Basics of Light Microscopy

Two concepts are necessary to get off to a good start: the importance of aligning the microscope and the electrical nature of light and matter.

A colleague once described the first microscope as "a piece of pipe with a bit of glass at each end." While today's microscopes are a bit more complicated, they can be simply described in terms of three pieces of glassware (the objective, condenser, and eyepieces) and two control mechanisms (the condenser aperture iris and the field iris). Understanding the function of each is the first step to good microscopy.

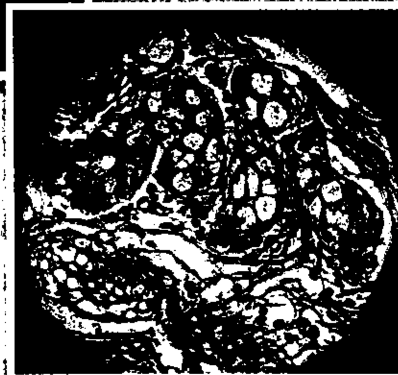
The objective is the mastermind of the microscope. Its engravings reveal its three major functions: the first step of magnification (the large number), the resolving power of the microscope (related to the numerical aperture, immediately following the magnification value), and contrast (PH for phase contrast, HMC for Hoffman Modulation Contrast, or DIC or IK for Nomarski/ interference contrast).

The condenser is the second member of the team. Its key role is to condition the light as it approaches the sample, typically by controlling the angle of approach and population of waves. Closing the iris isolates just those rays traveling directly up the axis of the microscope. Since these waves travel through the same optical path length, they will be in step or in phase with each other, creating coherent illumination.

This coherent axial illumination affects the sample in two ways. First, the pencil of light illuminates a great depth of field, well-suited for imaging thicker sections or overlaying structures. Secondly, highly coherent light is subject to diffraction at the edges of fine detail, causing the waves to change their phase relationship. When the diffracted waves meet again, those that are in step with each other will undergo constructive interference, resulting in a bright fringe, while those that are half a step out of phase will undergo destructive interference, resulting in a dark fringe that can be used to delineate an otherwise invisible edge.

Opening the condenser aperture iris broadens the population of rays. Some will travel the short path up the optical axis and others, the longer paths generated by the strong curvature of the bottom of the condenser lens. The farther from the axis, the longer the path. Opening the iris completely converts the axial pencil of light into a cone. Because of the diverse optical path lengths, the light is incoherent. With large aperture condensers, the cone has a sharp cross-over point that illuminates a shallow plane in the field, ideal for optical sectioning.

In reflected light microscopes, the objective also functions as the condenser. Since light does not occupy space, the same piece of glassware can serve both purposes—conditioning the light on the way down to the sample and then collecting the light on the way back. Although opening and closing the condenser increases and decreases the intensity, it is important to remember that the condenser controls coherence. Use neutral density filters to control intensity.



The field iris works with the baffle in the eyepieces to control the diameter of the field of view. The image (blurry, above) shows a defocused, off-center image of closed field iris, while a focused, centered image of the closed field iris is shown.

The eyepieces complete the microscope's two-stage magnification system (the large number). Most modern microscopes are fitted with Ramsden-type eyepieces, characterized by a rim or small ridge mounted about a third of the way up from the open bottom of the eyepiece. The inner diameter of that opening determines the diameter of the observable field of view and is annotated by a field number (mm), engraved next to the eyepiece magnification. To determine the actual field diameter, multiply the field number by 1,000 to convert to micrometers, then divide by the magnification of the objective being used.

The second control mechanism is the field iris, typically located either around the light port in the base or adjacent to it. While the condenser iris controls the angle of light approaching the sample, the field iris works with the baffle in the eyepieces to control the diameter of the field of view.

In the mid 1800s, microscopes made the transition from featureless sources, such as the sun or kerosene lamps, to the coiled tungsten filaments of electrical lamps causing serious artifacts in the image. August Koehler, a key influence at the Carl Zeiss works, suggested a new alignment procedure to solve the problem. Setting

Koehler is critical because it establishes a baseline for everything else you do in the microscope and should be done at least daily, then fine tuned for each view.

Set the focus on the eyepieces to zero and rack the condenser up so that it nearly touches the back of the slide. Adjust the spread of the binoculars so that you see one round field of view.

While looking outside the microscope, use the coarse focus to bring the objective very close to the specimen. Note which direction you turned the focus knob then, while peering into the eyepieces, turn the knob the other direction ("focus away"). This approach guarantees that you will always find the plane of focus and that you won't damage either the objective or the sample.

Close the field iris. Look into the microscope and, using the condenser focus, sharpen the image of the field iris against the image of the specimen (again, focus away). Next, using the condenser centration screws (located at 5 and 7 o'clock) center the image of the field iris. Check the centration by opening the iris until the edges of the leaves just hit the edge of the field of view, fine tuning if necessary. The field iris is typically set just outside the field of view but should be adjusted to fit the scatter from the sample.

This is also a good time to set the condenser aperture. Start with the aperture completely open then slowly close it until the edges of fine detail become well defined. When the aperture is correctly adjusted the image will have optimum clarity and sharpness.

Leave the diopter setting at zero for your dominant eye. Adjust the diopter for the other eye until it sees the same sharp image you see with the dominant eye.

Note that we have not included lamp centration in this particular Koehler setup. The lamp should be aligned every time you change a bulb, but should stay constant in between.

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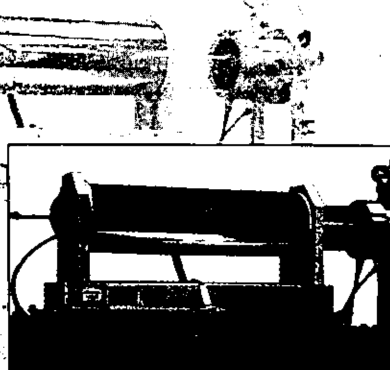

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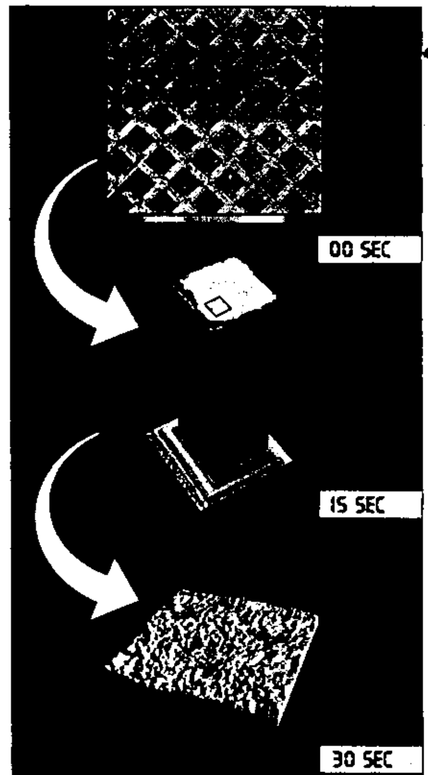

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Matter's electrical nature comes from the internal arrangement of atoms, molecules, and ions. If the internal electrical field is homogeneous in all directions, the matter can be classified as isotropic (same in all directions). On the other hand, if the electric field is stronger in one direction than another, the light will exhibit an electrical "grain," similar to that of wood, and is classified as anisotropic (not the same in all directions).

Light's electrical nature is derived from its description as electromagnetic radiation, traveling in a wave pattern through space. Our major concern is the electrical component.

When light interacts with matter, their electrical fields interact. The strength of the interaction is measured by refractive index, RI or n: the relationship of the velocity of light in vacuum or air (a constant, 300,000 km/sec) to the velocity of light in a material.

Although RI is not widely discussed, it is pivotal to microscopy. Differences in RI make boundaries between features and the background visible or invisible, forming the foundation for contrast enhancement techniques such as selective immersion, phase contrast, Hoffman Modulation contrast, and differential interference contrast. Selective immersion, for example, adjusts the contrast (visibility against the background) by increasing or decreasing the difference in RIs.

These differences between the sample and its surrounding also form the basis for refraction and define how lenses work. Difference between two internal RIs is central to the entire study of polarized light microscopy.

When the electric field in light interacts with the field at the edges of a feature, the light bends or diffracts, changing its optical path. Both the light diffracted by the sample and undiffracted background light are responsible for image formation.

The numerical aperture engraved on the objective describes the ability of that lens to collect the diffraction pattern and explains why an investment in good optics is important. Numerical aperture (NA) is the product of the sine of half the collecting angle and the refractive index of the fluid: $NA = n \sin \alpha$. The resolving power of the microscope depends on two factors, NA and the wavelength of light used:

$$R = \frac{(1.22 \times \lambda)}{(\text{NA objective} \times \text{NA condenser})}$$

Where R = resolution
λ = wavelength of light used

Resolution is defined as the smallest separation between two objects that permits them to still be imaged independently. When objects are closer than this limit, their imaging information merges, producing a blob.

—Barbara Foster

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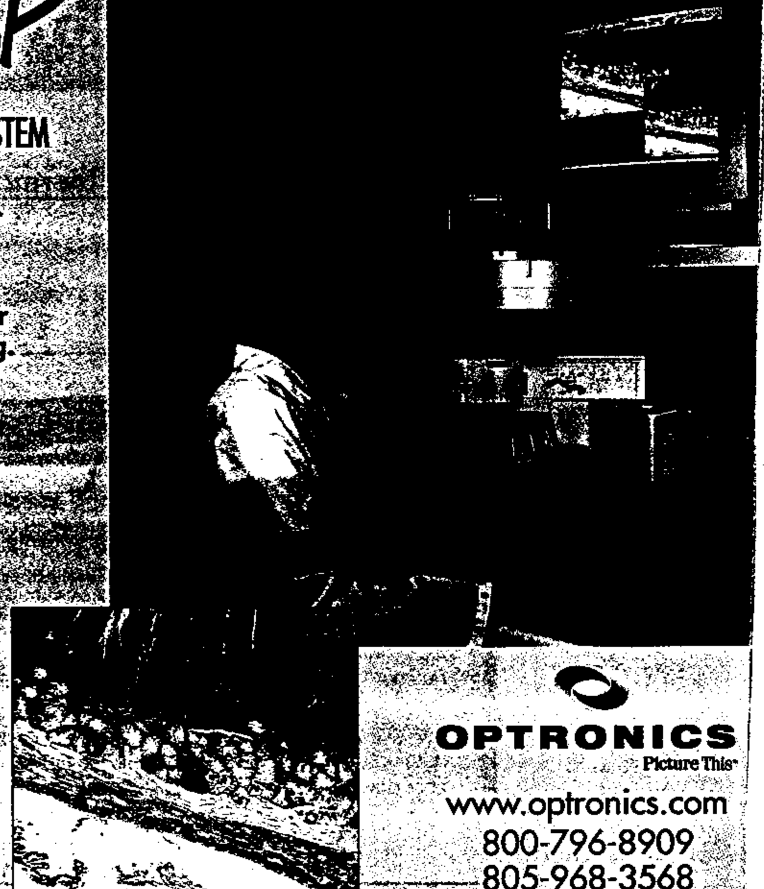

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