Introduction to Light Microscopy

Introduction

Light microscopes are important instruments not only for cell biologists but also for scientists in many other disciplines as well. Modern research requires the use of microscopes to observe objects too small to be resolved with the naked eye.

Magnification and Resolving Power of Microscopes

In the most general sense, lenses are devices for altering the direction of waves or particles (usually photons or electrons) in such a way as to form images.

The magnification of a lens or of a system of lenses is simply the factor by which the image is made larger than the original object. There is virtually no limit to the magnification that can be achieved with any microscope. For example, one can project a microscopic image on a wall and thus magnify it greatly. However, there is a real limit to useful magnification. Beyond a certain magnification, the image appears "fuzzy" and is said to have "empty magnification"; this fuzziness is due to the resolving power (or limit of resolution) of the particular lens system in use.

The resolving power of a microscope lens is defined as the ability of the lens to distinguish two particles and render them visible as two spots rather than one. The resolution is the smallest distance two objects can approach one another and still be resolved as two distinct objects.

Numerical aperture is said to be the "light gathering capability" of a lens. The refractive index is a measure of the extent to which light is slowed down by a given medium. For a given frequency and a given medium, we can measure the refractive index $n = \frac{V_0}{V}$, where $V_0$ = the velocity of light in a vacuum and $V$ = the velocity in the medium.

![Diagram of objective lens and specimen](image)

The resolution ($R$) of an objective lens depends upon two variables: the numerical aperture (N.A.) and wavelength ($\lambda$):

$$ R = \frac{0.6 \lambda}{N.A.} \quad \text{which is the same as} \quad R = \frac{0.6 \lambda}{n \sin \phi} $$

$$ \phi $$

objective lens  \quad NA = n \sin \phi

 specimen  \quad n = \text{refractive index of the medium} \quad n = \frac{V_0}{V}$

$$ n = \frac{\text{velocity of light in a vacuum}}{\text{velocity in the medium}} $$

$\phi$
The smaller the value of R, the greater the resolving power. In practical microscopy both the condenser and objective numerical apertures are important, and working aperture (W.A.) is substituted for N.A. in the above equation. The resolving power can be increased by about 50% by immersing the object and the lower objective surface in a medium of high refractive index such as oil. This increase in resolving power is due to the high refractive index of oil, 1.515, compared to air, 1.000 (see formulas above).

If a very thin "pencil" of parallel light rays strikes a microscopic object, some of the light continues in the original path as the "undiffracted light". Some of the light is scattered or diffracted by object details, and this light diverges at various angles from the undiffracted light. The finer the detail in the object, the greater the diffraction angle.

Figure 1: Light diffracted by a microscopic object in a bright-field microscope
Object detail appears in a microscope image only when the diffracted light from that detail passes into the objective lens. This is why the numerical aperture is so important in resolving power: the greater the N.A., the greater the angle at which light is accepted, and therefore, the smaller the R. This equals greater resolving power. The diffracted and undiffracted light interfere to produce contrast (lights and darks) in the image.

Contrast of Microscopic Objects

Microscopic objects must exhibit contrast in order to be perceived by the eye. Contrast is defined as a measure of the gradation in brightness that provides gray scale (or color) information under optimum conditions. The human eye can detect the presence of 2% contrast (50 gray levels). Contrast allows an object to be distinguished from its surroundings and can be just as important a limitation as resolving power.

Phase Contrast Microscopy

The bright-field microscope can be used for looking at brightly stained slices of fixed biological materials. However, most things that are alive, especially cells, are hardly detectable unless they are stained with a vital dye. An object that is visible only because it absorbs light is called an amplitude object. One that does not absorb, but rather bends, retards or advances light waves in relation to others nearby is said to be a phase object (because it phase-shifts light waves). Many objects are mixed phase-amplitude objects.

The bright-field microscope is mainly suitable for examining amplitude objects. The phase contrast microscope was the first contrast generating system readily available for the study of phase objects. Many kinds of microscopes are now available for generating contrast. We will be working with the phase contrast microscope in this lab.

Light Waves and What Phase Objects Do to Them

To understand how contrast generating systems work, we must consider light as waves rather than as rays of photons. A phase object is simply one composed of material with a refractive index unlike its surroundings (environment). The term "phase" object refers to the fact that the light waves passing through such an object are shifted in phase ("or phase shifted") relative to those passing near the object through the surrounding area (Fig. 2).
How a Phase Contrast Microscope Works

The phase contrast microscope works by separating the diffracted (light which passes through the specimen) and undiffracted light (light which passes by the specimen) so that we may then modify their relationship. We will then recombine these wavelengths to generate contrast and produce an image that can be seen by the eye.

Two components that allow the diffracted and undiffracted light to be separated, modified, and then recombined are added to a bright-field microscope. The first is a phase annulus, a black painted plate with an annular (ring-shaped) window, which is placed in front of the condenser (see Figure 3). The second component of a phase contrast microscope is the phase ring in the objective lens. The phase ring has two features. First, the glass in the phase ring is thinner than the rest of the glass in the objective lens. Second, the ring has been coated with silver to absorb light, which allows for better contrast.

The phase annulus can be seen by removing the condenser and turning it over. In our microscopes there is a separate annulus for each objective. When the annulus is in the light path, the specimen is illuminated with a hollow cone of light (your TA will demonstrate this for you).

Illuminating the specimen with a hollow cone of light, directs the undiffracted light to the phase ring. The undiffracted light is allowed to pass through the lens more quickly because this area of the objective lens is thinner. Therefore, the undiffracted light is advanced by 1/4 wavelength relative to the diffracted light.

Figure 2. Light waves passing through a cell: [A.] A fixed and stained cell (amplitude object) showing a reduction in amplitude of waves of light passing through it. Waves passing through the stained cytoplasm are reduced to half the background amplitude (\(l/2\)). Those passing through the heavily stained nucleus have been completely absorbed and emerge with zero amplitude. [B.] A living unstained cell (phase object) illustrating retardation. Those waves passing through the full thickness of the cytoplasm have been retarded by \(1/4\). [Note that these diagrams exaggerate the situation] (From K.F.A. Ross, Phase Contrast and Interference Microscopy, St. Martin's Press, Inc., 1967.)
Living, unstained preparations yield changes in the phase of the diffracted light as it passes through a specimen. In biological specimens, the phase of the light passing through the specimen is retarded (shifted) by an average of 1/4 wavelength. That is to say, the light passing through the specimen is retarded by 1/4 wavelength relative to the undiffracted light. This shift in phase occurs because the refractive index of the specimen is different than the refractive index of its surroundings. The eye cannot detect this phase shift.

The phase microscope has allowed us to advance the undiffracted light by 1/4 wavelength; the specimen has retarded the diffracted light by 1/4 wavelength. When the undiffracted light and the diffracted light are recombined, they will be 1/2 wavelength out of phase. This 1/2 wavelength phase shift converts the phase shift to an amplitude change. **The sum of the two wavelengths now results in a change in intensity, which can be seen by the eye.**

![Phase Contrast Microscope Diagram](image)

Figure 3: Components of a phase contrast microscope
The Importance of Köhler Illumination

In 1893, August Köhler introduced a simple way to adjust a microscope so that the best resolution, contrast, and image quality are obtained. This is accomplished by achieving uniform (parallel) illumination from nonhomogenous sources (such as the newly introduced electric lamp Köhler used rather than sunlight reflected off a flat mirror).

When sitting down to use a compound microscope, the first thing you should do is to align the illumination pathway of your microscope. A properly aligned microscope will insure that your specimen is illuminated with an evenly lit field of focused light and allow you to maximize the resolution capability of your microscope. This is accomplished by following the steps to Kohler illumination.

Directions for achieving Köhler illumination.

The steps to achieve Köhler illumination are easy to follow. For our purposes, the principles of Köhler illumination can be reduced to focusing the image of the field diaphragm in the plane of the specimen and adjusting the height of the condenser. Follow the five steps below, to achieve Köhler illumination.

1. Using the 10X objective, focus on a prepared slide of a specimen given to you by the TA.
2. Close the field diaphragm.
3. Adjust the condenser height so that a sharp image of the field diaphragm appears in the plane of the specimen.
4. Once the condenser height has been set correctly, center the image of the field diaphragm. To make fine adjustment to the centering, open the field diaphragm until the edge of the field diaphragm image is near the edge of your field of view. If the image of the field diaphragm is not centered, re-center the condenser.
5. Open the field diaphragm just beyond your field of view.

Ideally, Köhler illumination should be adjusted for each objective.

Precautions on Handling and Cleaning Microscopes

These microscopes are some of the finest microscopes available. Please take excellent care of these microscopes.

The greatest damage done to microscopes is usually during cleaning. The reason is that most kinds of tissue paper contain glass fragments (diatomaceous earth) as "filler". Rubbing such fragments (or dust) across the lower surface of an objective lens (or upper surface of a
condenser) can leave a scratch many times larger than the object detail being examined. One such unlucky wipe can "wipe out" a lens.

To avoid this disaster, clean by holding **lens paper only** flat and parallel to the lens surface. Deposit one drop of “Sparkle” solution on the paper and gently move the paper over the end of the objective causing the Sparkle to flow over the lens and cleaning it. Using a new piece of lens paper, gently draw the paper over the end of the objective to remove any residual Sparkle.

Ocular surfaces should be cleaned only with lens paper, which contains no filler. Oculars should be cleaned with 100% ethanol or Sparkle when you have finished using the microscope for the day.

Do not remove oculars, objective or condensers from the microscopes.

When focusing down close to the specimen with higher power objectives, be careful. Look at the preparation from the side and go slowly. Use only the fine focus when looking through the microscope.

Avoid wearing eye make-up or eye creams on laboratory days. Avoid fingerprints on lens surfaces.

If you have an eye infection, please speak with Professor Smith before using a microscope.

**References**


Laboratory Assignment:

Objective: In this laboratory you will generate images using bright-field, phase contrast, differential interference (DIC) and fluorescence microscopy. To generate these images you will learn how to use a Nikon E600 microscope, a digital camera and imaging software. You will not prepare a formal laboratory report for this exercise. It is important that you become comfortable working with these microscopes, as you will be using them throughout the term. Although several exercises are listed below, no specific instructions are included. Instructions will be given during class. Professor Smith, representatives from Nikon, and laboratory staff will be on hand to assist you. Our primary goal is for you to get acquainted with the microscope and have fun!

1. In the laboratory you will find prepared slides of various tissues that have been fixed, embedded, sectioned and stained. These slides will be used to practice bright-field microscopy and Köhler illumination.

2. A live culture of *Chlamydomonas reinhardtii* will available for preparing slides. This unicellular alga swims using two flagella. In unstained, living preparations, the two flagella are not visible using bright-field microscopy, but are easily viewed using phase contrast microscopy.

3. For fluorescence microscopy, we have purchased prepared slides of samples labeled with three fluorophores. When looking through the microscope, you will only be able to view one fluorophore at a time. In addition, the cameras are monochromatic, so the image you view on the computer monitor will be in shades of gray. These images can be colorized and merged using the imaging software.

4. To demonstrate DIC microscopy, you will prepare a slide of your own cheek cells. Compare the DIC image of the cells with a phase contrast image of these cells.

For more information about microscopy, check out: [http://www.microscopyu.com/](http://www.microscopyu.com/)
Parts of the Nikon E600 Microscope

A. eyepiece
B. fluorescence shutter
C. slider for fluorescence filter cubes
D. objectives
E. stage
F. condenser
G. field diaphragm
H. focus adjustment knob
I. X&Y stage controls
J. halogen lamp power switch
K. halogen lamp housing
L. mercury lamp housing
M. beam splitter