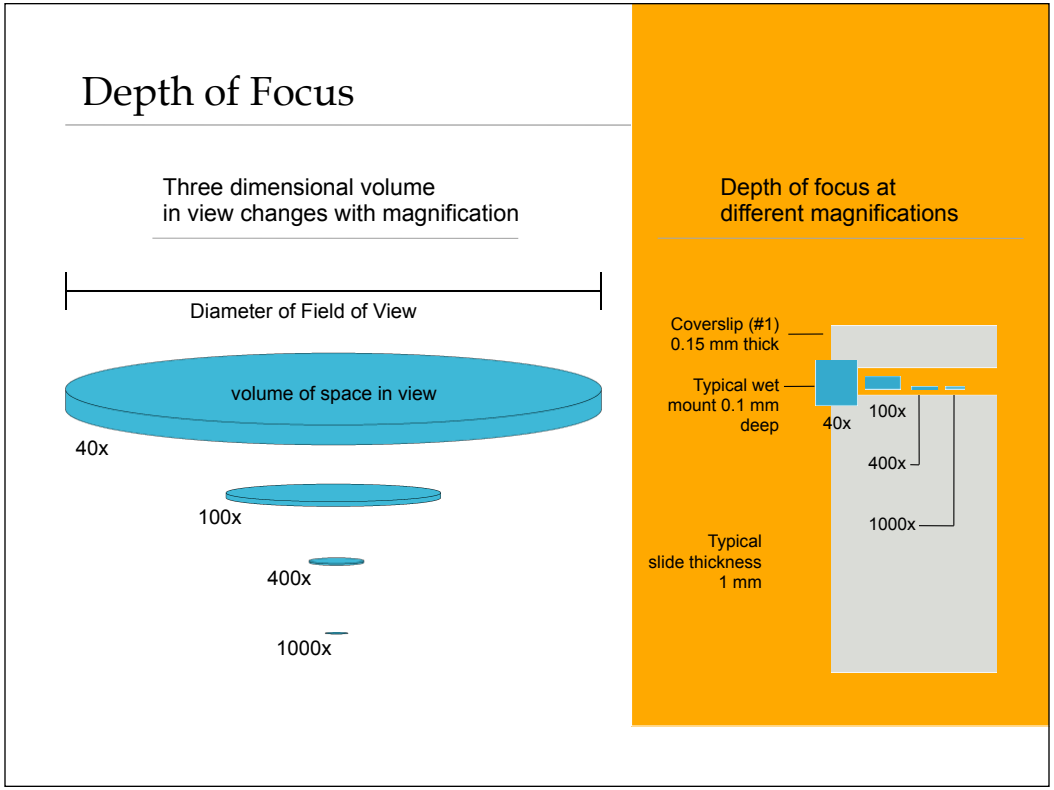

Depth of Focus

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.

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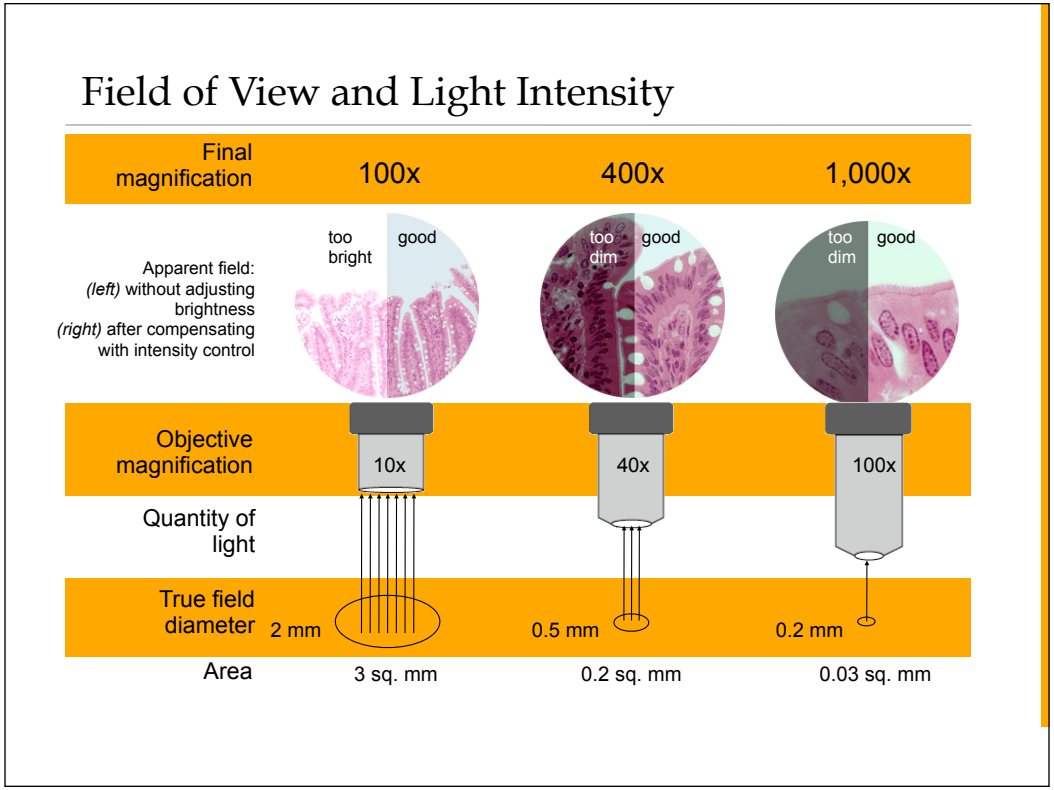

Field of View and Light Intensity

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.

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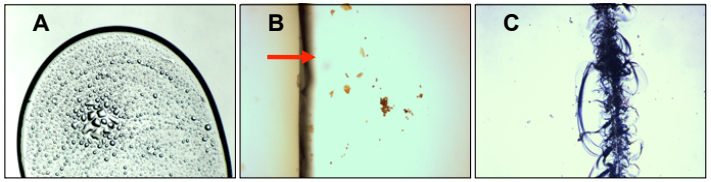
Image Reference:

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

What Do You See?

Can you identify the artifacts on this slide?



A: air bubble
B: coverslip edge
C: scratch on the slide

**All images 100x*

Find the Target at Low Power

The lowest power objective lens is often called the scanning lens. Scanning lenses are seldom of the highest quality and are not of much use in collecting information. Their purpose is primarily to find a specimen readily and to bring it to the center of the light path and roughly in focus.

In a typical microscope field at 40x (calculated by multiplying the power of the ocular lens by the power of the lens), the field diameter is 5 mm. The advantage of the scanning lens is depth of focus and large viewing area. Although you cannot see much detail, you should be able to find what you are looking for, provided (1) the image is visible in bright field and (2) you know what to look for.

The only concern with finding an object at a very low magnification is that a specimen may not be recognizable. Therefore, it is essential that you know something about your specimen before setting up to view it. Think about the size of the target, how much (or little) contrast it should have in bright field, and how the material is likely to be distributed on a slide. Here are a few suggestions for finding hard-to-locate objects.

Try stopping down the aperture diaphragm (in the condenser) to increase the contrast of the image. Objects will not be well resolved, but the goal at this point is to find them, not to take data. Try focusing on an artifact, such as an air bubble, the edge of a coverslip, or a piece of visible debris. Among the most difficult specimens that are suitable for bright field microscopy are very small Gram negative bacteria. Stained bacteria at low magnification resemble dust on the slide surface. You might use a glass marking tool to make a shallow scratch on the slide surface. (Obviously, you mustn't scratch a prepared slide that is meant to be re-used.) Just as the scratch begins to come into focus, you should be at the level of the specimen, although it still may be hard to find.

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