MICROBIOLOGY:
A Laboratory Manual

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PREFACE

Microbiology, by its very nature, is a dynamic science that is constantly evolving as more information is added to the continuum of knowledge. In view of the rapidity in which microbiological techniques are being modified and refined, the authors have endeavored to provide a blend of the staid traditional methodologies with the newer and more contemporary procedures in the second edition of *Microbiology: A Laboratory Manual*. In an attempt to meet the pedagogical needs of all students studying microbiology, the changes introduced in this edition include:

1. Expansion of the medical microbiology and immunology sections to include current, rapid testing methods for the isolation and identification of staphylococci, streptococci, and the etiological agents of sexually transmitted diseases, namely Herpes simplex and the chlamydiae. Also incorporated into the manual are new experiments pertaining to the preparation of blood cultures for the diagnosis of septicemia and the use of drug combinations to illustrate their synergistic effects.
2. Expansion of the introductory and principle sections of some experiments to provide greater clarity to facilitate student comprehension.
3. Consolidation of related experimetal procedures to produce a more workable sequence.
4. Addition of computer-assisted programs to the experiment concerned with the identification of unknown bacterial cultures for use in conjunction with the traditional protocol. IBM PC, Apple, or TRS-80 disk will be available to adopters upon request, from the publisher.
5. Incorporation of colored photographs of selected biochemical reactions to facilitate student interpretation of test observations.

The design of the manual has remained essentially the same as the earlier edition. Comprehensive introductory material is given at the beginning of each major area of study, and specific explanations and detailed directions precede each experimental exercise. We feel that this textual approach will augment, enhance, and reinforce course lectures, thereby enabling students more readily to comprehend the concepts and purposes of each experiment. This will be a further asset to students in institutions in which the laboratory and lecture sections are not taught concurrently. Finally, we feel that this book should reduce the time required for explanations at the beginning of each laboratory session and thus make more time available for students to spend on the experiments.

The experiments were critically selected and tested to instruct students effectively in the basic principles and techniques within a variety of microbiological areas. The protocols vary in content and complexity, providing the instructor with flexibility to mold the laboratory syllabus to the particular needs of the students within the confines and scope of the course. Furthermore, it provides a wide spectrum of exercises suitable for students in elementary and advanced general microbiology courses as well as those in allied health programs. Lastly, the design of each procedure was carefully planned so that the supplies, equipment, and instrumentation commonly found in undergraduate institutions would suffice for their successful execution.

The manual, consisting of 77 exercises, is arranged into 15 major sections covering the following topics essential to a general microbiology course:

Section I, on **microscopy,** introduces the students to the use and care of the microscope for the study of microorganisms.

Section II, on **bacterial staining,** instructs the students in procedures for visualization and differentiation of microorganisms and cell structures.

Section III, on **microbiological cultivation techniques,** stresses the various procedures used for the isolation and enumeration of microorganisms.

Section IV, on **cultivation of microorganisms,** acquaints students with the nutritional and physical requirements for microbial growth.

Section V, on **biochemical activities,** introduces the varied cellular enzymatic activities that may be used for differentiation and identification of specific groups of microorganisms.

Sections VI, VII, and VIII introduce the areas of **protozoology, mycology,** and **virology.**

Sections IX, **control of microbial growth,** acquaints students with the antimicrobial activities of various physical and chemical agents.

Sections X and XI are concerned with the sanitary aspects of **water** and **food,** as well as the fermentative role of microorganisms in the production of some beverages and food products.

Section XII, on **microbiology of soil,** discusses the role of soil microorganisms in the nitrogen cycle and antibiotic production.

Section XIII, on **bacterial genetics,** presents selected experiments to illustrate genetic principles using bacterial systems.

Sections XIV and XV, on **medical microbiology** and **immunology,** acquaint students with both the conventional and the more recent, rapid, clinical screening methodologies used for the isolation and identification of pathogenic microorganisms.

The format of each exercise is intended to facilitate presentation of the material by the instructor and to maximize the learning experience for the student. To this end, each experiment is designed as follows:

Purpose: Defines the specific principles and/or techniques to be mastered.

Principle: An in-depth discussion of the microbiological concept or technique and the specific experimental procedure.

Materials: To facilitate the preparation of all laboratory sessions, each procedure contains a list of the following:

Cultures: The selected test organisms chosen to demonstrate effectively the experimental principle or technique under study, as well as their ease of cultivation and maintenance in stock culture. A complete listing of the experimental cultures and prepared slides is presented in Appendix 4.

Media: The specific media and their quantities per designated student group. Appendix 1 lists the composition and method of preparation of all the media used in this manual.

Reagents: Biological stains and test reagents. The chemical composition and preparation of the reagents is presented an Appendixes 2 and 3.

Equipment: Supplies and instrumentation. The suggested equipment was selected to minimize expense.

Procedure: Explicit instructions are augmented by diagrams to aid students in the execution and interpretation of the experiments.

Observations and results: Tear-out sheets are located at the end of each exercise to facilitate interpretation of data by students and collection of the report sheets by the instructor.

Review questions: Questions are located on the report sheet to aid the instructor in determining the student's ability to understand the experimetal concepts and techniques.

Finally, it is our hope that this manual will serve as a vehicle for the development of manipulative skills and techniques essential for understanding the integrated complexity of the biochemical structure and function of the single cell. This will enable students to extend these principles to the better understanding of the more complex, higher forms of life. Ultimately, it is our hope that some might become sufficiently stimulated and excited by the happenings in the microbial world to pursue further the study of life at the molecular level, or to use and apply these laboratory skills in the vocational fields of applied microbiology and allied health.

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J.G.C.
N.S.

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Special thanks are extended to our microbiology laboratory technician, Joan Grace, for her assistance in prerunning these experiments to ensure their success when performed by the students. We are also indebted to Michael McKeon and Betty Sinowitz, Rockland Community College, for their unselfish assistance in the preparation of the computer-assisted programs for the identification of unknown bacterial cultures. Finally, we wish to extend our appreciation to our families for their patience and support during the initial writing and revision of this manual.

LABORATORY SAFETY: GENERAL RULES AND REGULATIONS

1. Upon entering the laboratory, coats, books, and other paraphernalia should be place in specified locations and never on bench tops.
2. At the beginning and termination of each laboratory session bench tops are to be wiped with a disinfectant solution provided by the instructor.
3. Smoking, eating, and drinking in the laboratory are absolutely prohibited.
4. Students should wear a lab coat or apron while working in the laboratory to protect clothing from contamination or accidental discoloration by staining solutions.
5. Long hair should be placed inside a paper cap or tied back to minimize fire hazard or contamination of experiments.
6. Removal of media, equipment, and especially bacterial cultures from the laboratory is absolutely prohibited.
7. All cultures should be handled as being potentially pathogenic, and the following precautions should be observed at all times:
   a. Cultures must always be carried in a test tube rack when the student is moving around the laboratory.
   b. Cultures must be kept in a test tube rack on the bench tops when not in use.
   c. Broth cultures must never be pipetted by mouth.
   d. Spilled cultures should be covered with paper towels and then saturated with disinfectant solution. Following 15 minutes of reaction time, the towels should be removed and disposed of in the manner indicated by the instructor.
8. Use of glassware markers:
   a. Non–water-soluble markers must be used (i.e., sharpies).
   b. If wax pencils are used, care must be taken not to grind broken tips into benchtops and the laboratory floor.
9. On completion of the laboratory session all cultures and materials to be discarded should be placed in the disposal area designated by the instructor.
10. Hands must be washed with detergent prior to leaving the laboratory.

CONTENTS

I

Microscopy

PURPOSES

1. To acquaint students with the evolutionary diversity of microscopic instruments.
2. To familiarize students with the components, use, and care of the compound brightfield microscope.
3. To instruct students in the correct use of the microscope for observation and measurement of microorganisms.

INTRODUCTION

Microbiology, the branch of science that has so vastly extended and expanded our knowledge of the living world, owes its existence to Antonj van Leeuwenhoek. In 1673, with the aid of a crude microscope consisting of a biconcave lens enclosed in two metal plates, Leeuwenhoek introduced the world to the existence of microbial forms of life. Over the years, microscopes have evolved from the simple, single-lens instrument of Leeuwenhoek, with a magnification of 300, to the present-day electron microscopes capable of magnifications greater than 250,000.

They are designated as either light microscopes or electron microscopes. The former use visible light or ultraviolet rays to illuminate specimens. They include the brightfield, darkfield, phase-contrast, and fluorescent instruments. Fluorescent microscopes use ultraviolet radiations whose wavelengths are shorter than those of visible light and not directly perceptible to the human eye. Electron microscopes use electron beams instead of light rays, and magnets instead of lenses to observe submicroscopic particles.

ESSENTIAL FEATURES OF VARIOUS MICROSCOPES

BRIGHTFIELD MICROSCOPE This instrument contains two main lens systems: The ocular lens in the eyepiece and the objective lens located in the nosepiece. The specimen is illuminated by a beam of tungsten light focused on it by a substage lens designated as a condenser. A major limitation of this system is the absence of contrast between the specimen and the surrounding medium, which makes it difficult to observe living cells. Therefore nonviable, stained preparations are used instead.

DARKFIELD MICROSCOPE This is similar to the ordinary light microscope; however, the condenser system is modified so that the specimen is not illuminated directly. The condenser directs the light obliquely so that the light is deflected or scattered from the specimen, which then appears bright against a dark background. Living specimens may be observed more readily with darkfield than with brightfield microscopy.

PHASE-CONTRAST MICROSCOPE Observation of microorganisms in an unstained state is possible with this microscope. Its optics include special phase-contrast objectives and a condenser that make visible cellular components that differ only slightly in their refractive indexes. As light is transmitted through a specimen with a refractive index different than that of the surrounding medium, a portion of this light is refracted (bent) due to slight variations in density and thickness of the cellular components. The special optics convert the difference between transmitted light and refracted rays, resulting in a significant variation in the intensity of light and thereby produce a discernible image of the structure under study. The image appears dark against a light background.

FLUORESCENT MICROSCOPE This microscope is used to visualize specimens that are chemically tagged with a fluorescent dye. The source of illumination is an ultraviolet (UV) light obtained from a high-pressure mercury lamp or hydrogen quartz lamp. The ocular lens is fitted with a filter that permits the longer ultraviolet wavelengths to pass, while the shorter wavelengths are blocked or eliminated. Ultraviolet radiations are absorbed by the fluorescent label and the energy is re-emitted in the form of a different wavelength in the visible light range. The fluorescent dyes absorb at wavelengths between 230 and 350 nm and emit orange, yellow, or greenish light. This microscope is used primarily for the detection of antigen-antibody reactions. Antibodies conjugated with a fluorescent dye are excited in the presence of ultraviolet light and the fluorescent portion of the dye becomes visible against a black background.

ELECTRON MICROSCOPE The instrument provides a revolutionary method of microscopy, with magnifications up to one million that permit visualization of submicroscopic cellular particles as well as viral agents. The lens and illumination system are similar to those of the light microscope except that in the electron microscope the specimen is illuminated by a beam of electrons rather than light, and the focusing is carried out by electromagnets instead of a set of optics. These components are sealed in a tube in which a complete vacuum is established. Transmission electron microscopes require thinly prepared, fixed, and dehydrated specimens for the electron beam to pass freely through them. As the electrons pass through the specimen, images are formed by directing the electrons onto x-ray film, thus internal cellular structures are made visible. Scanning electron microscopes are used for visualizing surface characteristics rather than intracellular structures. A narrow beam of electrons scans back and forth producing a three-dimensional image as the electrons are reflected off the specimen's surface.

It is apparent that the microscopist has a variety of optical instruments with which to perform routine laboratory procedures as well as sophisticated research. The compound brightfield microscope might justly be termed the workhorse, however, as it is commonly found in all biological laboratories. Although students should be familiar with the basic principles of microscopy, it is generally not possible for the average undergraduate to be exposed to this diverse array of complex and expensive equipment. Therefore only the compound brightfield microscope will be discussed in depth and used to examine specimens.

Microscopic Examination of Stained Cell Preparations

PURPOSES

To familiarize the students with:

1. The theoretical principles of brightfield microscopy.
2. The component parts of the compound microscope.
3. The use and care of the compound microscope.
4. The practical use of the compound microscope for visualization of cellular morphology from stained slide preparations.

PRINCIPLE

Microbiology is a science that studies living organisms that are too small to be seen with the naked eye. Needless to say, such a study must involve the use of a good compound microscope. There are many types and variations, however, they all fundamentally consist of a two-lens system, a variable but controllable light source, and mechanical adjustable parts for determining focal length between the lenses and specimen (Figure 1.1 on page 4).

Components of the Microscope

STAGE A fixed platform with an opening in the center allows for the passage of light from an illuminating source below to the lens system above the stage. This platform provides a surface for the placement of a slide with its specimen over the central opening. In addition to the fixed stage, most microscopes have a **mechanical stage** that can be moved vertically or horizontally by means of adjustment knobs. Less sophisticated microscopes have clips on the fixed stage and the slide must be positioned over the central opening manually.

ILLUMINATION The light source is positioned in the base of the instrument. Some microscopes are equipped with a built-in light source to provide direct illumination. Those that are not are provided with a mirror, one side flat and the other concave. An external light source such as a lamp is placed in front of the mirror to direct the light upward into the lens system. The flat side of the mirror is used for artificial light, and the concave side for sunlight.

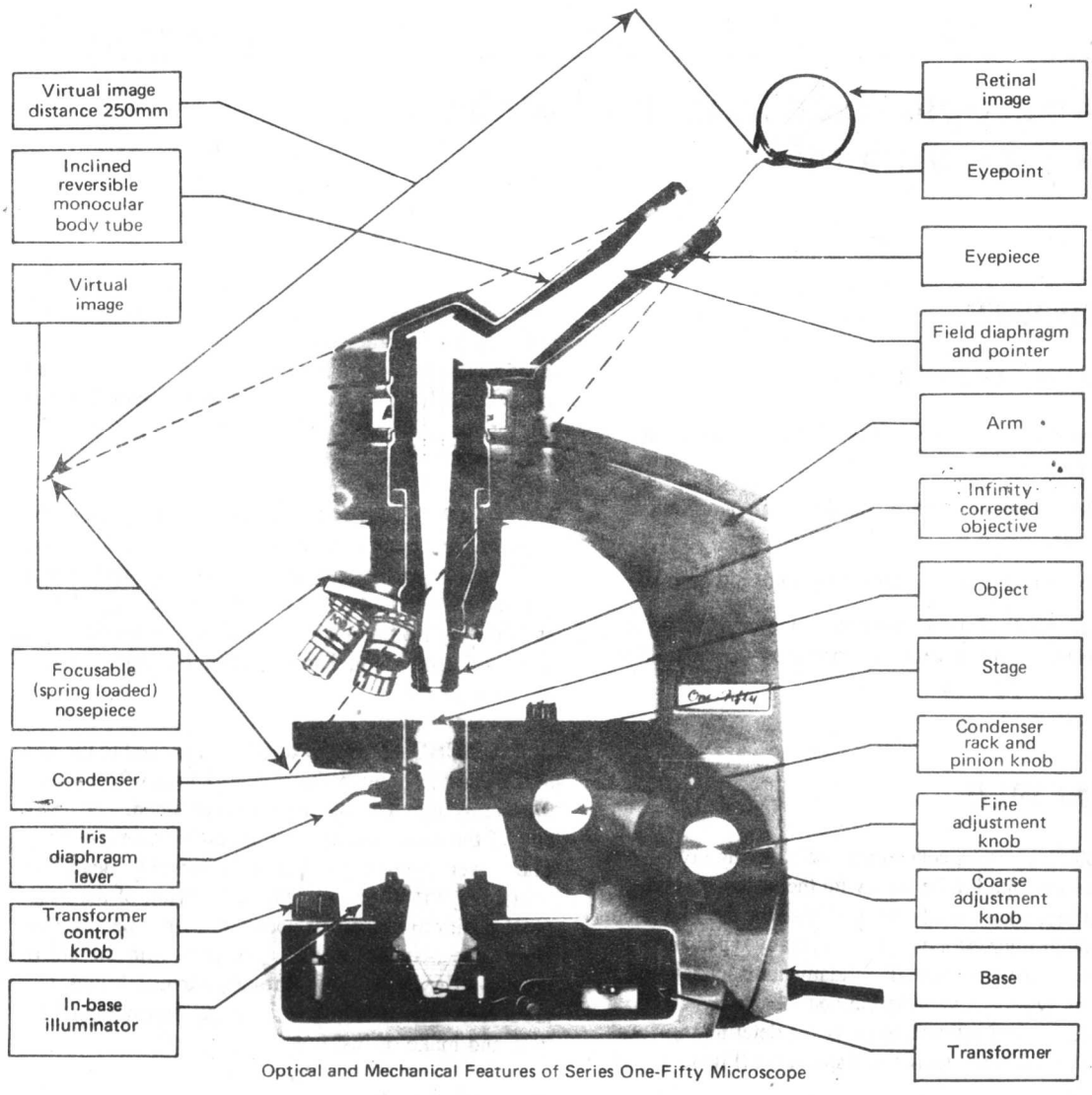
ABBE CONDENSER This is found directly under the stage and contains two sets of lenses that collect and concentrate light passing upward from the light source into the lens systems. The condenser is equipped with an **iris diaphragm,** which is a shutter controlled by a lever and used to regulate the amount of light entering the lens system.

BODY TUBE Above the stage and attached to the arm of the microscope is the body tube. This structure houses the lens system that magnifies the specimen. The upper end of the tube contains the **ocular** or **eyepiece** lens. The lower portion consists of a movable **nosepiece** containing the **objective lenses.** Rotation of the nosepiece positions objectives above the stage opening. The body tube may be raised or lowered with the aid of **coarse-adjustment and fine-adjustment knobs** that are located above or below the stage, depending on the type and make of instrument.

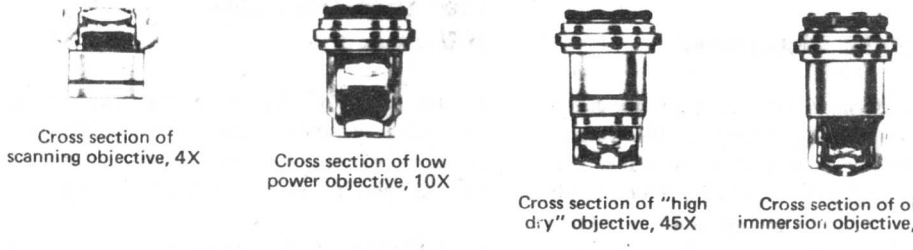
Theoretical Principles of Microscopy

To use the microscope with efficiency and minimal frustration, students should understand the basic principles of microscopy: magnification, resolution, numerical aperture, illumination, and focusing.

MAGNIFICATION Enlargement or magnification of a specimen is the function of a two-lens system; **the ocular lens** is found in the eyepiece and the **objective lens** is situated in a revolving nosepiece. These lenses are separated by the **body tube.** The objective lens is nearer the specimen and magnifies it, producing the **real image** that is projected up into the focal plane and then magnified by the ocular lens to produce the final image.



Optical and Mechanical Features of Series One-Fifty Microscope



Cross section of scanning objective, 4X

Cross section of low power objective, 10X

Cross section of "high dry" objective, 45X

Cross section of oil immersion objective, 100X

FIGURE 1.1. Optical and mechanical features of the microscope. (Courtesy of Reichert Scientific Instruments.)

TABLE 1.1. Overall linear magnification

Magnification		*Total Magnification*
Objective lenses	*Ocular lens*	*Objective multiplied by ocular*
Scanning 4 ×	10 ×	40 ×
Low-power 10 ×	10 ×	100 ×
High-power 45 ×	10 ×	450 ×
Oil immersion 97 ×	10 ×	970 ×

The most commonly used microscopes are equipped with a revolving nosepiece containing four objective lenses possessing different degrees of magnification. When these are combined with the magnification of the ocular lens, the total or overall linear magnification of the specimen is obtained. This is shown in Table 1.1.

RESOLVING POWER OR RESOLUTION Although magnification is important, students must be aware that unlimited enlargement is not possible by merely increasing the magnifying power of the lenses or by the use of additional lenses, because lenses are limited by a property called **resolving power.** By definition, resolving power is the ability of a lens to show two adjacent objects as discrete entities. When a lens cannot discriminate, that is, when the two objects appear as one, it has lost resolution. Increased magnification will not rectify the loss, and will, in fact blur the object. The resolving power of a lens is dependent on the wavelength of light used and a characteristic of the lens called the numerical aperture. This is expressed by the following formula:

$$\text{Resolving power} = \frac{\text{Wavelength of light}}{\text{Numerical aperture}}$$

With this formula it can be shown that the shorter the wavelength, the greater the resolving power of the lens. Thus short wavelengths of the electromagnetic spectrum are better suited than longer wavelengths in terms of the numerical aperture.

As with magnification, resolving power is also not without limits. The student might rationalize that merely decreasing the wavelength will automatically increase the resolving power of a lens. Such is not the case, because the visible portion of the electromagnetic spectrum is very narrow and borders on the very short wavelengths found in the ultraviolet portion of the spectrum. Resolving power, therefore, may only be increased by increasing the numerical aperture. The **numerical aperture** is defined as a function of the diameter of the objective lens in relation to its focal length. It is doubled by use of the substage condenser, which illuminates the object with rays of light that pass through the specimen obliquely as well as directly. Thus the larger the numerical aperture the greater the resolving power, which may be expressed mathematically, as follows:

$$\text{Resolving power} = \frac{\text{Wavelength of light}}{2\ (\text{numerical aperture})}$$

The relationship between wavelength and numerical aperture is only valid for increased resolving power when light rays are parallel. Therefore the resolving power is dependent on another factor, the **refractive index.** This is the bending power of light passing through air from the glass slide to the objective lens. The refractive index of air is lower than that of glass, and as light rays pass from the glass slide into the air they are bent or refracted so that they do not pass into the objective lens. This would cause a loss of light, which would reduce the numerical aperture and diminish the resolving power of the objective lens. Loss of refracted light can be compensated for by interposing mineral oil, which has the same refractive index as glass, between the slide and the objective lens. In this way decreased light refraction occurs and more light rays enter directly into the objective lens, producing a vivid image with high resolution (Figure 1.2 on page 6).

ILLUMINATION Effective illumination is required for efficient magnification and resolving power. Since the intensity of daylight is an uncontrolled variable, artificial light from a tungsten lamp is the most commonly used light source in microscopy. The light is passed through the condenser located beneath the stage. The condenser contains two lenses that are necessary to produce a maximum numerical aperture. The height of the condenser can be adjusted with the **condenser knob.** The condenser should always be kept close to the stage, especially when using the oil-immersion objective.

Between the light source and the condenser is the iris diaphragm that can be opened and closed by means of a lever, thereby regulating the amount of light en-

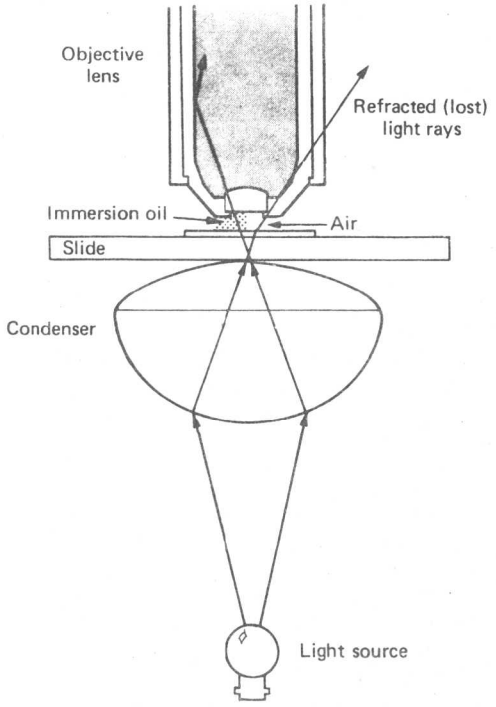


FIGURE 1.2. Refracted index in air and mineral oil

tering the condenser. Excessive illumination may actually obscure the specimen because of lack of contrast. The amount of light entering the microscope differs with each objective lens used. A rule of thumb is that **as the magnification of the lens increases, the distance between the objective lens and slide, called working distance, decreases and the angle of aperture of the objective increases** (Figure 1.3).

Use and Care of the Microscope

Students will be responsible for the proper care and use of microscopes. Since microscopes are expensive items of equipment the following regulations and procedures must be observed.

The instruments are housed in special cabinets and must be transported by students to the laboratory bench. The correct and only acceptable way to do this is to grip the microscope arm firmly with the right hand and the base with the left hand, and lift the instrument from the cabinet shelf. It is carried close to the body and gently placed on the laboratory bench. This will prevent collision with furniture or co-workers, and protect the instrument against damage.

Once the microscope is placed on the laboratory bench the following rules must be observed:

1. All unnecessary materials such as books, papers, purses, and hats should be removed from the laboratory bench.
2. The microscope's electric wire is uncoiled and plugged into an electrical outlet.
3. All lens systems are cleaned, as the smallest bit of dust, oil, lint, or eyelashes will decrease the efficiency of the microscope. The ocular, scanning, low-power, and high-power lenses may be cleaned by wiping several times with acceptable lens tissue. Paper toweling or cloth should never be used on a lens surface. If the oil-immersion lens is gummy or tacky, a moistened piece of lens paper saturated with xylol is used to wipe it clean. The xylol is immediately removed with a tissue moistened with 95 percent alcohol, and the lens is wiped dry with lens paper. **This procedure should only be used when necessary, as consistent use of xylol may loosen the lens.**

The following routine procedures must be followed to ensure correct and efficient use of the microscope.

1. Place the microscope slide with the specimen within the stage clips on the fixed stage. Move the slide to center the specimen over the opening in the stage directly over the light source.
2. Rotate the scanning lens or low-power lens into position. Lower the body tube with the coarse-adjustment knob to its lowest position. **Never lower the body tube while looking through the ocular lens.**
3. While looking into the ocular lens, with the coarse-adjustment knob slowly raise the stage until the specimen comes into focus. Using the fine-adjustment knob, bring the specimen into sharp focus.
4. Adjust the substage condenser and iris diaphragm to produce optimum illumination.
5. Most microscopes are **par focal,** which means that when one lens is in focus other lenses will also have the same focal length and can be rotated into position without further major adjustment. In practice, however, usually a half-turn of the fine-adjustment knob in either direction is necessary for sharp focus.
6. Once the specimen has been brought into sharp focus with a low-powered lens, preparation may

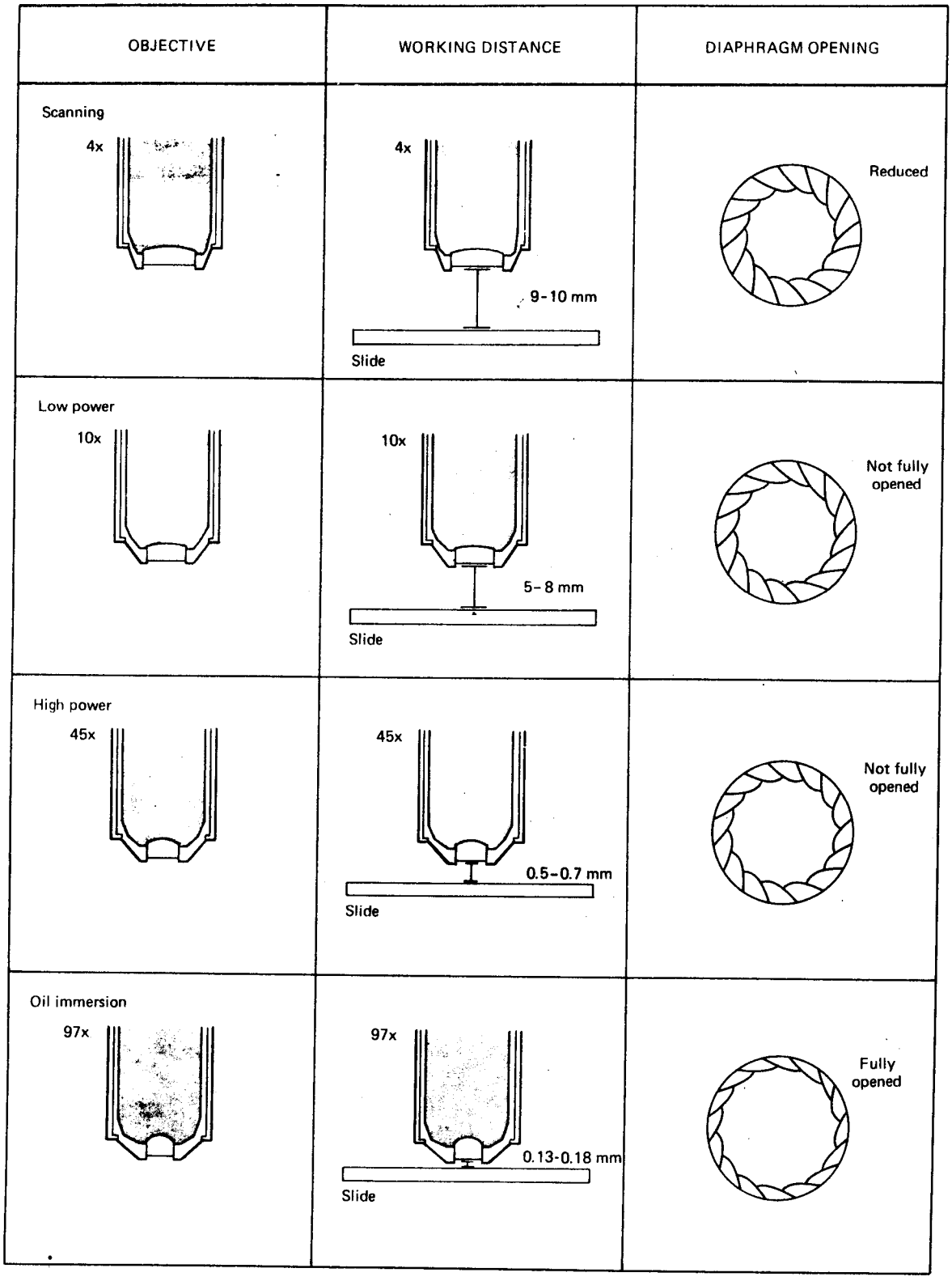


FIGURE 1.3. Relationship between working distance objective and diaphragm opening