READING ASSIGNMENT

WHAT IS LIFE?

What can we learn from a microscopic study of cell structure and behavior?

I. HOW DO MAGNIFICATION AND RESOLUTION DIFFER?

II. HOW CAN WE USE THE MICROSCOPE TO MEASURE SIZES?

III. WHAT DO WE NEED TO KNOW ABOUT CARE AND MAINTENANCE OF THE COMPOUND AND DISSECTING MICROSCOPES

I. HOW DO MAGNIFICATION AND RESOLUTION DIFFER?

After reading this section, you should be able to:
1) Distinguish between magnification and resolution.
2) Calculate resolution if given the formula.
3) Understand how to get maximum resolution from a light microscope.
4) Understand why an electron microscope can produce better resolution than a light microscope.

A. What Is magnification?

Objects can be magnified using lenses.

In general magnification is measured as:

$$\frac{\text{size of the image}}{\text{size of the object}} = \text{magnification}$$

If you've ever used a magnifying glass or lens, you know that you can vary the magnification by varying the distance of the lens from the object being magnified. In this case, the resolution capability of the lens remains constant, but the magnification used can vary.
There are no limits to magnification, you can always magnify an object and make it appear larger. On the other hand, there are specific limits to the resolution of any given lens or lens system.

When using a compound microscope (one with both an objective lens and an ocular lens), magnification can be calculated as:

Ocular Magnification X Objective Magnification = Total Magnification

10x X 10x = 100 x

Using this example, the microscope magnifies the image 100 times, or the diameter of the image is 100 times larger than the diameter of the specimen (Figure 1).

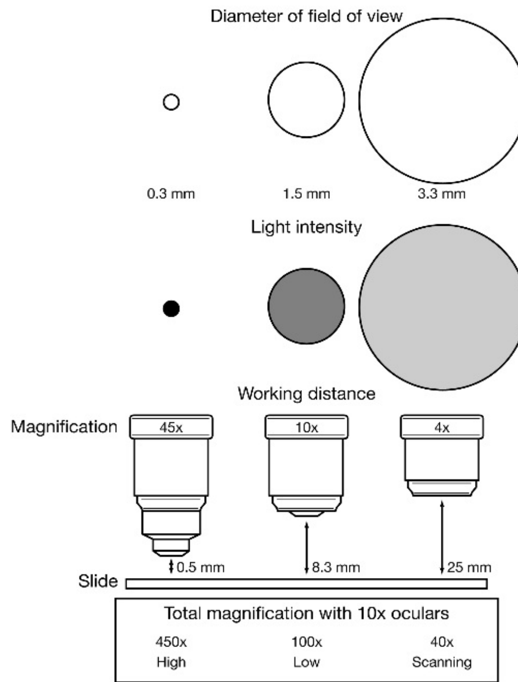
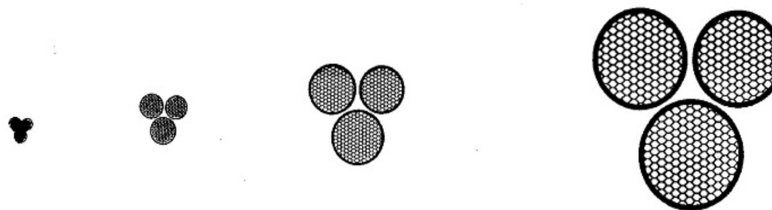


Figure 1. An example of how light intensity, working distance and field diameter vary with different power objectives.

B. What Is resolution?

Resolution is the ability to discern two dots (or images) as being separate from each other (Figure 2).

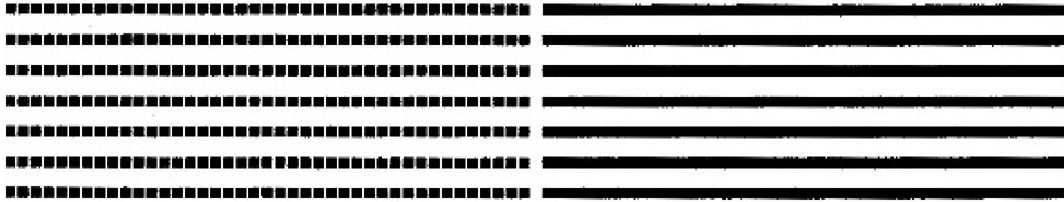
a) Increased magnification without increased resolution



b) Increased magnification and increased resolution

Figure 2: An example of the difference between increasing magnification alone (a) and increasing both magnification and resolution (b).

To give you a specific example of resolution, look at the dots and lines below with your eyes about 12 inches from the paper. Can you can see them as separate? If you now look at the page from a distance of 6 to 10 feet or more many of the dots and lines will no longer be seen as distinctly separate, therefore, they are not resolved.



The resolution distance of a microscope is determined by the wavelength of the source of illumination (light or electrons) and the numerical aperture of the lens used, such that:

$$R_D = lambda/2NA$$

R_D	=	resolution distance in nanometers
lambda (λ)	=	the wavelength of the source of illumination and
NA	=	numerical aperture

In microscopy, resolution is more important than magnification.

When light strikes a specimen on a slide, some light passes straight through and some is bent by the specimen and goes off at an angle. The finer the details in the specimen, the greater the angle of bending (Figure 3). The light rays that are bent by the specimen are the image forming rays. The more of these that can be gathered and focused by the objective, the better the image.

Numerical aperture is an expression of the ability of an objective to collect the image forming rays. The larger the numerical aperture, the more light rays captures and thus, the better the resolution.

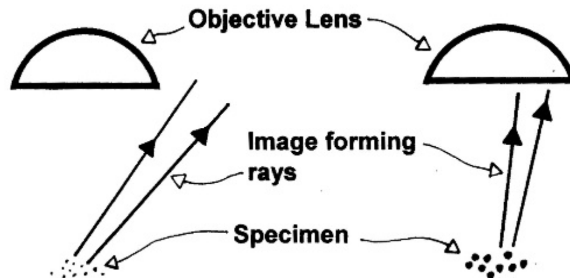


Figure 3: Effect of specimen detail on light rays. The finer the details in the specimen the greater the angle of bending. Very fine details can bend the light so much that it misses the objective altogether.

Numerical aperture can also be expressed as NA = n sine (AA/2).

The **refractive index (n)** is a constant for each medium the light passes through. For example,

the refractive index of air is 1.0, of water is 1.3 and of immersion oil is 1.5. The refractive index of immersion oil is about equal to that of the coverslip and the objective lens. Because of this, when immersion oil is placed between the coverslip and the objective, the image forming rays are bent less than they would be in air. This means, more of them enter the objective and contribute to image formation (Figure 4). As a result, resolution is better.

The **angular aperture (AA)** is the angle between the most divergent rays that can be accepted or collected by the objective lens to form a focused image. If the specimen under observation were touching the lens, the AA would be 180 degrees. In this case, the sine of the (AA/2) = sine of 90 degrees = 1. This is the maximum value of [sine (AA/2)] since the sine of any angle less than 180 degrees divided by 2 is less than 1. Generally, as the power of an objective increases, its AA increases. As a result, higher power lenses tend to have higher numerical apertures. For example, a 40X dry objective may have a numerical aperture of 0.65, while a 100X oil objective may have a numerical aperture of 1.25.

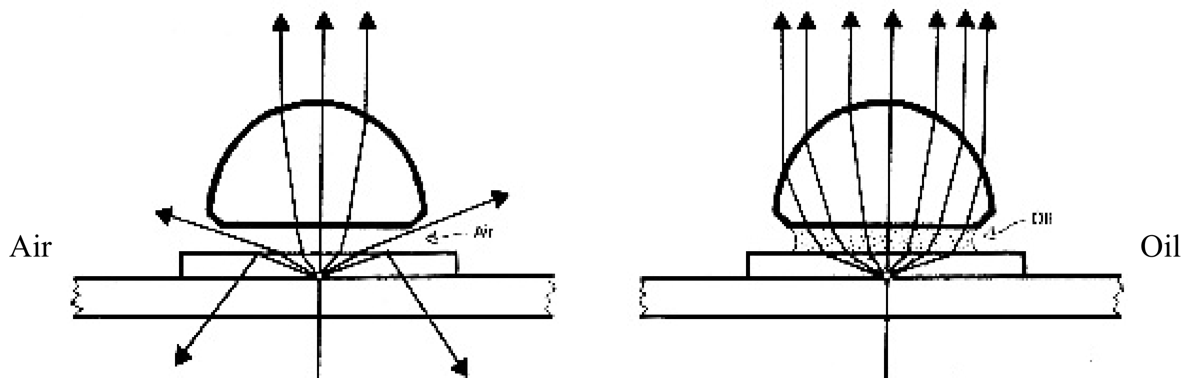


Figure 4: Numerical aperture and effect of refractive index of medium filling the space between the cover slip and the objective lens. Numerical aperture equals (n sine u', where u' equals the angular aperture divided by 2 (AA/2). On the left side of the figure, light rays from the specimen angles greater than about 20 degrees are bent so much when they pass from the coverslip to the air interface that they reenter the coverslip, that is, these image forming rays are lost to the objective. These rays (on the right side of the figure) are not bent as much when they pass from the coverslip glass to the oil and therefore can be captured by the objective lens to increase image detail or resolution.

Further improvements in resolution have been gained by addition of the **substage condenser**. The substage condenser controls the angle of the cone of light, or other illumination, which reaches the specimen. Light that enters the objective obliquely can distort lines and objects and decrease the accuracy of the image. In practice, resolving power is maximized when the condenser produces a cone of light which fills about 75% of the objective field (Figure 5).

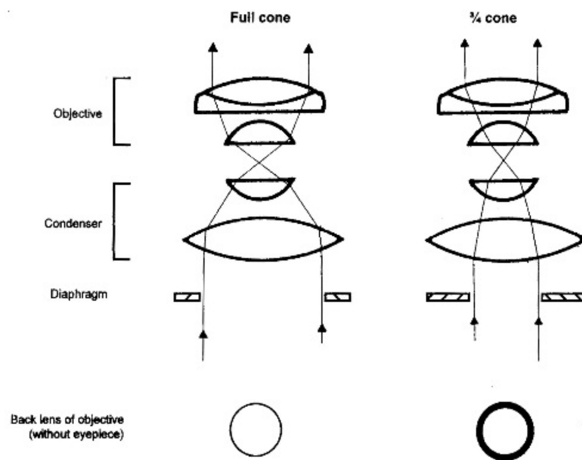


Figure 5: Illumination of an objective as controlled by the substage condenser diaphragm: (a) full cone, diaphragm open; (b) 3/4 cone (From Hodges, 1989)

There are specific limits on the resolution capacity of any microscope. In other words, whereas images can be magnified *ad infinitum*, the detail resolved will not increase beyond the limits set by the wavelength of illumination and the numerical aperture. To increase the amount of detail resolved, one must either decrease the wavelength of the illuminating source or increase the numerical aperture of the lens.

Light microscopes use light as the illumination source. Visible or white light is electromagnetic radiation with wavelengths between 400 nanometers (nm) (violet end) and 700 nm (red end) (Figure 6). White light can be separated into all of the different wavelength/colors of the spectrum by a prism or diffraction grating (as in a spectroscope).

The resolution of light microscopes may be increased by using filters which allow only the shorter wavelengths of the spectrum to pass through. For example, a blue filter would allow blue light (480nm) to pass through; a red filter would allow red light (700nm) to pass through.

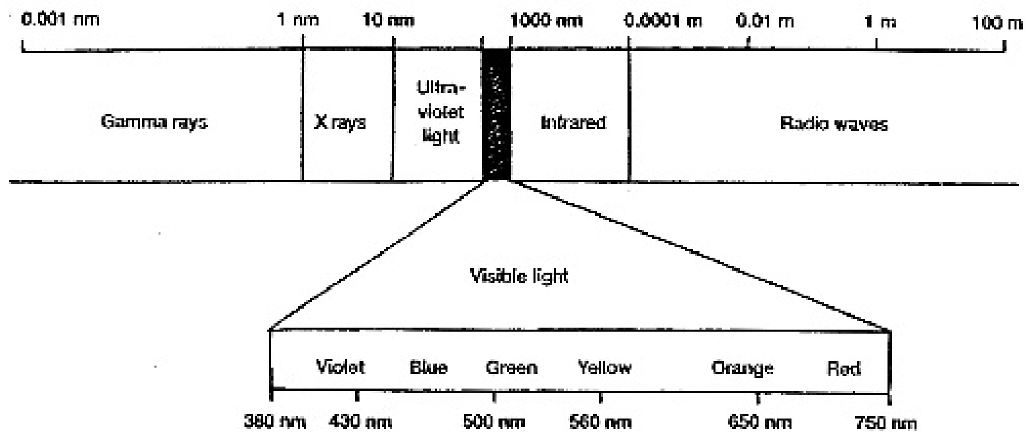


Figure 6: The wavelengths of light in the visible spectrum.

Electron microscopes use a beam of electrons as an illumination source, rather than visible light rays. The effective wavelength of the electron beam is much shorter than that of visible light and resolution power is much greater. Perhaps a better way of saying this is that R_D is even smaller because the wavelength of the electrons in an electron microscope is so much smaller.

II. HOW CAN WE MEASURE DISTANCES UNDER THE MICROSCOPE?

After reading this section you should know how to:
1) Determine field diameter using a clear ruler and a compound microscope.
2) Calculate field diameter for an objective, given the field diameter and power of another objective on the same compound microscope.
3) Examine depth of focus and understand how it can affect what you see under a microscope.

A. What Is Field Diameter and How Can It Be Used to Determine Specimen Size?

A compound microscope (the ALPHAPHOT) magnifies in two stages: at the level of the objective lens and at the level of the eyepiece or ocular lens.

The magnification of the objective lens is indicated on the side of the lens (4X, 10X, 40X). The final magnification equals the magnification of the objective lens times the magnification of the eyepiece lens (10X). A 10X objective and a 10X eyepiece would produce a total magnification of 100X. As noted before, the diameter of the image in this case would be 100x the actual diameter of the object. But what is the actual diameter? Determining size under the microscope can be a critical part of any microscopic investigation. There are several different methods available for determining size. Some examples are listed below.

To determine actual/real size of objects in your microscope field of view:

a. A micrometer disk can be placed in the eyepiece lens. By calibrating this with a stage micrometer (e.g. ruler) of known scale, specific millimeter values can be applied to the eyepiece micrometer scale. Note: These values would differ for each objective lens used.

b. Objects of known size can be placed on the slide with the specimen to be measured and a comparison can be made between the known and the unknown.

c. If the field diameter is known, a comparison between the specimen and the field diameter can be made. This is perhaps the easiest method. We will use this last method to determine size.

To determine field diameter of the lowest power objective, place a clear plastic ruler on the stage of the microscope and view it through the low power objective. Orient the millimeter marks on the ruler such that one of them lies on the left border of the field at about 9 o'clock. Count the number of millimeter marks that fall within the field to determine field diameter. For example, if you observed the following field, the field diameter would be 5.5 mm (Figure 7).

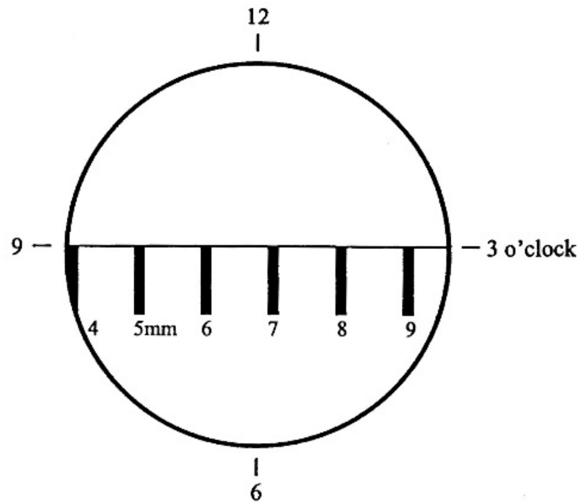


Figure 7: A clear plastic ruler viewed at low power.

You can calculate the field diameters for higher power objectives as follows.

$$\text{higher power field diam.} = \frac{\text{(diam. of low power field)(low power mag.)}}{\text{(higher power mag.)}}$$

4X objective and 10X ocular = 40X magnification and 5.5 mm field diameter

10 X objective and 10X ocular = 100 X magnification and x mm field diameter

$40X/100X = x/5.5 \qquad x = (40 \times 5.5)/100$

For higher power objectives, you will need to calculate the field diameter from this formula.

B. What Is Depth of Focus and How Does It Vary with Magnification?

As a general rule of thumb, as magnification of a lens increases, its focal length decreases. Focal length is the distance from the lens to the focal point. The focal point is the point at which light rays passing through the lens converge. This means that as magnification goes up, depth of field goes down. Therefore, if the thickness of the specimen is greater than the focal depth of the objective, the structure of the specimen in depth must be inferred by focusing up and down through successive images of the specimen.

Alternatively, serial sections of the specimen may be produced. To reconstruct the three dimensional structure from serial sections, one must first photograph each section at the same magnification. Exact measurements of section thickness are also determined. The images of the organelle are cut out of plastic film of a thickness proportional to that of each section and the magnification of the photo. Maintaining the proper orientation of each section, the individual images are then placed one on top of each other to obtain the 3-D image of the organelle.

III. WHAT DO WE NEED TO KNOW ABOUT CARE AND MAINTENANCE OF THE COMPOUND AND DISSECTING MICROSCOPES

Your instructor will provide you with a copy of the information describing care and maintenance procedures for the particular microscopes available in your laboratory.

After you read this material, you should know how to:
1) properly clean lenses.
2) replace the microscope lamp (bulb) and fuse.
3) mount a specimen on the stage and bring it into view and into focus.
4) adjust interpupillary distance and diopter.
5) adjust aperture diaphragm
6) find N.A. for each compound objective.
7) use the troubleshooting tables.
8) know eyepiece or ocular magnification.

IV. HOW DO THE TWO MAJOR CELL TYPES (PROKARYOTES AND EUKARYOTES) DIFFER?

After reading this section, you should know the major differences between eukaryotes and prokaryotes in:
1) cell structure
2) cell size

All living organisms are composed of cells. This is one of the most fundamental biological generalizations and was proposed only within the past 150 years. However, it took several decades and much laboratory experimentation to convince most people that all cells arise from pre-existing cells.

All cells can be subdivided into one of two categories based on subcellular structure and complexity. The prokaryotes lack true membrane-bound nuclei and membrane-bound organelles; the eukaryotes contain these structures. All prokaryotes are grouped into the Kingdom Monera. This kingdom includes two major subgroups, the Archaebacteria and the Eubacteria. The Eubacteria include what we commonly think of as the bacteria and cyanobacteria (blue green algae). The organisms in all of the other kingdoms of life are eukaryotes and have the eukaryotic cell structure.

Prokaryotes are structurally simple. A “typical” prokaryotic cell is bounded by a cell membrane,

contains ribosomes, 70s ribosomes, which are smaller than those of eukaryotes, and bare double stranded DNA as their genetic material or genome. The 70s indicates the sedimentation coefficient of these ribosomes during centrifugation. Eukaryotic ribosomes have a sedimentation coefficient of 80s. The 80s ribosomes are larger and heavier and sediment more rapidly than 70s ribosomes. Prokaryotic cells may also be bounded by a cell wall or a capsule. No naturally occurring sexual reproduction has been observed among the prokaryotes.

Most prokaryotes range in size from 1 to 10 µm in diameter. All are single celled or colonial in form. There are no protoplasmic connections between cells in colonies. The prokaryotic cell architecture or morphology is limited to three basic forms: coccus, bacillus and spirillus (sphere, rod and spiral shapes). On the other hand, the prokaryotes display a vast diversity of habitats. This occurs as a direct result of the vast variation to be found in the types of biochemical reactions that can be performed by the various species. In fact, there is almost nowhere on earth that prokaryotes of one or another species cannot be found. Among the different species of prokaryotes, one can find many heterotrophs, some photosynthetic forms and others which survive as chemoautotrophs.

There are no truly multicellular prokaryotes. Only eukaryotic cells appear in multicellular organisms displaying tissue differentiation.

Eukaryotes have more complex cellular structure. This includes a double membrane-bound nucleus containing chromosomes of DNA complexed with protein. Many of the other organelles found within eukaryotes are also membrane-bound, e.g., mitochondria, chloroplasts, vacuoles. These membranes are each selectively permeable. This allows for differential accumulation of substances and specialization of functions among the organelles of a single eukaryotic cell.

Most eukaryotes range in size from about 10 to 100µm in diameter. Whereas prokaryotes tend to be biochemically diverse and structurally similar; eukaryotes tend to be biochemically similar and structurally diverse. Only eukaryotes display true multicellularity and sexual reproduction.