Biomedical Engineering Laboratory I (Fall 2025)

Lab 1 Introduction to Microscopy[1]

OBJECTIVES

1. Be familiar with the components and operation of an optical microscope.
2. Determine the true magnification of a microscope objective lens.
3. Use optical diffraction techniques to examine the resolution and to alter the numerical aperture of a microscope objective.
4. Learn the utility of the phase contrast setting on an inverted microscope.

BACKGROUND

Microscopes are essential tools in biomedical imaging. You have probably used an optical microscope at some point during your education or prior research experience. Accordingly, you likely have some appreciation for the advantages of microscopic imaging. In this procedure, you will be introduced not only to the utility of microscopes, but also the underlying optical principles that govern the visualization of an object on a stage to the magnified image that is viewable via a scope's eyepieces. Upon completion of this procedure, you should be familiar with the components of a microscope, the underlying optical principles of microscopy and resolution, and the utility of the phase contrast setting in biomedical imaging.

History

There is no definitive inventor of the optical microscope; the earliest manifestations of using light manipulation to visualize small objects can be traced to approximately 1000 AD. In 1590, Dutch opticians Zaccharias and Hans Janssen (father and son) discovered that when multiple lenses were placed in a tube, images of objects in front of the tube were enlarged. This was the earliest implementation of compound microscopy: the use of multiple lenses to augment image visibility (Figure 1). The Janssens' work was the basis for the telescope, which was refined by Galileo in the early 17th century. During the 17th and 18th centuries Anton van Leeuwenhoek

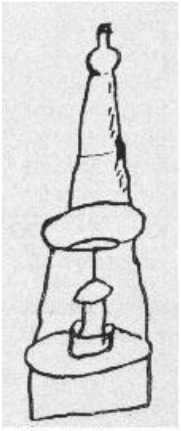

Figure 1. Earliest known drawing of a microscope.

[1] Adapted from Prof. Aaron M. Kyle, Columbia University.

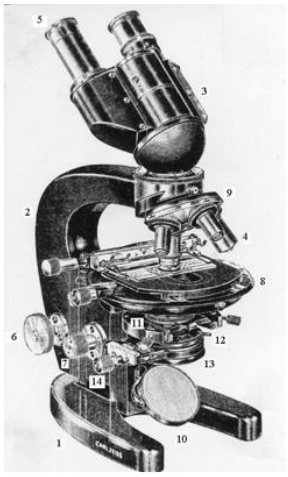

Figure 2. Zeiss Model L, forbear of modern optical microscopes.

developed lenses that were capable of identifying microscopic components such as yeast or blood constituents in capillaries. Van Leeuwenhoek's findings were expanded upon by Robert Hooke, who first coined the term "cell" to describe the structural mesh that he observed when imaging a slice of cork in a microscope. Hooke's work was the earliest identification of plant cells. Hooke's seminal work, Microphagia (1665), provided detailed accounts on his early experiments with compound microscopy. In the late 19th century, German instrument maker Carl Zeiss working with physicist Ernst Abbe wrote a comprehensive treatise on the theory of the microscope and lens design. This public disclosure along with the development of new types of glass elevated the ability of microscope/lens developers to maximize the resolution of their images and led to the formation of the modern optical microscope (Figure 2).

Basic Concepts and Components of a Microscope

1. Eyepiece – cylinder(s) containing lenses that are used to project images from the objective onto the retina.
2. Objective(s) – lenses mounted on a wheel to collect light from a sample and provide initial magnification.
3. Brightness Adjustment Knob – adjusts the intensity of the bright field light source.
4. Coarse and Fine Focusing Knobs – adjusts the vertical position of the objective with respect to the sample. Changing the knob position changes the focal plane of interest in the sample.
5. Stage – platform above the objective onto which the sample is placed. Stage has a hole through which light passes for illumination and image acquisition.
6. Light Bulb – provides bright field illumination to the sample.
7. Phase Contrast Slider – annular ring that focuses light for phase-contrast imaging or regular bright field imaging.

Figure 3. An Olympus CKX53 Inverted microscope.

Each element of the microscope will be used and manipulated throughout this procedure. Be sure to become familiar with each scope components and their purpose as we will continue to use this device in

subsequent procedures and courses.

Modern optical microscopes have a two-stage magnification scheme that utilizes lenses in the scope's objective and eyepiece. The objective is comprised of lenses that act in a fashion similar to a magnifying glass. A sample is placed on the microscope's stage and the objective is brought to a distance close to the sample, i.e., within the lens' working distance. Light that is reflected from or transmitted through the sample is captured by the objective and magnified into a real image that is larger than the original object. This inverted image is projected along a finite tube length and is focused onto a fixed plane in the eyepiece. Accordingly, the image that reaches the eyepiece is the magnified, inverted, real image projected by the objective. The image is further magnified by the eyepiece and projected as an erect image onto the retina. The combination of the objective lens and eyepiece form what is known as a finite tube length microscope (Figure 4a).

The finite length design is the basis for infinity corrected lenses, which are commonly used in modern microscopes. The sample is positioned at the front focal plane of the objective. Light from each azimuth of the object is brought into focus by a tube lens which is located between the objective and the eyepiece (Figure 4b). The resultant magnification from the objective and the tube lens is equal to the focal length of the tube lens divided by the focal length of the objective lens in use. In this procedure, you will determine the true magnification of an infinity corrected lens system using measurements at the eyepiece.

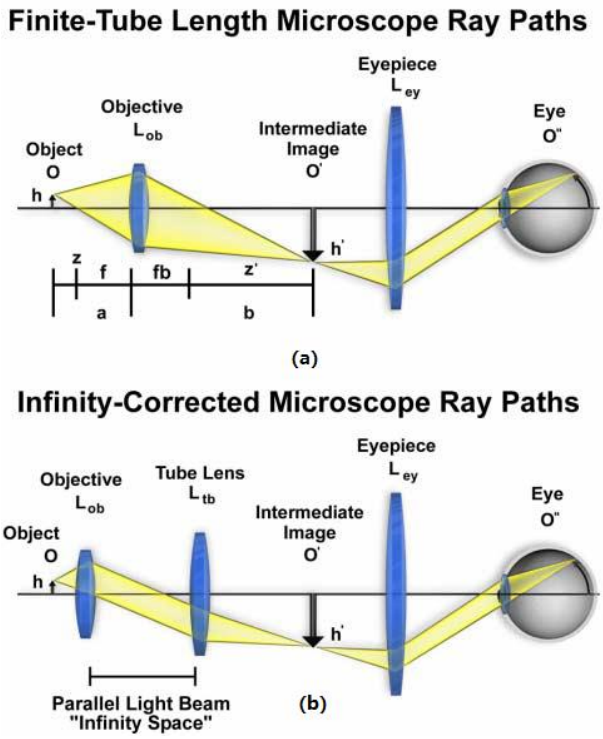

Figure 4. Schematic illustrations of (a) finite tube length and (b) infinite tube length microscopy ray paths from [2].

Resolution

The resolution of a microscope is the smallest distance between two distinguishable features. When a sample is examined at the resolution limits of a microscope objective, the resultant image may be distorted. This distortion may compromise your ability to accurately distinguish objects under microscopic examination. The resolvable distance between objects on a microscope is governed by a number of factors

including the coherence of illumination, the spectrum of light used to image the sample, or the type of specimen under examination.

Resolution is quantitatively represented by the numerical aperture of an objective. The numerical aperture (NA) is the measure of an objective's ability to gather light and resolve features of an object.

$$NA = nsin\theta \qquad (1)$$

Where n ~ the refractive index of the media between the sample and the objective and θ ~ angle of the cone at the sample.

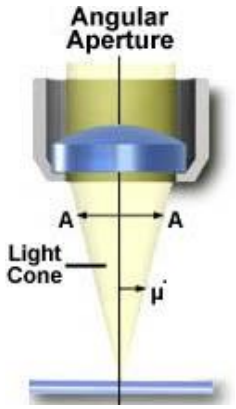

Figure 5. Illustration of cone of light from sample diffraction gathered by an objective lens. The NA quantifies the breadth of diffracted light gathered by the objective [3].

Light passing through a sample forms an inverted cone shape that is gathered by the objective (Figure 5). When there are multiple objects on a sample having light passed through them, e.g., a grating or organelles in a cell, the resultant cones create diffraction patterns and interferences in brightness. The NA represents an objective's ability to gather the light from these diffraction patterns: As the numerical aperture decreases, less of the diffracted light is collected and the objects become less resolvable. In essence, the NA is the objective's ability to collect information from the illuminated sample [2-3]. As the NA increases, more light is collected and the ability to resolve small objects is enhanced. The ability to resolve an image distance (d) at a wavelength (λ) is described by the Abbe diffraction limit:

$$d = \frac{1.22\lambda}{2NA} \qquad (2)$$

In this procedure, you will effectively alter the NA of a 10x objective and determine how these changes affect resolution and image quality.

Phase Contrast Microscopy

When light passes through certain materials, the diffracted light propagates more slowly. This slowing,

or phase shift, is undetectable to the naked eye. In the 1930s Frits Zernike developed a method to augment the phase shifts that arise as light passes through a specimen (a finding that resulted in Zernike receiving the 1953 Nobel Prize in Physics). This process is known as phase contrast microscopy. Phase contrast microscopy involves separating the undiffracted, direct light from light that is diffracted as it traverses a specimen. This is accomplished by passing condensed light from a phase ring (or condenser, Figure 3) through a sample, collecting the resultant direct and diffracted light with an objective, then passing the direct light through a phase plate, which creates an additional λ/4 phase shift between the direct and diffracted light (Figure 6). This shift causes the direct and diffracted light to destructively interfere, causing the sample to appear dark against a lighter background. Phase contrast effectively causes the phase differences between direct and diffracted light to appear as differences in intensity that are detectable by the eye. A more detailed discussion of phase microscopy can be found in [2].

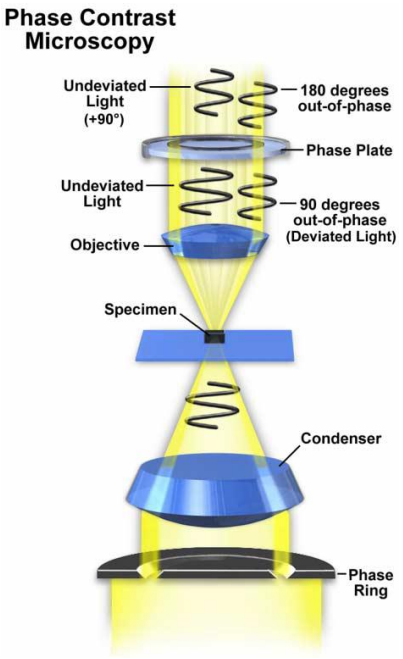

Figure 6. Illustration of direct (undeviated) and diffracted (deviated) light path from sample under phase contrast microscopy [2].

Phase contrast microscopy is particularly useful in biomedical imaging. Many biological materials, such as mammalian cells, single cell organisms, or organelles are transparent under bright field conditions. Phase contrast provides a means of discerning cells and cellular components. Additionally, phase contrast does not require any cell fixatives or staining, facilitating unperturbed examination of the cell cycle. In this laboratory you will examine samples under standard and phase contrast conditions to demonstrate the benefits of the latter.

Fluorescence Microscopy

Often when examining biological specimen, there are structures or processes that cannot be readily identified using the bright field alone. Fluorescence microscopy may be used when examining biological samples to uncover additional information about the sample. The governing principle of fluorescence microscopy is the Stokes' shift, that is, when certain materials are excited (irradiated) with light at a particular wavelength, the material will emit light at a higher wavelength due to energy lost as electrons

relax from an excited state to the resting state. In microscopic applications, materials called fluorophores (or fluorochromes) are integrated into a sample. The fluorophores have well-defined excitation-emission (Ex/Em) profiles (a listing of fluorophores commonly used for cell culture can be found here.) Briefly, a fluorophore is added to a sample by staining, protein tagging or immunofluorescence. The sample is placed on a microscope stage and irradiated with a specific band of light, exciting the fluorophore. The microscope is designed such that emitted light is exclusively collected and transmitted through the objective for visualization. In a well-configured scope, the regions where the fluorophore is present and emitting should exclusively transmit light to the eyepieces, causing the fluorescent regions to appear bright against a black background. This allows for selective identification and tracking of fluorescently-labeled areas of the sample.

Excitation and Emission

The light path for fluorescence microscopy differs from what occurs during bright field or phase imaging. Rather than using a bulb, the light source is usually a high intensity broadband (white) light source. In order to selectively illuminate a sample at a specific band, typically with UV, blue, or green wavelengths, the light is passed through an excitation filter, which is an optical bandpass filter that selectively passes light at a particular range of wavelength (color) while reflecting other wavelengths, allowing for specific excitation of the fluorophore. This light is reflected by a dichroic mirror through the objective and onto the sample, the objective act to focus the light onto a region of interest in the sample. Emitted light from the irradiated fluorophore is collected by the objective to form a fluorescence image. This light passes back through the dichroic mirror and is subsequently filtered by an emission filter, whose is selected such that reflected excitation light or any other light outside the emission band is not passed. The emitted light is project as a fluorescent image to the eyepieces of the microscope and/or the electronic detector (CCD camera) (Figure 7).

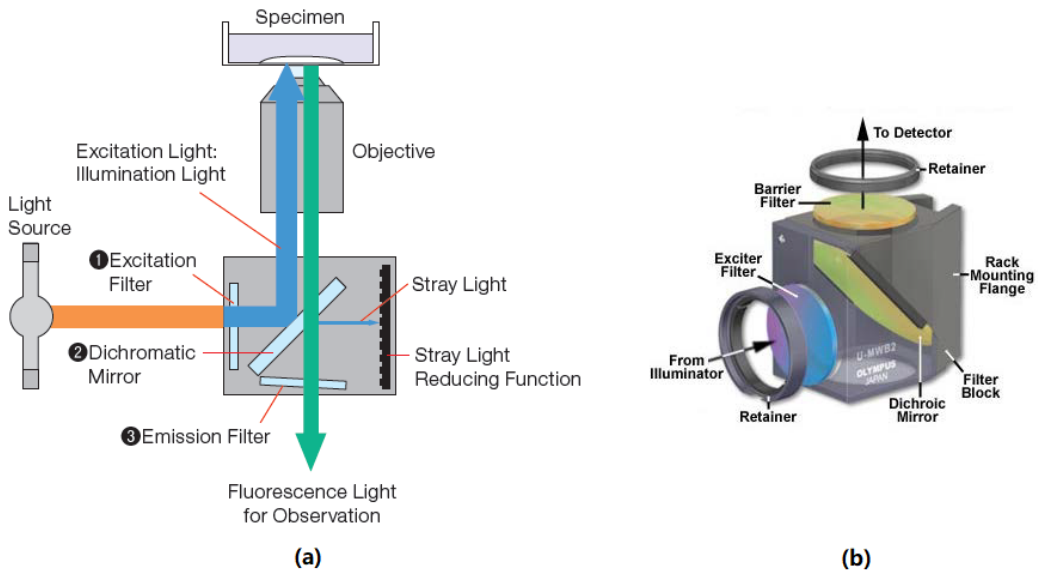

Figure 7. (a) Representative light path for fluorescent excitation of a sample and (b) filter cube through which excitation light is filtered and directed onto sample while emitted light is filtered and transmitted to ocular components.

MATERIALS AND METHODS

- Olympus CKX53 Inverted Microscope with 4x/10x/20x/40x (phase contrast) objectives
- Digital Camera
- Gratings (70 lines/mm & 110 lines/mm)
- Pinhole Apertures (Diameter = 200 µm, 500 µm, and 1000 µm)
- Microscope Slides
- MC3T3 Cells
- Fluorescent Labeled Cell Slides
- Rulers
- White paper

LABORATORY PROCEDURES

Procedure 1: Measure the True Magnification of an Objective

A microscope objective lens (the lens closest to the object or sample) is used to produce magnified images of small objects. These images are typically observed via the scope's eyepieces, but may also be projected onto a screen, a piece of paper, or the CCD of a digital camera.

1. Place a transparent ruler onto the stage of the microscope.
2. With a 4X objective, observe the ruler using the eyepieces. Use the coarse and fine focusing knobs to

obtain the correct focal distance.

3. Remove one of the eyepieces and put a piece of paper over the hole. Trace the projection of the image on the paper.
4. Measure the size of the magnified ruler image as it is projected onto the paper at the eyepieces. Use this measurement to determine the measured magnification (m_{meas}). *Have all your team members do the measurement and collect the m_{meas} (n= 4 or 3) and present the resultant average ± std. dev.*
5. Measure the distance between the objective lens and the sample (o).
6. Measure the distance between the objective and eyepieces (i). Use the equation (3) to calculate the theoretical magnification (m_{theo}):

$$|m| = \frac{i}{o} \quad (3)$$

Have all your team members do the measurement and collect the m_{theo} (n= 4 or 3) and present the resultant average ± std. dev.

Statistically compare the measured magnification to theoretical magnification. Do the values match? If not, explain what might account for any discrepancy.

Procedure 2: Alter the Numerical Aperture of an Objective

The numerical aperture of an objective quantifies how much optical information is collected from an illuminated sample. In this procedure, you will alter the NA of a 10X objective and examine the effects of these changes on the image quality.

1. Open either 'Ocular' or 'Capture' software.
2. Place a 70 lines/mm grating on a cover slide and place the resultant assembly on the stage of the microscope.
3. Using a 10X objective, bring the gratings into focus.
4. Capture a digital image of the grating. Note that additional adjustment might be necessary to obtain an in-focus image via the camera.
5. Remove the grating-cover slide and measure the distance from the objective to the position of the grating. **DO NOT ADJUST THE FOCUS FROM THIS POINT FORWARD!**
6. Place a 1000 µm pinhole aperture directly over the objective lens.
7. Put the grating-cover slide back and obtain another image of the grating.

8. Repeat Step 7 with a 500 µm and a 200 µm pinhole aperture respectively. *Comment on any changes in the appearance of the gratings with different aperture sizes. Why does the image change?*
9. Repeat steps 2 - 8 using a 110 lines/mm grating. *Comment on any image distortion that occurs with the various apertures and how this differs from the 70 lines/mm grating.*
10. *In your lab report, discuss what will happen to the images and how the acquisition of diffracted light is affected by covering the objective lens with various pinholes and why this distorts the images*.

Procedure 3: Phase Microscopy

Phase contrast microscopy is an essential technique in the microscopic imaging of biological materials. Most cells, single-cell organisms, and biological tissues appear transparent in bright field, and thus are difficult to visualize. Phase contrast microscopy effectively converts the phase shifts that occur as light diffracted through the specimen into light intensity differences that are detectable to the eye.

1. Examine a 20X objective on the microscope turret. What do you see? *Note the characteristics of the objective* (Figure 8 is a representative objective; you may not have all of the same labeling. More details about specifications engraved on the barrel of an objective can be found here).
2. Remove an eyepiece and sight down the microscope. You should be able to see the ring on the phase plate.
3. Move the phase contrast slider (Component 7 in Figure 3) to change the illumination scheme.

Figure 8. Representative image of a microscope objective [2]

4. Obtain the cell sample from TA. Examine the sample under the regular bright field and the phase contrast mode respectively. *Take digital photos and describe any difference between two images.*
5. After obtaining images of the sample with a particular objective, *apply the corresponding **scale bar** onto the image*. The scale bar calibration should be completed before the procedure. The scale image facilitates conversion of the image units, which are in pixels, into real world units of length. You should make scale bar measurement a regular part of microscopic measurements.

Procedure 4: Fluorescence Microscopy

1. Retrieve a fluorescently stained microscope slide from TA. *In your lab report, identify the host organism*

and location from which the cells were extracted. Provide the stains' typical excitation/emission colors (wavelengths) as well as subcellular structures to be identified.

2. Shut off the bright light and activate the mercury lamp. This lamp provides broadband (many wavelengths) light to the sample. At the current setting, the light is filtered such that ultraviolet light is directed towards the sample; emitted light is collected by the objective lens, and passed through a filter that only allows blue light to pass to the oculars.
3. Capture an image of the sample.
4. Switch to the blue excitation/green emission channel. Capture an image of cells. *What will occur in this fluorescence channel?*
5. Finally, switch to the green excitation channel and capture an image of the sample that emits red light.
6. *Using your camera software, create an RGB overlay of the three channels (Tint and merge). Include this in your lab report.*

Laboratory Report Guidelines

Be sure you include the following in your lab report:

- Answer/Discuss all of the italicized and underlined portions of the procedure.
- Include images that are requested in the procedure and any supplemental figures that you consider relevant with explanations.
- For the Alter the Numerical Aperture procedure, explain the distortion that occurs in the experiment in the context of diffraction and light information from diffraction cones, i.e., the image information collected by the objective.
- Address any sources of error in your experiments.

References/Relevant Reading

1. Davidson, M., Microscopy U: The Source for Microscopy Education. 2000-2018, Nikon.
2. Davidson, M. and M. Abramowitz, Optical Microscopy. 2009, National High Magnetic Field Laboratory, The Florida State University: Tallahassee.
3. Davidson, M., Optical Microscopy Primer. 1998-2018, The Florida State University.