Benson's

Microbiological Applications

Laboratory Manual in General Microbiology

Fifteenth Edition

Heidi R. Smith
Front Range Community College

Alfred E. Brown
Emeritus Professor, Auburn University

Mc Graw Hill

BENSON'S MICROBIOLOGICAL APPLICATIONS: LABORATORY MANUAL IN GENERAL MICROBIOLOGY,
FIFTEENTH EDITION

This book is printed on acid-free paper.

1 2 3 4 5 6 7 8 9 LMN 26 25 24 23 22 21

ISBN 978-1-260-25898-1 (bound edition)
MHID 1-260-25898-X (bound edition)
ISBN 978-1-260-42560-4 (loose-leaf edition)
MHID 1-260-42560-6 (loose-leaf edition)

Portfolio Manager: *Lauren Vondra*
Product Developer: *Erin DeHeck*
Director of Digital Content: *Michael G. Koot, PhD*
Marketing Manager: *Tami Hodge*
Content Project Managers: *Jessica Portz, Rachael Hillebrand*
Content Licensing Specialist: *Beth Cray*
Buyer: *Laura Fuller*
Designer: *David Hash*
Cover Image: *Shutterstock/Jarun Ontakrai*
Compositor: *MPS Limited*

mheducation.com/highered

About the Authors

Heidi Smith

Front Range Community College
B.A. Biology/Pre-Medicine, Taylor University, IN
M.S. Biology, Bowling Green State University, OH


Heidi Smith

Heidi Smith is the lead faculty for microbiology at Front Range Community College (FRCC) in Fort Collins, CO, and teaches a variety of biology courses each semester including microbiology and anatomy/physiology. Heidi also serves as the principal investigator on a federal grant program designed to increase student success in transfer and completion of STEM degrees at the local university. In that role, Heidi works directly with students to train them for and support them through summer undergraduate research experiences.

Student success is a strategic priority at FRCC and a personal passion of Heidi's. She continually works to develop professionally in ways that help her do a better job of reaching this important goal. For more than 10 years, Heidi has had the opportunity to collaborate with faculty throughout the United States in developing digital tools that measure and improve student learning outcomes, such as SmartBook, Virtual Labs, and Connect. This collaborative experience with these tools has revolutionized her approach to teaching in face-to-face, hybrid, and fully online courses. The use of digital technology has given Heidi the ability to teach courses driven by real-time student data and with a focus on active learning and critical thinking activities.

Heidi has been an active member of the American Society for Microbiology. She has presented instructional technology and best online and face-to-face teaching practices on numerous occasions at the annual conference for undergraduate educators. She also served as a member of the ASM Task Force on Curriculum Guidelines for Undergraduate Microbiology Education, assisting in the identification of core microbiology concepts as a guide to undergraduate instruction.

Off campus, Heidi spends as much time as she can enjoying the beautiful Colorado outdoors and youth sports with her husband and four teenagers.

Alfred Brown

Emeritus Professor of Microbiology
Auburn University
B.S. Microbiology, California State College, Long Beach
Ph.D. Microbiology, UCLA


Alfred Brown

Teaching Dr. Brown has been a member of the American Society for Microbiology for 50 years, and he has taught various courses in microbiology over a teaching career that spans more than 30 years. Courses have included general microbiology, medical microbiology, microbial physiology, applied and environmental microbiology, photosynthesis, microbiological methods, and graduate courses such as biomembranes. In 2008, Dr. Brown retired from the Auburn University faculty as an emeritus professor of microbiology. Dr. Brown has won numerous awards for his excellence in teaching microbiology.

Administration During his tenure at Auburn University, Dr. Brown served as the director of the University Electron Microscope Facility. He also served as the chair of the Department of Botany and Microbiology and the chair of the Department of Biological Sciences.

Research My research has focused on the physiology of the purple nonsulfur bacteria. This has involved how bacteriochlorophyll and photosynthetic membrane synthesis are coordinated. Herbicides, such as atrazine, have been used to determine the binding site for ubiquinone in photosynthetic electron transport. Binding occurs on the L-subunit, a protein in the photosynthetic reaction center. Resistance to atrazine involves a single amino acid change in the L-subunit that prevents the herbicide from binding to the protein and inhibiting electron transport. This is comparable to how atrazine inhibits electron transport in plants and how resistance to these herbicides develops. My laboratory also investigated how the sulfonylurea herbicides inhibit acetolactate synthase, a crucial enzyme in the pathway for branched-chain amino acids. Recently, I consulted for a company that manufactures roofing shingles. Because of the presence of calcium carbonate in shingles, cyanobacteria can easily grow on their surface, causing problems of contamination. My laboratory isolated various species of cyanobacteria involved in the problem and taxonomically characterized them. We also tested growth inhibitors that might be used in their control.

Dr. Brown and his wife have traveled extensively in Europe since his retirement. His three children have followed him in science, having earned doctorates in physics, chemistry, and anatomy.

Contents

Indicates a Connect Virtual Labs activity is available for all or part of this exercise. For more information, visit https://www.mheducation.com/highered/connect/virtual-labs

Preface

Benson's Microbiological Applications has been the gold standard of microbiology laboratory manuals for more than 35 years. This manual has a number of attractive features that resulted in its adoption in universities, colleges, and community colleges for a wide variety of microbiology courses. These features include user-friendly diagrams that students can easily follow, clear instructions, and an excellent array of reliable exercises suitable for beginning or advanced microbiology courses.

In revising the lab manual for the 15th edition, we have tried to maintain the proven strengths of the manual and further enhance it. We have updated the introductory material in many exercises to reflect changes in scientific information and increase relevancy for students. Critical thinking questions have also been added to increase the Bloom's level of the laboratory reports. Finally, the names and biosafety levels of microorganisms used in the manual are consistent with those used by the American Type Culture Collection (ATCC). This is important for those users who rely on the ATCC for a source of cultures.

Guided Tour Through a Lab Exercise

Learning Outcomes

Each exercise opens with Learning Outcomes, which list what a student should be able to do after completing the exercise.

Learning Outcomes

After completing this exercise, you should be able to

1. Prepare a thin smear of bacterial cells and stain them with a simple stain.
2. Understand why staining is necessary to observe bacteria with a brightfield microscope.
3. Observe the different morphologies of bacterial cells.

Introduction

The introduction describes the subject of the exercise or the ideas that will be investigated. It includes all of the information needed to perform the laboratory exercise. The 15th edition has improved its student relevancy message within these introductions, explaining to students why they should care about the lab.

Why It Matters

How can you apply this exercise to the real world?

It is often essential to determine the number of bacteria in a sample. Sometimes, it is important to know how many bacteria are present in food or water to ensure safety. For example, the grading of milk is based on the number of bacteria present. In healthcare, bacterial numbers can also be an important indicator of infection. Whether a patient has a bladder infection is dependent on a certain threshold level of bacteria present in a urine sample.

First and Second Periods

In many cases, instructions are presented for two or more class periods so you can proceed through an exercise in an appropriate fashion.

First Period

(Inoculations and Incubation)

During this period, each group will inoculate five FTM tubes and two nutrient agar plates. All of the plates will be placed in a GasPak jar. Both the GasPak jar containing the plates and the FTM tubes that you inoculate will be incubated at 37°C.

Second Period

(Culture Growth Evaluations)

Gather your tubes and the GasPak jar from the incubator, and remove the lid from the GasPak jar to obtain your agar plates. When transporting the plates and tubes to your desk, *take care not to agitate the FTM tubes*. The position of growth in the medium can be easily changed if handled carelessly.

Materials Needed

This section lists the laboratory materials that are required to complete the exercise.

Materials

per student:

- 1 tube of nutrient broth
- 1 trypticase soy agar (TSA) plate
- 1 blood agar plate
- 3 sterile cotton swabs

Procedures

The procedures and methods provide a set of detailed instructions for accomplishing the planned laboratory activities.

Procedures

If your microscope has three objectives, you have three magnification options: (1) low-power, or 100× total magnification with the 10× objective, (2) high-dry magnification, which is 400× total with a 40× objective, and (3) 1000× total magnification with a 100× oil immersion objective.

Whether you use the low-power objective or the oil immersion objective will depend on how much

Illustrations

Illustrations provide visual instructions for performing steps in procedures or are used to identify parts of instruments or specimens.

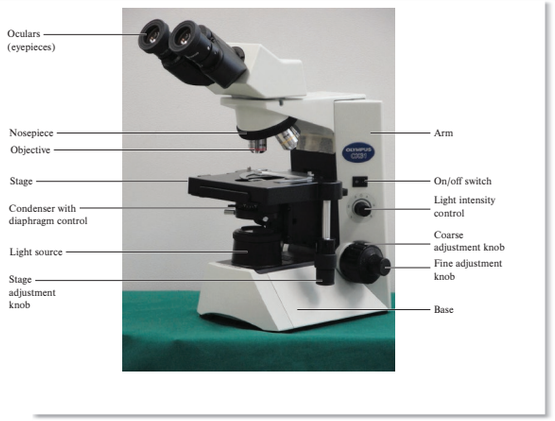
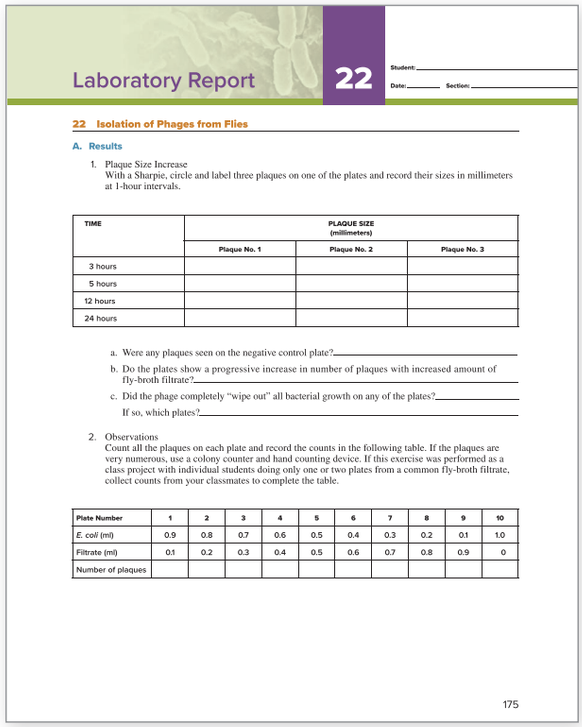


Figure 1.2 Components of a compound brightfield microscope.
James Redfearn/McGraw-Hill Education



Laboratory Report 22

Student: _____

Date: _____ Section: _____

22 Isolation of Phages from Flies

A. Results

1. Plaque Size Increase
With a Sharpie, circle and label three plaques on one of the plates and record their sizes in millimeters at 1-hour intervals.

TIME	PLAQUE SIZE (millimeters)		
	Plaque No. 1	Plaque No. 2	Plaque No. 3
3 hours			
5 hours			
12 hours			
24 hours			

a. Were any plaques seen on the negative control plate?_____

b. Do the plates show a progressive increase in number of plaques with increased amount of fly-broth filtrate?_____

c. Did the phage completely "wipe out" all bacterial growth on any of the plates?_____
If so, which plates?_____

2. Observations
Count all the plaques on each plate and record the counts in the following table. If the plaques are very numerous, use a colony counter and hand counting device. If this exercise was performed as a class project with individual students doing only one or two plates from a common fly-broth filtrate, collect counts from your classmates to complete the table.

Plate Number	1	2	3	4	5	6	7	8	9	10
E. coli (ml)	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	1.0
Filtrate (ml)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	0
Number of plaques										

175

Laboratory Reports

A Laboratory Report to be completed by students immediately follows most of the exercises. These Laboratory Reports are designed to guide and reinforce student learning and provide a convenient place for recording data. These reports include various types of review activities, tables for recording observations and experimental results, and questions dealing with the analysis of such data.

As a result of these activities, students will increase their skills in gathering information by observation and experimentation. By completing all of the assessments in the Laboratory Reports, students will be able to determine if they accomplished all of the learning outcomes.

Safety

In the 15th edition, we have followed the recent recommendations of the American Society for Microbiology concerning the biosafety levels of organisms used in the exercises in this laboratory manual. The Basic Microbiology Safety section in the introductory pages of the lab manual has been completely revised to align with ASM's *Guidelines for Biosafety in Teaching Laboratories* released in 2012. The BSL classifications of all organisms used in each exercise have been updated according to ATCC. Where possible, BSL-1 organisms replace BSL-2 organisms in many exercises. However, in some exercises, it is necessary to use BSL-2 organisms such as specific staphylococci, streptococci, and some of the *Enterobacteriaceae* due to specific tests, stains, and concepts that involve these organisms. For these exercises, we recommend that safety procedures be followed as suggested by ASM, such as the use of biosafety cabinets.

Changes to This Edition

Exercises

- A "Why It Matters" box has been added to every exercise, highlighting the relevance of the exercise. to everyday life.
- A new exercise has been added on the CRISPR-Cas9 system with easy-to-access materials and straight forward preparation.
- Many photographs and some diagrams have been replaced, upgraded, or revised for currency and relevance.
- Procedures were modified if possible for minimal lab preparation and materials as well as for safety considerations (including changes to the organisms used).

Part 1 Microscopy

Exercise 1 Brightfield Microscopy

- Updated microscope photos and reorganized for easier-to-follow information about microscopy.

Exercise 4 Microscopic Measurements

- Revised figures and added photos to clarify the calibration process.

Part 2 Survey of Microorganisms

- Updated diagram of Domains Bacteria, Archaea, and Eukarya.

Exercise 6 Ubiquity of Bacteria

- Revised procedure and data table to allow more flexibility for exposure methods.
- Removed reference to and data collection of molds to focus on the ubiquity of bacteria.

Part 3 Manipulation of Microorganisms

Exercise 8 Aseptic Technique

- Revised figure for accuracy and added a close-up drawing of the zigzag pattern of slant inoculation.

Exercise 9 Pure Culture Techniques

- Focused on two quadrant streak methods as the most commonly used patterns.

Part 4 Staining and Observation of Microorganisms

Exercise 11 Simple Staining (Observation of Bacterial Morphology)

- New figures have been added to clarify the common bacterial shapes and arrangements.
- Labels have been added to photomicrographs to highlight important content.

Exercise 12 Negative Staining

- Photomicrograph has been magnified and labeled to highlight important content.

Exercise 14 Gram Staining

- Revised the procedural figure for greater clarity.
- Revised the overall procedure to minimize lab preparation and focus on controls for determining Gram reaction.
- Adjusted laboratory report to align with new procedure.
- Added questions about clinical relevance to the laboratory report.

Exercise 15 Endospore Staining

- Added figure of sporulation cycle.
- Focused exercise on the more commonly used Schaeffer-Fulton method but retained Dorner method as an alternative.

Exercise 17 Motility Determination

- Updated figure on flagellar arrangements.
- Streamlined entire exercise for easier implementation.

Part 5 Culture Methods

Exercise 19 Enumeration of Bacteria: The Standard Plate Count

- Completely revised the exercise to incorporate microtube dilution method (former procedure retained as an alternative) and added updated photos for currency of equipment and materials.

Part 7 Environmental Influences and Control of Microbial Growth

Exercise 24 Effects of Oxygen on Growth

- Focused exercise and laboratory report on FTM tubes and anaerobic jar plates for ease and economy of implementation.
- Adjusted plate inoculation figure for better results gathering.

Exercise 25 Temperature: Effects on Growth

- Revised procedure to be implemented per group rather than per student to minimize materials and preparation time.
- Added photo of *Serratia* incubated at room temperature.

Exercise 30 Evaluation of Alcohol: Its Effectiveness as an Antiseptic

- Revised procedural figure for greater clarity.
- Added hand sanitizer to test options and edited the laboratory report data collection table accordingly.

Exercise 31 Antimicrobic Sensitivity Testing: The Kirby-Bauer Method

- Updated the information on the historical discovery of penicillin.
- Added figure depicting major cellular targets of antimicrobials.

Part 9 Miniaturized Multitest Systems

Exercise 40 *Enterobacteriaceae* Identification: The API® 20E System

- Clarified procedure and adjusted figures to align.

Exercise 41 *Enterobacteriaceae* Identification: The EnteroPluri-*Test* System

- Edited table descriptions of reactions for easier understanding.

Part 10 Applied Microbiology

Exercise 43 Bacterial Counts of Foods

- Updated introduction to the exercise adding examples and photos.

Exercise 47 Temperature: Lethal Effects

- Revised procedure for groups instead of individual students to minimize cost and preparation time.

Part 11 Bacterial Genetics and Biotechnology

Exercise 50 Polymerase Chain Reaction for Amplifying DNA

- Revised explanation of PCR diagnosis figure for clarity.

Exercise 52 CRISPR-Cas9 System

- Added this new exercise exploring one of the most current advances in the genetics field and its relationship to microbiology and the treatment of disease.
- Based on a kit from Carolina Biological company for easy access, preparation, and instruction.

Part 12 Medical Microbiology

Exercise 53 The Staphylococci: Isolation and Identification

- Upgraded photomicrograph of staphylococcus.
- Removed excess media for specimen collection and primary isolation.
- Revised tables in Laboratory Report for better data collection and to eliminate student identity information.
- Added detailed note regarding safety concerns with human microbiota.

Exercise 54 The Streptococci and Enterococci: Isolation and Identification

- Upgraded photo of blood agar plate hemolysis.
- Added detailed note regarding safety concerns with human microbiota.

Exercise 55 Gram-Negative Intestinal Pathogens

- Revised data collection table to clarify observations versus interpretation of results.

Exercise 56 A Synthetic Epidemic

- Added information about the COVID-19 pandemic.

Part 13 Immunology and Serology

Exercise 60 Enzyme-Linked Immunosorbent Assay (ELISA)

- Revised exercise (including photo and figure) to match the current commercial kit results.

We would like to thank all the people at McGraw-Hill for their tireless efforts and support on this project. They are professional and competent and always a pleasure to work with on this manual. A special and deep thanks to Erin DeHeck, our product developer. Once again, she kept the project focused, made sure we met deadlines, and made suggestions that improved the manual in many ways. Thanks as well to Lauren Vondra, portfolio manager; Jessica Portz, content project manager; Tami Hodge, marketing manager; David Hash, designer; Rachael Hillebrand, assessment content project manager; Beth Cray, content licensing specialist; and many who worked "behind the scenes."

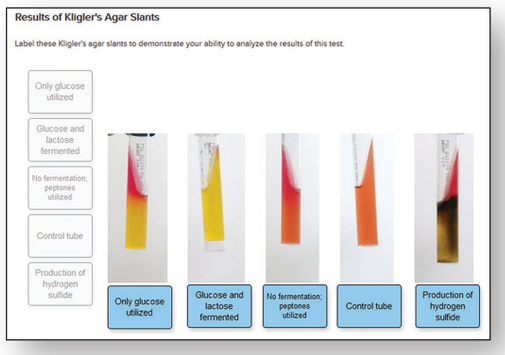
Digital Tools for Your Success

Save Time with Auto-Graded Assessments. Gather Powerful Performance Data.

McGraw-Hill Connect for Benson's Microbiological Applications Laboratory Manual in General Microbiology provides assignment and assessment solutions, connecting your students with the tools and resources they'll need to achieve success.

Homework and Assessment

With **Connect for Benson's Microbiological Applications Laboratory Manual in General Microbiology**, you can deliver auto-graded assignments, quizzes, and tests online. Choose from a robust set of interactive questions and activities using high-quality art from the lab manual and animations. Assignable content is available for every Learning Outcome and is categorized according to the ASM Objectives. As an instructor, you can edit existing questions and author entirely new ones.



Auburn University Photographic Services/McGraw-Hill Education

Instructor Resources

Customize your lecture with tools such as animations and images. An instructor's manual is also available.

Lecture Capture

McGraw-Hill Tegrity® records and distributes your class lecture with just a click of a button. Students can view this lecture material anytime, anywhere via computer or mobile device. The material is indexed as you record, so students can use keywords to find exactly what they want to study.

Virtual Labs and Lab Simulations

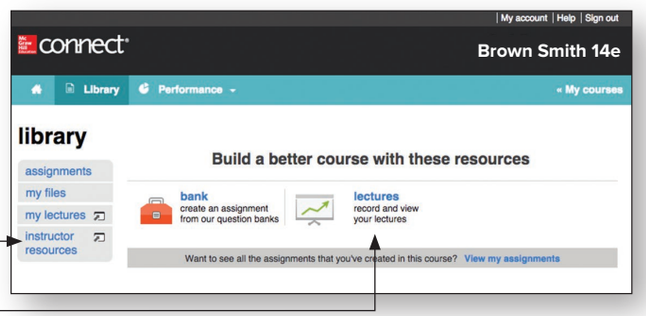
While the biological sciences are hands-on disciplines, instructors are now often being asked to deliver some of their lab content online, as full online replacements, supplements to prepare for in-person labs, or make-up labs.

These simulations help each student learn the practical and conceptual skills needed, then check for understanding and provide feedback. With adaptive pre-lab and post-lab assessment available, instructors can customize each assignment.

From the instructor's perspective, these simulations may be used in the lecture environment to help students visualize complex scientific processes, such as DNA technology or Gram staining, while at the same time providing a valuable connection between the lecture and lab environments.

Detailed Reports

Track individual student performance—by question, by assignment, or in relation to the class overall—with detailed grade reports. Integrate grade reports easily with your Learning Management Systems (LMS).



Olga Kovalenko/Hemera/360/Getty Images

Basic Microbiology Laboratory Safety

Every student and instructor must focus on the need for safety in the microbiology laboratory. While the lab is a fascinating and exciting learning environment, there are hazards that must be acknowledged and rules that must be followed to prevent accidents and contamination with microbes. The following guidelines will provide every member of the laboratory the information required to assure a safe learning environment.

Microbiological laboratories are special, often unique environments that may pose identifiable infectious disease risks to persons who work in or near them. Infections have been contracted in the laboratory throughout the history of microbiology. Early reports described laboratory-associated cases of typhoid, cholera, brucellosis, and tetanus, to name a few. Recent reports have documented laboratory-acquired cases in laboratory workers and healthcare personnel involving *Bacillus anthracis, Bordetella pertussis, Brucella, Burkholderia pseudomallei, Campylobacter, Chlamydia*, and toxins from *Clostridium tetani, Clostridium botulinum,* and *Corynebacterium diphtheriae*. In 2011, the CDC traced an outbreak of *Salmonella* to several undergraduate microbiology laboratories, prompting further discussion about safety guidelines for the lab classroom setting.

The "Biohazard" symbol must be affixed to any container or equipment used to store or transport potentially infectious materials.

Centers for Disease Control and Prevention (CDC)

The term *containment* is used to describe the safe methods and procedures for handling and managing microorganisms in the laboratory. An important laboratory procedure practiced by all microbiologists that will guarantee containment is **aseptic technique**, which prevents workers from contaminating themselves with microorganisms, ensures that others and the work area do not become contaminated, and also ensures that microbial cultures do not become unnecessarily contaminated with unwanted organisms. Containment involves personnel and the immediate laboratory environment. Containment also guarantees that infectious agents do not escape from the laboratory and contaminate the environment external to the lab. Containment, therefore, relies on good microbiological technique and laboratory protocol as well as the use of appropriate safety equipment.

Biosafety Levels (BSL)

The biosafety level classifications of microorganisms represent the potential of the organism to cause disease and the conditions under which the organism should be safely handled. The CDC classifies organisms into four levels, which take into account many factors such as virulence, pathogenicity, antibiotic resistance patterns, vaccine and treatment availability, and other factors. The four biosafety levels are described in the table on page xii.

All microorganisms used in the exercises in this manual are classified as BSL-1 or BSL-2. Note: Although some of the organisms that students will culture and work with are classified as BSL-2, these organisms may be laboratory strains that do not pose the same threat of infection as primary isolates of the same organism taken from patients in clinical samples. Hence, these laboratory strains can, in most cases, be handled using normal procedures and equipment found in the vast majority of student teaching laboratories. However, it should be emphasized that many bacteria are opportunistic pathogens, and therefore all microorganisms should be handled by observing proper techniques and precautions.

Each of the biosafety levels indicates that certain laboratory practices and techniques, safety equipment, and laboratory facilities should be used when working with organisms in that classification. Each combination is specifically appropriate for the operations performed and the documented or suspected routes of transmission of the infectious agents. In response to the *Salmonella* outbreaks in undergraduate laboratories, the American Society for Microbiology set out to define a clear set of safety practices for laboratories based on the use of BSL-1 or BSL-2 organisms. In 2013, the *Guidelines for Biosafety in Teaching Laboratories* was published in the *Journal of Microbiology Education*.

Standard Laboratory Practices (Based on ASM *Guidelines for Biosafety in Teaching Laboratories*)

BSL-1 Guidelines

Although BSL-1 organisms pose very little risk of disease for healthy students, they are still capable of causing infection under certain circumstances. These guidelines indicate the recommended best practices in the laboratory for the protection of students and the community.

Personal Protection

- It is recommended that lab coats be worn in the laboratory at all times. Lab coats can protect a students from contamination by microorganisms that they are working with and prevent contamination from stains and chemicals. At the end of

Biosafety Levels for Selected Infectious Agents

BIOSAFETY LEVEL (BSL)	TYPICAL RISK	ORGANISM
BSL-1	Not likely to pose a disease risk to healthy adults.	*Achromobacter denitrificans* *Alcaligenes faecalis* *Bacillus cereus* *Bacillus subtilis* *Corynebacterium pseudodiphtheriticum* *Micrococcus luteus* *Neisseria sicca* *Staphylococcus epidermidis* *Staphylococcus saprophyticus*
BSL-2	Poses a moderate risk to healthy adults; unlikely to spread throughout the community; effective treatment readily available.	*Enterococcus faecalis* *Klebsiella pneumoniae* *Mycobacterium phlei* *Salmonella enterica var. Typhimurium* *Shigella flexneri* *Staphylococcus aureus* *Streptococcus pneumoniae* *Streptococcus pyogenes*
BSL-3	Can cause disease in healthy adults; may spread to the community; effective treatment readily available.	*Blastomyces dermatitidis* *Chlamydia trachomatis* *Coccidioides immitis* *Coxiella burnetii* *Francisella tularensis* *Histoplasma capsulatum* *Mycobacterium bovis* *Mycobacterium tuberculosis* *Pseudomonas mallei* *Rickettsia canadensis* *Rickettsia prowazekii* *Yersinia pestis*
BSL-4	Can cause disease in healthy adults; poses a lethal risk and does not respond to vaccines or antimicrobial therapy.	Filovirus *Herpesvirus simiae* Lassa virus Marburg virus Ebola virus

the laboratory session, lab coats are usually stored in the lab in a manner prescribed by the instructor.

- You may be required to wear gloves while performing the lab exercises. They protect the hands against contamination by microorganisms and prevent the hands from coming in direct contact with stains and other reagents. This is especially important if you have open wounds.
- Wash your hands with soap and water before and after handling microorganisms.
- Safety goggles or glasses should be worn while you are performing experiments with liquid cultures, splash hazards, or while spread plating. They must also be worn when working with ultraviolet light to prevent eye damage because they block out ultraviolet (UV) rays.
- Sandals or open-toe shoes are not to be worn in the laboratory. Accidental dropping of objects or cultures could result in serious injury or infection.
- Lab coats, gloves, and safety equipment should not be worn outside of the laboratory unless properly decontaminated first.
- Students with long hair should tie the hair back to avoid accidents when working with Bunsen burners/open flames. Long hair can also be a source of contamination when working with cultures.
- Avoid wearing dangling jewelry or scarves to lab.
- If you are immune-compromised (including pregnancy) or provide care for someone who is immune-compromised, please consult with your physician about your participation in these laboratory exercises.

Laboratory Environment and Equipment

- Most importantly, read the exercise and understand the laboratory protocol before coming to the laboratory. This way, you will be familiar with potential hazards in the exercise. Unless directed

to do so, do not subculture any unknown organisms isolated from the environment as they could be potential pathogens.
- Students should store all books and materials not used in the laboratory in areas or receptacles designated for that purpose. Only necessary materials such as a lab notebook and the laboratory manual should be brought to the student work area. Use only institution-provided writing instruments.
- Avoid handling personal items such as cell phones and calculators while performing laboratory exercises. Students must also avoid handling contact lenses or applying makeup while in the laboratory.
- Eating, drinking (including water), gum, chewing tobacco, and smoking are not allowed in the laboratory.
- Know the location of exits and safety equipment such as the eye wash and shower stations, first aid kit, and fire extinguisher in the event of an accident that requires the use of this equipment.
- The door to the laboratory must remain closed, and only enrolled students should be allowed to enter the laboratory classroom.
- Before beginning the activities for the day, work areas should be wiped down with the disinfectant that is provided for that purpose. Likewise, when work is finished for the day, the work area should be treated with disinfectant to ensure that any contamination from the exercise performed is destroyed. To avoid contaminating the work surface, do not place contaminated pipettes, loops/needles, or swabs on the work surface.
- Always use correct labeling procedures on all containers.
- If possible, use a microincinerator or disposable loops for transferring microorganisms from one container to another. If these are unavailable, please use extreme caution when working with the open flame of a Bunsen burner. The flame is often difficult to see.
- Caution is imperative when working with alcohol and open flames. Alcohol is highly flammable, and fires can easily result when using glass rods that have been dipped in alcohol.
- Always make sure the gas is turned off before leaving the laboratory.
- Pipetting by mouth is prohibited in the lab. All pipetting must be performed with pipette aids.
- Use test tube racks when transporting cultures throughout the laboratory.
- You may be required to sign a safety agreement stating that you have been informed about safety issues and precautions and the hazardous nature of microorganisms that you may handle during the laboratory course.

BSL-2 Guidelines

BSL-2 organisms pose a moderate risk of infections, but the diseases caused by these organisms are treatable and usually not serious. Before working with these organisms, students should already show proficiency in following all of the guidelines for BSL-1 organisms. Additional precautions that should be taken when working with BSL-2 organisms include:

- Wear face shields or masks, along with proper eye wear, when working with procedures that involve a potential splash hazard. Alternatively, conduct all work with these organisms in a biological safety cabinet.
- Laboratory coats are required when working with these organisms.
- Use microincinerators or disposable loops rather than Bunsen burners.

Emergencies

- Report all spills, accidents, or injuries immediately to the laboratory instructor.
- Do not handle broken glass with your hands.
- Follow institutional policy in documenting all injuries and other emergency situations.

Disposal of Laboratory Materials

Dispose of all contaminated materials properly and in the appropriate containers. Your instructor will give you specific instructions for your laboratory classroom.

- Biohazard containers—Biohazard containers are to be lined with clearly marked biohazard bags; disposable agar plates, used gloves, and any materials such as contaminated paper towels should be discarded in these containers; no glassware, test tubes, or sharp items are to be disposed of in biohazard containers.
- Sharps containers—Sharps, needles, and Pasteur pipettes should be discarded in these containers.
- Autoclave shelf, cart, or bin—Contaminated culture tubes and glassware used to store media and other glassware should be placed in these areas for decontamination and washing.
- Trash cans—Any noncontaminated materials, paper, or trash should be discarded in these containers. Under no circumstances should laboratory waste be disposed of in trash cans.
- Slides and broken glass may be disposed of in a sharps container, a beaker filled with disinfectant, or a labeled cardboard box. Listen carefully to your instructor's directions for these items.

Microorganisms Used or Isolated in the Lab Exercises in This Manual

ORGANISM	GRAM STAIN AND MORPHOLOGY	HABITAT	BSL	LAB EXERCISE
Alcaligenes faecalis ATCC 8750	Negative rod	Decomposing organic material, feces	1	26, 39
Bacillus cereus ATCC 14579	Positive rod	Soil	1	15
Bacillus coagulans ATCC 7050	Positive rod	Spoiled food, silage	1	48
Bacillus megaterium ATCC 14581	Positive rod	Soil, water	1	10, 11, 12, 15, 28, 47
Bacillus subtilis ATCC 23857	Positive rod	Soil, decomposing organic matter	1	24, 37
Chromobacterium violaceum ATCC 12472	Negative rod	Soil and water; opportunistic pathogen in humans	2	9
Clostridium sporogenes ATCC 3584	Positive rod	Soil, animal feces	1	24, 48
Corynebacterium xerosis ATCC 373	Positive rods, club-shaped	Conjunctiva, skin	1	10, 11
Enterobacter aerogenes ATCC 13048	Negative rods	Feces of humans and animals	1	36, 39
Enterococcus faecalis ATCC 19433	Positive cocci in pairs, short chains	Water, sewage, soil, dairy products	2	24, 39, 59
Enterococcus faecium ATCC 19434	Positive cocci in pairs, short chains	Feces of humans and animals	2	59
Escherichia coli ATCC 11775 (or 8677 or HB101 as noted in exercise)	Negative rods	Sewage, intestinal tract of warm-blooded animals	1	8, 9, 14, 19, 21, 22, 24, 25, 26, 27, 29, 31, 32, 36, 37, 38, 39, 47, 48, 51, 52
Geobacillus stearothermophilus ATCC 12980	Gram-positive rods	Soil, spoiled food	1	25, 48
Halobacterium salinarium ATCC 33170	Gives gram-negative reaction; rods	Salted fish, hides, meats	1	27
Klebsiella pneumoniae ATCC 13883	Negative rods	Intestinal tract of humans; respiratory and intestinal pathogen in humans	2	13, 39
Micrococcus luteus ATCC 12698	Positive cocci that occur in pairs	Mammalian skin	1	9, 29, 39
Moraxella catarrhalis ATCC 25238	Negative cocci that often occur in pairs with flattened sides	Pharynx of humans	1	14

Microorganisms Used or Isolated in the Lab Exercises in This Manual (continued)

ORGANISM	GRAM STAIN AND MORPHOLOGY	HABITAT	BSL	LAB EXERCISE
Mycobacterium smegmatis ATCC 19420	Positive rods; may be Y-shaped or branched	Smegma of humans	1	16
Proteus vulgaris ATCC 29905	Negative rods	Intestines of humans, and animals; soil and polluted waters	2	37, 38, 39, 55
Pseudomonas fluorescens ATCC 13525	Negative rods	Soil and water; opportunistic pathogen in humans	2	14, 17, 31, 36, 39
Saccharomyces cerevisiae ATCC 18824	Yeast	Fruit, used in beer, wine, and bread	1	26
Salmonella enterica subsp. enterica serovar Typhimurium ATCC 700720	Negative rods	Most frequent agent of *Salmonella* gastroenteritis in humans	2	39, 55, 57
Serratia marcescens ATCC 13880	Negative rods	Opportunistic pathogen in humans	1	9, 25, 31, 39
Shigella flexneri ATCC 29903	Negative rods	Pathogen of humans	2	55
Staphylococcus aureus ATCC 12600	Positive cocci, irregular clusters	Skin, nose, GI tract of humans, pathogen	2	23, 39, 53, 54, 58
Staphylococcus epidermidis ATCC 14990	Positive cocci that occur in pairs and tetrads	Human skin, animals; opportunistic pathogen	1	10, 11, 12, 14, 16, 17, 26, 27, 28, 29, 31, 32, 36, 37, 38, 39, 47, 53
Staphylococcus saprophyticus ATCC 15305	Positive cocci that occur singly and in pairs	Human skin; opportunistic pathogen in the urinary tract	1	53
Streptococcus agalactiae ATCC 13813	Positive cocci; occurs in long chains	Upper respiratory and vaginal tract of humans, cattle; pathogen	2	54, 59
Streptococcus bovis ATCC 33317	Positive cocci; pairs and chains	Cattle, sheep, pigs; occasional pathogen in humans	1	54, 59
Streptococcus dysagalactiae subsp. equisimilis ATCC 12394	Positive cocci in chains	Mastitis in cattle	2	54

Microorganisms Used or Isolated in the Lab Exercises in This Manual (continued)

ORGANISM	GRAM STAIN AND MORPHOLOGY	HABITAT	BSL	LAB EXERCISE
Streptococcus mitis ATCC 49456	Positive cocci in pairs and chains	Oral cavity of humans	2	54
Streptococcus mutans ATCC 25175D-5	Positive cocci in pairs and chains	Tooth surface of humans, causes dental caries	1	54
Streptococcus pneumoniae ATCC 33400	Positive cocci in pairs	Human pathogen	2	54
Streptococcus pyogenes ATCC 12344	Positive cocci in chains	Human respiratory tract; pathogen	2	54, 59
Streptococcus salivarius ATCC 19258	Positive cocci in short and long chains	Tongue and saliva	1	54
Thermoanaerobacterium thermosaccharolyticum ATCC 7956	Negative rods; single cells or pairs	Soil, spoiled canned foods	1	48

PART 1

Microscopy

Although there are many kinds of microscopes available to the microbiologist today, this unit focuses on several types, including brightfield, darkfield, and phase-contrast microscopes. If you have had extensive exposure to microscopy in previous courses, these exercises may not be of great value to you; however, if the study of microorganisms is a new field for you, there is a great deal of information that you need to properly use these instruments.

JGI/Blend Images

Microscopes in a college laboratory represent a considerable investment and require special care to prevent damage to the lenses and mechanical parts. A microscope may be used by several people during the day and moved from the work area to storage, which results in a much greater chance for damage to the instrument than if the microscope were stationary and used by only a single person.

The complexity of some microscopes also requires that certain adjustments be made periodically. Knowing how to make these adjustments to get the equipment to perform properly is very important. This unit will teach you how to use specific features of different microscopes to operate them successfully.

Microscopy should be as fascinating to the beginner as it is to the long-time professional; however, only with knowledge and a little practice can the beginner get the most out of a microscopy experience.

EXERCISE 1

Brightfield Microscopy

Learning Outcomes

After completing this exercise, you should be able to

1. Identify the basic components of a brightfield microscope and understand the function of each component in proper specimen observation.
2. Examine a specimen using the low-power, high-dry, and oil immersion lenses.
3. Understand the proper use, care, and storage of a microscope.

Figure 1.1 **A model of a Leeuwenhoek microscope from the late 1600s.**
Tetra Images/Alamy Stock Photo

A microscope that allows light rays to pass directly to the eye is called a ***brightfield microscope.*** This is the most common type of instrument encountered by students in beginning biology courses. All brightfield microscopes have certain things in common, yet they differ somewhat in mechanical operation. Similarities and differences of various models are discussed in this exercise so that you will know how to use the instrument that is available to you. *Before attending the first laboratory session in which the microscope is used*, read over this exercise and answer all the questions on the Laboratory Report.

Why It Matters

How can you apply this exercise to the real world?

Prior to the development of the microscope, medical doctors believed that epidemic diseases such as cholera and plague were caused by "bad air." They did not know that microorganisms existed until the late 1600s when Anton van Leeuwenhoek discovered them using the microscopes he had designed (figure 1.1).

Today, scientists routinely use microscopes as one of many tools to learn about the microbes that cause infectious diseases and to diagnose patients suffering from these diseases.

Components

The labels in figure 1.2 indicate the general parts of a common brightfield microscope. Your microscope should have these same components, but they may look slightly different or be located in different areas of the microscope. Take time to familiarize yourself with where these components can be found on your particular model.

Light Source A light source of some kind is positioned in the base of most microscopes. Ideally, the light should have an **intensity control** knob or slider to vary the intensity of light. This is often found near the on/off switch. The microscope in figure 1.2 has a knob under the on/off switch to regulate the voltage supplied to the lightbulb. If you turn on your microscope and the light does not appear to be working, check this control knob to make sure the intensity hasn't been turned down so far that the light appears to be out.

Lens Systems All compound microscopes have three lens systems: the oculars, the objectives, and the condenser. Figure 1.3 illustrates the light path through these three systems.

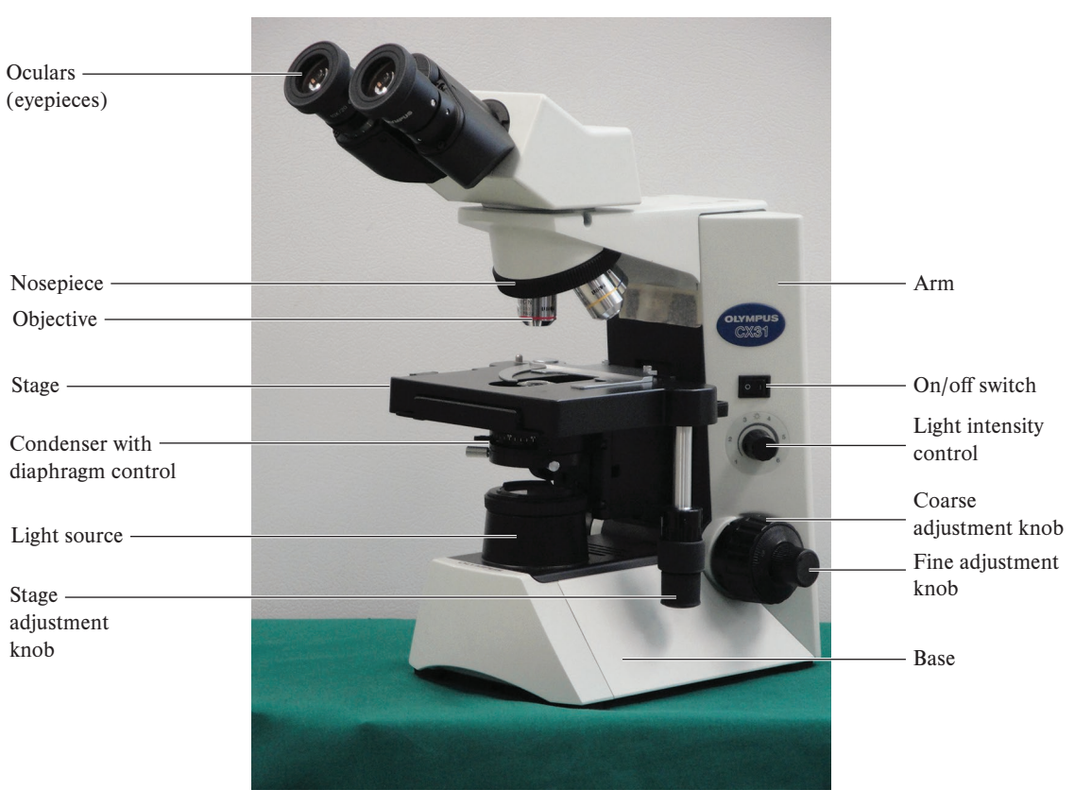


Figure 1.2 **Components of a compound brightfield microscope.**
James Redfearn/McGraw-Hill Education

The **ocular,** or eyepiece, is a complex piece, located at the top of the instrument, that consists of two or more internal lenses and usually has a magnification of 10×. Most modern microscopes have two ocular (binocular) lenses.

Three or more **objectives** are usually present. Note that they are attached to a rotatable **nosepiece,** which makes it possible to move them into position over a slide. Objectives on most laboratory microscopes have magnifications of 10×, 40×, and 100×, designated as **low-power, high-dry,** and **oil immersion,** respectively. Some microscopes will have a fourth objective (4×) for rapid scanning of a slide.

The **total magnification** of a compound microscope is determined by multiplying the power of the ocular lens times the power of the objective lens used. Thus, the magnification of a specimen when the oil immersion lens is being used is:

10 (ocular lens) × 100 (oil immersion lens) = 1000

The object is now magnified 1000 times its actual size.

The third lens system is the **condenser,** which is located under the stage. It collects and directs the light from the light source to the slide being studied. Unlike the ocular and objective lenses, the condenser lens does not affect the magnifying power of the compound microscope. The condenser can be moved up and down by a knob under the stage. A **diaphragm** within the condenser regulates the amount of light that reaches the slide. On some microscope models, the diaphragm is controlled by turning a knob, whereas on others, a diaphragm lever is present. Figure 1.2 illustrates the location of the condenser and diaphragm.

Focusing Knobs The concentrically arranged **coarse adjustment** and **fine adjustment knobs** on the side of the microscope are used for bringing objects into focus when studying an object on a slide. The coarse adjustment knob moves the stage up and down considerably, but the fine adjustment knob only moves it very slightly. Therefore, it is critical that the correct knob is used when focusing under the different objectives lenses.

Figure 1.3 **The light pathway of a microscope.**

Ocular Adjustments On binocular microscopes, one must be able to change the distance between the oculars and to make changes for right and left eye differences (diopter adjustments). On most microscopes, the interocular distance is changed by simply pulling apart or pushing together the oculars.

To make diopter adjustments, one focuses first with the right eye only. Without touching the focusing knobs, diopter adjustments are then made on the left eye by turning the **diopter adjustment ring** on the left ocular until a sharp image is seen. One should now be able to see sharp images with both eyes.

Resolution

It would appear that the magnification of a microscope is only limited by the magnifying power of a lens system. However, in reality, the limit for most light microscopes is 1000×, which is set by an intrinsic

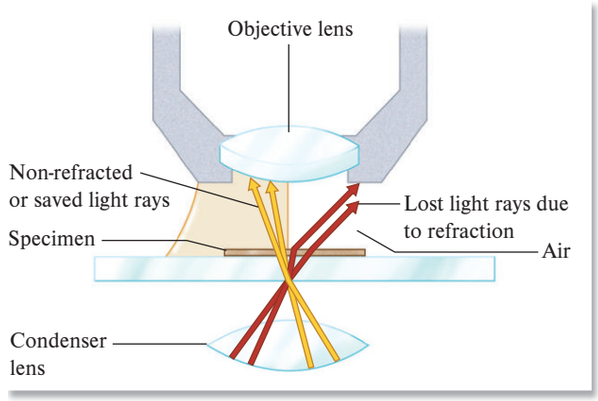


Figure 1.4 **Immersion oil, having the same refractive index as glass, prevents light loss due to refraction.**

property of lenses called **resolving power.** The resolving power of a lens is its ability to completely separate two objects in a microscopic field. The resolving power is given by the formula $d = 0.5\ \lambda/\text{NA}$. The limit of resolution, d, or the distance between the two objects, is a function of two properties: the wavelength of the light used to observe a specimen, λ, and a property of lenses called the **numerical aperture,** or NA. Numerical aperture is a mathematical expression that describes how the condenser lens concentrates and focuses the light rays from the light source. Its value is maximized when the light rays are focused into a cone of light that then passes through the specimen into the objective lens. However, because some light is refracted or bent as it passes from glass into air, the refracted light rays are lost, and as a result the numerical aperture is diminished (figure 1.4). The greater the loss of refracted light, the lower the numerical aperture. The final result is that the resolving power is greatly reduced.

For any light microscope, the limit of resolution is about 0.2 µm. This means that two objects closer than 0.2 µm would not be seen as two distinct objects. Because bacterial cells are about 1 µm, the cells can be resolved by the light microscope, but that is not the case for internal structures in bacterial cells that are smaller than 0.2 µm.

In order to maximize the resolving power from a lens system, the following should be considered:

- A blue filter can be placed over the light source because the shorter wavelength of the resulting light will provide maximum resolution.
- The condenser should be kept at the highest position that allows the maximum amount of light to enter the objective lens and therefore limit the amount of light lost due to refraction.

- The diaphragm should not be closed too much. While closing the diaphragm improves the contrast, it also reduces the numerical aperture.
- **Immersion oil** must be used between the slide and the 100× objective lens. This is a special oil that has the same refractive index as glass. When placed between the specimen and objective lens, the oil forms a continuous lens system that limits the loss of light due to refraction (figure 1.4).

The bottom line is that for magnification to increase, resolution must also increase. Thus, a greater magnification cannot be achieved simply by adding a stronger objective lens.

Care of the Instrument

Microscopes represent considerable investment and can be damaged easily if certain precautions are not observed. The following suggestions cover most hazards.

Transport When carrying your microscope from one part of the room to another, use both hands to hold the instrument, as illustrated in figure 1.5. If it is carried with only one hand and allowed to dangle at your side, there is always the danger of collision with furniture or some other object. *Under no circumstances should one attempt to carry two microscopes at one time.*

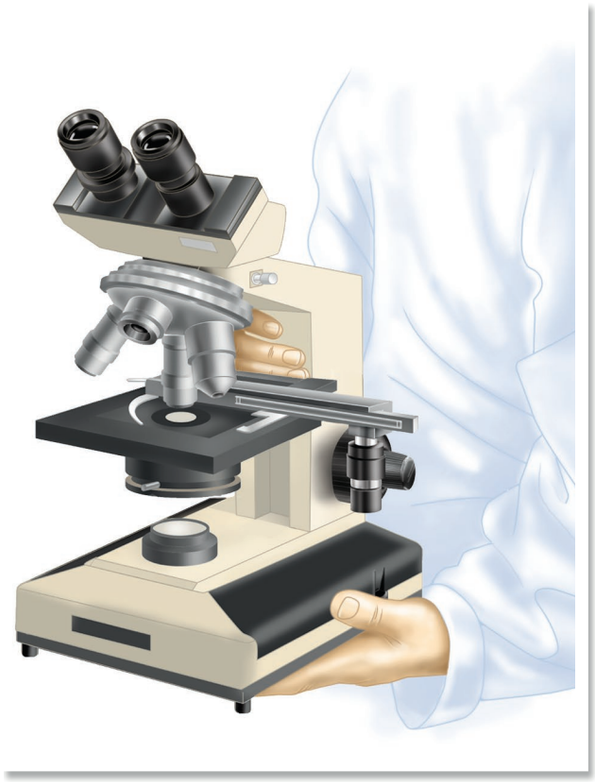


Figure 1.5 **The microscope should be held firmly with both hands while being carried.**

Clutter Keep your workstation uncluttered while doing microscopy. Keep unnecessary books and other materials away from your work area. A clear work area promotes efficiency and results in fewer accidents.

Electric Cord Microscopes have been known to tumble off of tabletops when students have caught their foot in a dangling electric cord. Do not let excess cord hang off the workbench.

Dust Protection In most laboratories, covers are used to protect the instruments during storage. If one is available, place it over the microscope at the end of the period.

Lens Care

Keeping the lenses of your microscope clean is a constant concern. Unless all lenses are kept free of dust, oil, and other contaminants, they cannot achieve the degree of resolution that is intended. At the beginning of each laboratory period, check the lenses to make sure they are clean. At the end of each lab session, be sure to wipe any immersion oil off the immersion lens if it has been used. Consider the following suggestions for cleaning the various lens components:

Cleaning Tissues and Solvents Only lint-free, optically safe tissues should be used to clean lenses. Tissues free of abrasive grit fall in this category. Booklets of lens tissue are most widely used for this purpose. Various liquids can be used for cleaning microscope lenses. *Use only the type of tissue and cleaning liquids that are recommended by your instructor.*

Cleaning the Oculars The best way to determine if your eyepiece is clean is to rotate it as you look through the microscope. If objects seen through the microscope move with the rotation, this is evidence of dirt and artifacts on the oculars. If cleaning the top lens of the ocular with lens tissue fails to remove the debris, try cleaning the lower lens with lens tissue.

Cleaning the Objectives Objective lenses often become soiled by materials from slides or fingers. A piece of lens tissue moistened with an approved solvent or water will usually remove whatever is on the lens. At any time that the image on the slide is unclear or cloudy, assume at once that the objective you are using is dirty.

Cleaning the Condenser Dust often accumulates on the top surface of the condenser; thus, wiping it off occasionally with lens tissue is desirable.

Procedures

If your microscope has three objectives, you have three magnification options: (1) low-power, or 100× total magnification with the 10× objective, (2) high-dry magnification, which is 400× total with a 40× objective, and (3) 1000× total magnification with a 100× oil immersion objective.

Whether you use the low-power objective or the oil immersion objective will depend on how much magnification is necessary. Generally speaking, however, it is best to start with the low-power objective and progress to the higher magnifications as your study progresses. Obtain a specimen slide from your instructor and use the following suggestions to make microscopic observations.

Low-Power Examination The main reason for starting with the low-power objective is to enable you to explore the slide to look for the object you are planning to study. Once you have found what you are looking for, you can proceed to higher magnifications. Use the following steps when exploring a slide with the low-power objective:

1. Position the slide on the stage with the material to be studied on the *upper* surface of the slide. The slide must be held in place by the mechanical stage lever.
2. Turn on the light source, using a *minimum* amount of voltage. If necessary, reposition the slide so that the stained material on the slide is in the *exact center* of the light source.
3. Check the condenser to see that it has been raised to its highest point.
4. If the low-power objective is not directly over the center of the stage, rotate it into position. Be sure that as you rotate the objective into position, it clicks into its locked position.
5. Turn the coarse adjustment knob to lower the objective until it stops. A built-in stop will prevent the objective from touching the slide.
6. While looking down through the oculars, bring the object into focus by turning the coarse adjustment knob very slowly or using the fine adjustment focusing knob. If you are using a binocular microscope, it will also be necessary to adjust the interocular distance and diopter adjustment ring to match your eyes.
7. For optimal viewing, it is necessary to focus the condenser and adjust it for maximum illumination. This procedure should be performed each time the objective lens is changed. Raise the iris diaphragm to its highest position. Close the iris diaphragm until the edges of the diaphragm image appear fuzzy. Lower the condenser using its adjustment knob until the edges of the diaphragm are brought into sharp focus. You should now clearly see the sides of the diaphragm expand beyond the field of view. Refocus the specimen using the fine adjustment. Note that as you close the iris diaphragm to reduce the light intensity, the contrast improves.
8. Once an image is visible, move the slide about to search out what you are looking for. The slide is moved by turning the stage adjustment knobs that move the mechanical stage.
9. Check the cleanliness of the ocular, using the procedure outlined earlier.
10. Once you have identified the structures to be studied and wish to increase the magnification, you may proceed to either high-dry or oil immersion magnification. However, before changing objectives, *be sure to center the object you wish to observe.*

High-Dry Examination To proceed from low-power to high-dry magnification, all that is necessary is to rotate the high-dry objective into position and open up the diaphragm somewhat. It may be necessary to make a minor adjustment with the fine adjustment knob to sharpen up the image, but *the coarse adjustment knob should not be touched.*

Figure 1.6 **When you are using the high-dry or oil immersion lens, use only the fine adjustment knob.**
©Adam Gault /AGE fotostock

Good quality modern microscopes are usually both **parfocal** and **parcentral.** This means that the image will remain both centered and in focus when changing from a lower-power to a higher-power objective lens.

When increasing the lighting, be sure to open up the diaphragm first instead of increasing the intensity of the light source; the reason is that bulb life is greatly extended when used at low voltage. If the field is not bright enough after opening the diaphragm, feel free to increase the intensity. A final point: Keep the condenser at its highest point.

Oil Immersion Techniques The oil immersion lens derives its name from the fact that a special mineral oil is placed between the specimen and the 100× objective lens. As stated previously, this reduces light refraction and maximizes the numerical aperture to improve the resolution. The use of oil in this way enhances the resolving power of the microscope. Figure 1.4 reveals this phenomenon.

With a parfocal microscope, one can go directly to oil immersion from either low-power or high-dry. However, going from low-power to high-power and then to oil immersion is usually better. Once the microscope has been brought into focus at one magnification, the oil immersion lens can be rotated into position without fear of striking the slide.

Before rotating the oil immersion lens into position, however, a drop of immersion oil must be placed directly on the slide. An oil immersion lens should never be used without oil.

When using the oil immersion lens, more light is necessary to adequately visualize an image. Opening the diaphragm increases the resolving power of the microscope at higher magnifications. Thus, the iris diaphragm must be opened wider when using the oil immersion lens. Also, do not forget to refocus the condenser when moving from lower-power to higher-power objectives.

Since the oil immersion lens will be used extensively in all bacteriological studies, it is of paramount importance that you learn how to use this lens properly. Using this lens takes a little practice due to the difficulties usually encountered in manipulating the lighting. It is important for all beginning students to appreciate that the **working distance** of a lens, the distance between the lens and microscope slide, decreases significantly as the magnification of the lens increases. Hence, the potential for damage to the oil immersion lens because of a collision with the microscope slide is very great. A final comment: *Do not rotate the high-dry (40×) lens back into place after oil has been placed on the slide.* If you must go back to a lower magnification, clean oil completely off the slide and oil immersion lens before proceeding.

Putting It Away

When you take a microscope from the cabinet at the beginning of the period, you expect it to be clean and in proper working condition. The next person to use the instrument after you have used it will expect the same consideration. A few moments of care at the end of the period will ensure these conditions. Check over the following list of items at the end of each period before you return the microscope to the cabinet.

1. Remove the slide from the stage and clean or dispose of it according to your instructor's directions.
2. If immersion oil has been used, wipe it off the lens and stage with lens tissue. Also, make sure that no immersion oil is on the 40× objective. This lens often becomes contaminated with oil as a result of mistakes made by beginning students.
3. Rotate the low-power objective into position.
4. Adjust the mechanical stage so that it is centered and as low as possible.
5. If the microscope has a long attached electric cord, secure it with a rubber band or wrap it around the base.
6. Replace the cover.
7. Return the microscope to its correct place in the cabinet.

Laboratory Report

Before the microscope is to be used in the laboratory, answer all the questions on Laboratory Report 1. Preparation on your part prior to going to the laboratory will greatly facilitate your understanding. Your instructor may wish to collect this report at the *beginning of the period* on the first day that the microscope is to be used in class.

Laboratory Report 1

Student: _____

Date: _____ Section: _____

1 Brightfield Microscopy

A. Short-Answer Questions

1. Describe the position of your hands when carrying the microscope to and from your laboratory bench.

_____

_____

2. Differentiate between the limit of resolution of the typical light microscope and that of the unaided human eye.

_____

_____

3. (a) What two adjustments can be made to the condenser? (b) What effect do these adjustments have on the image?

_____

_____

4. Why are condenser adjustments generally preferred over the use of the light intensity control?

_____

_____

5. When using the oil immersion lens, what four procedures can be implemented to achieve the maximum resolution?

_____

_____

_____

6. Why is it advisable to start first with the low-power lens when viewing a slide?

_____

_____

7. Why is it necessary to use oil in conjunction with the oil immersion lens and not with the other objectives?

_____

_____

8. What is the relationship between the working distance of an objective lens and its magnification power?

_____

_____

B. Matching Questions

Match the lens to its description. Choices may be used more than once.

A. Condenser
B. High-dry objective
C. Low-power objective
D. Ocular
E. Oil immersion objective

1. This objective lens provides the highest magnification.
2. This objective lens provides the second-highest magnification.
3. This objective lens provides the lowest magnification.
4. This objective lens has the shortest working distance.
5. The coarse focus knob should be adjusted only when using this objective lens.
6. This lens collects and focuses light from the light source onto the specimen on the slide.
7. This lens, also known as the eyepiece, often comes in pairs.
8. Diopter adjustments can be made to this lens.
9. A diaphragm is used to regulate light passing through this lens.

C. True-False

Evaluate each statement, and write T for true and F for false.

1. Only lint-free, optically safe tissue should be used to wipe off microscope lenses.
2. The total magnification capability of a light microscope is only limited by the magnifying power of the lens system.
3. The coarse focus knob can be used to adjust the focus when using any of the objective lenses.
4. Once focus is achieved at one magnification, a higher-power objective lens can be rotated into position without fear of striking the slide.

D. Multiple Choice

Select the answer that best completes the following statements.

1. The resolving power of a microscope is a function of
   a. the magnifying power of the lenses.
   b. the numerical aperture of the lenses.
   c. the wavelength of light.
   d. Both (a) and (b) are correct.
   e. Both (b) and (c) are correct.
2. The coarse and fine focus knobs adjust the distance between
   a. the objective and ocular lenses.
   b. the ocular lenses.
   c. the ocular lenses and your eyes.
   d. the stage and the condenser lens.
   e. the stage and the objective lens.

3. A microscope that maintains focus when the objective magnification is increased is called
   a. binocular.
   b. myopic.
   c. parfocal.
   d. refractive.
   e. resolute.
4. The total magnification achieved when using a 100× oil immersion lens with 10× binocular eyepieces is
   a. 10×.
   b. 100×.
   c. 200×.
   d. 1000×.
   e. 2000×.
5. The most useful adjustment for increasing image contrast in low-power magnification is
   a. closing down the diaphragm.
   b. closing one eye.
   c. opening up the diaphragm.
   d. placing a drop of oil on the slide.
   e. turning the light source on as high as possible.
6. Before the oil immersion lens is rotated into place, you should
   a. center the object of interest in the preceding lens.
   b. lower the stage with use of the coarse focus adjustment knob.
   c. place a drop of oil on the slide.
   d. Both (a) and (c) are correct.
   e. All are correct.

EXERCISE 2

Darkfield Microscopy

Learning Outcomes

After completing this exercise, you should be able to

1. Visualize a specimen using a darkfield microscope.
2. Understand the difference between the illumination systems for the darkfield microscope and the brightfield microscope.

Why It Matters

How can you apply this exercise to the real world?

Delicate transparent living organisms can be more easily observed with darkfield microscopy than with conventional brightfield microscopy. This method is particularly useful when one is attempting to identify bacteria in body fluid from someone that has syphilis. **Figure 2.1** illustrates the appearance of these spirochetes under such darkfield illumination.

A brightfield microscope can be converted to produce the darkfield effect. This is accomplished by placing a darkfield stop below the regular condenser or by replacing the condenser with a specially constructed one. This is necessary to alter the light rays to approach the objective in such a way that only oblique rays strike the objects being viewed. The obliquity of the rays must be so extreme that if no objects are in the field, the background is completely light-free. Objects in the field become brightly illuminated by the rays that are reflected up through the lens system of the microscope.

Although there are several different methods for producing a dark field, only two devices will be described here: the star diaphragm and the cardioid condenser. The availability of equipment will determine the method to be used in this laboratory.

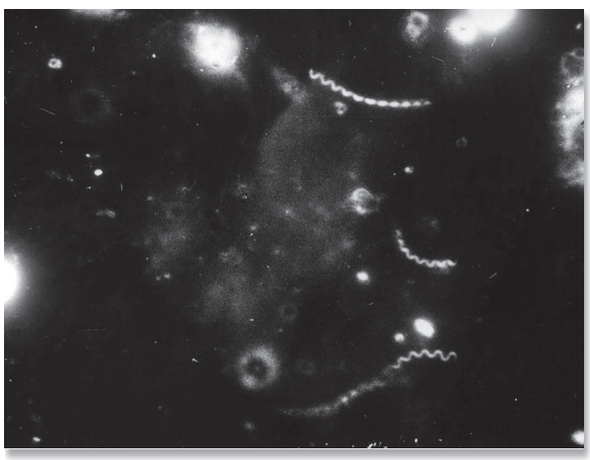


Figure 2.1 **Darkfield image of *Treponema pallidum*, the bacterium that causes syphilis.**
Source: Susan Lindsley/Centers for Disease Control and Prevention

The Star Diaphragm

One of the simplest ways to produce the darkfield effect is to insert a star diaphragm into the filter slot of the condenser housing, as shown in figure 2.2. This device has an opaque disk in the center that blocks the central rays of light. If your lab does not have this equipment, your instructor may demonstrate how to make your own star diaphragm. Figure 2.3 reveals the effect of this stop on the light rays passing through the condenser.

Figure 2.2 **The insertion of a star diaphragm into the filter slot of the condenser will produce a dark field suitable for low magnifications.**
Auburn University Photographic Services/McGraw-Hill Education

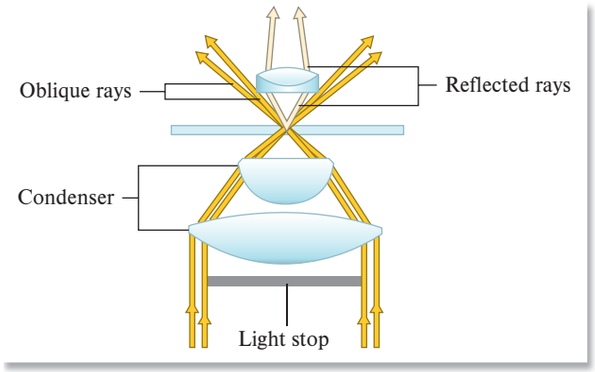


Figure 2.3 **The star diaphragm allows only peripheral light rays to pass up through the condenser. This method requires maximum illumination.**

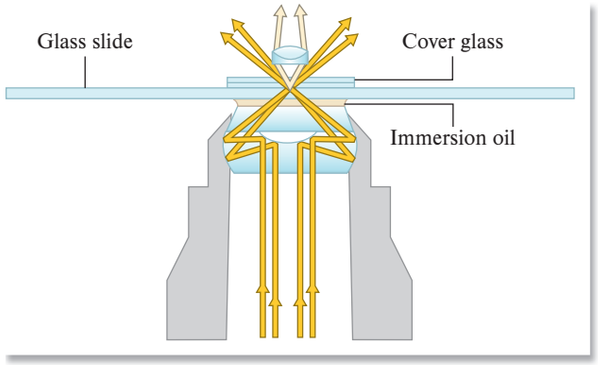


Figure 2.4 **A cardioid condenser provides greater light concentration for oblique illumination than the star diaphragm.**

In setting up this type of darkfield illumination, it is necessary to keep these points in mind:

1. Limit this technique to the study of large organisms that can be seen easily with low-power magnification. *Good resolution with higher-powered objectives is difficult with this method.*
2. Keep the diaphragm wide open and use as much light as possible. If the microscope has a light intensity control, you will find that the higher light intensity will produce better results.
3. Be sure to center the stop as precisely as possible.
4. Move the condenser up and down to produce the best effects.

The Cardioid Condenser

The difficulty that results from using the star diaphragm with high-dry and oil immersion objectives is that the oblique rays are not as carefully metered as is necessary for the higher magnifications. Special condensers such as the cardioid or paraboloid types must be used. Since the cardioid type is the most frequently used type, its use will be described here.

Figure 2.4 illustrates the light path through such a condenser. Note that the light rays entering the lower element of the condenser are reflected first off a convex mirrored surface and then off a second concave surface to produce the desired oblique rays of light. Once the condenser has been installed in the microscope, the following steps should be followed to produce ideal illumination.

1. Adjust the upper surface of the condenser to a height just below stage level.
2. Place a clear glass slide in position over the condenser.
3. Focus the 10× objective on the top of the condenser until a bright ring comes into focus.
4. Center the bright ring so that it is concentric with the field edge by adjusting the centering screws on the darkfield condenser. If the condenser has a light source built into it, it will also be necessary to center it as well to achieve even illumination.
5. Remove the clear glass slide.
6. If a funnel stop is available for the oil immersion objective lens, remove the oil immersion objective and insert the funnel stop. (This stop serves to reduce the numerical aperture of the oil immersion objective to a value that is less than that of the condenser.)
7. Place a drop of immersion oil on the upper surface of the condenser and place the slide on top of the oil. The following preconditions in slide usage must be adhered to:
   - Slides and cover glasses should be optically perfect. Scratches and imperfections will cause annoying diffractions of light rays.
   - Slides and cover glasses must be free of dirt or grease of any kind.
   - A cover glass should always be used.
8. If the oil immersion lens is to be used, place a drop of oil on the cover glass.
9. If the field does not appear dark and lacks contrast, return to the 10× objective and redo step 4. If contrast is still lacking after these adjustments, the specimen is probably too thick.
10. If sharp focus is difficult to achieve under oil immersion, try using a thinner cover glass and adding more oil to the top of the cover glass and bottom of the slide.

Laboratory Report

After reading over this exercise and completing any special assignments made by your instructor, answer the questions in Laboratory Report 2 about darkfield microscopy.

Laboratory Report 2

Student: _____

Date: _____ Section: _____

2 Darkfield Microscopy

Short-Answer Questions

1. For which types of specimens is darkfield microscopy preferred over brightfield microscopy?

2. If a darkfield condenser causes all light rays to bypass the objective, where does the light come from that makes an object visible in a dark field?

3. What advantage does a cardioid condenser have over a star diaphragm?

EXERCISE 3

Phase-Contrast Microscopy

Learning Outcomes

After completing this exercise, you should be able to

1. Understand how the phase-contrast microscope takes advantage of density differences in cell components to produce an image.
2. Align a phase-contrast microscope.
3. Properly use a phase-contrast microscope to obtain an image of living cells.

If one tries to observe cells without the benefit of staining, very little contrast or detail can be seen. Because cells are composed of mostly water, they will appear transparent against an aqueous background. Staining increases the contrast between the cell and its surrounding medium, allowing the observer to see more cellular detail, including some inclusions and various organelles. However, staining usually results in cell death, which means we are unable to observe living cells or their activities, and staining can also lead to undesirable artifacts. A microscope that is able to differentiate transparent internal cellular structures and enhance the contrast between a cell and its surroundings, without the necessity of staining, is the **phase-contrast microscope.** In this exercise, you will learn to use the phase-contrast microscope and observe the activities of living cells.

Why It Matters

How can you apply this exercise to the real world?

The phase-contrast microscope was developed by the Dutch physicist Frits Zernike in the 1930s. For this discovery, he was awarded the Nobel Prize in Physics in 1953. Today, it is the microscope of choice for viewing living cells and their activities such as motility. **Figure 3.1** is a phase-contrast image of an amoeba, displaying some amazing internal details of this unstained protozoan.

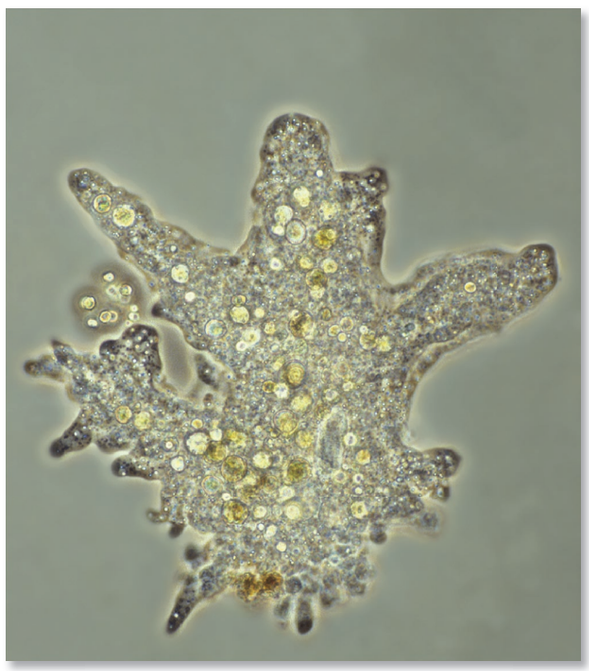


Figure 3.1 **Phase-contrast image of *Amoeba proteus*.**
Stephen Durr

Properties of Light

To understand how a phase-contrast microscope works, it is necessary to review some of the physical properties of light and how it interacts with matter such as biological material. Light energy can be represented as a waveform that has both an amplitude and a characteristic wavelength (illustration 1, figure 3.2). Some objects can reduce the amplitude of a light wave, and they would appear as dark objects in a microscope. In contrast, light can pass through matter without affecting the amplitude, and these objects would appear transparent in a microscope. However, as light passes through some of the transparent objects, it can be slowed down by $\frac{1}{4}$ wavelength, resulting in a **phase shift** of the light's wavelength (illustration 2, figure 3.2). For a cell, the phase shift without a reduction in amplitude results in the cell having a different refractive index than its surroundings. However, the phase shifts caused by biological

EXERCISE 3 Phase-Contrast Microscopy

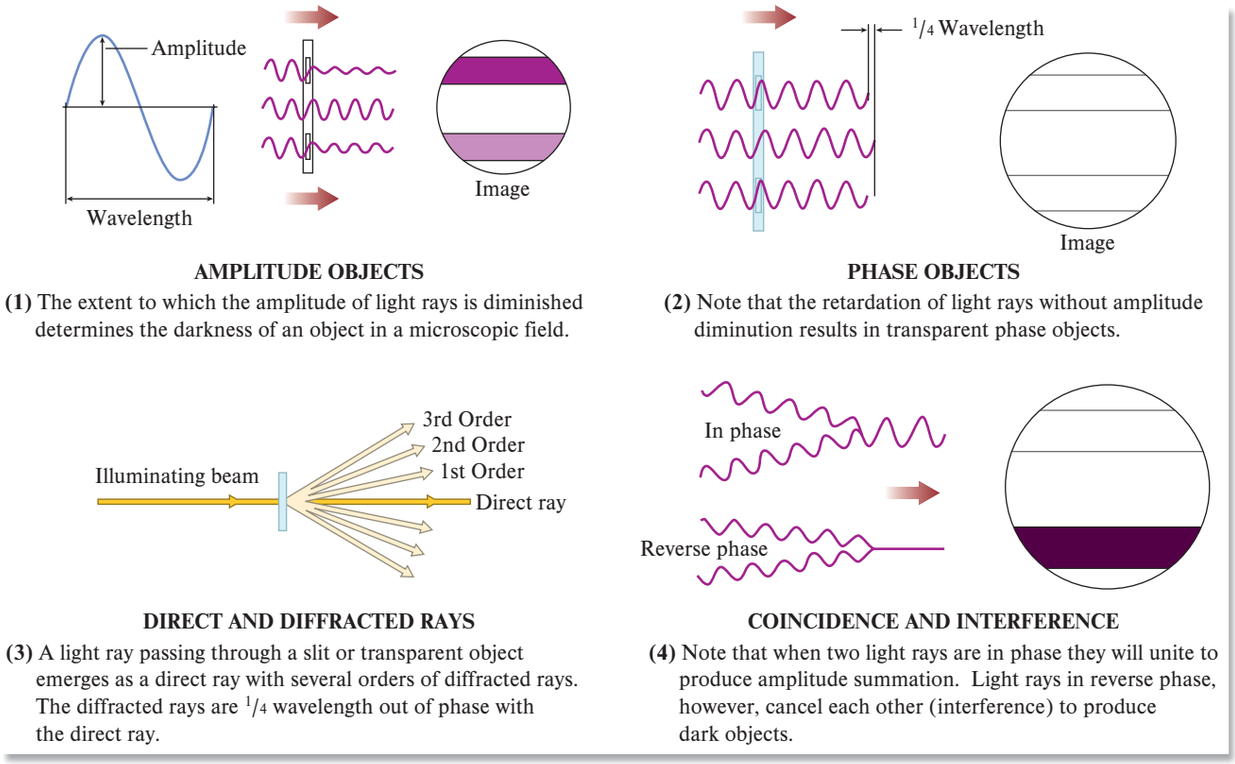


Figure 3.2 **The utilization of light rays in phase-contrast microscopy.**

material are usually too small to be seen as contrast differences in a brightfield microscope. Therefore, in a brightfield microscope, cells appear transparent rather than opaque against their surroundings. Since biological material lacks any appreciable contrast, it becomes necessary to stain cells with various dyes in order to study them. However, Zernike took advantage of the $\frac{1}{4}$ wavelength phase shift to enhance the small contrast differences in the various components that comprise a cell, making them visible in his microscope. This involved manipulating the light rays that were shifted and those that were unchanged as they emerged from biological material.

Light rays passing through a transparent object emerge as either direct or diffracted rays. Those rays that pass straight through unaffected by the medium are called **direct rays.** They are unaltered in amplitude and phase. The rays that are bent because they are retarded by the medium (due to density differences) emerge from the object as **diffracted rays.** It is these specific light rays that are retarded $\frac{1}{4}$ wavelength. Illustration 3, figure 3.2, shows these two types of light rays.

If the direct and diffracted light waves are brought into exact phase with each other, the result is **coincidence** with the resultant amplitude of the converged waves being the sum of the two waves. This increase in amplitude will produce increased brightness of the object in the field. In contrast, if two light waves of equal amplitude are in reverse phase ($\frac{1}{2}$ wavelength off), their amplitudes will cancel each other to produce a dark object. This is called **interference.** Illustration 4, figure 3.2, shows these two conditions.

Phase-Contrast Microscope

In constructing his first phase-contrast microscope, Zernike experimented with various configurations of diaphragms and various materials that could be used to retard or advance the direct light rays. Figure 3.3 illustrates the optical system of a typical modern phase-contrast microscope. It differs from a conventional brightfield microscope by having (1) a different type of diaphragm and (2) a phase plate.

The diaphragm consists of an **annular stop** that allows only a hollow cone of light rays to pass through the condenser to the specimen on the slide. The **phase plate** is a special optical disk located on the objective lens near its rear focal plane. It has a **phase ring** that advances or retards the direct light rays $\frac{1}{4}$ wavelength.

Note in figure 3.3 that the direct rays converge on the phase ring to be advanced or set back $\frac{1}{4}$ wavelength.

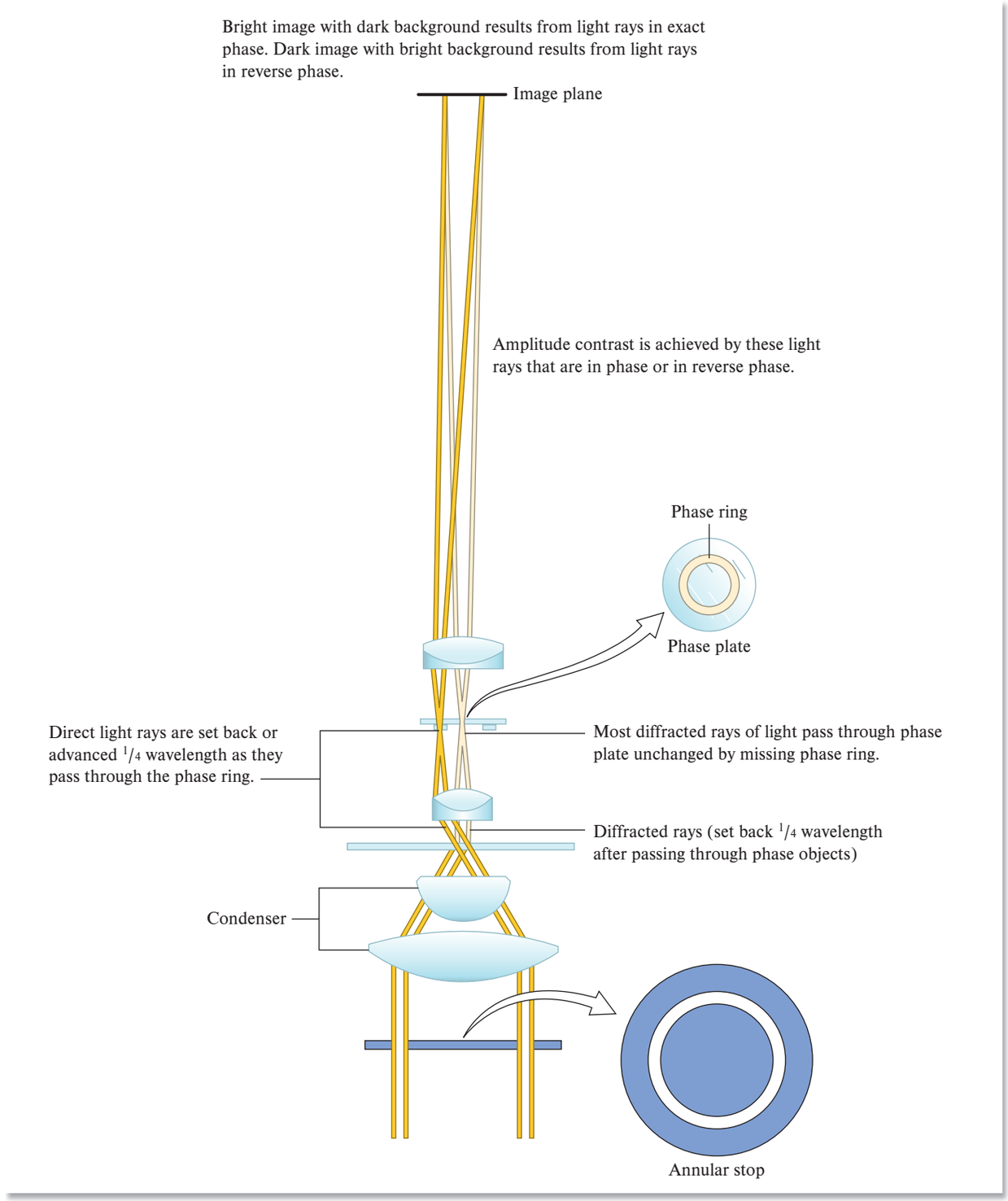


Figure 3.3 **The optical system of a phase-contrast microscope.**

These rays emerge as solid lines from the object on the slide. This ring on the phase plate is coated with a material that will produce the desired phase shift. The diffracted rays, on the other hand, which have already been set back $\frac{1}{4}$ wavelength by the phase object on the slide, completely miss the phase ring and are not affected by the phase plate. It should be clear, then, that depending on the type of phase-contrast microscope, the convergence of diffracted and direct rays on the image plane will result in either a brighter image

(*amplitude summation*) or a darker image (*amplitude interference* or *reverse phase*). These are referred to as **bright-phase** microscopy and **dark-phase** microscopy, respectively. The apparent brightness or darkness, incidentally, is proportional to the square of the amplitude; thus, the image will be four times as bright or dark as one seen through a brightfield microscope.

Microscope Adjustments

If the annular stop under the condenser of a phase-contrast microscope can be moved out of position, this instrument can also be used for brightfield studies. Although a phase-contrast objective has a phase ring attached to the top surface of one of its lenses, the presence of that ring does not impair the resolution of the objective when it is used in the brightfield mode. It is for this reason that manufacturers have designed phase-contrast microscopes in such a way that they can be quickly converted to brightfield operation.

To make a microscope function efficiently in both phase-contrast and brightfield situations, one must master the following procedures:

- lining up the annular ring and phase rings so that they are perfectly concentric,
- adjusting the light source so that maximum illumination is achieved for both phase-contrast and brightfield usage, and
- being able to shift back and forth easily from phase-contrast to brightfield modes.

Alignment of Annulus and Phase Ring

Unless the annular ring below the condenser is aligned perfectly with the phase ring in each objective lens, good phase-contrast imagery cannot be achieved. Figure 3.4 illustrates the difference between nonalignment and alignment. If a microscope has only one phase-contrast objective, there will be only one annular stop that has to be aligned. If a microscope has two or more phase objectives, there must be a substage unit with separate annular stops for each phase objective, and the alignment procedure must be performed separately for each objective and its annular stop.

Since the objective cannot be moved once it is locked in position, all adjustments are made to the annular stop. Since the method of adjustment varies from one brand of microscope to another, follow the instructions provided by the manufacturer. On many models, the annular rings are moved into position with special knobs on the substage unit (figure 3.5). Once the adjustments have been made, they are rigidly set and needn't be changed unless someone inadvertently disturbs them.

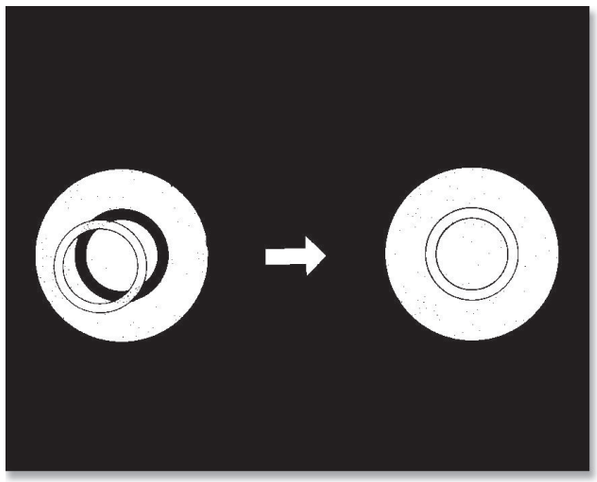


Figure 3.4 **The image on the right illustrates the appearance of the rings when perfect alignment of phase ring and annulus diaphragm has been achieved.**

Figure 3.5 **Alignment of the annulus and phase ring is achieved by adjusting the two knobs as shown.**
Auburn University Photographic Services/McGraw-Hill Education

To observe ring alignment, one can replace the eyepiece with a **centering telescope,** as shown in figure 3.6. With this unit in place, the two rings can be brought into sharp focus by rotating the focusing ring on the telescope. Refocusing is necessary for each objective and its matching annular stop.

Light Source Adjustment

For both brightfield and phase-contrast modes, it is essential that optimum lighting be achieved. Two

Figure 3.6 **If the ocular of a phase-contrast microscope is replaced with a centering telescope, the orientation of the phase ring and annular ring can be viewed.**
Auburn University Photographic Services/McGraw-Hill Education

suggestions that highlight some of the problems and solutions follow:

1. Since blue light provides better images for both phase-contrast and brightfield modes, a blue filter can be placed in the filter holder that is positioned in the light path. If the microscope has no filter holder, placing the filter over the light source on the base will help.
2. Brightness of field under phase-contrast is controlled by adjusting the light intensity or the iris diaphragm on the base. Considerably more light is required for phase-contrast than for brightfield since so much light is blocked out by the annular stop.

Working Procedures

Once the light source is correctly adjusted and the phase elements are centered, you are finally ready to examine slide preparations. Keep in mind that from now on, most of the adjustments described earlier should not be altered; however, if misalignment has occurred due to mishandling, it will be necessary to refer back to the alignment procedures. The following guidelines should be adhered to in all phase-contrast studies:

- Use only optically perfect slides and cover glasses.
- Be sure that slides and cover glasses are completely free of grease or chemicals.
- Use wet mount slides instead of hanging drop preparations. Culture broths containing bacteria or protozoan suspensions are ideal for wet mounts.
- In general, limit observations to unstained living cells. In most instances, stained slides are not satisfactory.

The first time you use phase-contrast optics to examine a wet mount, follow these suggestions:

1. Place the wet mount slide on the stage and bring the material into focus *using brightfield optics* at low-power magnification.
2. Once the image is in focus, switch to phase optics at the same magnification. Remember, it is necessary to place in position the matching annular stop.
3. Adjust the light intensity, first with the iris diaphragm and then with the light intensity control. In most instances, you will need to increase the amount of light for phase-contrast.
4. Switch to higher magnifications in the same way you do for brightfield optics, except that you will have to rotate a matching annular stop into position.
5. If an oil immersion phase objective is used, add immersion oil to the top of the condenser as well as to the top of the cover glass.
6. Don't be disturbed by the "halo effect" that you observe with phase optics. A bright halo around objects and their surroundings is normal.

Laboratory Report

This exercise may be used in conjunction with Part 2 when studying various types of organisms. Organelles in protozoans and algae will show up more distinctly using phase-contrast microscopy rather than brightfield optics. After reading this exercise and completing any special assignments made by your instructor, answer the questions in Laboratory Report 3.

Laboratory Report 3

Student: _____

Date: _____ Section: _____

3 Phase-Contrast Microscopy

A. Short-Answer Questions

1. Staining of cells is often performed to enhance images acquired by brightfield microscopy. Phase-contrast microscopy does not require cell staining. Why is this advantageous?

_____

_____

2. As light passes through a transparent object, how are direct and diffracted light rays produced? How much phase shift occurs?

_____

_____

3. How do coincidence and interference of light rays differ? What type of image does each produce? How does that contribute to a sharper image?

_____

_____

_____

4. Differentiate between bright-phase and dark-phase microscopy in terms of phase shift.

_____

_____

_____

B. Multiple Choice

Circle the answer that best completes the following statements.

1. A phase-contrast microscope differs from a brightfield microscope by having a
   a. blue filter in the ocular lens.
   b. diaphragm with an annular stop.
   c. phase plate in the objective lens.
   d. Both (b) and (c) are correct.
   e. All are correct.
2. Which of the following bacterial characteristics would be best observed using phase-contrast microscopy?
   a. Motility of cells
   b. Bacterial nucleoid
   c. Cell wall
   d. Glycocalyx

3. Amplitude summation occurs in phase-contrast optics when both direct and diffracted rays are
   a. in phase.
   b. in reverse phase.
   c. off $\frac{1}{4}$ wavelength.
   d. None of these is correct.
4. The phase-contrast microscope is best suited for observing
   a. living organisms in an uncovered drop on a slide.
   b. stained slides with cover glasses.
   c. living organisms in hanging drop slide preparations.
   d. living organisms on a slide with a cover glass.

EXERCISE 4

Microscopic Measurements

Learning Outcomes

After completing this exercise, you should be able to

1. Calibrate an ocular micrometer using a stage micrometer.
2. Use an ocular micrometer to accurately measure the dimensions of stained cells.

Why It Matters

How can you apply this exercise to the real world?

How small is a micrometer? If you picture a meter stick, every one of those very small graduated marks represents a millimeter. A millimeter is 1000 times bigger than a micrometer, so it becomes impossible to visualize just how small a micrometer is with the naked eye.

Determining the size of microbes requires measurement to be done under the microscope by adding a ruler to an eyepiece of the microscope. However, when we change the total magnification, the actual size of the ruler divisions changes. This exercise allows you to determine the measurement of each division of an **ocular micrometer** at different levels of magnification, so that you can measure microbes using any objective lens.

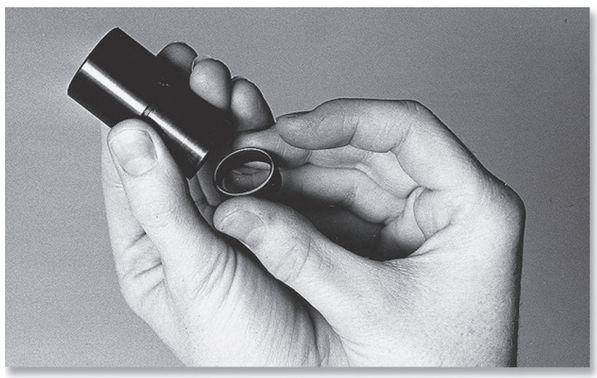


Figure 4.1 **The ocular micrometer with retaining ring is inserted into the base of the eyepiece.**
©Harold J. Benson

An ocular micrometer consists of a circular disk of glass that has graduations engraved on its upper surface. These graduations appear as shown in illustration (a), figure 4.3. On most microscopes, the ocular micrometer is simply inserted into the bottom of the ocular, as shown in figure 4.1.

Calibration of the Ocular Micrometer

The distance between the lines of an ocular micrometer is an arbitrary value that changes with different levels of magnification. Before one can use the micrometer, it is necessary to calibrate it for each of the objectives by using a stage micrometer. A **stage micrometer** (figure 4.2) is a slide with lines inscribed on it that are exactly 0.01 mm (10 µm) apart. Illustration (b), figure 4.3, shows these graduations. The stage micrometer is placed on the microscope stage and centered over the light source.

To calibrate the ocular micrometer for a given objective, it is necessary to superimpose the two micrometers and determine how many of the ocular divisions coincide with one division on the scale of the stage micrometer. Figure 4.3 illustrates the appearance of the divisions on the ocular micrometer (a) and the stage micrometer (b).

Figure 4.2 **An example of a stage micrometer.**
©Aaron Roeth Photography

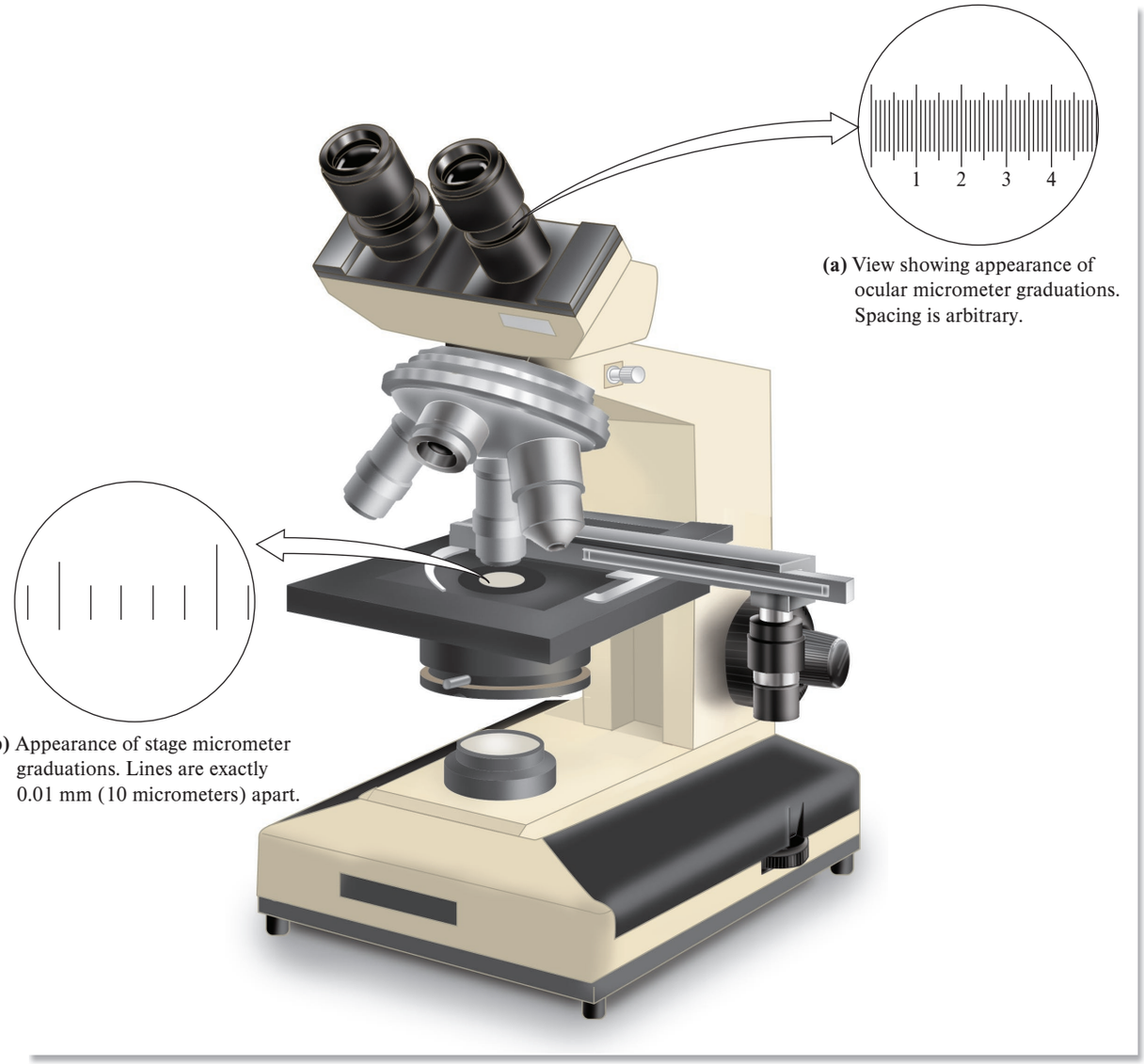


Figure 4.3 **Appearance of the stage and ocular micrometers under the microscope.**

Figure 4.4 shows how the two micrometers appear when they are properly aligned in the microscopic field. In this example, 2 stage micrometer divisions of 0.01 mm each match up with 8 ocular micrometer divisions (small graduated lines in the image) to give an ocular value of 2/8 × 0.01 mm, or 0.0025 mm. Since there are 1000 micrometers in 1 millimeter, the distance of one ocular division is 2.5 µm.

With this information known, the stage micrometer can then be replaced with a slide of organisms to be measured using the same magnification. Figure 4.5 shows how a field of microorganisms might appear with the ocular micrometer in the eyepiece. To determine the size of an organism, then, it is a simple matter to count the ocular divisions and multiply this number by the known size of each ocular division at that particular magnification.

In this example, the yeast cell on the right spans 15 ocular divisions. According to the calibration in figure 4.4, each division is 2.5 µm so the length of this yeast cell is 15 × 2.5 µm = 37.5 µm. This assumes that the calibration was done using the same objective lens as the one being used to view and measure this yeast cell. Can you calculate the length of the microbe on the left using the same process?

Procedure

Follow the steps below to calibrate your ocular micrometer at all levels of magnification.

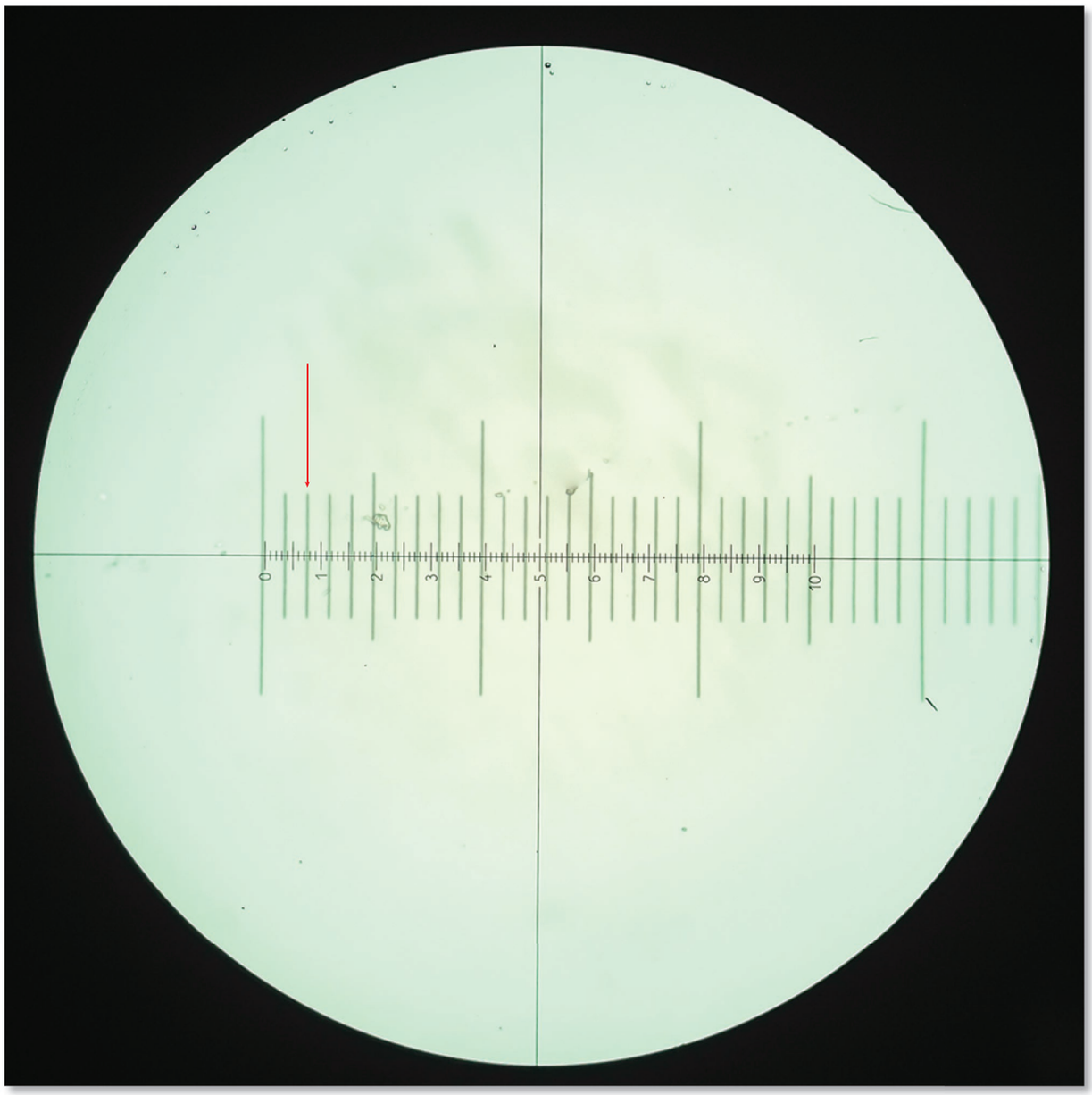


Figure 4.4 **Alignment of the ocular (smaller numbered scale) and stage (larger scale) micrometers for calibration.**
MR.BEE Photographer/Shutterstock

1. If your microscope does not already have an eyepiece with an ocular micrometer, follow your instructor's directions to insert one into your microscope.
2. Place the stage micrometer on the microscope stage and center it exactly over the light source.
3. With the low-power (10×) objective in position, bring the graduations of the stage micrometer into focus.
4. Rotate the eyepiece until the graduations of the ocular micrometer lie parallel to the lines of the stage micrometer.
5. Move the stage micrometer laterally until the lines at one end coincide. Then look for another line on the ocular micrometer that coincides *exactly* with one on the stage micrometer. Occasionally, one stage micrometer division will include an even number of ocular divisions. In most instances, however, several stage divisions will be involved. In this case, divide the number of stage micrometer divisions by the number of ocular divisions that coincide. This value must then be multiplied by 0.01 mm to get the amount of each ocular division.

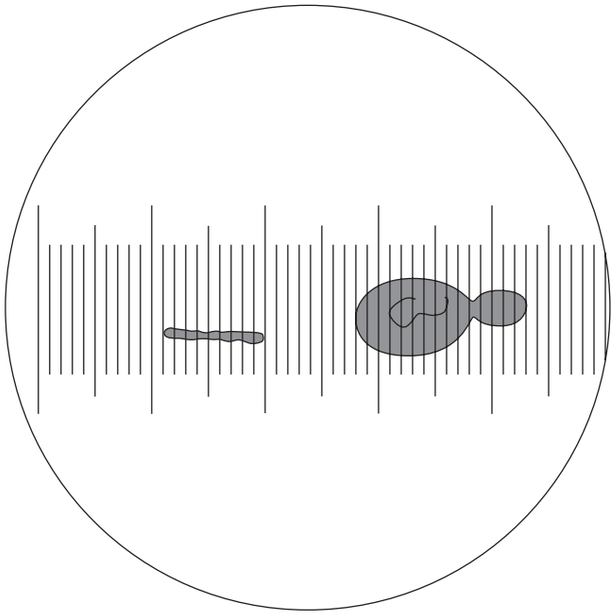


Figure 4.5 **Using the calibrated ocular micrometer to measure organisms.**

Example: 3 divisions of the stage micrometer line up with 20 divisions of the ocular micrometer.

$$\begin{aligned}\text{Each ocular division} &= \tfrac{3}{20} \times 0.01 \\ &= 0.0015 \text{ mm} \\ &= 1.5\,\mu\text{m}\end{aligned}$$

Record your values for the low-powered objective lens into the table of the Laboratory Report.

6. Repeat the process using the high-dry objective (40×) and record your values in the table.
7. To calibrate the oil immersion lens (100×), place a drop of immersion oil on the stage micrometer, swing the oil immersion lens into position, and bring the lines into focus. Repeat the process and record your values in the table.
8. Use the calibrated ocular division values to measure microorganisms according to your instructor's assignments.

Laboratory Report

Answer the questions in Laboratory Report 4.

Laboratory Report 4

Student: _____

Date: _____ Section: _____

4 Microscopic Measurements

A. Calibration Values

Objective Lens	Stage Divisions	Ocular Divisions	Calculation of Ocular Division in mm (stage/ocular × 0.01 mm)	Ocular Division in µm
10×				
40×				
100×				

B. Short-Answer Questions

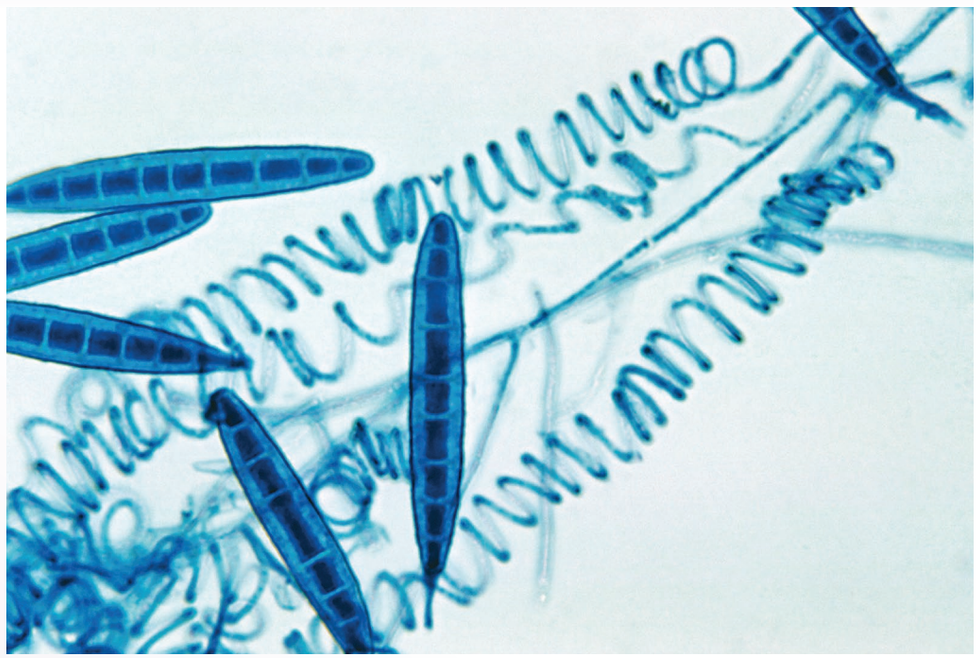
1. How do the graduations differ between ocular and stage micrometers?

2. If 13 ocular divisions line up with two divisions of the stage micrometer, what is the diameter (µm) of a cell that spans 16 ocular divisions using the same objective lens? Show your calculations below.

3. Why must the entire calibration procedure be performed for each objective?

PART 2

Survey of Microorganisms

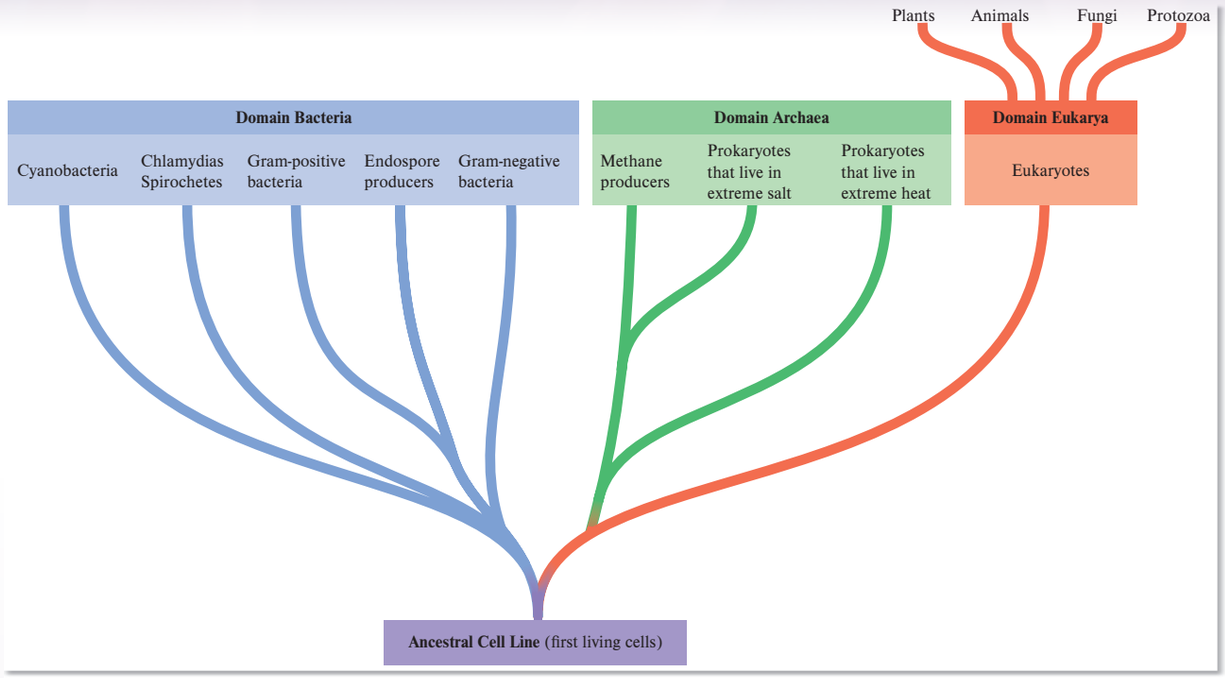


CDC/Dr. Lucille K. Georg

Microorganisms abound in the environment. Eukaryotic microbes such as protozoa, algae, diatoms, and amoebas are plentiful in ponds and lakes. Bacteria are found associated with animals, occur abundantly in the soil and in water systems, and have even been isolated from core samples taken from deep within the earth's crust. Bacteria are also present in the air where they are distributed by convection currents that transport them from other environments. The Archaea, modern-day relatives of early microorganisms, occupy some of the most extreme environments such as acidic-volcanic hot springs, anaerobic environments devoid of any oxygen, and lakes and salt marshes excessively high in sodium chloride. Cyanobacteria are photosynthetic prokaryotes that can be found growing in ponds and lakes, on limestone rocks, and even on the shingles that protect the roofs of our homes. Fungi are a very diverse group of microorganisms that are found in most common environments. For example, they degrade complex molecules in the soil, thus contributing to its fertility. Sometimes, however, they can be nuisance organisms; they form mildew in our bathroom showers and their spores cause allergies. The best way of describing the distribution of microorganisms is to say that they are ubiquitous, or found everywhere.

Intriguing questions to biologists include, How are the various organisms related to one another, and where do the individual organisms fit in an evolutionary scheme? Molecular biology techniques have provided a means to analyze the genetic relatedness of the organisms that comprise the biological world and determine where the various organisms fit into an evolutionary scheme. By comparing the sequence of ribosomal RNA molecules, coupled with biochemical data, investigators have developed a phylogenetic tree that illustrates the current thinking on the placement of the various organisms into such a scheme. This evolutionary scheme divides the biological world into three domains.

Domain Bacteria These organisms have a prokaryotic cell structure. They lack organelles such as mitochondria and chloroplasts, are devoid of an organized nucleus with a nuclear membrane, and possess 70S ribosomes that are inhibited by many broad-spectrum antibiotics. The vast majority of organisms are enclosed in a cell wall composed of peptidoglycan. The bacteria and cyanobacteria are members of this domain.



Domain Eukarya Organisms in this domain have a eukaryotic cell structure. They contain membrane-bound organelles such as mitochondria and chloroplasts, an organized nucleus enclosed in a nuclear membrane, and 80S ribosomes that are not inhibited by broad-spectrum antibiotics. Plants, animals, and microorganisms such as protozoa, algae, and fungi belong in this domain. Plants have cell walls composed of cellulose and fungi have cell walls composed of chitin. In contrast, animal cells lack a cell wall structure.

Domain Archaea The Archaea exhibit the characteristics of both the Bacteria and Eukarya. These organisms are considered to be the relatives of ancient microbes that existed during Archaean times. Like their bacterial counterparts, they possess a simple cell structure that lacks organelles and an organized nucleus. They have 70S ribosomes like bacteria, but the protein makeup and morphology of their ribosomes are more similar to eukaryotic ribosomes. Like eukaryotes, the ribosomes in Archaea are not sensitive to antibiotics. They have a cell wall but its structure is not composed of peptidoglycan. The principal habitats of these organisms are extreme environments such as volcanic hot springs, environments with excessively high salt, and environments devoid of oxygen. Thus, they are referred to as "extremophiles." The acido-thermophiles, the halobacteria, and the methanogens (methane bacteria) are examples of the Archaea.

In the exercises of Part 2, you will have the opportunity to study some of these organisms. In pond water, you may see amoebas, protozoa, various algae, diatoms, and cyanobacteria. You will sample for the presence of bacteria by exposing growth media to various environments. The fungi will be studied by looking at cultures and preparing slides of these organisms. Because the Archaea occur in extreme conditions and also require specialized culture techniques, it is unlikely that you will encounter any of these organisms.

EXERCISE 5

Microbiology of Pond Water—Protozoa, Algae, and Cyanobacteria

Learning Outcomes

After completing this exercise, you should be able to

1. Prepare wet mounts of samples from aquatic environments.
2. Identify various eukaryotic microorganisms belonging to the algae and protozoan groups.
3. Differentiate between prokaryotic cyanobacteria and eukaryotic algae.

The purpose of this exercise is to study the diverse organisms that can occur in pond water (figure 5.1). This will include eukaryotic organisms classified as algae and protozoa as well as the prokaryotic cyanobacteria. Illustrations and descriptions are provided to assist you in identifying some of these organisms. In addition, you may also observe small nematodes (roundworms), insect larvae, microcrustaceans, rotifers, and other invertebrates. Your instructor may supply you with additional materials to identify these organisms, as they are not covered in this manual.

Why It Matters

How can you apply this exercise to the real world?

Even though water can look crystal clear to the naked eye, it is often surprising how many microorganisms exist when water is investigated under the microscope. Cool mountain water might look like a tasty refreshment, but disease-causing organisms such as *Giardia* can bring an unwelcome outcome to indulging in that cool drink!

Materials

- bottles of pond water
- clean microscope slides and coverslips
- transfer pipettes and forceps
- additional reference books
- optional: prepared slides of protozoa and algae

To observe these organisms, you will make wet mounts of various samples of pond water. This is achieved by pipetting a drop of pond water onto a microscope slide and covering the drop with a coverslip. To achieve the best results, use the following guidelines:

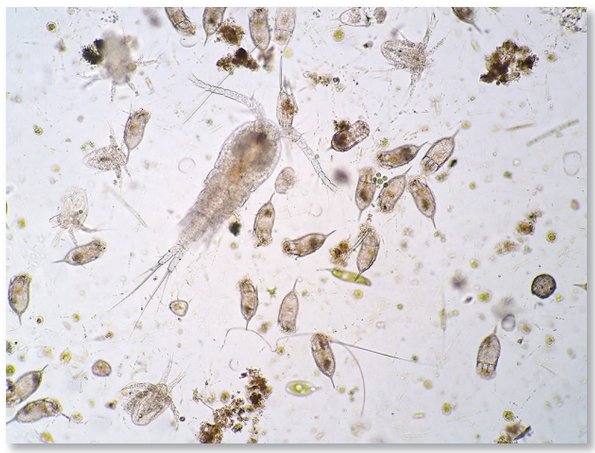


Figure 5.1 Freshwater plankton under the microscope.
Shutterstock/Choksawatdikorn

1. Use a clean slide and coverslip.
2. Insert the pipette into the *bottom* of the pond water bottle to obtain the maximum number of organisms. Fewer will occur at mid-depth.
3. Remove filamentous algae using forceps. Avoid using too much material.
4. Focus first with the low-power objective. Reduce the illumination using the iris diaphragm to provide better contrast.
5. When you find an organism of interest, switch to the high-dry objective and adjust the illumination appropriately.
6. Refer to figures 5.2 to 5.7 and the accompanying text to help in your identification.
7. Record your observations on the Laboratory Report.

Survey of Organisms

You will likely see a variety of eukaryotic microorganisms as well as possibly cyanobacteria in pond water used in this exercise. You will identify and categorize these organisms based on differences in

Table 5.1 **Classification of Organisms**

PROKARYOTES (DOMAIN BACTERIA)	EUKARYOTES (DOMAIN EUKARYA)
Cyanobacteria (figure 5.8)	Protozoa Diplomonads and Parabasalids Euglenozoans Euglenids (figure 5.3, illustrations 1, 2) Kinetoplastids Alveolates Ciliates Dinoflagellates (figure 5.4, illustrations 10, 11) Apicomplexans Stramenophiles Diatoms (figure 5.7) Golden algae (figure 5.4, illustrations 1–4) Amoebozoans Gymnamoebas (figure 5.2, illustration 6) Entamoebas Algae Red and green algae Unicellular red algae Unicellular green algae (figures 5.4–5.6)

morphological characteristics. Traditionally, morphology was a primary means for constructing formal classification schemes for these organisms. However, true evolutionary relationships cannot be determined by simply observing morphological characteristics. Nowadays molecular genetics is used to more clearly establish the taxonomic and evolutionary relationships among these organisms. Observable morphological differences do exist between these organisms, and therefore you can use an informal system based on morphology to characterize and categorize the various organisms that you observe. Table 5.1 will help you understand some of the major morphological groups of the organisms that you may see. **Please keep in mind that these are not formally recognized taxonomic groups,** but they are useful for identifying and categorizing organisms in the laboratory based on specific physical traits.

Eukaryotes

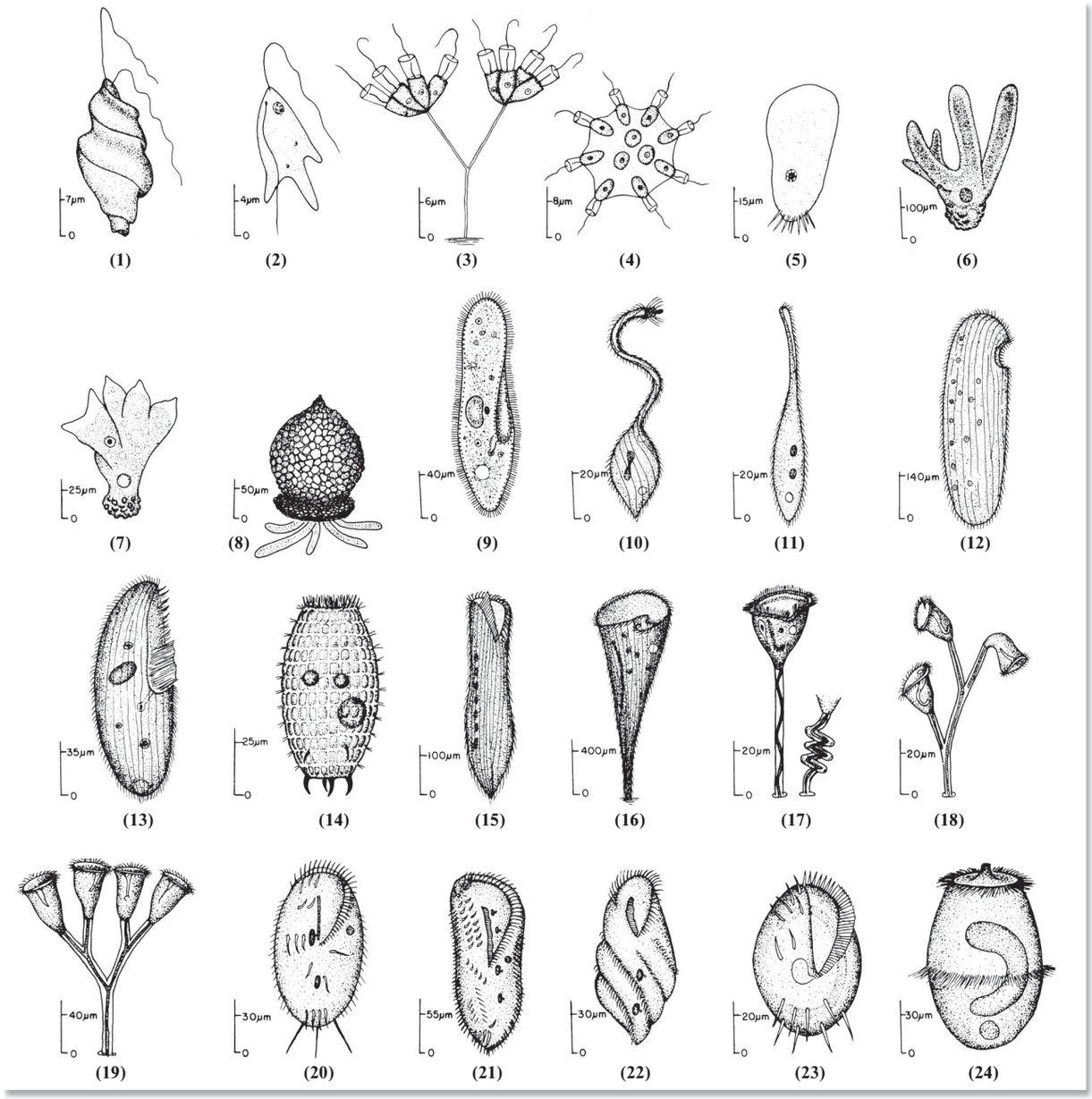
Protozoa

The Protozoa are protists, which means "first animals." They were some of the original microorganisms observed by Leeuwenhoek more than 300 years ago when he studied samples of rain water with his simple microscopes. Protozoa were initially classified as unicellular, colorless organisms, lacking a cell wall, and were further subdivided based on their modes of motility. However, the application of modern genetic analysis has shown that these organisms are a diverse group that have little relationship to one another. They are mostly heterotrophic, unicellular organisms that lack a rigid cellulose cell wall characteristic of algae or a chitinous cell wall characteristic of fungi. Figure 5.2 illustrates many different examples of these organisms.

Their specialized structures for motility such as cilia, flagella, or pseudopodia were important in previous classification schemes, but genetic analysis subdivides them into the following groups with the accompanying characteristics:

Diplomonads and Parabasalids These organisms contain two nuclei and organelles called **mitosomes,** which are reduced mitochondria that lack electron transport components and Krebs cycle constituents. *Giardia lamblia* is a diplomonad that causes giardiasis, a diarrheal disease acquired by campers drinking contaminated water from lakes and streams. The parabasalids are characterized by the presence of a **parabasal body** that is associated with the Golgi apparatus. They lack mitochondria but instead contain hydrogenosomes that carry out anaerobic respiration, releasing hydrogen gas. Some inhabit the guts of termites where they digest cellulose for the insect. They are also serious pathogens of vertebrates, causing infections in the intestinal or urogenital tracts. *Trichomonas vaginalis* is a parabasalid that causes a sexually transmitted disease in humans.

Euglenozoans These organisms are a diverse group that are unicellular and contain a unique crystalline rod associated with their flagellum. Some are pathogens

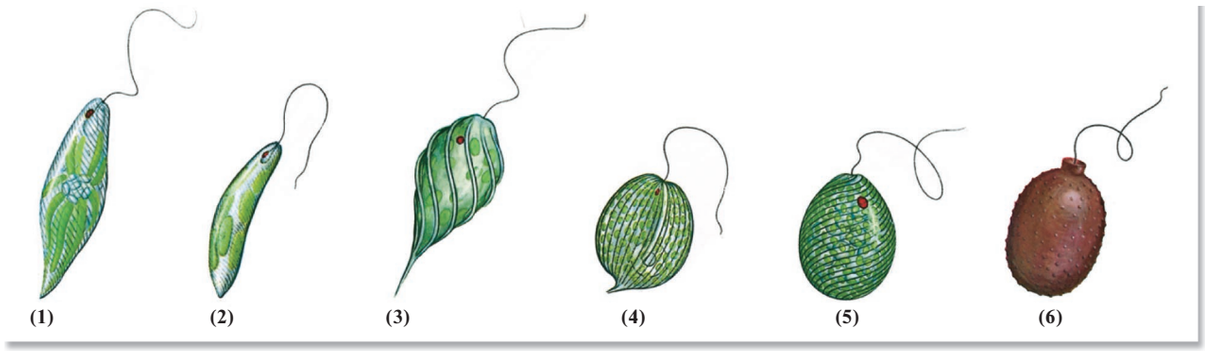


(1) *Heteronema*
(2) *Cercomonas*
(3) *Codosiga*
(4) *Protospongia*
(5) *Trichamoeba*
(6) *Amoeba*
(7) *Mayorella*
(8) *Difflugia*
(9) *Paramecium*
(10) *Lacrymaria*
(11) *Litonotus*
(12) *Loxodes*
(13) *Blepharisma*
(14) *Coleps*
(15) *Condylostoma*
(16) *Stentor*
(17) *Vorticella*
(18) *Carchesium*
(19) *Zoothamnium*
(20) *Stylonychia*
(21) *Onychodromus*
(22) *Hypotrichidium*
(23) *Euplotes*
(24) *Didinium*

Figure 5.2 **Protozoa.**

while others are free living. They are comprised of the kinetoplastids and the euglenids.

- **Kinetoplastids**
  The kinetoplastids have a single large mitochondrion in the cell that contains a kinetoplast, which is a large mass of DNA. Some are found in aquatic habitats while others are very serious pathogens in humans. The trypanosomes are responsible for several diseases in humans, such as African sleeping sickness caused by *Trypanosoma brucei*, Chagas' disease caused by *Trypanosoma cruzi*, and leishmaniasis caused by *Leishmania major*. Trypanosomes are crescent-shaped and possess a single flagellum that originates at a basal body and is enclosed by part of the cytoplasmic membrane, thus forming an undulating structure over the cell body.



(1) *Euglena* (700×) **(2)** *Euglena* (700×) **(3)** *Phacus* (1000×) **(4)** *Phacus* (350×) **(5)** *Lepocinclis* (350×) **(6)** *Trachelomonas* (1000×)

Figure 5.3 **Flagellated euglenids.**
The U.S. Environmental Protection Agency, Office of Research & Development, Cincinnati, OH 45268

- **Euglenids**
  The euglenids can grow either heterotrophically or phototrophically because they contain chloroplasts. They are nonpathogens that occur primarily in aquatic habitats, where many subsist on bacteria. Food is taken in by the process of **phagocytosis** when the cytoplasmic membrane surrounds the food and brings it into the cell for digestion. *Euglena* (figure 5.3, illustrations 1 and 2) is a member of this group.

Alveolates The alveolates are comprised of the ciliates, the dinoflagellates, and the apicomplexans. Members of this group contain sacs called **alveoli** associated with the cytoplasmic membrane that may function in maintaining the osmotic balance of the cell. The apicomplexans are obligate parasites.

- **Ciliates**
  Ciliates are characterized by having two kinds of nuclei, *micronuclei* and *macronuclei*. Micronuclei genes function in sexual reproduction, whereas genes encoding for cellular functions are associated with the macronuclei. An example of a ciliate is *Paramecium* (figure 5.2, illustration 9). Reproduction occurs by the process of conjugation, in which two *Paramecium* cells fuse and exchange their micronuclei. The ciliates are usually covered with cilia that are responsible for motility of the cell. Cilia are short, hairlike structures that have the same structure as flagella, that is, a 9 + 2 arrangement of microtubules. Motility occurs when cilia beat in a coordinated fashion to propel the cell forward or backward. Cilia also function in digestion. They line the cytostome or oral groove of the organism to direct food such as bacteria to the cell mouth, where it is enclosed in a vacuole and taken into the cell by phagocytosis. Many *Paramecium* harbor endosymbiotic prokaryotes in their cytoplasm or in the macronucleus; for example, methanogens produce hydrogen gas in the hydrogenosome of the *Paramecium*. Some species can attach to solid surfaces—for example, *Vorticella* and *Zoothamnium* (figure 5.2, illustrations 17 and 19). Ciliates can be pigmented: *Stentor* (figure 5.2, illustration 16), blue; *Blepharisma* (figure 5.2, illustration 13), pink; and *Paramecium busaria* (figure 5.2, illustration 9), green. The green color is due to the presence of endosymbiotic algal cells. The ciliates are important inhabitants of the forestomach of ruminant animals such as cattle. In the rumen (stomach), they degrade cellulose and starch, which can be used by the animal for nutrition. They also feed on rumen bacteria to maintain their numbers in the rumen. The ciliate *Balantidium coli* is an intestinal pathogen of domestic animals that occasionally infects humans. It causes an intestinal disease similar to *Entamoeba histolytica*.

- **Dinoflagellates**
  The dinoflagellates (figure 5.4, illustrations 10, 11) are characterized by the presence of two flagella of different lengths. The flagella encircle the cell and when they beat, they cause the cell to whirl or spin. These organisms occur in both marine and freshwater habitats. *Gonyaulax* is a dinoflagellate that occurs in marine coastal waters. Some species are bioluminescent and can cause luminescence when waves break on the shore or boats move through the water. The organisms produce "red tides" caused by xanthophyll in the cells. Blooms of these organisms can occur naturally or by increased pollution in coastal waters. These blooms can result in fish kills and poisoning in humans when contaminated shellfish are consumed. *Gonyaulax* produces a neurotoxin, called **saxitoxin,** that is responsible for the symptoms of dizziness, numbness of the lips, and difficulty breathing.

- **Apicomplexans**
  The apicomplexans are obligate parasites in animals and humans. In humans they are the cause of malaria (*Plasmodium* spp.), toxoplasmosis (*Toxoplasma gondii*), and coccidiosis (*Eimeria*). They produce resting stages called *sporozoites* that facilitate transmission of the pathogen. The cells contain structures called *apicoplasts*, which are vestiges of chloroplasts that have degenerated. They contain no photosynthetic pigments, but they do have some genes that encode for fatty acid and heme synthesis.

Stramenophiles The stramenophiles are composed of the oomycetes (water molds), the diatoms, the golden algae, and the multicellular paeophytes, which includes the seaweeds. The unicellular stramenophiles have flagella with short, hairlike extensions. The name is derived from this feature. *Stramen* is Latin for straw and *pilos* means hair.

- **Diatoms**
  Diatoms (figure 5.7) are phototrophic freshwater and marine organisms that are unique in the biological world because they synthesize a cell wall composed of silica. The external portion of the wall is called a *frustule* and is very diverse in its shape. The frustule consists of two halves, the epitheca and the hypotheca. It is analogous to a box with a lid, with the epitheca fitting over the hypotheca. Pores in the frustule called *areolae* function as passageways for gases and nutrients. The frustules remain after the death of the organism and are referred to as diatomaceous earth, which is used in many applications such as a polishing abrasive in toothpaste. They do not decay when the organism dies, and hence they are a preserved fossil record of diatoms. Based on fossil records of the frustules, it is estimated that diatoms were present on the earth about 200 million years ago. *Nitzschia* is a common diatom (figure 5.7, illustration 7).

- **Golden Algae**
  The *chrysophytes* or golden (brown) algae are classified with the stramenophiles because genetic analysis has determined that they are more closely related to the diatoms and oomycetes (water molds) than to the other unicellular algae. They are inhabitants of fresh and marine waters. Some species are also chemoorganotrophs, which means they can derive food from the transport of organic compounds across the cytoplasmic membrane instead of carrying out photosynthesis. They are motile by means of two flagella. Their gold or brown color is the result of the carotenoid, *fucoxanthin*. They also contain chlorophyll *c* rather than the chlorophyll *a* found in other algae. *Dinobryon* is a colonial golden alga that occurs in fresh water (figure 5.4, illustration 9).

Amoebozoa The amoebozoa occur in both terrestrial and aquatic habitats. They are often found in pond water. This group is composed of the gymnamoebas, the entamoebas, the slime molds, and the cellular slime molds. In this exercise, we will only focus on the gymnamoebas and mention the entamoebas because of their importance as pathogens.

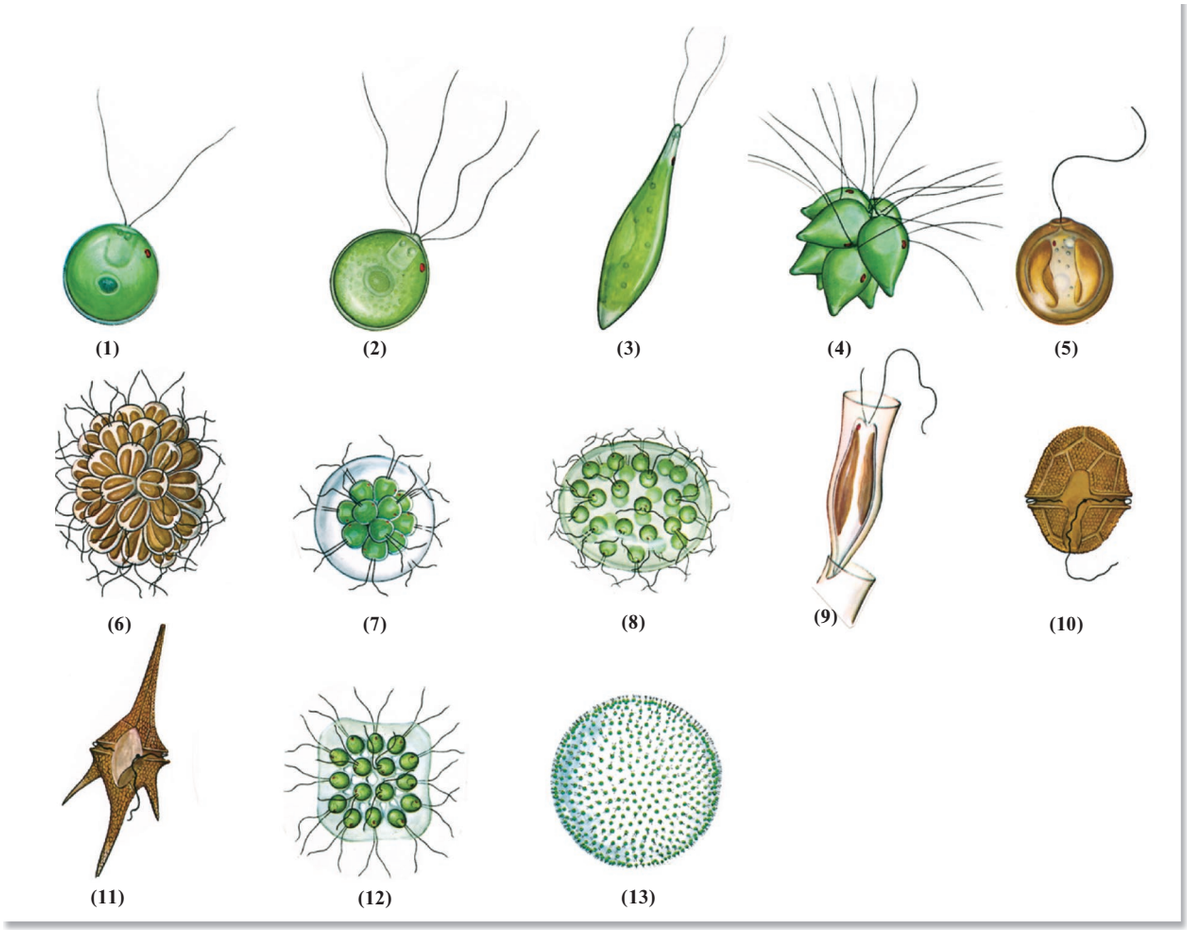
- **Gymnamoebas**
  These amoebozoa are primarily free living, occurring in aquatic habitats and in the soil. They achieve motility by amoeboid movement in which pseudopodia are extended and the cell cytoplasm streams into the tip of the pseudopodium. Microfilaments associated with the cytoplasmic membrane aid in the overall process. Pseudopodia are also utilized by the amoebas for entrapping and surrounding food by phagocytosis. They can range in size from 15µ to above 700µ. An example is shown in figure 5.2, illustration 6.

- **Entamoebas**
  These amoebas are strictly parasites of invertebrates and vertebrates. An important pathogen in humans is *Entamoeba histolytica,* which causes amoebic dysentery. The organism is transmitted in a cyst form by fecal contamination of water and food. In the intestine it causes ulceration, resulting in a bloody diarrhea.

Algae

The algae are a diverse group of organisms that obtain their carbon requirements from oxygenic photosynthesis in which carbon dioxide is fixed into cellular materials and water is split to evolve oxygen. They are typically smaller and less complex in their structure than land plants, but they are similar to plants because they possess photosynthetic pigments such as chlorophyll and carotenoids that harvest light energy from the sun. They range in size from microscopic unicellular forms to the seaweeds that form giant kelp beds in the oceans. The microscopic algae form filaments or colonies that are comprised of several individuals loosely held together in an organized fashion. Many of the unicellular algae are motile by means of a flagellum. Reproduction can occur by both asexual and sexual mechanisms.

The algae are diverse in their ecology. They occur primarily in aquatic habitats such as freshwater lakes and streams and in the oceans, where they are important members of the phytoplankton. Algae also occur in terrestrial habitats. Some grow on snow, imparting a pink color. They enter into unique symbiotic



(1) *Chlamydomonas* (1000×)
(2) *Carteria* (1500×)
(3) *Chlorogonium* (1000×)
(4) *Pyrobotrys* (1000×)
(5) *Chrysococcus* (3000×)
(6) *Synura* (350×)
(7) *Pandorina* (350×)
(8) *Eudorina* (175×)
(9) *Dinobryon* (1000×)
(10) *Peridinium* (350×)
(11) *Ceratium* (175×)
(12) *Gonium* (350×)
(13) *Volvox* (100×)

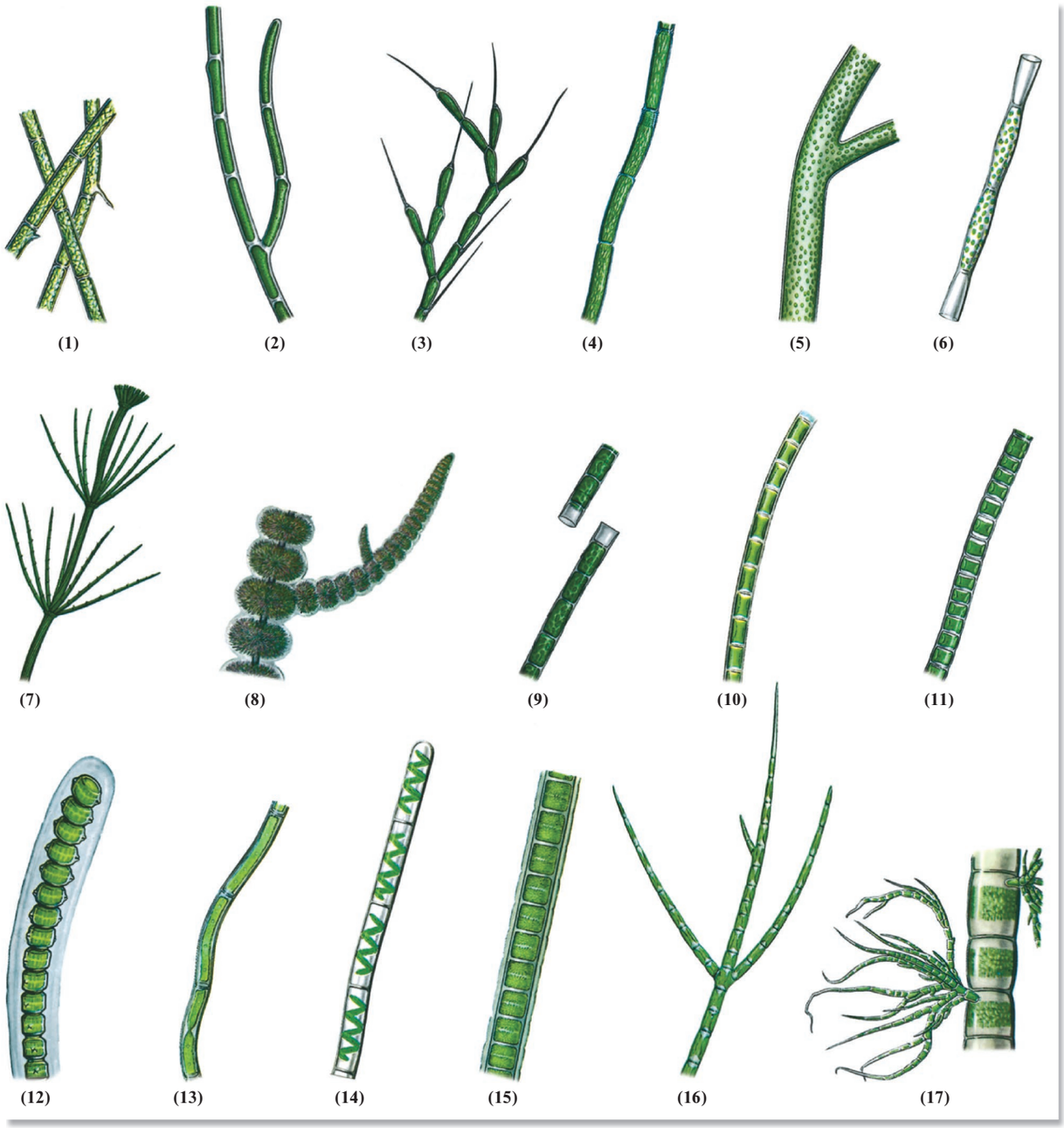
Figure 5.4 **Flagellated green algae.**
The U.S. Environmental Protection Agency, Office of Research & Development, Cincinnati, OH 45268

relationships with fungi called lichens, which are found on trees and rocks. Although unique morphologically, the algae are related genetically to other protists.

Red and Green Algae The red and green algae belong to the *rhodophytes* and *chlorophytes*, respectively. They may be unicellular (figure 5.4, illustrations 1–5), colonial (figure 5.4, illustrations 6, 7, 8, 12, 13), or filamentous (figure 5.5). Many seaweeds are included in the red algae. Agar used in microbiological media is extracted from a seaweed, *Gelidium*, a member of the red algae.

- **Unicellular Red Algae**
  The unicellular red algae occur primarily in marine habitats, with a few freshwater varieties. Cells may have one or more flagella. They contain chloroplasts, which carry out photosynthesis. Their primary photosynthetic pigment is chorophyll *a*; however, they are unique because they lack chorophyll *b*. In addition, they also have phycobiliproteins, which assist in harvesting light. Their red coloration is in fact due to the presence of *phycoerythrin*, one of the phycobiliproteins. These pigments are also found in the cyanobacteria.

- **Unicellular Green Algae**
  The majority of algae observed in fresh water such as ponds and lakes are green algae and hence, these will be the ones that you will most likely encounter in this exercise. Like green plants, they contain chlorophyll *a* and *b*. They store starch granules as an energy reserve. *Chlamydomonas* is an excellent example of a unicellular green alga (figure 5.4, illustration 1). This organism has been the object of extensive studies, especially involving photosynthesis. Colonial forms of green algae include



(1) *Rhizoclonium* (175×)
(2) *Cladophora* (100×)
(3) *Bulbochaete* (100×)
(4) *Oedogonium* (350×)
(5) *Vaucheria* (100×)
(6) *Tribonema* (300×)
(7) *Chara* (3×)
(8) *Batrachospermum* (2×)
(9) *Microspora* (175×)
(10) *Ulothrix* (175×)
(11) *Ulothrix* (175×)
(12) *Desmidium* (175×)
(13) *Mougeotia* (175×)
(14) *Spirogyra* (175×)
(15) *Zygnema* (175×)
(16) *Stigeoclonium* (300×)
(17) *Draparnaldia* (100×)

Figure 5.5 **Filamentous algae.**
The U.S. Environmental Protection Agency, Office of Research & Development, Cincinnati, OH 45268

Pandorina, Eudorina, Gonium, and *Volvox* (figure 5.4, illustrations 7, 8, 12, 13). *Vaucheria* and *Tribonema* (figure 5.5, illustrations 5 and 6) are not green algae but have been recently reclassified as Tribophyceae based on genetic analysis.

A unique group of green algae are the "desmids," whose cells are composed of two halves, called semicells (figure 5.5, illustration 12, and figure 5.6, illustrations 16–20). The two halves of the cell are separated by a constriction called the isthmus. Some of the