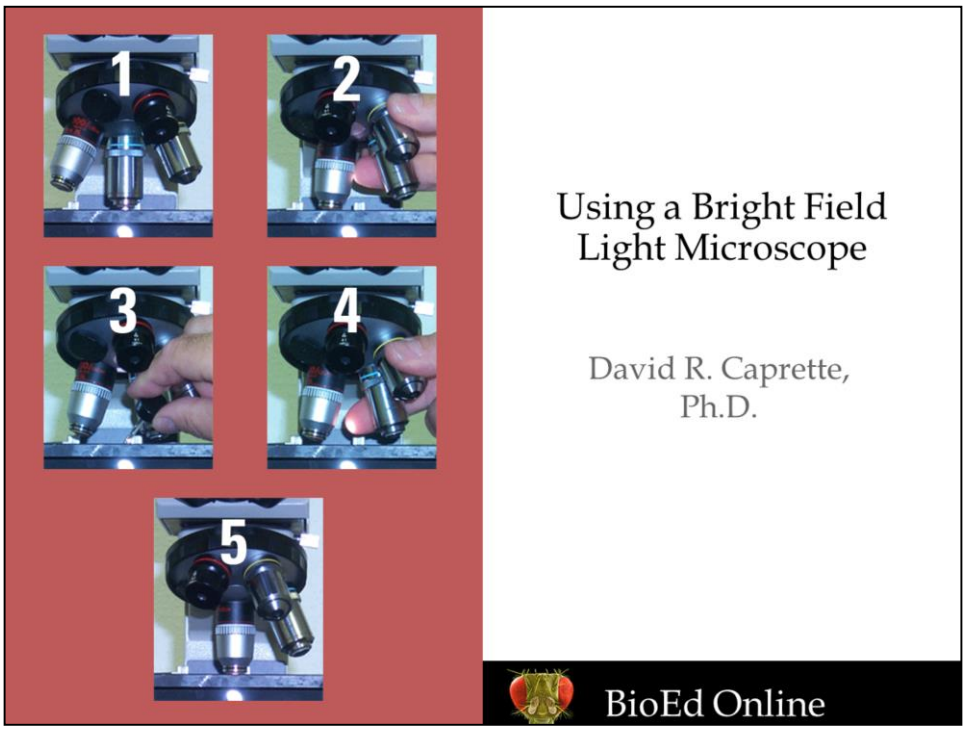

Using a Bright Field Light Microscope

This presentation will take us through the steps required to view a specimen in a bright field light microscope. We will cover proper mounting of a specimen, adjusting the condenser and oculars, and strategies for finding a target and for working up in magnification. We will discuss how the thickness of a specimen can limit the range of useful magnifications, and how to use oil immersion objective lenses to obtain the best resolution possible in a bright field microscope.

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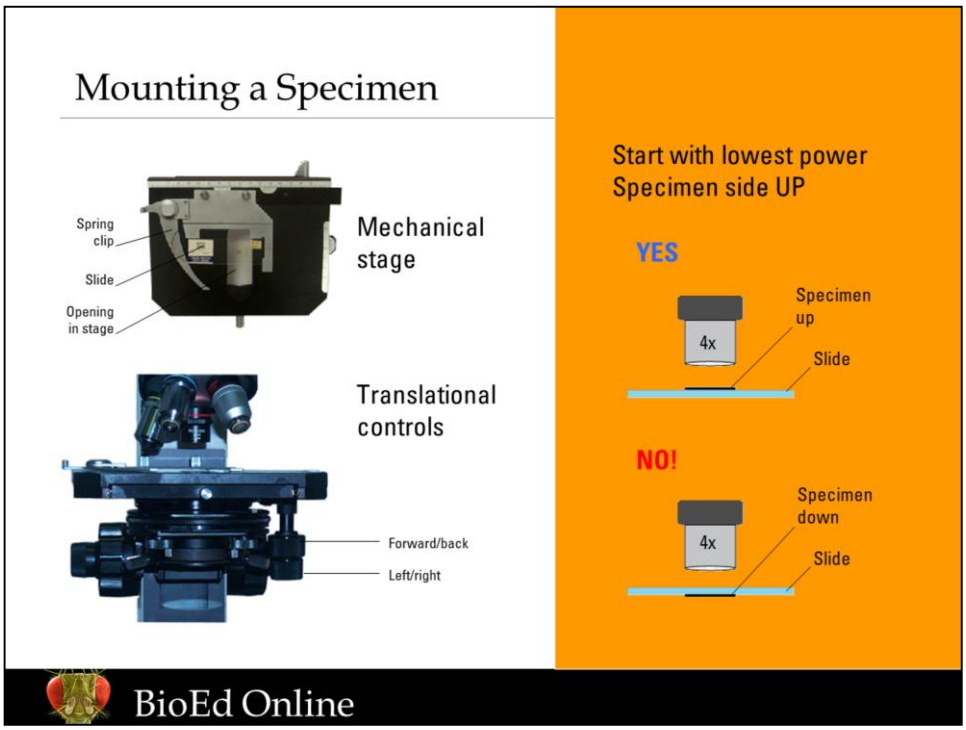

Mount a Specimen

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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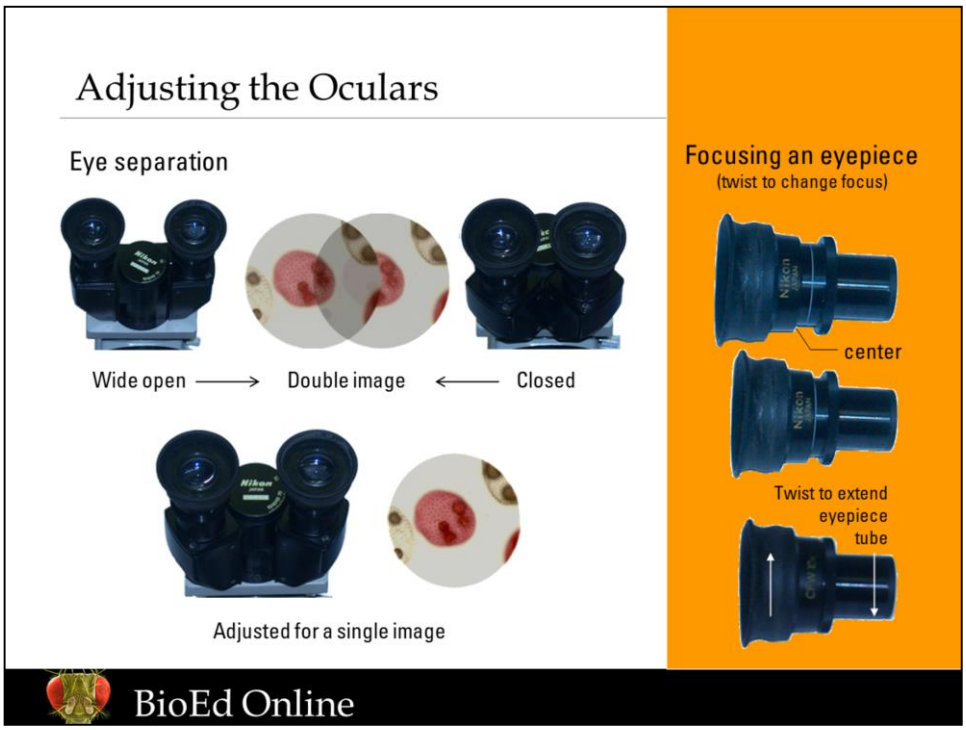

Adjusting the Oculars

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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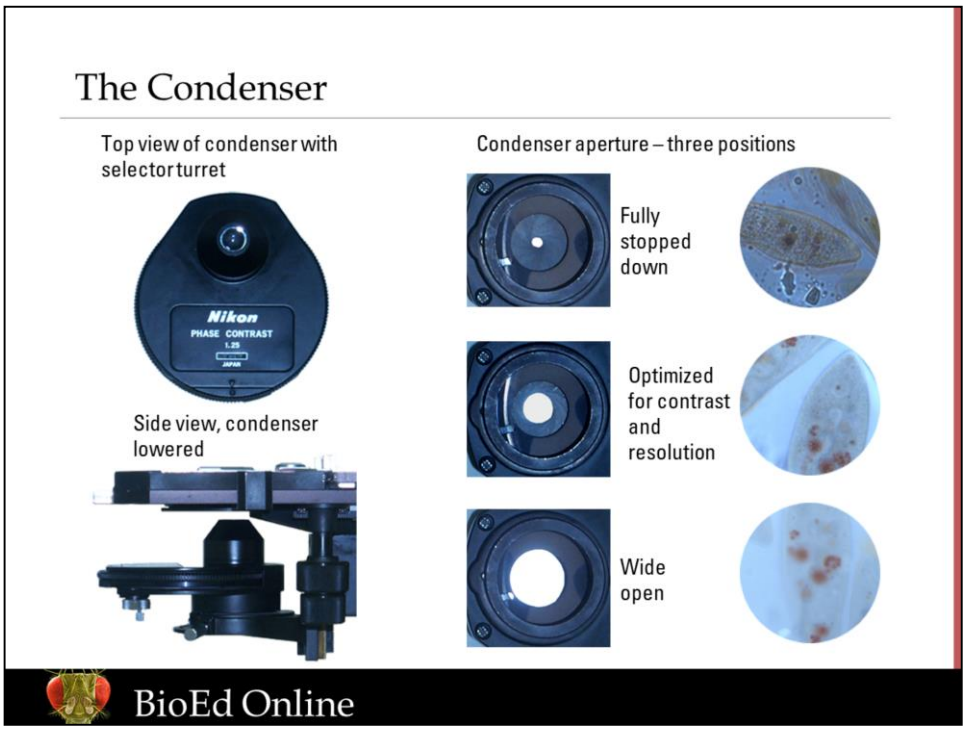

The Condenser

To obtain a sufficiently bright image at high magnification, we need a very intense light beam. The condenser lens gathers light from a wide cross-sectional area and concentrates it in the area of the specimen, giving us a far more intense light beam than we could obtain directly from the source. With a good quality illumination system, one usually has to reduce the intensity of the light source to view a specimen at low magnification.

As important as the condenser is for intensifying the light beam, its primary function is to condition the beam before it enters the specimen and objective lens. To obtain full resolution from a given objective, the condenser should be adjusted so that the back lens of the objective lens is filled with light. Such adjustment is accomplished using an aperture diaphragm control that is built into the condenser. The aperture diaphragm is similar to the aperture of a camera lens. The size of the opening is adjustable so that the diameter of the light beam as it passes through a particular plane can be varied.

For any given objective lens, there is an ideal position for the aperture diaphragm. “Stopping down” the aperture (making it smaller in diameter) beyond the ideal diameter dims and distorts the image. “Opening up” the aperture increases glare and washes out the image. Usually, to reduce glare, we sacrifice some resolution and stop down the aperture so that about 75% of the back lens of the objective is illuminated.

Inexperienced users may employ the aperture diaphragm control in the condenser to regulate the amount of light reaching the eye. However, misusing a condenser that way will sacrifice the resolving power of the microscope.

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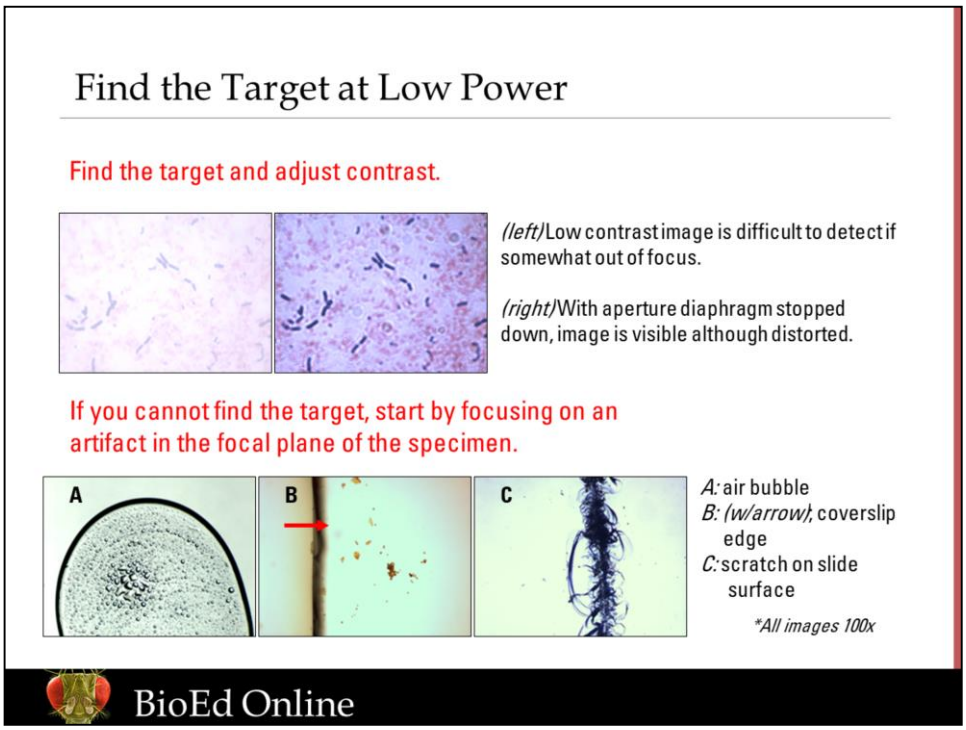

Find the Target at Low Power

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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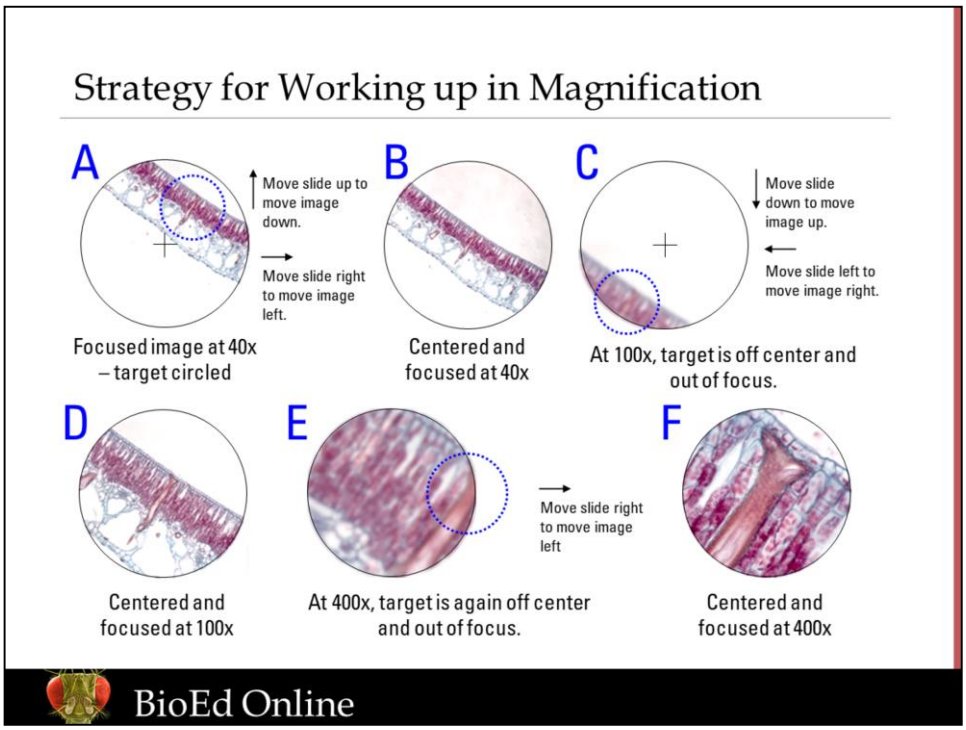

Strategy for Working up in Magnification

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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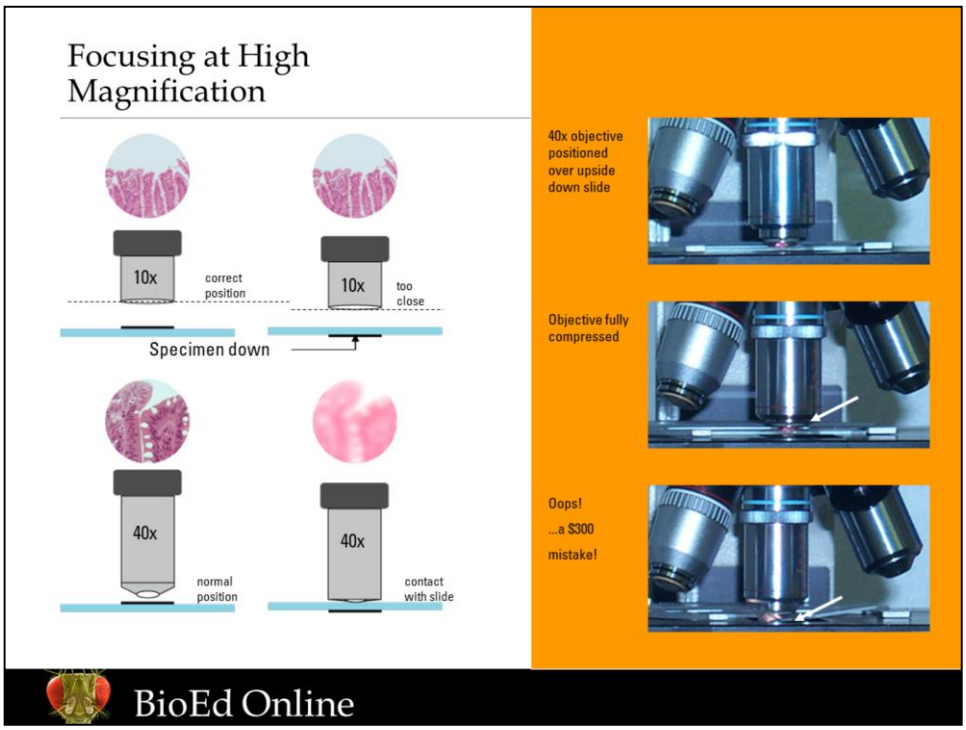

Focusing at High Magnification

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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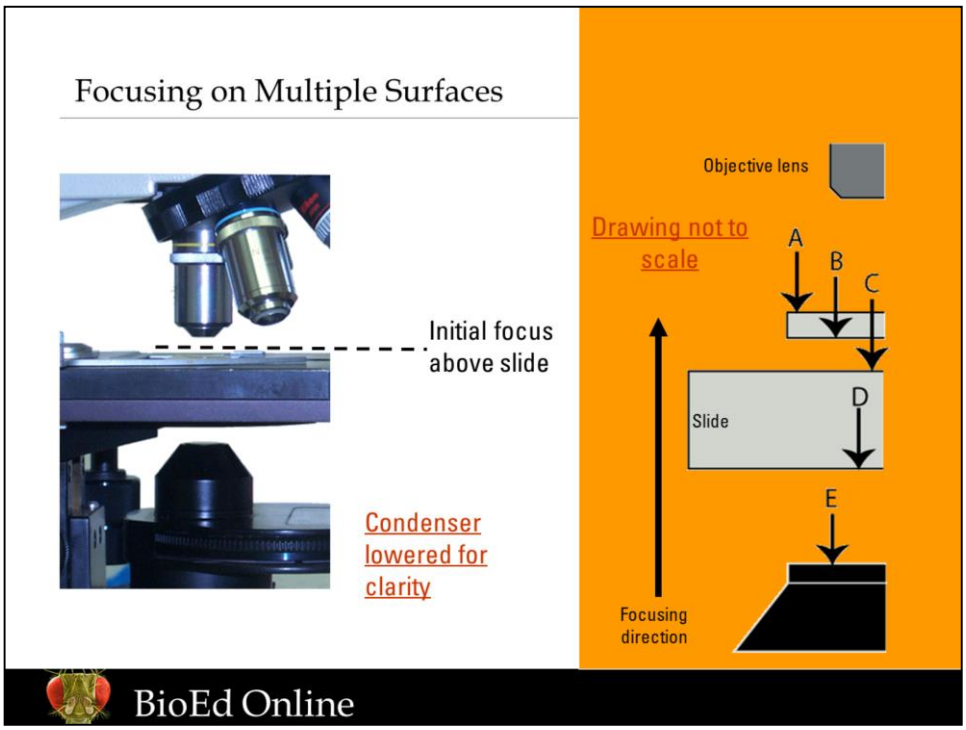

Focusing on Multiple Surfaces

Even with parfocal objectives, each time you change lenses, you will have to make some adjustment to focus. When you go to high dry magnification, and especially to an oil immersion lens, the depth of focus is so narrow that the specimen may be faint or invisible. If you lose track of a specimen completely, it usually is fruitless to remain at high power and search. You are searching in three dimensions, and your quarry occupies only a small volume in the three dimensional search area. It is best to enlarge your field of view in all three dimensions by going back to a lower magnification, re-focusing and re-centering a specimen before returning to high magnification.

A trick for spotting your target when it is out of focus is to jiggle the slide with the mechanical control and focus on any object that appears to be moving. You should be aware, though, that your slide consists of multiple surfaces, and that you naturally must focus on the surface that bears your specimen.

With the objective lens well away from the specimen so that no part of the slide can be in focus, suppose you begin moving the stage toward the objective. The first surface that comes into focus is the top of the coverslip (A), although in bright field you may not see anything without stopping down the aperture diaphragm in the condenser. You may see scratches, dust, and fibers on the surface. The next surface is where the coverslip contacts the specimen. In a wet mount, the bottom of the coverslip (B) might be above the target material. To focus on a specimen at the surface of the slide (C) you would need to raise the stage. The next surface is the bottom of the slide (D). If you raise the stage too far, you actually may focus on the surface of the condenser lens (E). You can tell if you are focused on the condenser by jiggling the slide. Anything on the slide will be seen to move, but the condenser image will remain still.

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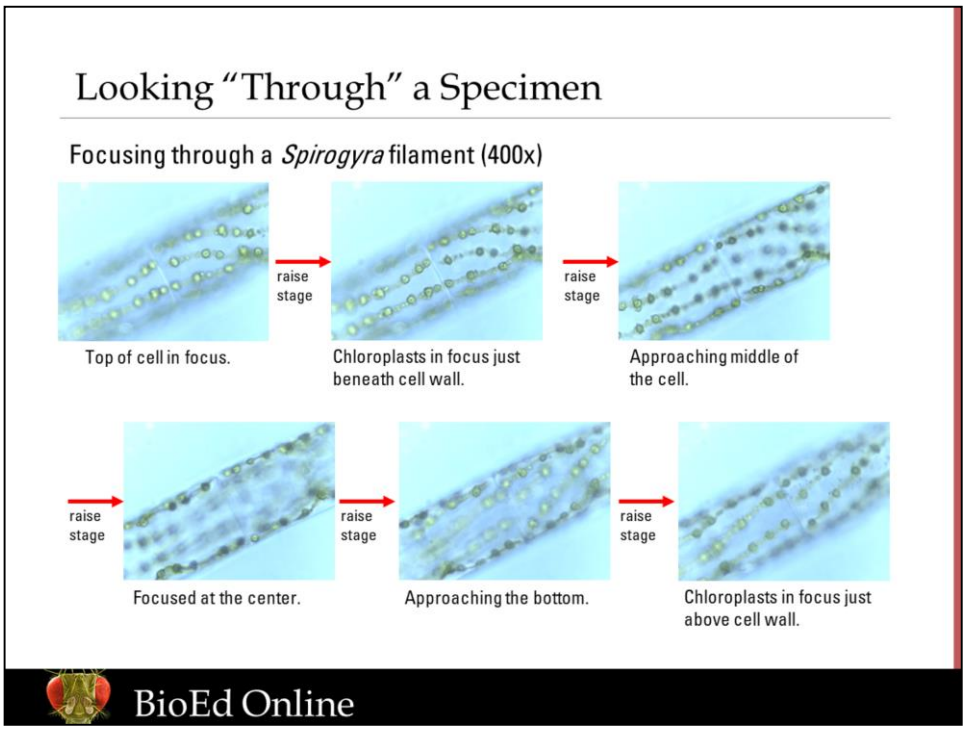

Looking "Through" a Specimen

The thickness of a specimen limits the maximum functional magnification with which we can expect to obtain useful information. Objective lenses, as you may recall, have limited depth of focus, so while one part of a thick specimen is in focus, the other parts that are out of focus make it difficult to see detail. But the structures of some specimens are arranged in such a way that one can focus on one level at a time and obtain much more information than by using low magnification to see everything at once.

For example, a strand of the filamentous alga Spirogyra is, for the most part, in focus at 40 power. At 100 power, some part of the cell, either toward or away from the objective lens, is out of focus at any given distance. At 400 power, so little of a cell is in focus at any given time that one can begin with the specimen below the focal plane and optically section the filament by slowly raising the stage. First, the chloroplasts just beneath the cell wall appear in focus. As the stage is raised, chloroplasts come into and go out of focus in a spiral pattern. A middle view of a cell shows chloroplasts in focus only at the edges, revealing that the chloroplasts of Spirogyra are arranged near the cell walls and are not found in the centers of the cells. Raising the stage further brings the bottommost chloroplasts into focus. Unless you are aware of the direction in which you are moving the stage, you cannot tell the difference between top and bottom.

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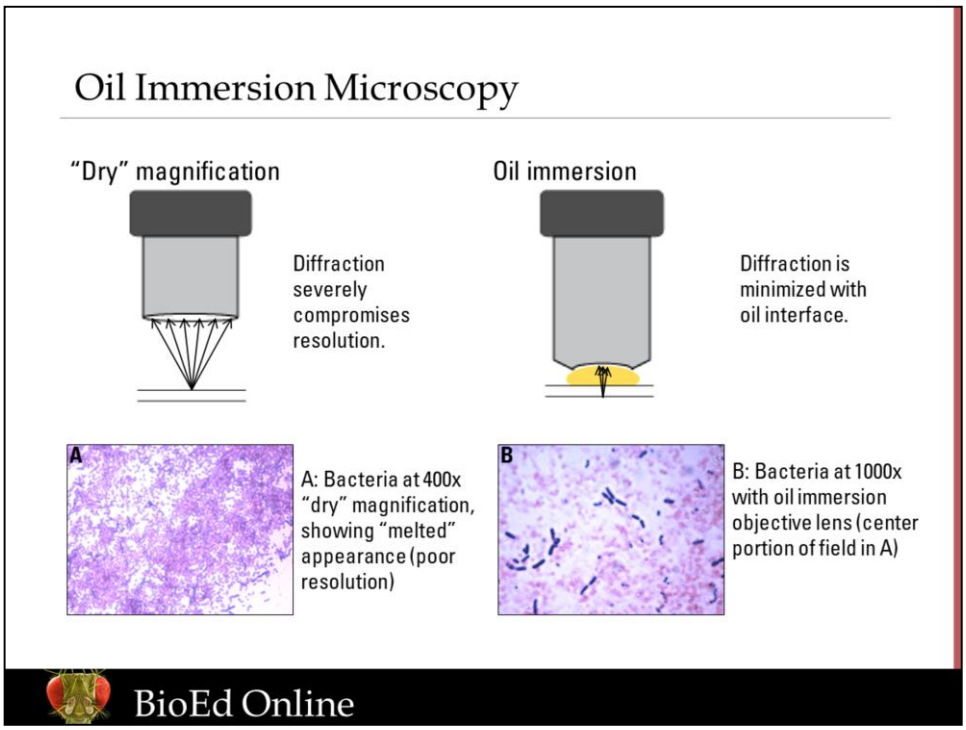

Oil Immersion Microscopy

When a stream of photons travels through a medium, such as air, and strikes or exits a surface of a different medium, such as glass, its velocity and its direction change. The extent of deviation from the original path depends on the angle of the light ray from the perpendicular, and also varies with wavelength. White light consists of a spectrum of wavelengths, so on its way through a specimen, through glass, and into air, light passing through any one point is fairly well scattered. Scattering may not be noticeable at low magnification, but at magnifications above 200x, it will make what should be a discrete point look like a fuzzy ball. Similarly, what should be separate cells, such as in a chain of bacterial rods, will look like a melted thread.

Immersion oil typically has a refractive index* of 1.5, nearly the same as that of glass. That means that as light passes between glass and oil, it does not change velocity significantly, and does not bend as much. Using oil, we can exercise much greater control in concentrating light on the back lens of an objective lens, matching its numerical aperture and improving resolution tremendously.

By the way, light passing at 90 degrees to a surface doesn't bend at all. We obtain very good resolution with parallel light rays (i.e., with light coming in at right angles to the surface of a lens). At the center of a lens, light rays are parallel. This is the basis for the pinhole camera, and is the reason why near-sighted people can see more clearly when they squint.

* refractive index = n = (velocity of light in a vacuum)/(velocity of light in a transmitting medium)

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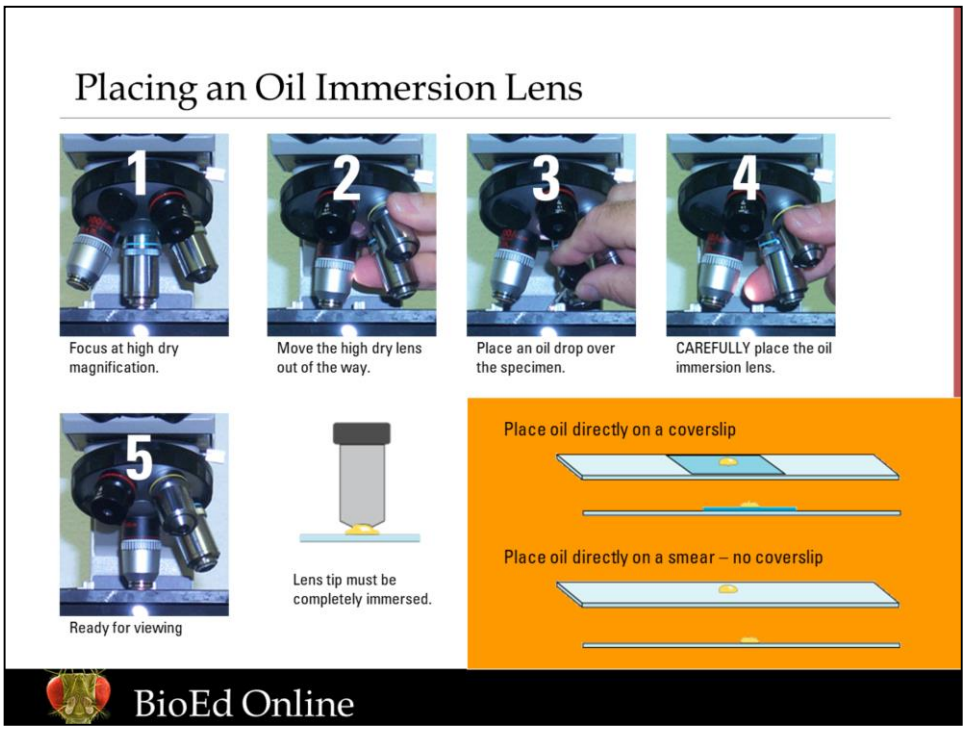

Placing an Oil Immersion Lens

When a lens is marked with the word "oil," the tip of the lens itself is made to be dipped into a transparent oil drop placed in the light path directly on a coverslip or dried smear. Light passes through the specimen and/or coverslip, into oil, and then into the objective without passing through air. The result is a dramatic improvement in resolution.

A typical oil immersion lens magnifies between 95x and 100x. The lens must be brought extremely close to the specimen and the depth of focus is very shallow. Unless one is very experienced and is working with familiar specimens, it is essential to start at low magnification and work up as described previously. The specimen should be viewed, centered, and focused using the high dry lens before adding the oil and moving the oil immersion lens into position.

To place the oil immersion lens, move the high dry lens out of the way and place a generous drop of oil directly on top of the slide. You should have a convex drop in the center of the light path. Carefully rotate the oil immersion lens into place so that the end of the lens contacts the oil. You should be looking directly at the lens and specimen at this point, not through the eyepieces.

It probably will be necessary to increase the illumination to maximum, because the illuminated surface area is now very small. The light path must be well aligned. If the elements of your microscope are adjustable, your manual should indicate how to optimize alignment for maximum brightness. If the images are still too dim, be sure that the lamp is putting out enough light and replace if necessary.

Because the depth of focus is so shallow with a 100x lens, there probably will be no sign of the specimen at first, even with parfocal lenses. Even knowing the correct direction to focus, if you go too fast you might move right through the focal plane and not even recognize the specimen. Students in microbiology frequently encounter this problem when trying to view Gram negative bacteria.

You may use the stage translational controls with an oil immersion lens, just as you would any other lens. But if you move too far from the center of the drop, the lens may lose contact with

the oil. If that happens you can carefully move the lens out of the way, add more oil, and replace the lens without losing your focus. The hard part is finding the focal plane in the first place.

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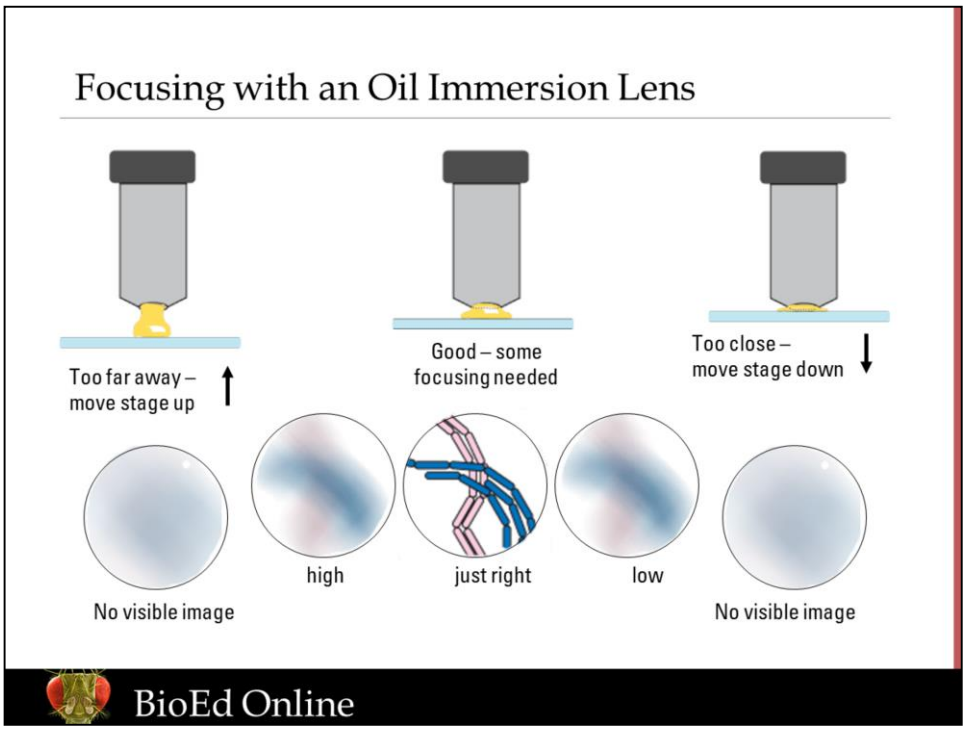

Focusing with an Oil Immersion Lens

The first time you use an oil immersion lens, it might help to have someone watch the objective lens while you attempt to focus. As with any high power lens, use the FINE FOCUS CONTROL ONLY. It may take many turns to bring the specimen into focus, so be patient. The lens will nearly touch the specimen before you reach focus. If it contacts the slide, it will begin to telescope, and your observer should warn you that you have gone too far. Moving in the other direction will bring the specimen into focus. However, if the gap between the lens and specimen reaches a couple of millimeters, you are too far above the specimen, and have missed the focal plane completely.

Now what? Here's what not to do: do NOT go back to the high dry lens. It will contact the oil, fouling the surface of the lens, which is meant to be used only in air, and not in oil.

Check that your specimen is indeed on top. Every time I teach microbiology, someone, sooner or later, puts a bacterial smear on a stage upside down and I spend quite a bit of time trying to help him or her focus before I realize what happened. If you cannot find the focal plane, you may go back to a low magnification objective, such as 4x or 10x, to refocus and re-center the specimen. The direction needed to bring the specimen into focus at low magnification may give you a clue about how far out you were with the high power lens, and in what direction. To protect your high dry objective, you will have to "jump" directly to back to the oil immersion lens.

When you go back to the oil lens, make sure your oil drop is big enough to accept it; you may need another drop. This time, with illumination turned way up, try stopping down the aperture diaphragm in your condenser. The image will be distorted, but if there is anything to be seen, it will have greater contrast and you are more likely to find it. Slowly rotate the fine focus control. It may help to move the mechanical stage slightly back and forth. The eye can detect movement more readily than it can see a stationary image. When you identify your specimen, you can readjust the aperture to optimize resolution and contrast.

One more strategy is to place the lens as close to the surface as you dare, and then slowly move the stage away from the objective. This method requires patience because you may begin far out of focus. But at least you know you are moving in the right direction.

When you finish using an oil immersion lens, you must dab off the oil with good quality lens tissue. Dried oil will interfere with viewing and can be difficult to remove.

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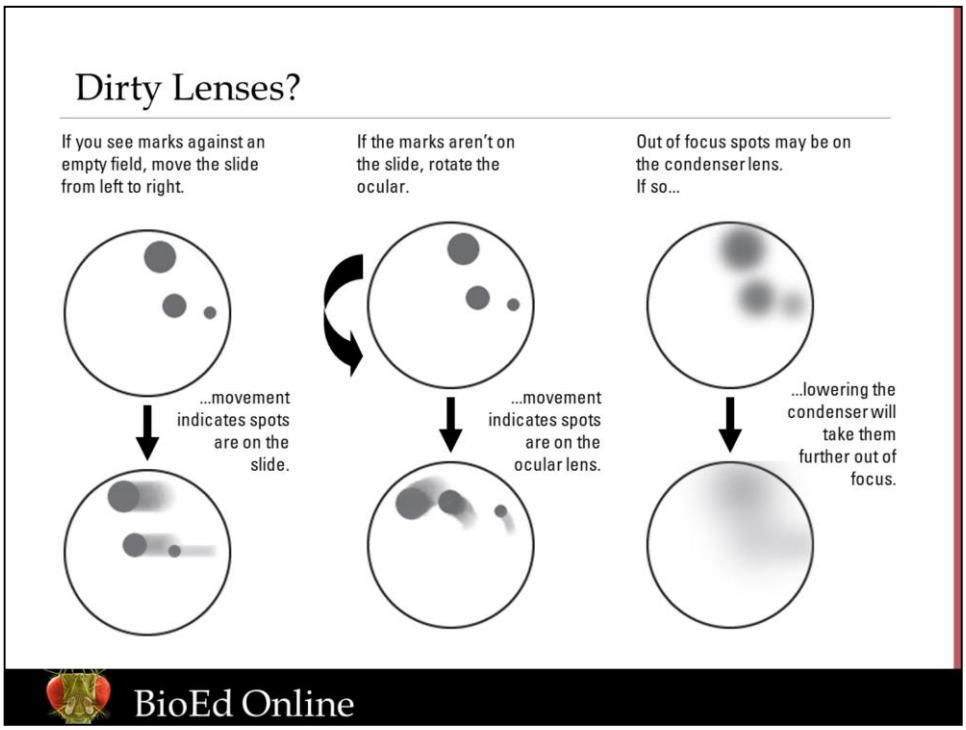

Dirty Lenses?

Unless you are working in an ultra-clean laboratory, none of your optical surfaces will be spotless. A small amount of dust shouldn't interfere with viewing at optimum resolution and contrast in bright field. However, when you have persistent shadows in the field of view, it probably is time to clean one or more of your lenses. Rather than spend the time to clean every optical surface on the microscope, you might try to determine which lens is the culprit.

Move the slide. Obviously, anything moving with the slide itself is on it and not on one of your lenses. If the slide appears to be clean, rotate an ocular in the eyepiece tube. Do the shadows move? If so, your ocular should be cleaned. If not, try the other one. Are there shadows that don't move? Is there a persistent shadow in the same place with all objectives? Chances are, your condenser lens is dirty. If you can adjust the position of your condenser, move it down. If the shadow changes, the condenser exit lens is the culprit. Otherwise, try cleaning the condenser and see if the marks go away.

If the contamination cannot be identified with condenser, slide, or oculars, then the answer by default is to clean your objective. Always clean gently, and dab rather than rub the surface. Use a 100% cotton applicator or good quality lens tissue with distilled water or very dilute acetic acid if necessary. Sometimes, a user will mistakenly leave oil on a lens surface, possibly even the high dry lens. Dried oil should be loosened by removing the objective and placing it on a surface with lens up. Place a drop of distilled water or dilute acetic acid on top and dab off after a minute or two.

If no surface can be identified as the source of the contamination, there may be internal contamination. In this case, it is time to take the instrument to the repair shop. Taking apart a lens is not recommended. A user should clean only the outside surface of any lens, including objectives, condenser, and oculars. If there is contamination inside a lens, it should be taken for professional cleaning.

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