

BIOL 448 Eukaryotic
Microbiology
Laboratory Manual

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EUKARYOTIC MICROBIOLOGY LABORATORY SCHEDULE

Dates	Lab Number	Page Number	Topic(s)
Sept. 5 - 7	No Lab	NA	No Lab
Sept. 10 - 14	Lab 1	Pg. 18	Making a van Leeuwenhoek-Type Microscope
Sept. 17 - 21	Lab 2	Pg. 44	Discovering and Classifying Eukaryotic Microbes Part 1: Environmental Sampling
Sept. 24 - 28	Lab 3	Pg. 52	Discovering and Classifying Eukaryotic Microbes Part 2: Microscopic Observation, Isolation and Cultivation
Oct. 1 - 5	Lab 4	Pg. 60	Discovering and Classifying Eukaryotic Microbes Part 2 Continued: Microscopic Observation, Isolation and Cultivation
Oct. 8 – 12 (Oct 8 th Thanksgiving)	Lab 5	Pg. 64	Discovering and Classifying Eukaryotic Microbes Part 3: Observation and Description of a New Species Classification Assignment Due Oct 12th 9:00 am
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Laboratory Schedule

			Species and Culturing of Amoebae
Oct. 29 – Nov. 2	Lab 8	Pg. 69	Unikonts: Amoebozoa Description of a New Species Draft Materials and Methods Due Oct 29th
Nov. 5 - 9	Lab 9	Handouts will be provided.	Archaeplastida Description of a New Species Draft Results Due Nov 5th
Nov. 12 – 16 (Nov. 12 th Remembrance Day)	Lab 10	Handouts will be provided.	Excavates Description of a New Species Draft Introduction Due Nov 13th
Nov. 19 - 23	Lab 11	Handouts will be provided.	Chromalveolates / Rhizaria: Internal and External Skeletons
Nov. 26 - 30	Lab 12	Handouts will be provided.	Chromalveolates / Rhizaria: Alveolates Description of a New Species Final Paper Due Nov. 26th

Section 1

Labs and Assignments

EUKARYOTIC MICROBES - DISCOVERY LABS

OUTLINE OF THE DISCOVERY LABS PROJECTS

"The current number of described protist species, including fungi, is widely acknowledged to be a fraction of the total diversity in nature. Many geographic regions have not been sampled at all and most regions and habitats are insufficiently sampled. The rate of discovery of new species from environmental samples remains high. Indeed, most soil, freshwater, or marine samples collected contain a multitude of undescribed species that are found through microscopy or environmental DNA samples. Owing to insufficient environmental sampling and reisolation, the geographical distribution of most species remains unknown."

(Adl et al., 2005)

The development of microscopy opened up a previously unexplored, diverse world for biologists. Even after hundreds of years of study, most species have not been formally described so chances are that some of the organisms you will collect or isolate in the upcoming series of labs have not yet been described.

Being new to the study of protists, you are in a very similar position to early microscopists like Antonie van Leeuwenhoek; making some sense of the incredible diversity found in a single drop of water by grouping organisms based on similarities in morphology, behavior and habitat. People learn by doing. Therefore, you will begin your study of eukaryotic microbes by observing organisms you collect from habitats around the main campus of UBC.

The environmental samples you collect in Lab 2 "Part 1: Environmental Sampling" (pg. 44) will be used in two separate projects: i) a Classification Assignment (pg. 6) and ii) a Species Description Assignment (pg. 8). At this point, the objective is not to find specific organisms, but for you to get a sense of the ecological and morphological diversity of eukaryotic microbes. Along the way, you will learn about the techniques used to study eukaryotic microbes, the morphological variation in microbes and hopefully you will begin to appreciate the incredible diversity found in a drop of water, a few particles of soil or grains of sand. For your Classification Assignment (pg. 6) you will emulate early researchers, by construct and justify a classification system for the organisms you collect from around the UBC campus. For the Species Description Assignment (pg. 8) you will attempt to isolate or enrich a single organism from one of your samples and write a formal description of the organism.

CLASSIFICATION ASSIGNMENT

Due Oct 12th 9:00 am

OBSERVATIONS AND CLASSIFICATION OF PROTISTS FROM FIELD MATERIAL

Observations (Labs 3-5)

Note: BIOL 448 students will work independently.

You will need to hand in drawings of 10-20 organisms and your notes on each organism (minimum of 5 per student for BIOL203; 10 per student for BIOL 448 students) and submit a written justification for your classification system. The section Observation Methods for Eukaryotic Microbes (pg. 127) has some useful tips for making observations of eukaryotic microbes from different substrates or media.

- 1) Begin by observing your sample using the stereomicroscope using the methods outlined in the Stereo- or Dissecting Microscopes section (pg. 105).
- 2) Make wet mounts of field material following the instructions in the Making a Wet Mount section (pg. 127).
- 3) Follow the steps outlined in the Using the Compound Microscope section (pg. 107) to properly adjust your microscope and observe material in your wet mount.
- 4) Review the Observation Methods for Eukaryotic Microbes section (pg. 127) for tips on methods appropriate for the substrate or media you sampled. You may need to try a few different methods.
 - a. If you are having difficulty finding cells, you may consider trying some fixing and staining procedures from the Simple fixing and staining on a slide section (pg. 129) or
 - b. Try observing your sample using Phase Contrast (pg. 121).
- 5) Draw and make notes on 10-20 different organisms present in your sample. You must hand in a minimum of 5 drawings (BIOL 203), 10 drawings for BIOL 448 students. Please refer to the Biological Drawings section (pg.54) for instructions and tips on making biological drawings.

Justification of Your Classification System

You will also hand in a 1 page written justification for the classification system you developed.

Classification Assignment

Your classification system will need some basic characteristics:

- 1) The basic unit in your classification system is the species.
 - a. You will need to decide on the important characteristics you used to identify the species and distinguish it from other species.
 - b. You will need to describe the important characteristics as part of the justification for your classification system.
 - c. Assign your species a descriptive name (scientific names are not required).
- 2) Arrange your species into logical groups.
 - a. As with your species groups you need to describe the important characteristics used to unite species within a group.
- 3) Each student will hand in a minimum of five (10 for BIOL 448) of their own drawings labeled with the species names used in the group's classification system.
 - a. Follow the instructions for Biological Drawings, pg. 54. Grading of drawings will follow the Biological Drawings Grading Rubric, pg. 58.
 - b. Students will receive an individual grade for their drawings.
 - c. Drawings must be on unlined, plain white paper
 - d. Drawings must be made in lab, not from photographs taken in lab.

SPECIES DESCRIPTION ASSIGNMENT

Draft Materials and Methods Due Oct 29th

Draft Results Due Nov 5th

Draft Introduction Due Nov 13th

Final Paper Due Nov. 26th

Note: BIOL 448 students will work independently.

WHAT DO BIOLOGISTS DO WHEN THEY DISCOVER A NEW SPECIES?

When biologists discover an organism that is new to science, they document the characteristics of the new species, give the new organism a name, publish a formal description of the organism and designate a type specimen. Formal descriptions are generally published in scientific journals such as *The Journal of Eukaryotic Microbiology*. The formal description contains a detailed enough description of the characteristics of the organism that other biologists can recognize the organism and understand how the organism was classified. For example, many new species will be placed in existing genera, which have in turn already been placed in higher-level groups such as Families and Orders. In some cases, the new organism will be placed in a new genus within a described family or, in rare cases; the organism may be placed in an entirely new Family or Order.

You will document the characteristics of the organism(s) you isolate or enrich in Labs 2-7 and produce a formal description of the organism.

Making Observations to Create a Species Description

Written Description and Observations

The type of organism you culture, the time you have available and materials available in the lab will largely dictate the types of observations you can make. While attempting to assign your specimen to a specific group (e.g. family or genus), you need to make specific types of observations of your specimen. Some generally useful types of observations include:

- 1) Measurements of the length and width of cells and size of other structures like flagella can be important characteristics used to distinguish species.
 - a. When making measurements you want to get an idea of the average size of a cell or structure. This will require making the same measurements of at least three cells.

Species Description Assignment

- b. The more measurements you make the better your estimates of the average size of a structure and the variability in size of a structure. Make sure to record the number of different cells measured.
- 2) Counting the number of different types of structures such as the number of flagella, if present.
- 3) Orientation of structures, for example which way is the flagella pointing?
- 4) Staining reactions
- 5) Behavior of the living organism

Photographs and Drawings

The most important records of the observations you make will be in the form of drawings and any photographs you might take. Drawings should summarize all of the important features you observed. A single drawing may represent accumulated knowledge from observing a single cell through multiple planes of focus (i.e. focusing up and down through the cell to observe multiple structures) and your accumulated knowledge from observing multiple cells. You may include multiple drawings to illustrate different aspects of cellular architecture, anatomy or staining reactions. Please refer back to the section on Biological Drawings (pg. 54) for information on drawings and the Biological Drawings Grading Rubric (pg. 58) for information on how drawings will be evaluated.

- 1) You may need several drawings to capture all of the details of the organism, especially organisms that are not symmetrical.
- 2) If you include photographs, you will likely need to take several photographs in order to get one or two images that capture the essential details of the organism.
 - a. You should experiment with phase-contrast (pg. 121) bright field (110) and staining (pg. 129) to resolve as much of the structure of your organism as possible in your photographs.

As you compare your specimen to descriptions in reference-books, keys or primary literature resources, you may make additional specific observations – record these in your notes.

You must make at least one line drawing of the organism that will be included as a figure in the final paper. Drawings will receive individual grades and should be made in class, not from photographs taken during class.

Assigning Your Organism to a Taxonomic Group

Using Literature Resources and Keys

After you have made careful observations of your organism, you need to begin comparing your organism to published descriptions of organisms. You can now begin using published keys and literature resources such as "The Illustrated Guide to the Protozoa" (J. J. Lee, Hutner, & Bovee, 1985) or other resources that are habitat specific such as "Free Living Freshwater Protozoa" (Patterson, 1996). Using these types of keys as a first step, you should be able to determine which group(s) your organism your organism likely belongs to.

The keys you will be using may be dichotomous or pictorial or have elements of both. A dichotomous key is a series of questions about the characteristics of the organism. You work through the key by answering a question and moving on to the next question indicated by your answer to the first question. As you work through keys, it is important to take notes on the choices you make in case you need to go back. Alternatively you may compare your organism to pictures in a pictorial key that show general features of a group of organisms.

The next step is to carefully compare your observations to the published description(s) of the organisms that seem most similar to your organism. You need to carefully and critically compare the observations you made of your organism to the published description; just because you followed a key and arrived at a name does not mean that you have arrived at the correct name for your organism. To find published descriptions, you can try searching the library literature databases (e.g. Web of Science) for information on your organism or using the National Centre for Biotechnology Information (NCBI) taxonomy database (pg. 10).

In your write-up, you will need to justify your assignment to a particular group (e.g. genus or family).

Using The National Centre for Biotechnology (NCBI) Taxonomy Database

Often keys or other literature resources may lead you to a family, species or generic name. Finding information on current classification at taxonomic ranks above and below the rank of the name you have found can be challenging. A valuable resource for finding taxonomic information is the NCBI Taxonomy Database. This resource is an entry point into the taxonomic literature by providing information on taxonomic ranks above and below a search term.

For example, if you identify your organism as *Paramecium caudatum*, you may not have a lot of information on the higher taxonomic groups to which this species belongs especially since you are new to the study of eukaryotic microbes. As well, if the key you are using is old, the classification presented in the resource you are using may

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not reflect current classification. This is where the NCBI Taxonomy Database becomes useful.

- 1) Go to the NCBI website (<http://www.ncbi.nlm.nih.gov/>)
- 2) Enter your species, genus or family name in the search box (Figure 1, pg. 11)
- 3) From the dropdown menu, select "Taxonomy" (Figure 1, pg. 11)
- 4) Click search
- 5) A results list from your search will be displayed (Figure 2, pg. 11).
- 6) Clicking on the *Paramecium caudatum* link will bring up a page containing lineage information for the name (Figure 3, pg. 12).
- 7) Clicking on the *Paramecium caudatum* link on the lineage information page will link to another page providing links to additional taxonomic and DNA sequence resources (Figure 4, pg. 12).

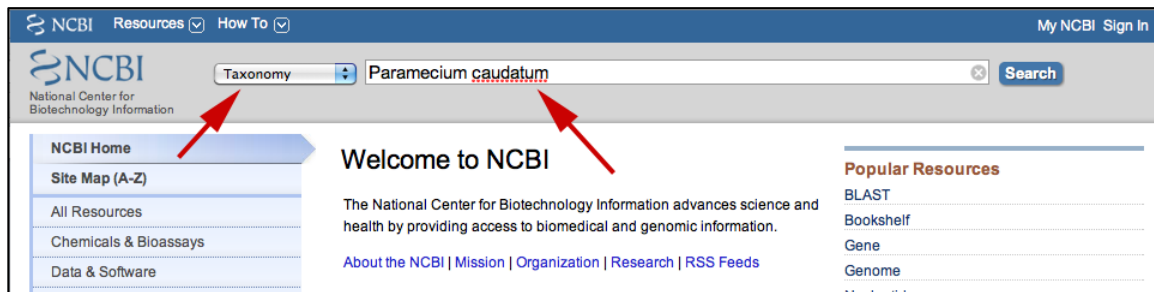


Figure 1 Searching for taxonomic information on *Paramecium caudatum* using the National Centre for Biotechnology (NCBI) database.

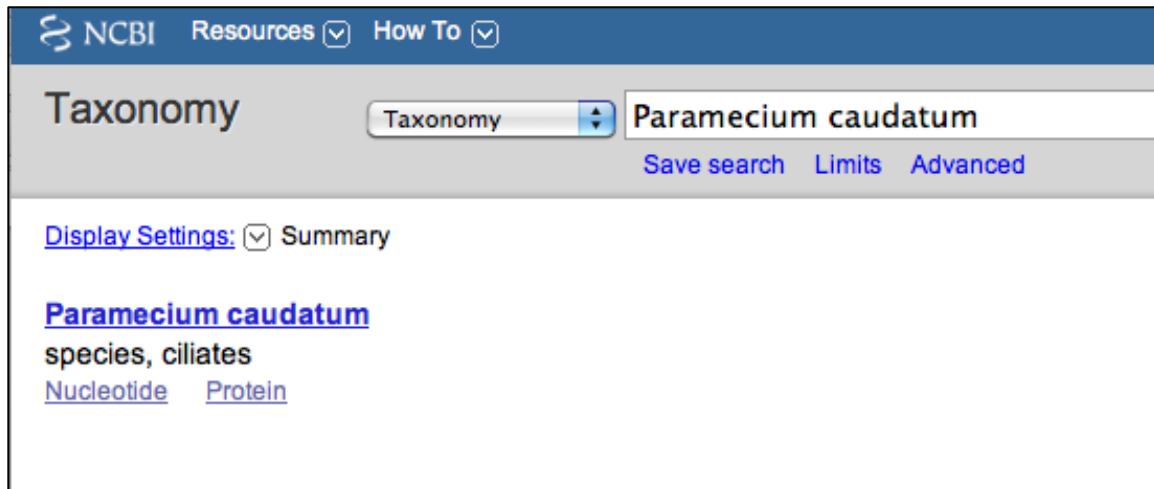


Figure 2 Results list from a search of the National Centre for Biotechnology (NCBI) taxonomy database for *Paramecium caudatum*.

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The screenshot shows the NCBI Taxonomy Browser interface. At the top, there are navigation tabs for Entrez, PubMed, Nucleotide, Protein, Genome, Structure, PMC, Taxonomy, and Books. A search bar contains 'as complete name' and 'lock' options. Below the search bar, there are checkboxes for various database types like Nucleotide, Protein, etc. The main content area displays the lineage for *Paramecium caudatum* as follows: **Lineage (full):** [root](#); [cellular organisms](#); [Eukaryota](#); [Alveolata](#); [Ciliophora](#); [Intramacronucleata](#); [Oligohymenophorea](#); [Peniculida](#); [Parameciidae](#); [Paramecium](#). Below this, a list shows **Paramecium caudatum** and its subgenus **Paramecium caudatum syngen 3**. A disclaimer at the bottom states: "The NCBI taxonomy database is not an authoritative source for nomenclature or classification - please consult the relevant scientific literature for the most reliable information."

Figure 3 The complete lineage for *Paramecium caudatum* from the most inclusive groups [cellular organisms; Eukaryota;] to genus level [Paramecium].

The screenshot shows the NCBI Taxonomy Browser resource page for *Paramecium caudatum*. The search bar contains 'as complete name' and 'lock' options. The main content area displays the following information:

- Paramecium caudatum**
- Taxonomy ID: 5885
- Inherited blast name: ciliates
- Rank: species
- Genetic code: [Translation table 6 \(Ciliate Nuclear; Dasycladacean Nuclear; Hexamita Nuclear\)](#)
- Mitochondrial genetic code: [Translation table 4 \(Mold Mitochondrial; Protozoan Mitochondrial; Coelenterate Mitochondrial; Mycoplasma; Spiroplasma\)](#)
- Lineage (full): [cellular organisms](#); [Eukaryota](#); [Alveolata](#); [Ciliophora](#); [Intramacronucleata](#); [Oligohymenophorea](#); [Peniculida](#); [Parameciidae](#); [Paramecium](#)

On the right side, there is a table titled "Entrez records":

Database name	Subtree links	Direct links
Nucleotide	145	144
Protein	195	194
Structure	2	2
Genome Sequences	1	1
Popset	1	1
PubMed Central	124	124
Gene	49	49
SRA Experiments	1	1
Genome Projects	1	1
Taxonomy	2	1

Below the table, there is a section titled "External Information Resources (NCBI LinkOut)" with a table:

LinkOut	Subject	LinkOut Provider
Paramecium caudatum	taxonomy/phylogenetic	Catalog of Life
Paramecium caudatum Ehr.	taxonomy/phylogenetic	Encyclopedia of life
Paramecium caudatum Ehr.	taxonomy/phylogenetic	Integrated Taxonomic Information System
Wikipedia	taxonomy/phylogenetic	iPhylo

Figure 4 The National Centre for Biotechnology Information (NCBI) resource page for *Paramecium caudatum*.

NEW SPECIES DESCRIPTION WRITE UP

For the final part of your new species description assignment, you will write up a report in the format of a paper in the Journal of Eukaryotic Microbiology. You will be following formatting and manuscript presentation instructions that are based on the

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instructions to authors for the Journal of Eukaryotic Microbiology and use an article by (Jiang, Warren, & Song, 2011) as an example of how your paper should look.

To access the sample article by (Jiang, Warren, & Song, 2011), follow the instructions below.

- 1) Go to the UBC library website (<http://www.library.ubc.ca/welcome.html>), click on the Journals tab in the search frame. Type in "Journal of Eukaryotic Microbiology" (Figure 5, pg. 13)
- 2) Click on the "Journal of Eukaryotic Microbiology [electronic resource]" link in the search results list (Figure 6, pg. 14).
- 3) In the "Actions" frame at the right of the screen click on the "Check UBC eLink for FullText link" (Figure 7 pg. 14).
- 4) On the website for the Journal of Eukaryotic Microbiology, search for the article Jiang, J., Warren, A., and Song, W. (2011) Morphology and Molecular Phylogeny of Two New Marine Euplotids, *Pseudodiophrys nigricans* n. g., n. sp., and *Paradiophrys zhangii* n. sp. (Ciliophora: Euplotida). The Journal of Eukaryotic Microbiology, 58(5): 437-445.



Figure 5 Searching for a journal title using the University of British Columbia library website (<http://www.library.ubc.ca/welcome.html>).

Species Description Assignment

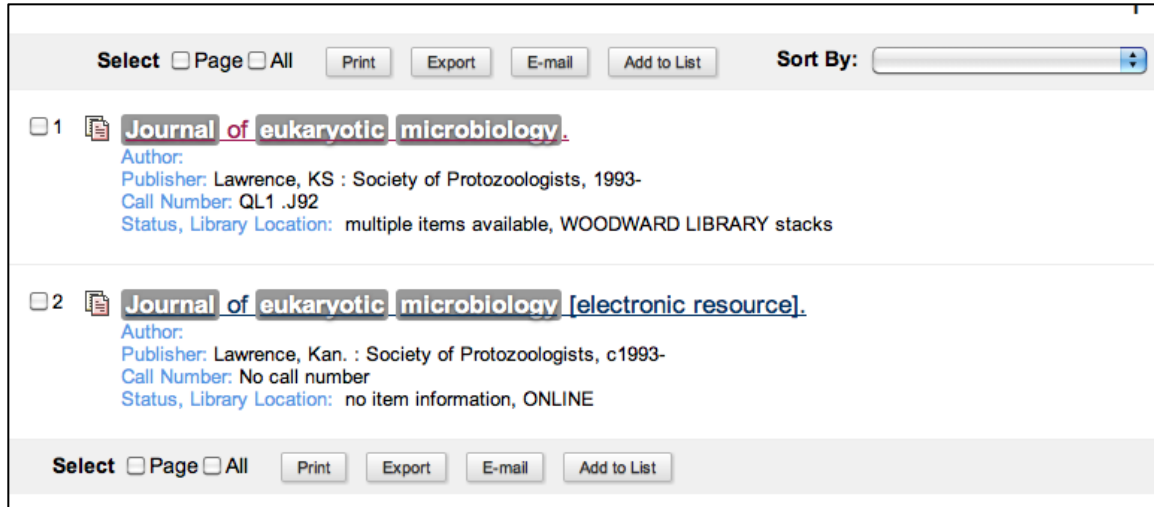


Figure 6 Results list from the journal title search.



Figure 7 Accessing the online version of the Journal of Eukaryotic Microbiology.

Checklist for Preparing Your Paper

Formatting for your paper is loosely based on the basic guidelines outlined in the "Checklist for Manuscript Preparation" from the Journal of Eukaryotic Microbiology available from the journal website (<http://www.wiley.com/bw/submit.asp?ref=1066-5234>). Use the outline below as a checklist for the manuscript your group will hand in for grading.

General Formatting

- Papers must be in English.
- The paper size is standard letter size (8.5 x 11 inch; 21.59 x 27.94 cm).
- All pages should have a 1-inch (2.54 cm) margin on all sides
- All text should be 1 ½ spacing.
- Use the font "Times New Roman" in 12-point for all text.
- Number pages consecutively beginning with the title page
- Sections should be presented in the order: 1) TITLE PAGE 2) ABSTRACT, 3) INTRODUCTION 4) MATERIALS AND METHODS, 5) RESULTS AND

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DISCUSSION 5) LITERATURE CITED and 6) FIGURES AND CAPTIONS 7) TABLES AND CAPTIONS.

- Section headings should appear in capitals.
- All figures should be numbered and only one complete figure with a caption should appear per page. Figures should not be embedded within the text.

Title Page

- The title should be descriptive.
- The first letter of each word in the title should be capitalized with the exception of articles, prepositions, and conjunctions.
- The Authors' names must appear in alphabetical order, in all capitals and in the form: First Name (Given Name) Middle Initial Last Name (Family Name). For example, SALLY R. SMITH and JOHN B. JONES.

Abstract

- The heading ABSTRACT should appear in all caps and be followed by a one paragraph of 200 words or less.
- Include brief statements of:
 - The purpose of the paper
 - The methods used
 - The major results, and overall significance.
 - The abstract should not contain references, abbreviations or acronyms.

Text

- INTRODUCTION** Begin text with the heading INTRODUCTION in caps followed by a brief 3-4 paragraph introduction. Spacing should be 1 ½ lines. Your introduction should include:
 - The purpose of your study.
 - A brief description of your approach (detailed methodology belongs in the materials and methods section).
 - A description of the group of organisms to which your new species belongs including information on habitat, life history, ecology etc.
 - A description of the major characteristics (morphological, biochemical and ultrastructural) of the major taxonomic group to which your organism belongs.
 - A description of the Supergroup to which your organism belongs based on (Keeling et al., 2005) or (Adl et al., 2005).
- MATERIALS AND METHODS** must include the subsections: *Sampling*, *Isolation* and *Microscopic Observations*. Subheadings should be in italics and underlined. Additional subheadings can be added if needed. This section should be detailed

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enough that another knowledgeable researcher could repeat the experiment but should not be a detailed protocol. Please see the sample paper as a model.

- Sampling
 - A description of the sampling location where you collected the sample from which your organism was isolated.
 - How physical or chemical measurements of the environment were made.
 - A description of the method(s) used to collect the sample from which your organism was isolated.
 - A description of how the sample was handled and stored.
- Isolation
 - A description of the method(s) used to enrich or isolate your organism in the lab.
- Microscopic Observations
 - A brief description of microscopic, fixing and staining methods used.

□ RESULTS AND DISCUSSION

- The bulk of your results section will comprise a description of the species. The species name followed by a list of figures and tables should appear as in the sample paper.
- If you are describing a new species, you will need to give it a scientific name and indicate that this is a new species by including "n. sp." (**new species**) following the species name. If your species has been already been described, do not include the "n. sp." designation.
- As in the sample paper, the results should include description(s) of all the characteristics you observed for the species. With specific reference to appropriate figures and data tables.
 - **Each group member must make at least one line drawing of the organism and at least one drawing from each group member must be included in the final manuscript. Drawings will receive individual grades.**
 - **The figure caption should indicate the name of the person who made the drawing.**
- As part of the justification for a new species or inclusion of your specimen within an existing species you will need to present:
 - A detailed comparison between your specimen and a description of a closely related species from the literature.
 - Highlight the similarities or differences between your specimen and the description.
 - If you feel that the species you observed has been described, you should provide a detailed comparison of your specimen to the original species description.
 - A brief discussion of additional observations you could make to further support your argument. For example, you may not have

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been able to perform some useful types of staining procedures due to time, difficulty or chemical hazards.

- Figures and tables cited in the text should be in numerical order.
- Throughout the manuscript the abbreviation 'Fig.' should be used.

Text Citations

- In-text citations and citations appearing in figure or table legends must appear in the LITERATURE CITED section. RefWorks is a useful program for creating in-text citations, bibliographies and managing references. UBC has a license for RefWorks (<http://guides.library.ubc.ca/refworks>).
- All references listed in the LITERATURE CITED section must be cited in the text.
- Citations are written as author-year in parentheses without a comma: (e.g. Smith 1980; Smith and Jones 1984).
- For materials with more than two authors use '*et al.*' (e.g. Smith *et al.* 1986).
- Multiple citations should be in alphabetical order and separated by semicolons.
- Multiple works by the same authors in the same year must be distinguished by the (e.g. a, b, c) following the year of publication. For example, (Fenchel 1987; Smith and Jones 1984a, b).

Literature Cited

- Following the heading LITERATURE CITED, list the references using 1 ½ spacing throughout.
- Using the last name of the first author, alphabetically arrange references.
- Single-author works precede multi-authored works by the same author.
- The format for references should follow the examples from the sample article.

Figure and Tables

- Figure and tables legends appear with the individual figures or tables following the LITERATURE CITED section.
- The figure captions should be brief and self-explanatory (i.e. the figure should be intelligible without referring back to the body of the text). Please see the figure legends in the sample article for examples.
 - **Since individual grades will be assigned to figures, the name of the person who made the figure should be included in the legend.**
- The table legends should be brief and self-explanatory (i.e. the figure should be intelligible without referring back to the body of the text).

Supplemental Material

- Field notes from Lab 2 collecting trip.

LAB 1 - MAKING A VAN LEEUWENHOEK- TYPE MICROSCOPE

LEARNING OUTCOMES

By the end of this lab you should be able to:

Demonstrate observational and note-taking skills and can use lab equipment for making measurements and observations of protists by:

- a) Building and using a simple microscope based on van Leeuwenhoek's design.
 - b) Calculate or measure the field of view, focal length and the power of your lenses.
 - c) Describe the relationship between field of view, focal length and lens power for lenses produced by you and your classmates.
 - d) Make observations using the replica microscope and identify how aberration, contrast magnification, resolution, working distance, focal length, and, field of view affect the image produced by a simple microscope.
-

PRE-LAB PREPARATION

1) Read the introduction and procedure for Lab 1, especially the "Basic Microscopy Concepts" section (pg. 32).

LAB OUTLINE

- 1) Building The Microscope
- 2) Activity 1 - Contrast
- 3) Activity 2 (Field of View)
- 4) Activity 3 Determining the Power and Focal Length of the Lens
- 5) Graphing the Relationship Between Lens Diameter and Other Lens Parameters

INTRODUCTION

In the spirit of exploration and discovery that is the basis for the lab portion of BIOL203, you will begin at the beginning, exploring the world of biology invisible to the unaided eye with the tools used by the first explorers.

Antonie van Leeuwenhoek was a wealthy cloth merchant who lived in the city of Delft, in the Netherlands, from 1632 to 1723. He is best known for his pioneering work on microscopy. From 1673 onwards he created as many as 500 microscopes and from these made numerous significant discoveries, including determining the existence of single-celled organisms. Ironically, the discovery of single celled organisms brought his scientific credibility into question for some time.

The success of the simple microscopes used by van Leeuwenhoek and others can be attributed to many things, but a number of technical matters stand out. First, his microscopes relied on a single lens to provide magnification. Compound microscopes, those with more than one lens in the light path, theoretically provide better resolution and greater magnification, but they are also much more technically challenging to fabricate. As well, van Leeuwenhoek devised a relatively simple means to produce his lenses. In particular, his methodology appears to have reduced the need for precise grinding techniques; grinding was a laborious and technically difficult process.

The few examples of Leeuwenhoek's microscopes that remain today are elegant creations of brass or silver with many working parts. Although much less complex than modern microscopes, replicas of metal with the same working features are challenging to build and require some technical skill. An example of a replica of one of van Leeuwenhoek's microscopes is shown in Figure 8 (pg. 20). However, the basic functional aspects of the design and lens production can be replicated in a few minutes, using a few simple materials.

Lab 1 – Making a van Leeuwenhoek Microscope

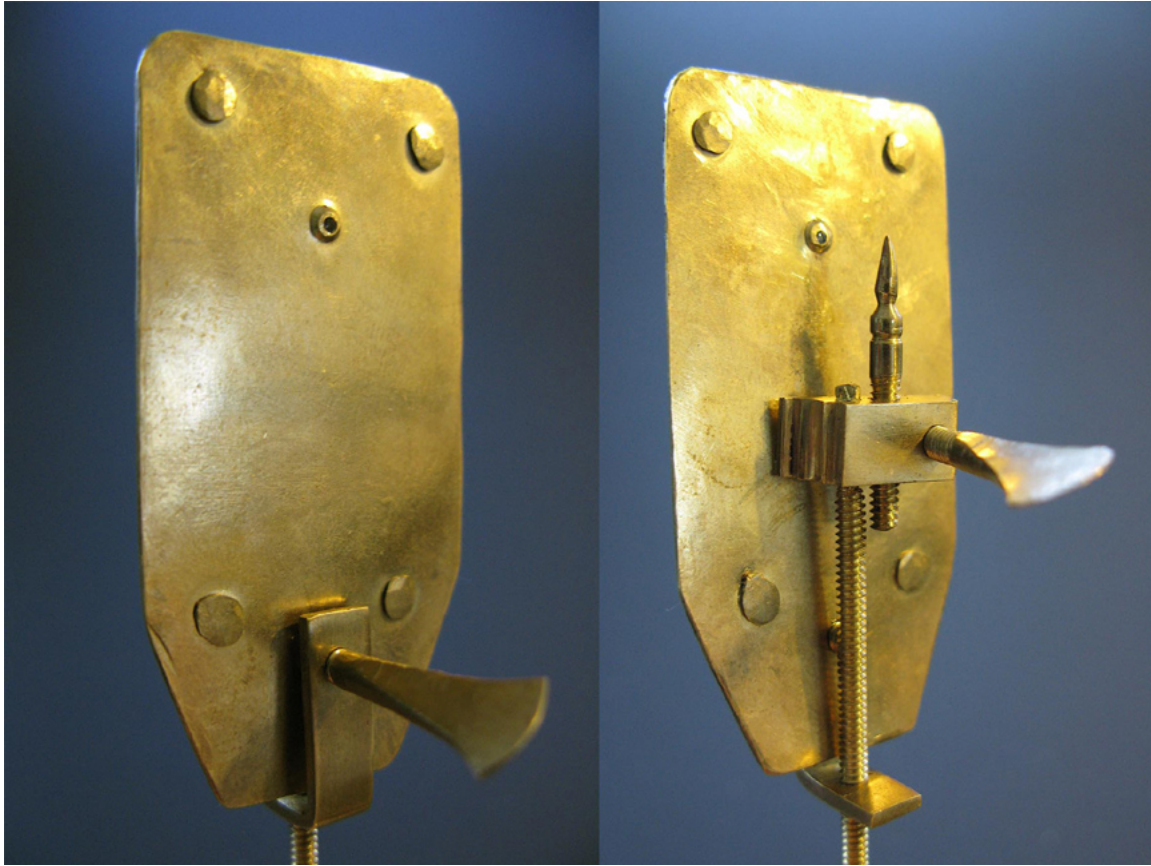


Figure 8 Front and back views of a brass replica of a van Leeuwenhoek microscope. Building such a replica requires a few tools and some skill with them.

Following the steps available here, you will be able to construct a working microscope using van Leeuwenhoek's general design and method of lens production. From this, you can then measure the size of the lens you make, and calculate its magnification. Lenses capable of magnifying a sample 100x to 200x actual size are not too difficult to produce. For example, if you viewed a cell that was 0.02mm in diameter using a lens with a magnification of 150x the cell would appear to be $0.02 \times 150 = 3\text{mm}$ in diameter. You will build a microscope and lenses then examine some of the basic properties of your lens (size, magnification, field of view and focal length) and make observations using your microscope.

It's amazing to consider how we often take microscopy for granted. However, when you use the microscope you build yourself, try to imagine what it must have been like to peer through one of these creations and discover a completely unknown realm of life; your instrument will reproduce the microbial world as it would have looked like using the technology of the seventeenth century.

BUILDING THE MICROSCOPE

Materials

- 1 glass Pasteur pipette or capillary tube, or whatever glass source is handy
- 5 x 10 cm piece of 1mm posterboard (thick side)
- 5 x 10 cm piece of cardstock (thin side)
- 1 small dab about the size of a small marble of tacky putty or chewing gum
- Wood block (e.g. small piece of 2 x 4 or particle board)
- Razor blade or X-Acto knife
- Goggles

Tools

- Drill with 1/16 bit
- Stapler
- Flame (portable plumbing torch or Bunsen burner)
- Caliper

INSTRUCTIONS

Making The Microscope Plates

1. **Cut out microscope plates.** Cut out two roughly equal sized pieces of posterboard and cardstock about 6 X 3 cm, with a slight taper at one end (Figure 9, pg. 22)

Lab 1 – Making a van Leuwenhoek Microscope

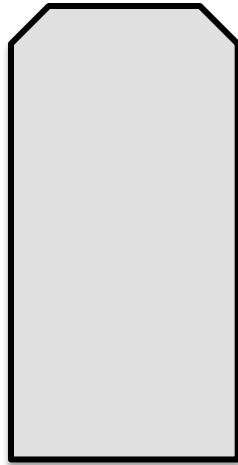


Figure 9 Cardboard plates for the body of the van Leeuwenhoek microscope.

2. Drill light path. Sandwich the poster board and cardstock together and place on the wood block. Drill a 1 mm diameter hole (1/16 inch) approximately 1.5 cm from each side and the top, and about 4.5 cm from the bottom (Figure 10 pg.22). With the razor blade or X-Acto knife, shave off any rough paper around the holes.



Figure 10 Drilling a hole to hold the lens and create a light path.

Creating The Lens

The lens will be a sphere of glass whose diameter dictates the magnification. You will be making two lenses, one “large” and one “small”. Aim for a lens of about 2mm in diameter since this is big enough to work with and gives a decent magnification. There are two main steps to this stretching the glass and forming the lens.

Stretch some glass

1. Put on safety goggles and light the torch or Bunsen burner.
2. Holding the glass rod or pipette at both ends, place the center in the flame and hold it there until the glass melts. It helps to roll it in the flame to equally expose all sides of the heated area (Figure 11, pg. 23).
3. When the glass is soft and will stretch without breaking, remove it from the flame and immediately pull the two ends apart to stretch the glass very thinly. You are aiming for a glass tube of $>0.5\text{mm}$. Too thick and your lens tends to be teardrop shaped rather than spherical, too thin and you have to feed a lot of glass into the flame during the actual formation of the lens (Figure 12, pg. 24).

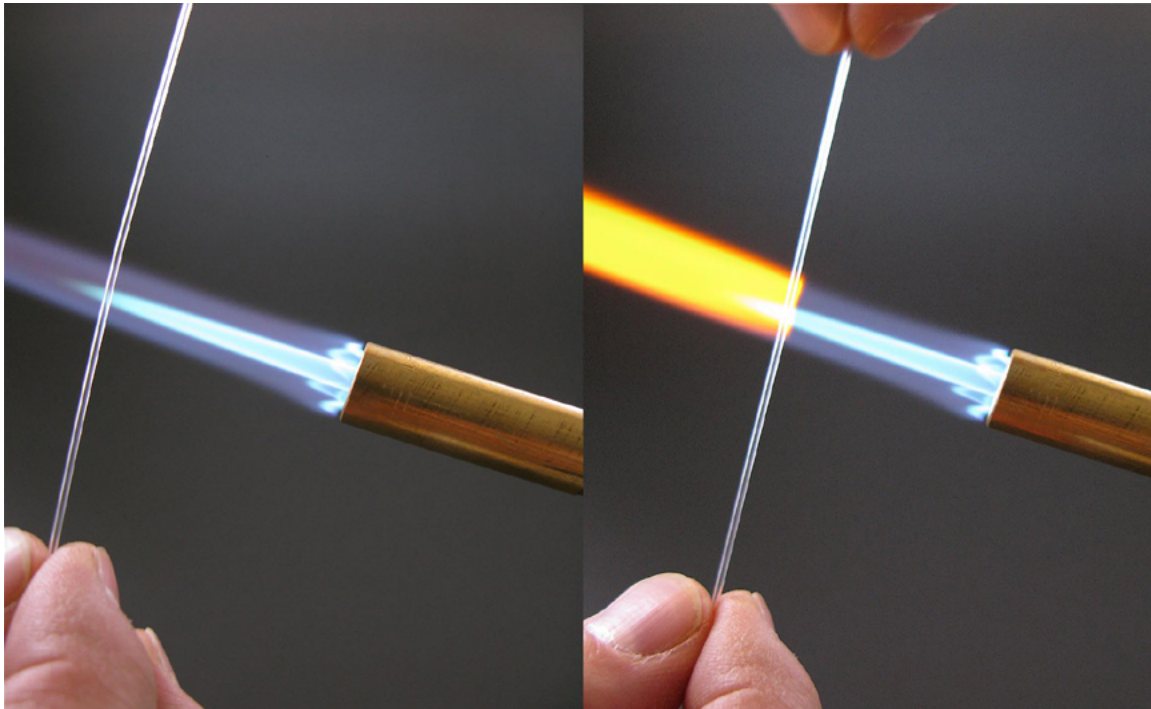


Figure 11 To stretch the glass, first place it in the flame, rolling it to heat it evenly until it is quite soft and wobbly.

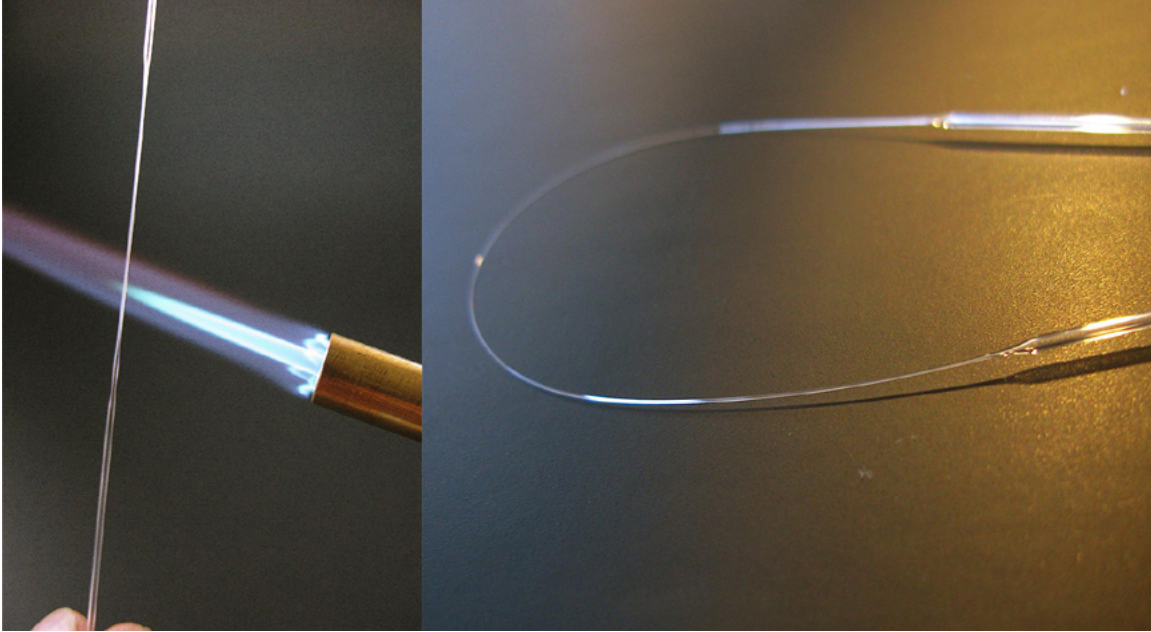


Figure 12 Once the glass is thoroughly soft, remove it from the flame and immediately pull apart the two ends so the softened part is stretched to a uniformly thin filament or tube.

Forming The Lens

1. Keep your safety goggles on.
2. Once the stretched glass has cooled sufficiently to handle, break it somewhere in the middle of the stretched portion.
3. Position the flame horizontally, and slowly feed the stretched glass into the flame from above (Figure 13, pg. 25).
4. A small, white-hot glass sphere will grow at the tip of the tube as you feed it into the flame.
 - a. Do not remove the sphere from the flame and let it cool before the lens has reached the size you want. If you move the lens in and out of the flame, bubbles will form in the glass ruining the lens.
5. Keep feeding the tube into the flame until the sphere is about 2 mm in size (Figure 13 pg.25).
 - a. A nice trick to help keep this motion constant and the sphere in the flame is to feed the stretched glass through the finger loop of a pair of scissors. Position the glass tube so that about 5 cm of glass extends from the of the finger loop of the scissors. Hold the scissors by the blade and twist slightly so there is mild tension on the glass. This will hold the end of the glass steady as you feed it into the flame.
6. Once the sphere is the size you want, remove it from the flame, let it cool completely, and break the tube off about 0.5 cm from the sphere.
 - a. The short piece of tubing gives you a handle so you can avoid touching the lens during later construction and will help you align the lens to avoid

Lab 1 – Making a van Leuwenhoek Microscope

looking through the 'wound' inevitably caused by breaking the lens from the tube.

7. Make a couple of lenses and choose two of different sizes for constructing your microscopes.

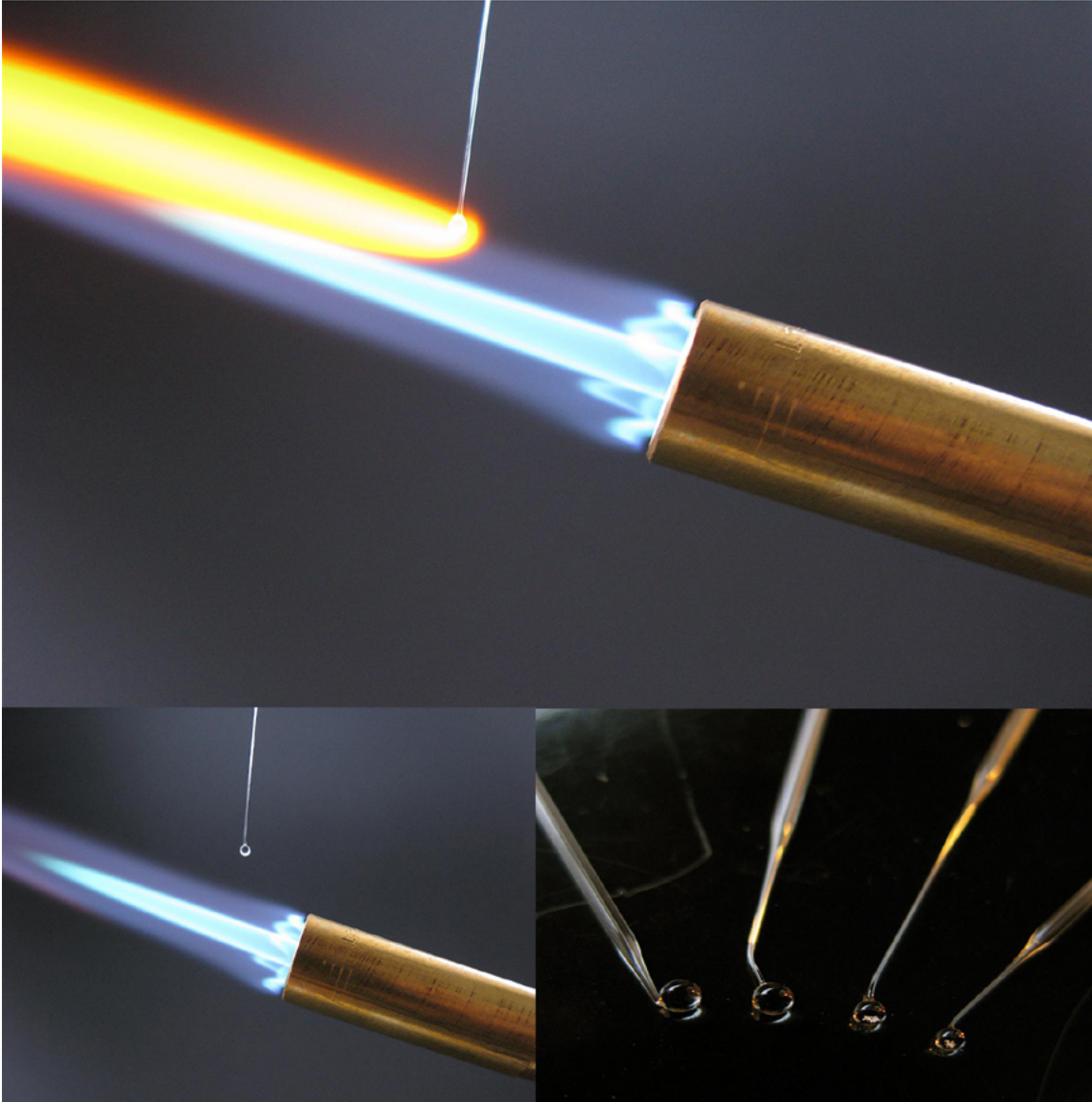


Figure 13 Slowly feed the thin glass filament into the flame. The tube will melt and a small ball will form at the end of the filament (upper panel). Once you are happy with the size of the ball remove it (bottom left). These are easy to make, so make several and chose the best sphere with no visible imperfections (like bubbles). Snap off the sphere leaving a short length of tube attached to it.

Lab 1 – Making a van Leuwenhoek Microscope

Troubleshooting Forming the Lens

1. Your lens should be a sphere, and not a teardrop shape. If you get a teardrop your glass was likely not stretched sufficiently, so go back to step one and stretch a new one to be thinner.

Measuring the Diameter of the Lens

1. Before assembling the microscope, you will need to measure the diameter of your lens.
2. Record your lens diameters in Table 1 (pg. 39) and write the lens diameter on one of the microscope plates for future reference.

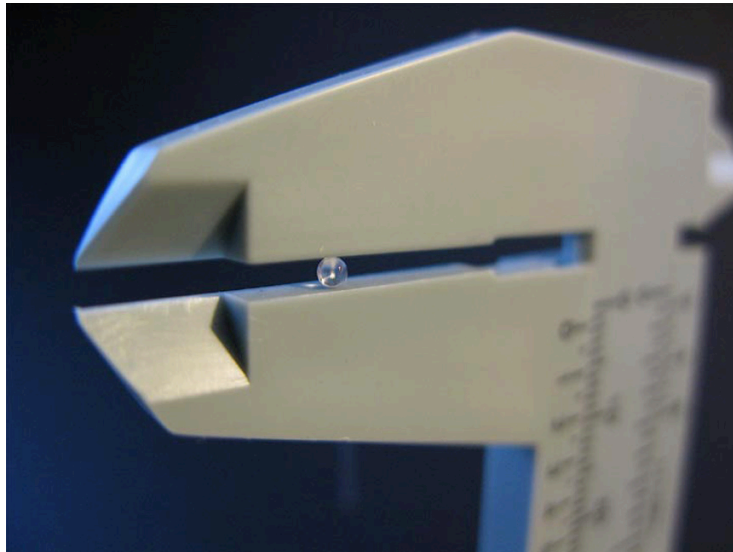


Figure 14 Measure the diameter of the lens using a caliper.

Assembling the Microscope

1. Hold your lens by the protruding glass tube and place in the hole you drilled in the posterboard. The glass tube should lie flat on the inside surface of the posterboard (Figure 15, pg. 27).
2. Set the cardstock on top so the lens and handle are sandwiched between the two layers.
3. Hold the two layers firmly together with the holes and lens lined up.
4. Staple the cardboard plates together with two staples 0.5 cm from either side of the hole (Figure 16, pg. 27) with the cardstock side up.

Lab 1 – Making a van Leuwenhoek Microscope

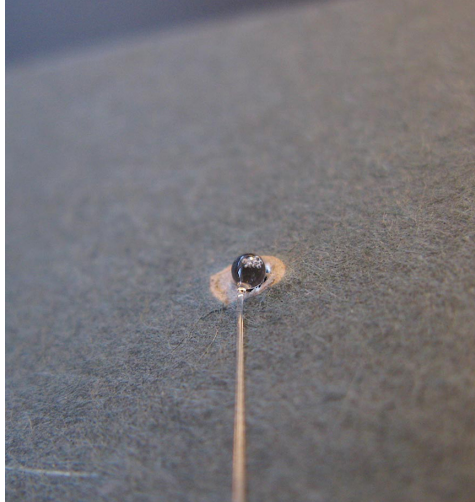


Figure 15 Place the lens in the hole on the inside of the posterboard, lying the remaining tube flat on the surface.

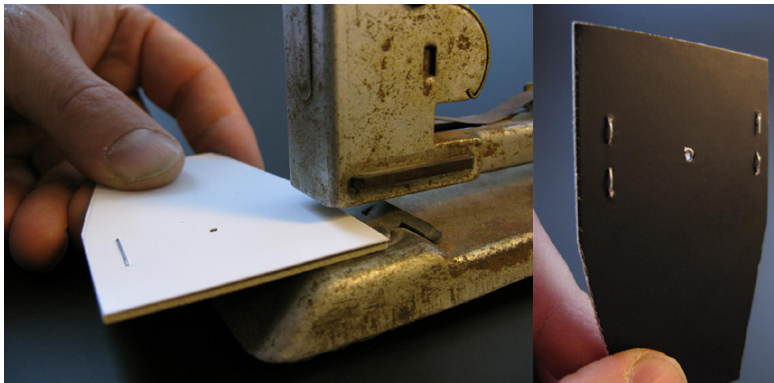


Figure 16 Staple the two pieces of paper together with the lens between them, and your microscope is assembled.

Troubleshooting Assembling the Microscope

1. Your microscope only works in one direction. You look through the posterboard side and place your sample on the cardstock side. The directionality of the microscope is due to the short working distances of the lenses (the distance between the object you are viewing and the lens). As a result, your sample needs to be very close to the lens. The thinner cardstock allows the sample to be brought close enough to the lens to be viewed.

Making Your Focus Mechanism

The biggest challenge to making a microscope from paper is how to focus your specimen. You will mount your sample in tacky putty to mount the specimen and adjust the distance of the sample from the lens to bring the sample into focus. To illustrate

Lab 1 – Making a van Leuwenhoek Microscope

how to mount and focus a sample, you will observe a barb of a feather. But you can also mount a variety of other kinds of samples.

1. To start, put your feather barb on a flat surface.
2. Take a dab of putty about the size of a pea and press it over one edge of the feather barb (Figure 17, pg. 28). Pick the putty off the surface, and the barb will stick to the putty and project from it.
3. Stick the putty to the cardstock side of the microscope just below the hole so the barb is right over the hole (Figure 18, pg. 29). Be careful not to get putty on your lens. This will be your stage, focusing device, and your movement controls.
4. To view the specimen, hold the microscope as close to your eye as is comfortable, looking from the posterboard side directly into a light source a light bulb, or a bright sky.

Never look directly at the sun with any optical device.

5. To focus, place your thumb on the putty. Pushing the putty up towards the top of the microscope will cause the sample to pivot closer to the lens, pulling down will cause it to pivot away from the lens. Movement from side to side will position the sample over the lens as desired (Figure 19, pg. 29).
6. Use the same approach to mount and view other kinds of samples.

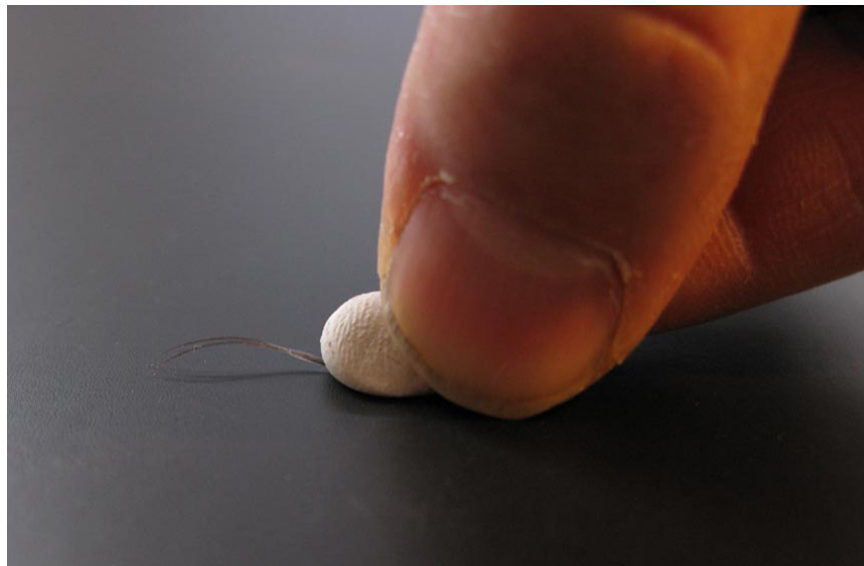


Figure 17 Take a pea size piece of tacky putty and press it on the subject (a feather barb is shown). The subject should stick to the putty when you remove it from the surface.

Lab 1 – Making a van Leuwenhoek Microscope



Figure 18 Press the putty with subject attached to the cardstock (thin) side of the microscope with the subject lying over the lens.

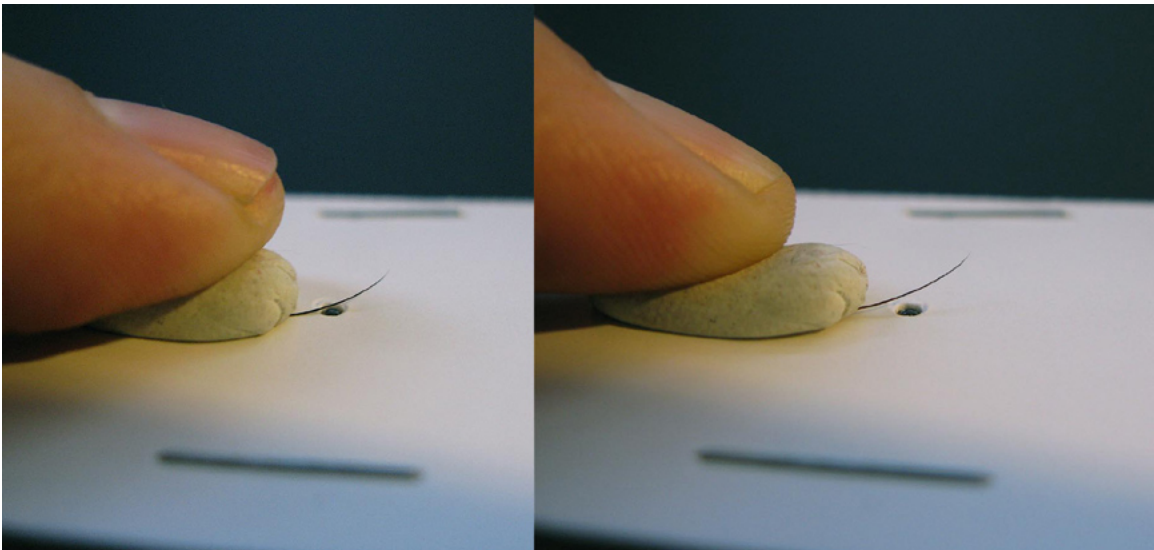


Figure 19 To focus, place your thumb on the putty. Pushing up will pivot the sample towards the lens, while pulling down will pull the sample away from the lens.

Mounting a Dry, Solid Specimen

- 1) Place the specimen on a surface and press the putty over it and attach it to the microscope as described. Feather barbs, insect wings, or onion skin work well.

Mounting a Wet Specimen On a Single Coverslip

- 1) Place a single glass coverslip on a surface and mount it with the putty.
- 2) Drop your sample on the coverslip and hold the microscope horizontally with the coverslip side up (so your sample does not drip) and look from the bottom (Figure 20, pg. 30).

Lab 1 – Making a van Leuwenhoek Microscope

Mounting a Wet Specimen Between Two Coverslips

Wet samples can also be mounted by sandwiching a drop of the sample between two coverslips and mounting the coverslips as described for a single coverslip. Sandwiched between coverslips the sample will be much thinner than mounting a drop on the surface of a single coverslip. It will likely be easier to focus on organisms in a thin sample than in a thick droplet.

Samples containing diatoms are easily viewed sandwiched between cover slips since diatoms are relatively large, regularly shaped and have cell walls. Samples containing foraminiferans (aquatic amoeba-like organisms with shells) or radiolarians (aquatic amoebae with mineral skeletons) are also good, but not as easy to find in nature.

- 1) Mount a single glass coverslip on the microscope.
- 2) Place a drop of sample on the mounted coverslip.
- 3) Place a second coverslip over the first.

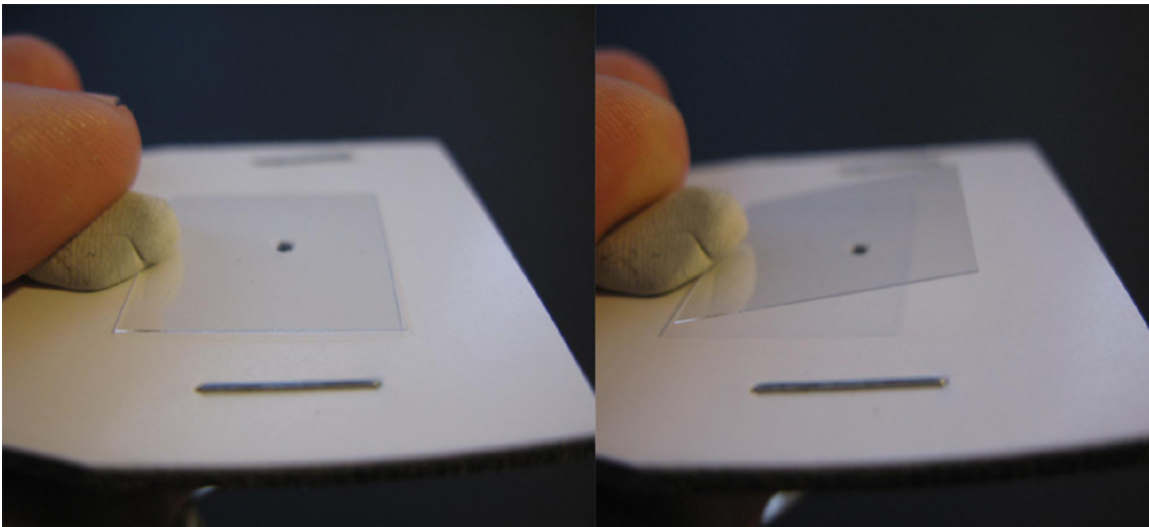


Figure 20 To view a liquid sample, follow the procedure of Figures 1-8 to 1-10 using a glass cover slip, and once attached to the microscope, drop the liquid sample on to the glass.

ACTIVITY 1 - CONTRAST

Contrast is the difference in color or intensity (light or dark) between the specimen and the background or between structures within the sample. Low contrast is when there is little difference in color or intensity between an object and the background or adjacent objects. Figure 70 (pg. 121) shows the effect of light intensity on contrast.

1. Observe chicken feathers stained different colors (unstained white feathers, black, red etc.).
2. Which color or colors of feathers were easiest to view?

3. What features made viewing this sample easier than the others?

4. Sketch barbs of the feather in the space below.

5. Describe some of the difficulties you had when trying to view and draw the specimens. Did you observe any Aberrations (pg. 41) or difficulties resolving separate structures (pg. 42).

ACTIVITY 2 (FIELD OF VIEW)

Field of view is the area of a sample you can observe with a particular microscope objective (lens); i.e. the distance across the circle of light you see when you look through a microscope. The diameter of the field of view is related to magnification. The useful field of view can be reduced by image distortion or aberrations that occur towards the edges of lenses. For a discussion of common aberrations please refer to pg. 41.

1. Affix a slide containing a basic micrometer to your stage.
2. Measure the field of view by determining the number of hash marks there are from one edge of the field of view to the opposite edge (Figure 21, pg. 32). Each hash mark is 0.01 mm (= 10 μm).
3. Record your results in the Table 1 (pg. 39).

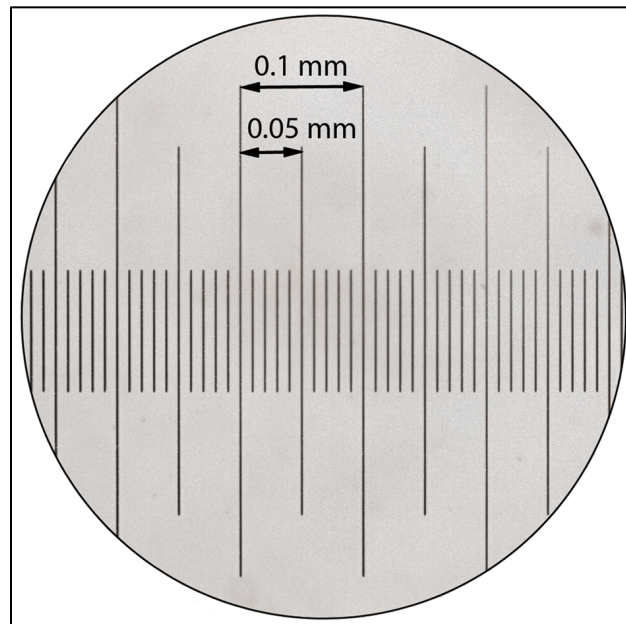


Figure 21 An example of measuring the field diameter of a 20 \times objective for a compound microscope using a stage micrometer. The circle represents the field of view. The distance between each of the small hatch marks is 0.01 mm (10 μm). The field diameter is 0.94 mm (940 μm ; 940 micrometers)

ACTIVITY 3 DETERMINING THE POWER AND FOCAL LENGTH OF THE LENS

In addition to using your microscope to look at tiny things, it is a good exercise in optics to determine the power of the lens. In this activity you will determine the power of your lens and compare it with other students in the class. Because everyone will make slightly different sized lenses, you will be able to see the relationship between the size of the lens and the power.

Magnification or Lens Power

Magnification is the ratio of the size of the image produced by a lens and the actual size of an object (Equation 1, pg. 33). The power of the lens is inversely related to the focal length (Equation 3, pg. 34; Figure 22, pg. 33). The focal length (Equation 2, pg. 34) is the distance from the middle of a lens to the focal point (the point at which parallel rays of light converge after passing through a lens).

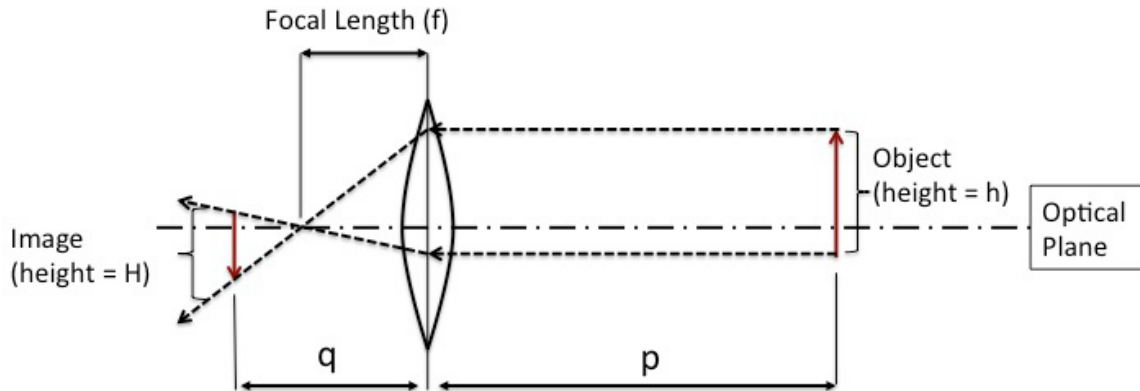


Figure 22 The relationship between image height (H) and object height (h) for a converging lens. The focal length of a lens is the point at which light rays converge on the optical plane.

$$\frac{q}{p} = \frac{H}{h}$$

Equation 1 Relationship between the object height (h), image height (H), distance between the lens and the object (p) and the lens and the image (q).

M is the magnification of the lens

q is the distance between the lens and the image

p is the distance between the lens and the object

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H is the image height

h is the object height

$$\frac{1}{f} = \frac{1}{p} + \frac{1}{q}$$

$$f = \frac{p \times q}{p + q}$$

Equation 2 The focal length (f) of a lens.

By convention, lens power is calculated at a distance of 250 mm from the lens since this is the closest distance the average human eye can focus on an object. When you view an object under the microscope you are actually seeing a virtual image (an image that can't be captured by a camera) that appears as if it is ~250mm in front of the lens.

$$I = \frac{250 \text{ mm}}{F} = \frac{250 \text{ mm} (p + q)}{pq}$$

Equation 3 The magnifying power of a lens.

Materials

- Micrometer
- Laser pointer
- Transmission electron microscope (EM) grid, 100-mesh (e.g. [Pelco product 1GC100](#))
- Measuring tape

Instructions

Project a Laser Beam Through TEM Grid and Lens

1. To measure the power of the lens, set up the components in this order: the laser source, then the EM grid, then the lens, then a surface, with the EM grid and the lens situated very close to one another.

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2. The EM grid is a small, usually copper disk with a fine metal mesh, the size of which is known exactly. For the 100-mesh grid suggested above, the width of each hole is $204\mu\text{m}$ and width of each bar is $50\mu\text{m}$.
3. When the laser beam is passed through the grid and lens, it projects a greatly enlarged image of the grid pattern on to the surface. If you then measure the distance between two grid bars on projected image and compare it to the known size of the grid (i.e. $204\mu\text{m}$), taking into account the distance it has been projected, you can calculate the power of the lens using the following equations.

For the lenses you have made, it is difficult to accurately measure the distance between the lens and the object (p in Equation 1). Rearranging Equation 1 to solve for p gives:

$$p = \frac{qh}{H}$$

Equation 4 Calculation of p , the distance between the object being viewed and the middle of a lens.

Which can be substituted into Equation 3 to give:

$$I = \frac{250\text{mm} \times (h + H)}{hq}$$

Equation 5 Calculating the magnification of a hand made spherical lens.

I is the magnification of the lens

q is the distance between the lens and the image

H is the image height (e.g. the width of projected the hole or bar you measured)

h is the object height (e.g. the width of each hole is $204\mu\text{m}$ and width of each bar is $50\mu\text{m}$)

Lab 1 – Making a van Leuwenhoek Microscope



Figure 23 The basic set up for a stand to measure the power of a lens using a laser.

Lab 1 – Making a van Leuwenhoek Microscope

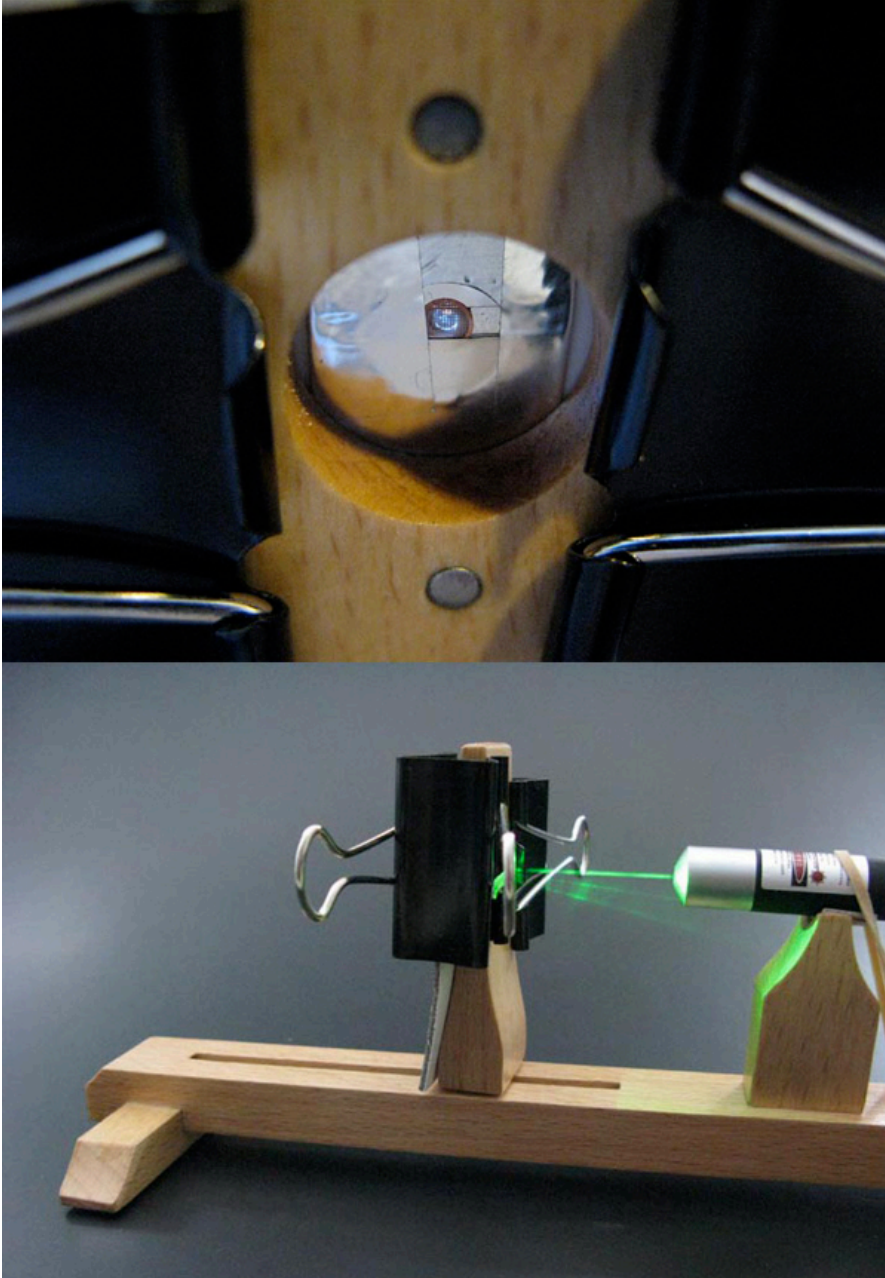


Figure 24 When the beam is centered; clamp the microscope onto the far side of the EM grid, so that the lens is directly behind the grid.

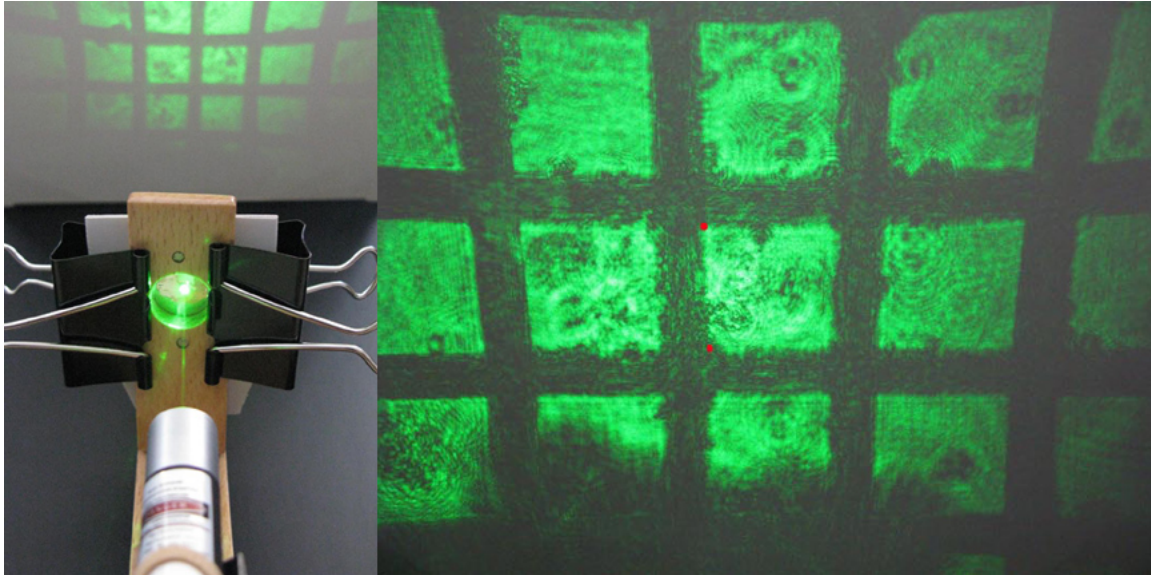


Figure 25 Turn on the laser, and the beam will pass through the grid, then the lens, projecting a magnified image of the grid onto a surface. Measure the distance between two points on this projected image (where the actual size on the grid is known), and measure the distance from the lens to the surface. For example, measure between the positions indicated by two red dots, which are 0.204mm apart on the actual grid.

Measuring Lens Power and Calculating Focal Length

1. A stand (Figure 23, Figure 24 and Figure 25; pgs. 36 to 38) is used to hold the laser steady and provides a platform to attach the EM grid.
2. The laser beam is first aimed at the center of the grid by placing paper shims under the pointer to align the beam, with the switch held on by the elastic that holds the pointer in place (Figure 23).
3. When the laser is aligned (Figure 24), the microscope is mounted using bulldog clips so that the lens is against the grid.
4. Turn on the laser and point it at a surface to measure the projected image size and projected distance (Figure 25).
5. Using Equation 3 (pg. 34), calculate the lens power for each of your lenses and enter your data in Table 1 (pg. 39).
6. Use Equation 4 (pg. 35) to calculate p , the distance between your lens and the EM grid.
7. Use Equation 2 (pg. 34) to calculate the focal length of the lens and enter your data in Table 1 (pg. 39).
8. After completing Table 1 (pg. 39), add your results to the class data table.

Lens Number	Lens Diameter	Field Diameter	Power	Focal Length

Table 1 Measurement of lens parameters.

Graphing the Relationship Between Lens Diameter and Other Lens Parameters

- 1) Download the aggregated class data from the course website.
- 2) Using Excel or graphing paper, graph the relationship between lens diameter (x axis; the independent variable) and lens power (y axis; the dependent variable).
- 3) Using Excel or graphing paper, graph the relationship between lens diameter (x axis, the independent variable) and field diameter (y axis, the dependent variable).
- 4) Using Excel or graphing paper, graph the relationship between lens power (x axis, the independent variable) and focal length (y axis, the dependent variable).
- 5) Using Excel or graphing paper, graph the relationship between lens power (x axis, the independent variable) and field of view (y axis, the dependent variable).
- 6) For each graph, write a 1-2 sentence describing the pattern(s) you observe in each graph.
- 7) For a modern compound microscope, how would you expect the focal length and field diameter to change as you increase magnification from the 10x to 40x objectives.

SOME EXAMPLES IMAGES

These were taken with a Canon G9 point and shoot camera and do not do the image quality justice because to get the field of view to a reasonable size you must zoom in fully and Canon cameras do not function in macro mode while zoomed in (so the depth of field is bad).

Lab 1 – Making a van Leuwenhoek Microscope

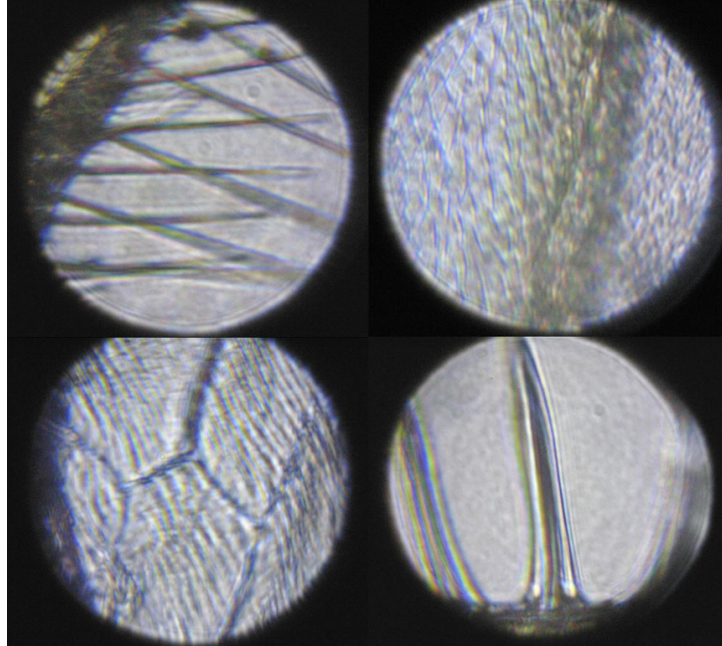


Figure 26 Clockwise from upper left: the hairs on the trailing edge of an insect wing, cells in an onion skin, and the spikes on the surface of a radiolarian skeleton and a vein of an insect wing.

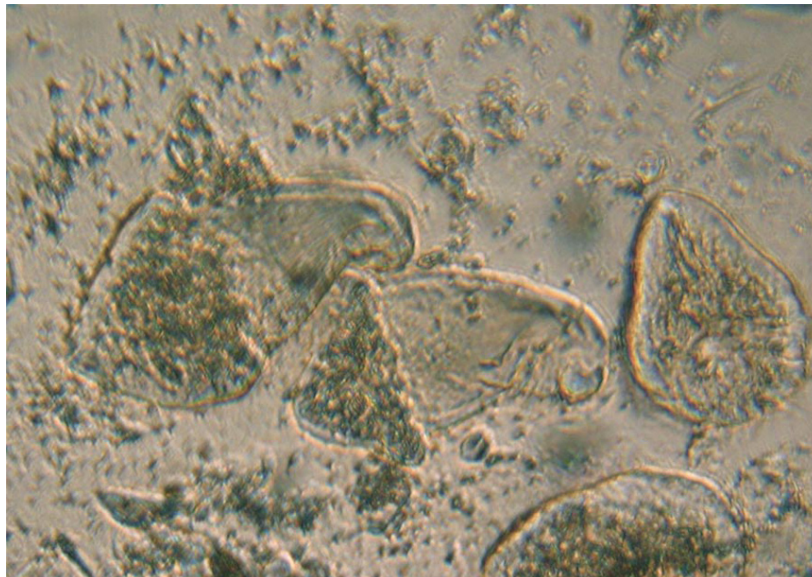


Figure 27 An example of an image with a lower magnification lens of live cells in liquid on a cover glass. This is the hypermastigote parabasalid *Trichonympha* from the hindgut of the termite *Zootermopsis angusticollis*.

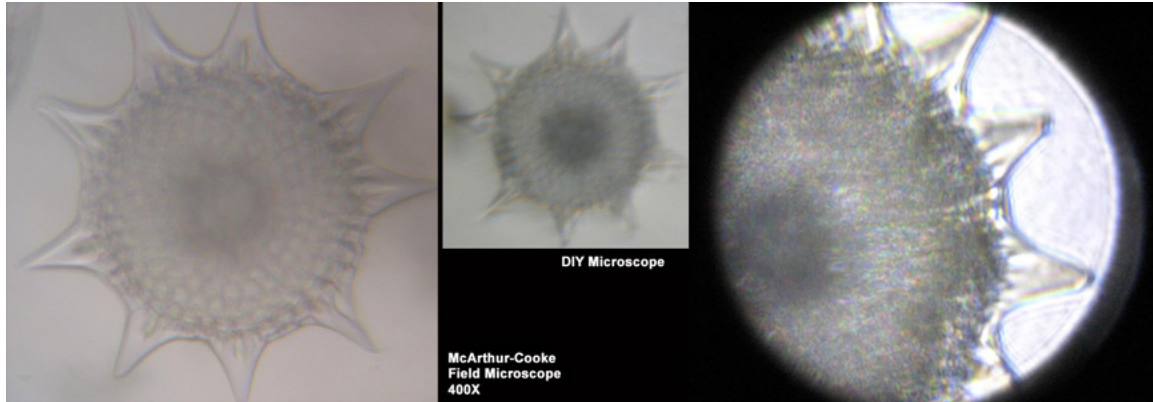


Figure 28 Comparison of the same Radiolarian skeleton with three different microscopes. At left is a commercial McArthur-Cooke field microscope set at 400X. In the center is the same skeleton magnified using a home made lens photographed with the same camera and at the same scale (showing the power of this lens to be about 200X). On the right is the same sample again but photographed using the Canon G9 and a different microscope with a much higher power lens.

BASIC MICROSCOPY CONCEPTS

Aberrations

Aberrations are various types of image distortion due to the interaction of light with a lens or lens system. Aberrations reduce the resolution (pg. 42) of a lens system and the quality of the image produced. In modern microscopes, lens and light systems are engineered to reduce the effect of aberrations on image quality. Some types of aberrations that you may encounter are: chromatic, spherical, and field curvature.

Chromatic Aberrations

Chromatic aberrations result in colored halos around objects when samples are viewed using a light source composed of a number of wavelengths (e.g. white light). This is due to images created with different wavelengths being focused at different points. Filters can be used to reduce the effect of chromatic aberrations.

Spherical Aberrations

Spherical aberrations reduce image sharpness, clarity and ultimately reduces the resolving power or resolution of a lens. Spherical aberrations are caused by differences in the focal point of light passing through the center of the lens and the edge of the lens.

Field Curvature

Field curvature results in an image that is either in focus in the center of the field of view (pg. **Error! Bookmark not defined.**) but not the edge of the field of view or the image is in focus at the edge of the field of view but not the center of the field of view. This type of aberration is due to the curvature of the lens. For a curved lens, the distance from the sample to the middle of the lens will be less than the distance from the sample to the edge of the lens. Since focus is determined by adjusting the distance from the sample to the lens, if the portion of the sample viewed with the middle of the lens is in focus, the greater distance from the sample to the edge of the lens results in the portions of the sample viewed with the edge of the lens to being out of focus.

Resolution

The ability to perceive adjacent objects or structures as distinct (Figure 29 pg. 42). The resolution of a lens system is related to the ability of an optical system to correct for aberrations and the light gathering ability of an objective lens. The numerical aperture (NA) of a lens is a quantitative measure of light gathering ability of a lens which is in turn related to the working distance (pg. 43) of the objective; the smaller the working distance the higher the numerical aperture, the greater the resolving power of the objective.

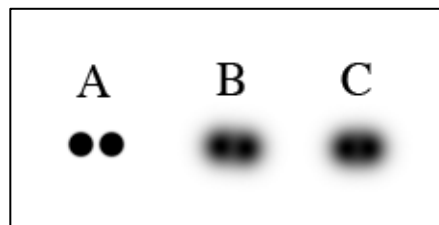


Figure 29 A diagrammatic illustration of the concept of resolution. **A)** A highly resolved image, the two points appear as distinct. **B)** A less resolved image, the two points appear somewhat distinct but the boundaries between the points are blurred making them appear to be part of a single object. **C)** A poorly resolved image, the two points appear to be a single object not two distinct objects.

Useful Magnification

Useful magnification is the upper limit of magnification that provides useful information or resolution of image detail. Beyond this point, additional magnification does not produce more resolution of a sample. For example, if increasing magnification of image C from Figure 29 does not result in improved resolution as in image A, then the limit of useful magnification has been reached. Increasing magnification beyond the resolving power of a lens system actually leads to image degradation.

Working Distance

Working distance is the distance between the object you are viewing and the lens. Working distance is related to magnification; the greater the magnification of a lens the shorter the working distance.

FOR THIS WEEK

1. Hand in Activities 1 – 3.
2. Download class data and graph the relationships between lens size and other lens parameters.
3. Graph the relationships between lens power and other lens parameters (pg. 39).

FOR NEXT WEEK

1. Read the Eukaryotic Microbes - Discovery Labs (pg. 5).
2. Read Lab 2 - Discovering and Classifying Eukaryotic Microbes (pg. 44).
3. Prepare a sampling plan for your assigned habitat (Preparing Your Sampling Plan) (pg. 48) and the Sampling Field Notes you will need to make (pg. 49).
4. Look at the map of your sampling site Figure 30 (pg.47).
5. Next week you will meet your group at the assigned sampling site rain or shine. Check the weather forecast the night before (http://www.weatheroffice.gc.ca/canada_e.html) and dress appropriately for the weather!
 - a. If you are sampling at Wreck Beach, check the tide tables (<http://www.tides.gc.ca/eng>).
6. Read Using a Field Microscope – Basic Setup (pg. 103) and Making a Wet Mount (pg. 127)

LAB 2 - DISCOVERING AND CLASSIFYING EUKARYOTIC MICROBES

PART 1: ENVIRONMENTAL SAMPLING

LEARNING OUTCOMES

By the end of this lab you should be able to:

Identify and apply basic field techniques for the collection and observation of protists by:

- a) Choosing and applying appropriate environmental sampling techniques suitable for a terrestrial or marine environment.
- b) Using standard field equipment for measuring environmental variables (pH, salinity, location etc.) for sampling sites.

Demonstrate observational and note-taking skills by:

- a) Making field notes on sampling techniques, sampling location and specific samples taken etc.
- b) Using a field microscope to observe organisms taken directly from sampling sites.

PRE-LAB PREPARATION

1. **Meet your group at the assigned sampling site rain or shine. Please be on time, collecting will begin at 15 minutes after the lab begins.**
2. Read the Eukaryotic Microbes - Discovery Labs (pg. 5).
3. Read Lab 2 - Discovering and Classifying Eukaryotic Microbes (pg. 44).
4. Prepare a sampling plan for your assigned habitat (Preparing Your Sampling Plan) (pg. 48) and the Sampling Field Notes you will need to make (pg. 49).
5. Look at the map of your sampling site Figure 30 (pg.47).
6. Next week you will meet your group at the assigned sampling site rain or shine. Check the weather forecast the night before (http://www.weatheroffice.gc.ca/canada_e.html) and dress appropriately for the weather!
 - a. If you are sampling at Wreck Beach, check the tide tables (<http://www.tides.gc.ca/eng>).

Lab 3 – Microscopic Observation Isolation and Cultivation

7. Read Using a Field Microscope – Basic Setup (pg. 103) and Making a Wet Mount (pg. 127).

LAB OUTLINE

- 1) Meet at assigned collecting site (Figure 30 pg. 47).
- 2) Collect and label samples. Make field notes on samples.
- 3) Make observations of microbes using the field microscope (Using a Field Microscope – Basic Setup, pg. 103 and Making a Wet Mount, pg. 127).
- 4) Return to lab and place labeled samples in the fridge.

COLLECTING AND GROWING EUKARYOTIC MICROBES

Where do eukaryotic microbes occur? Just about everywhere. They may be free-living, or form symbiotic relationships with other organisms. When thinking about where to collect eukaryotic microbes and how to grow them in the lab, you need to consider two important factors: i) the likely niche occupied by the organism and ii) how organisms obtain organic nutrients (like sugars) and inorganic nutrients (nitrogen, phosphorous, trace minerals) for growth and reproduction.

Free-living eukaryotic microbes are abundant in moist habitats for example, wet soil, mud, ponds, and marine environments. Drying out (desiccation) is a problem for organisms inhabiting terrestrial environments or environments where moisture availability varies. Although moisture availability is very important, other physical, chemical and biotic factors will interact to define the niche occupied by a particular organism. Symbionts occur in association with another organism and the specific niche they occupy can vary from general (e.g. anywhere on the surface of the organism) to very specific (e.g. specific locations on the surface of a host, gut, circulatory system or reproductive organs).

Eukaryotic microbes can be photosynthetic autotrophs; heterotrophs or they may even have a mixture of nutritional modes (mixotrophic) depending on the species or the environment. Photosynthetic organisms require light so will be found in environments that receive at least some sunlight. The light quality and intensity will determine which organisms can live in a particular environment.

Heterotrophic organisms have a variety of ways of obtaining nutrients. They can be predators, saprobes (living on dead organisms or organic material), or symbionts (living in association with other living organisms and acquiring nutrition from that organism). In general predators are larger than their prey, for example, an amoeba feeding on smaller bacteria. Parasitism can be thought of a special case of predation where the predator is smaller than the prey. The symbiotic relationships between eukaryotic microbes and other organisms can range from parasitic (where the other organism is harmed) to commensal (where the other organism is not harmed but does not benefit) to mutualistic where both organisms benefit.

Lab 3 – Microscopic Observation Isolation and Cultivation

In general, organisms that are saprobes or predators are often easier to cultivate in the laboratory than symbiotic organisms. Saprobes may require specific nutrients but are not cultivated in association with another living organism. In the lab, the nutritional requirements for saprobes are provided by the medium in which organisms are grown. Media for cultivating a saprobe can vary from simple (e.g. aqueous extracts from plants, animals or fungi) to complex chemically defined solutions. Not all saprobes can be easily cultivated in the lab; some saprobes require highly specialized conditions that have so far prohibited successful cultivation.

To cultivate predators, conditions must be present to promote the growth of the prey organism(s) or the food organism(s) must be cultivated separately and fed to the predator. For example, *Paramecium* species are often cultivated in a medium such as hay infusion (basically a tea made from grass) that promotes the growth of bacteria that are then eaten by the *Paramecium*.

Predators may be generalists, able to consume a wide variety of prey items, or specialists that rely on one or a few prey species. Cultivation of specialized predators may be restricted by the ability to cultivate the required food source. Predators must also be provided with fresh prey at regular intervals. Mutualists can be difficult or impossible to cultivate because they require a living host and generally can't live separated from their specific host. For example, cultivation of the malaria parasite, *Plasmodium falciparum*, requires growing the asexual state of the parasite in human red blood cells.

There are a wide variety of methods for collecting and isolating eukaryotic microbes. Collection and isolation methods will depend on the type(s) of eukaryotic microbes to be studied and the type of environment to be sampled. For your project, the basic approach will be to collect samples from habitats likely to contain organisms, observe the samples to identify some of the organisms present then attempt to isolate a single organism or enrich the sample so that one organism becomes dominant.

Collection Locations

For the purposes of this lab, the class will be sampling two different ecosystems: 1) marine intertidal and 2) terrestrial. The terrestrial ecosystem can be further divided into habitats such as soil, rotten wood, ponds and streams. The marine intertidal ecosystem can be further divided into habitats such as rock surfaces, sand, open water etc.

The intertidal marine sampling site will be at Wreck Beach (Figure 30, pg. 47). Please note that Tower and Wreck Beaches are clothing optional beaches. Depending on the weather, people may still be sunbathing. If this is potentially offensive, please arrange to sample in the terrestrial group. Please check tide information (<http://www.tides.gc.ca/eng>) prior to the lab since the tide level will can affect your sampling plan

Lab 3 – Microscopic Observation Isolation and Cultivation

The terrestrial sampling site will be in the UBC Botanical Garden and Centre for Plant Research (Figure 30, pg. 47). This area contains a variety of habitats including a number of permanent ponds and streams.

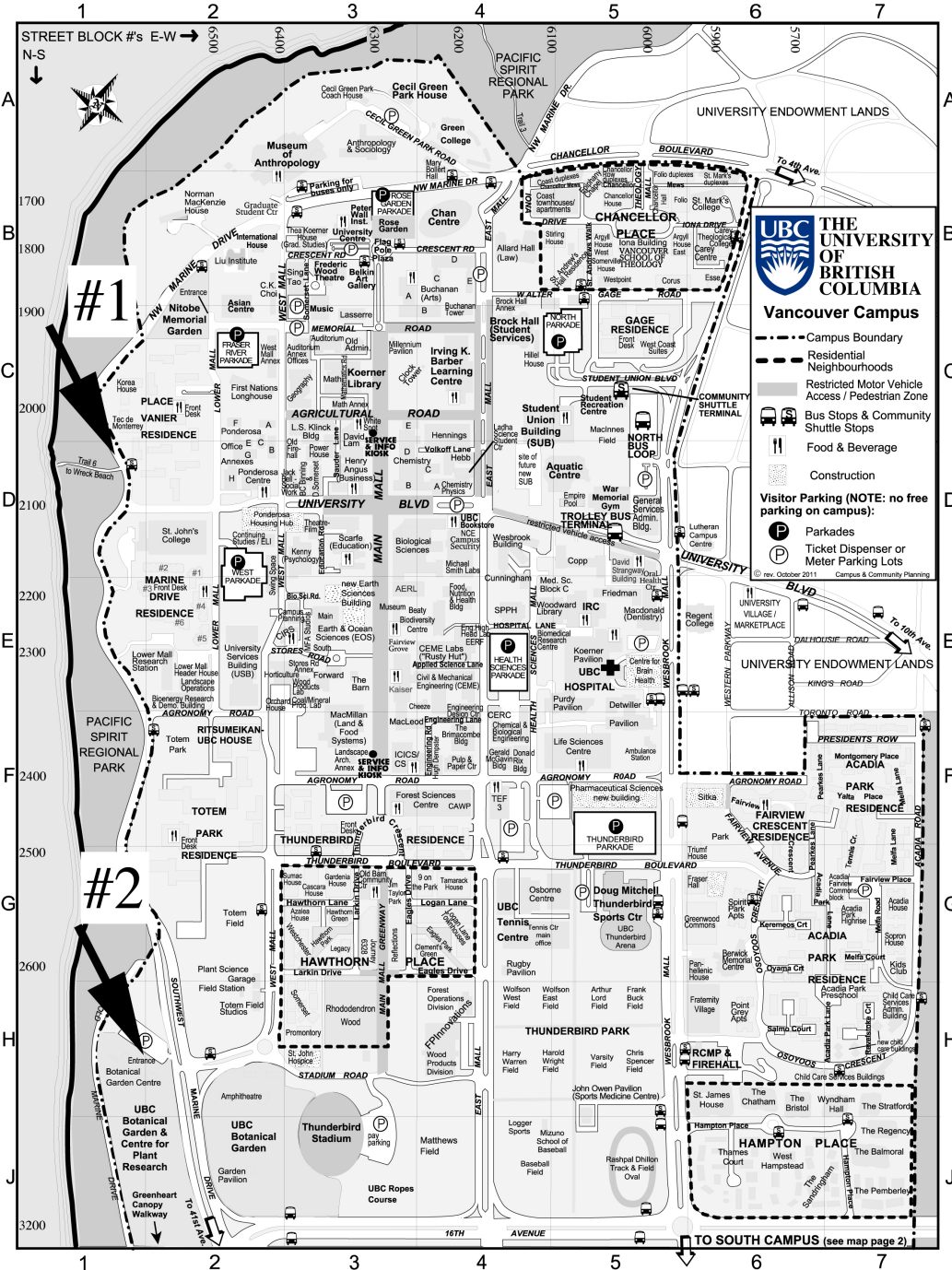


Figure 30. Sampling sites for collection of material for observation and isolation of eukaryotic microbes. Sampling site #1 is the “marine intertidal”. Students will meet with their TA or instructor at the top of the stairs to Wreck Beach head down to the beach as a group. Sampling site #2 is the “terrestrial” habitat. Students will meet with the TA or instructor at the entrance to the Botanical Gardens.

Preparing Your Sampling Plan

The two objectives of your collecting trip are to collect material containing as many different kinds of eukaryotic microbes as possible for use in your Classification Assignment (pg. 6) and Species Description Assignment (pg. 8). Keeping these objectives in mind, you need to come up with a sampling plan to achieve these objectives. Check off the boxes as you go.

- Read the "Collection Methods For Eukaryotic Microbes and Sampling Methodology " (pg. 98).
- Decide on the type of sampling you will employ e.g. point sampling or transect sampling.
- Decide on the approximate amount of material you plan to collect that you will designate a single sample for point or transect sampling. For example, if you plan to collect water, soil or sand, what is the approximate volume you will collect at each point? If you are doing a point sample of plant or animal material, what is a reasonable collection unit? For example, if you choose to sample organisms associated with clamshells, how many shells do you plan to collect at a specific location?
- Decide on how many samples in total you want to collect. If you only collect one small sample of sand do you think is it likely to contain many different species that can be then isolated or enriched in the lab? If you collect 100 samples, will you be able to look at them all over the next few weeks or carry them back to the lab?
- What materials will you need to make your collections? For example, bags, bottles, shovels etc.? The list of available equipment is given in the Sampling and Measurement Equipment section (pg. 101).
- What measurements of the physical and chemical properties of the environment will you make in the field?
- What measuring devices are available to make measurements of the physical or chemical properties of the environment?
- What material(s) (pens, pencils, paper etc.) will you need to make field notes on the environment you are sampling? What will you do if it rains?
- How will you label your samples in the field so that you can relate a specific sample back to your field notes? Unlabeled samples will be thrown out.
- Make up a sampling form to record information on your sampling site, sample numbers etc. See the template form (Figure 31 pg. 50). You will be handing in your completed sampling sheets as part of your final project.

If you think you will need extra materials, please bring them with you!

Sampling Field Notes

Although you are coming to the field site prepared to take samples, you may need to alter your sampling plan in the field depending on the conditions you encounter. You will need to make careful notes on the actual sampling techniques used.

The notes made in the sampling sheet (Figure 31 pg. 50) are fairly complete, some additional information could be included to make the notes more useful for both the collector and anyone who will be using the collections in the future. You never know what observations will be important in the future and you can't rely on your memory to fill in the holes after the fact.

1. The information on the collection site could be more complete. For example, the collecting site could have been listed as "Wreck Beach, Point Gray, British Columbia, Canada". Someone not familiar with Vancouver may have any way of knowing your collections were made at the local "Wreck Beach". They may incorrectly assume your samples came from Wreck Beach in Australia or Ship Wreck Beach in Angola.
2. A map or sketch of the beach could be included with specific sampling sites indicated.
3. The description of the substrate could be more detailed and include observations about how wet or dry the material was when collected.
4. Since the collector could distinguish different types of or organic material in the sample, multiple samples could have been collected representing different alga in the drift.
5. No mention was made of observations using the field microscope nor were sketches or any organisms included.
6. Don't be afraid to add supplemental information to your notes once you return to the lab. For example, you can look up predicted tide heights, weather conditions on the day of sampling (e.g. amount of rain in mm) and weather or environmental conditions in the days prior to sampling.
7. Include pictures of your sampling site! Most cell phones have cameras, use them to supplement your field notes or bring a digital camera with you.

Collection Information

Date Oct 28/11 Collector(s) B. Coucl
Sample Number BC 001

Geographical Location / Description
Wreck Beach high tide mark in alga/
drift zone

Physical / Chemical Properties of Environment
Temp: 8°C Instrument/Method Alcohol thermometer
Salinity: ~~15 ppt~~ Instrument/Method Not measured (ocean 15 ppt)
on site
pH not measured Instrument/Method _____

Description of Sampled Substrate / Medium
algae from the drift were collected -
species included mostly Fucus with some
Ulva and other unidentified material

Collection Method(s)
- Material was collected by hand - enough
to fill a small ziplock bag. -> bag sealed
- point sample

Other Observations (e.g. organisms observed with field microscope)
- some alga quite decayed and stinky/slimy
- some arthropods seen in seaweed and through
living bag. - most Fucus intact but
slimy
-> weather overcast and rainy
-> Time ~ 4:00pm - tide 3.8m (checked on
Fisheries + Oceans Website <http://www.tides.gc.ca>)

Figure 31 An example collection form for sampling of substrate or media containing eukaryotic microbes.

FOR THIS WEEK

4. After returning to the lab, hand in your field notes to your TA for grading and comments. You will be graded on three main criteria:
 - a. Completeness: Is the sampling methodology and information about the sample complete enough that the instructor can understand what was sampled, where samples were obtained and how samples were made.
 - b. Neatness and Clarity: The notes are legible and understandable to the instructor.
 - c. Correspondence With a Sample: The sampling notes should correspond with one or more labeled samples and the sample description should match what is in the actual sample.
5. Place your labeled sample containers in the cold room. Open the lids or seals on your sample containers slightly to allow some air circulation; remember these are mostly aerobic organisms and require oxygen to live.
6. Check again that your samples are properly labeled. The instructor will throw samples that are not labeled in the garbage.

FOR NEXT WEEK

- 1) Watch the how to use a compound microscope videos on the course website.
- 2) Read Lab 3 - Discovering and Classifying Eukaryotic Microbes (pg. 52).
- 3) Read Using Laboratory Microscopes (pg. 105).
- 4) Read Direct Observations Of Eukaryotic Microbes From Field Material (pg. 127).
- 5) Read the Methods for Observation of Protists From Different Habitats appropriate for the samples you collected in the field (beginning on pg. 133).
- 6) Review the Common, Non-protist Stuff You Might See Under the Microscope section (pg. 135).
- 7) Read the Culturing or Enriching Eukaryotic Microbes from Environmental Samples section appropriate for the samples you collected in the field (beginning on pg.141) and decide on two methods you will use to enrich organisms from your environmental samples.
- 8) Review your field notes from Lab 2.

LAB 3 - DISCOVERING AND CLASSIFYING EUKARYOTIC MICROBES

PART 2: MICROSCOPIC OBSERVATION, ISOLATION AND CULTIVATION

LEARNING OUTCOMES:

By the end of this lab you should be able to:

Demonstrate observational and note-taking skills and use lab equipment for making measurements and observations of protists.

- a) You can use a working replica of a van Leeuwenhoek microscope to make observations.
- b) You can use modern compound and dissecting microscopes to make observations of protists and estimate organism size.
- c) You can make wet mount slides and apply simple fixation and staining techniques to environmental samples and lab cultures.
- d) You can effectively summarize microscopic observations in the form of diagrams, tables, charts or written descriptions.

Identify and apply basic lab techniques for the isolation, cultivation and identification of a protist from an environmental sample.

- a) Working in groups (individually for BIOL 448), students can choose and apply an appropriate method or methods for enriching eukaryotic microbes from their environmental sample(s).
-

PRE-LAB PREPARATION

- 1) Watch the how to use a compound microscope videos on the course website.
- 2) Read Lab 3 - Discovering and Classifying Eukaryotic Microbes (pg. 52).
- 3) Read Using Laboratory Microscopes (pg. 105).
- 4) Read Direct Observations Of Eukaryotic Microbes From Field Material (pg. 127).
- 5) Read the Methods for Observation of Protists From Different Habitats appropriate for the samples you collected in the field (beginning on pg. 133).
- 6) Review the Common, Non-protist Stuff You Might See Under the Microscope section (pg. 135).
- 7) Read the Culturing or Enriching Eukaryotic Microbes from Environmental Samples sections appropriate for the samples you collected in the field

Lab 3 – Microscopic Observation Isolation and Cultivation

(beginning on pg.141) and decide on two methods you will use to enrich organisms from your environmental samples.

- 8) Review your field notes from Lab 2.

LAB OUTLINE

- 1) Using Your van Leuwenhoek Microscope, observe field material and make a drawing of one organism.
- 2) Observe Field material and begin making observations for your Classification Assignment (pg. 6). You will need to hand in at least two drawings at the end of lab.
- 3) Begin developing a classification system for the organisms you observe for your Classification Assignment (pg. 6).
- 4) Isolation Of Protists from Field Material. Begin enrichment or isolation procedures for eukaryotic microbes from environmental samples using two of the methods appropriate for you sample (Culturing or Enriching Eukaryotic Microbes from Environmental Samples, pg. 141).

USING YOUR VAN LEUWENHOEK MICROSCOPE

Make observations of material you collected last week using the van Leeuwenhoek microscope you from your field sample. Refer back to Lab 1 - Making a Van Leeuwenhoek-Type Microscope (pg. 18) for instructions on how to mount specimens for observation using the microscope you made (Mounting a Wet Specimen On a Single Coverslip, pg. 29; Mounting a Wet Specimen Between Two Coverslips, pg. 30).

- 1) Draw one organism you observe with your microscope and hand in your drawing to your instructor. Instructions for drawings are given in the "Biological Drawings" section (pg. 54).

OBSERVATIONS OF FIELD MATERIAL

The objective of this activity is for you to explore and document the diversity of eukaryotic microbes present in the environmental samples you collected last week. Each group member will make at least 5 drawings (pg. 54) (10 Drawings for BIOL448 students) of organisms observed in environmental samples collected during Lab 2. As a group (individually for BIOL 448), you will then develop a classification system for all of the organisms observed by the group member and write a short justification for your classification system (Classification Assignment, pg. 6).

Lab 3 – Microscopic Observation Isolation and Cultivation

The section Methods for Observation of Protists From Different Habitats (pg. 133) has some useful tips for making observations of eukaryotic microbes from different substrates or media.

- 1) Make wet mounts of samples of the material you collected (Making a Wet Mount, pg. 127).
- 2) Begin by observing your sample using the stereomicroscope using the methods outlined in the Using Laboratory Microscopes section (pg. 105).
- 3) Follow the steps outlined in the Using the Compound Microscope section (pg. 107) to properly adjust your microscope and observe material in your wet mount. The microscopy videos on the course website are another useful guide for setting up your microscopes.
- 4) Review the sections in Direct Observations Of Eukaryotic Microbes From Field Material (pg. 127) appropriate for the substrate or media you sampled and use this as a guide for preparing samples for observation. You may need to try a few different methods.
- 5) With your group (individually for BIOL 448), draw and make notes on 10-20 different organisms present in your sample. Each group member must hand in a minimum of five drawings (10 for BIOL 448). Please refer to the section Biological Drawings (pg. 54) for instructions and tips on making biological drawings.

ISOLATION OF PROTISTS FROM FIELD MATERIAL

The objective of this section of the lab is to have you attempt to culture or at least concentrate and maintain a protist in the lab for a period of time. The organism you isolate or concentrate in culture will be the basis of your Species Description Assignment (pg.8).

This week you will choose and employ two basic enrichment techniques to promote the growth of organisms from the complex mixture of organisms in your samples. The exact procedure will depend on the medium or substrate you collected. Read the Culturing or Enriching Eukaryotic Microbes from Environmental Samples section (pg. 141) and choose two enrichment procedures you would like to try.

Biological Drawings

Why Do Biologists Draw?

Two of the most important reasons for drawing the organisms you observe under the microscope are: i) to practice observational skills and ii) to create a record of

Lab 3 – Microscopic Observation Isolation and Cultivation

your observations that will be important in preparing for your lab exam (no lab exam for BIOL 448). Drawing requires you to actively observe and summarize your observations. Drawings are also critical for communicating information about an organism's structure since morphological features are used to identify, classify and categorize organisms

I can't draw very well, why can't I just take a picture?

Drawing is an intellectual activity for the observer. Drawing forces you to carefully observe organisms and think carefully about what you see. You need to translate what you see into a reasonable, recognizable representation of the organism being observed. Often drawings summarize knowledge gained from observing multiple samples or views.

In order to accurately represent the three dimensional structure of an organism, you will often need to focus up and down through a sample since the depth of field is limited and adjust lighting. In making a drawing you will be summarizing all of the observations you make. A photograph can be useful but is only a two dimensional representation of the structure. Drawings also focus a reader's attention on specific aspects of an organism's anatomy.

Elements of a Biological Drawing

A biological drawing should be as accurate as possible and accurately show relative proportions, relationships among structures and patterns. A biological drawing is NOT a work of art; the purpose of drawings are: to convey information by summarizing important or notable features of an organism. In order to effectively summarize the features of an organism you need to think ahead: i) observe the organism, ii) decide what are the most important features and iii) think how can you best represent these features in a drawing

You should also include labels of as many structures as possible and notes in your drawing (e.g. how the organism moves).

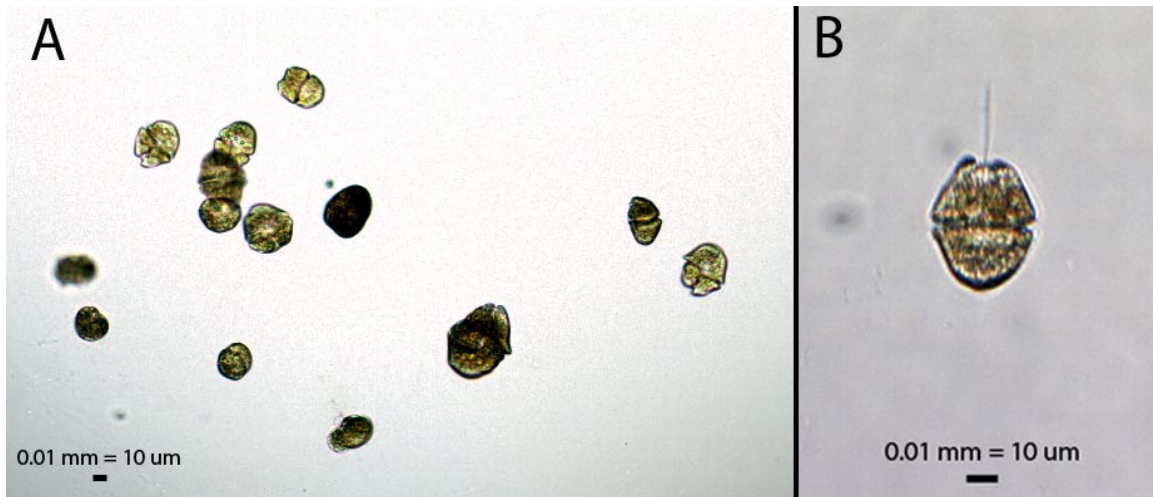


Figure 32 The marine dinoflagellate, *Akashiwo sanguinea* (Alveolata; Dinophyceae) viewed with a light microscope.

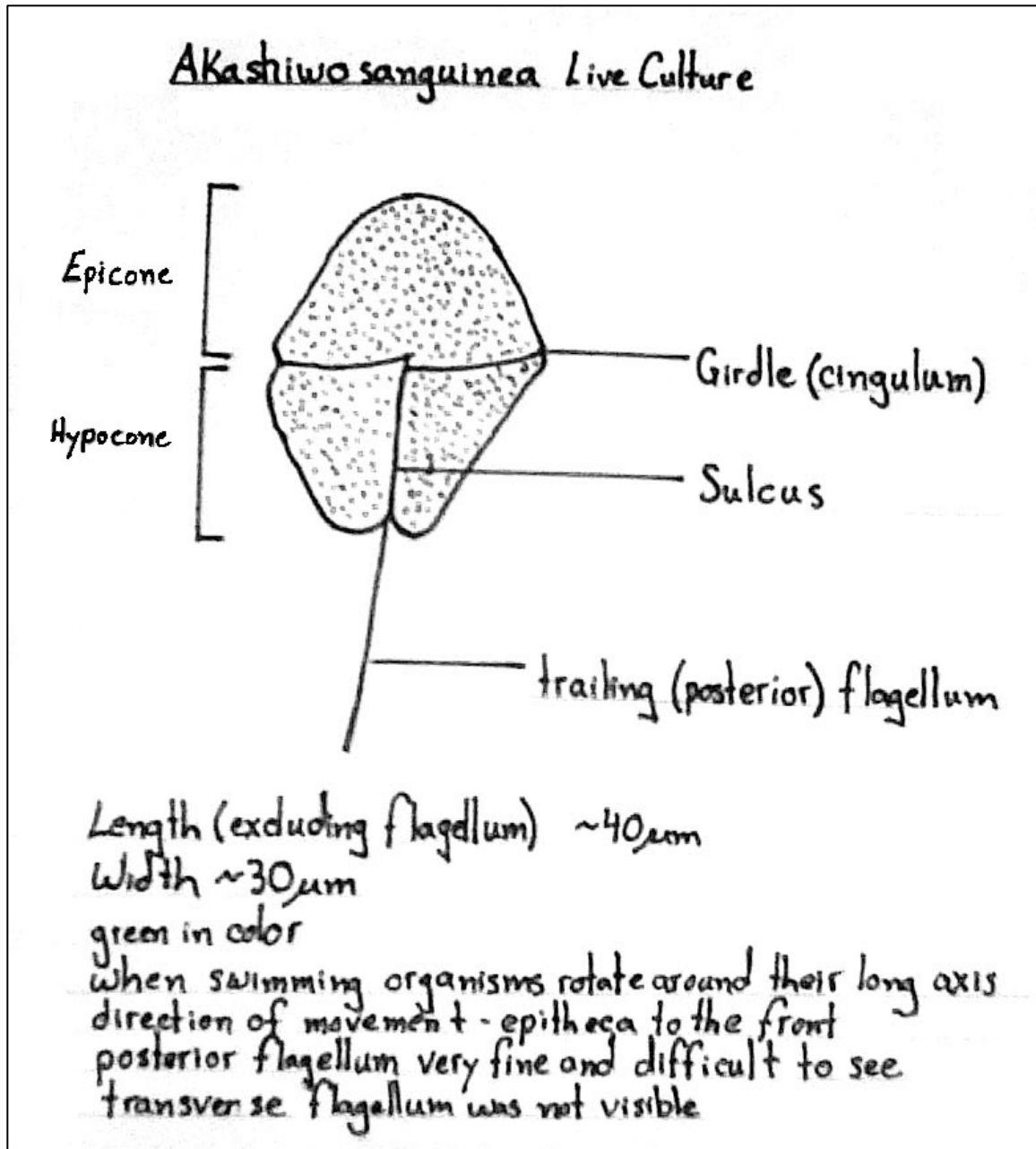


Figure 33 Line drawing of the marine dinoflagellate, *Akashiwo sanguinea* (Alveolata; Dinophyceae) combining observations from both images in Figure 32 (pg. 57).

BIOLOGICAL DRAWINGS GRADING RUBRIC

Grade /10	Characteristics
9-10	<p>Very neat, precise drawing of the organism or biological structure. The organism is clearly recognizable based on the drawing. Relative proportions of organism (length/width) and all structures are correct. All visible features are included with correct proportions and relative positions. All structures neatly labeled where possible. Additional notes are included to supplement drawing</p>
7-8	<p>The organism is clearly recognizable based on the drawing. Relative proportions of organism (length/width) and all structures are correct. Visible features are included with correct proportions and relative positions. All structures labeled where possible. Additional notes are included to supplement drawing</p>
5-6	<p>The organism somewhat recognizable based on the drawing. Some structure or visible features are missing or positions and proportions are not completely correct. Relative proportions of the organism (length/width) relative proportions of organism (length/width) and all structures are not completely correct. Not all structures are labeled where possible. Additional notes are absent or cursory</p>
0-4	<p>The organism is not recognizable based on the drawing. Drawing contains major inaccuracies in visible features, proportions and generally demonstrates lack of attention or a rushed effort. Additional notes are absent, cursory or demonstrate lack of attention or a rushed effort.</p>

FOR THIS WEEK

- 1) Hand in a drawing you made of material observed using your van Leeuwenhoek microscope.
- 2) Hand in two drawings of organisms present in the field material you collected in Lab 2.

FOR NEXT WEEK

- 1) Watch the how to use a compound microscope videos on the course website.
- 2) If you have not already done so, please read the following sections:
 - a. Using Laboratory Microscopes (pg. 105).
 - b. Direct Observations Of Eukaryotic Microbes From Field Material (pg. 127).
 - c. Methods for Observation of Protists From Different Habitats (beginning on pg. 133).
 - d. Common, Non-protist Stuff You Might See Under the Microscope section (pg. 135).
 - e. Culturing or Enriching Eukaryotic Microbes from Environmental Samples (beginning on pg.141).
- 3) Read Lab 4 - Discovering and Classifying Eukaryotic Microbes (pg. 60).
- 4) Read the Sterile Technique & Practices For the Safe Handling of Microorganisms (pg. 139).
- 5) Complete the Sterile Technique and Safe Handling of Microorganisms quiz.
- 6) Read the Bulk Transfer of Enrichment Culture to Fresh Media (pg. 143).
- 7) Read the Direct Isolation and Serial Dilution section (pg. 144).

LAB 4 - DISCOVERING AND CLASSIFYING EUKARYOTIC MICROBES

PART 2 CONTINUED: MICROSCOPIC OBSERVATION, ISOLATION AND CULTIVATION

LEARNING OUTCOMES

By the end of this lab you should be able to:

Demonstrate observational and note-taking skills and can use lab equipment for making measurements and observations of protists.

- a) You can use modern compound and dissecting microscopes to make observations of protists and estimate organism size.
- b) You can make wet mount slides and apply simple fixation and staining techniques to environmental samples and lab cultures.
- c) You can effectively summarize microscopic observations in the form of diagrams, tables, charts or written descriptions.

Identify and apply basic lab techniques for the isolation, cultivation and identification of a protist from an environmental sample.

- a) Demonstrate practices for the safe handling and disposal of microbial cultures.
 - b) Choose and apply an appropriate method for isolating eukaryotic microbes from enrichment culture.
-

PRE-LAB PREPARATION

- 1) Watch the how to use a compound microscope videos on the course website.
- 2) If you have not already done so, please read the following sections:
 - a. Using Laboratory Microscopes (pg. 105).
 - b. Direct Observations Of Eukaryotic Microbes From Field Material (pg. 127).
 - c. Methods for Observation of Protists From Different Habitats (beginning on pg. 133).
 - d. Common, Non-protist Stuff You Might See Under the Microscope section (pg. 135).

Lab 4 – Microscopic Observation, Isolation and Cultivation Continued

- e. Culturing or Enriching Eukaryotic Microbes from Environmental Samples (beginning on pg.141).
- 3) Read Lab 4 - Discovering and Classifying Eukaryotic Microbes (pg. 60).
- 4) Read the Sterile Technique & Practices For the Safe Handling of Microorganisms (pg. 139).
- 5) Read the Bulk Transfer of Enrichment Culture to Fresh Media (pg. 143).
- 6) Read the Direct Isolation and Serial Dilution section (pg. 144).

LAB OUTLINE

- 1) Complete the “Sterile Technique and Safe Handling Of Microorganisms” quiz.
- 2) Continue making observations of eukaryotic microbes from your environmental samples and continue to work on your Classification Assignment (pg. 6).
- 3) Begin the Species Description Assignment (pg. 8) by observing the enrichment cultures you set up last week for the presence of microorganisms.
- 4) Draw 1-2 organisms from your enrichment culture and hand it in at the end of lab for grading. The drawing should include:
 - a. Title, Date, Your Name.
 - b. Sample information (e.g. source of inoculum from enrichment culture).
 - c. Labels and notes.
 - d. Estimate of size.
- 5) Perform a bulk transfer a sample of your enrichment culture to fresh media (Bulk Transfer of Enrichment Culture to Fresh Media, pg. 143).
- 6) Attempt to isolate a single organism from your enrichment culture or environmental sample using either the direct isolation or dilution method and transfer it to fresh media (Direct Isolation and Serial Dilution, pg. 144).

OBSERVATIONS OF FIELD MATERIAL

The objective of this activity is for you to explore and document the diversity of eukaryotic microbes present in the environmental samples you collected in Lab 2. Each group member will make at least 5 drawings (pg. 54) (10 Drawings for BIOL448 students) of organisms observed in environmental samples collected during Lab 2. As a group (individually in BIOL 448), you will then develop a classification system for all of the organisms observed by the group member and write a short justification for your classification system (Classification Assignment, pg. 6).

The section “Methods for Observation of Protists From Different Habitats” (pg. 133) has some useful tips for making observations of eukaryotic microbes from different substrates or media.

Lab 4 – Microscopic Observation, Isolation and Cultivation Continued

- 1) Make wet mounts of samples of the material you collected (Making a Wet Mount, pg. 127).
- 2) Begin by observing your sample using the stereomicroscope using the methods outlined in the Using Laboratory Microscopes section (pg. 105).
- 3) Follow the steps outlined in the Using the Compound Microscope section (pg. 107) to properly adjust your microscope and observe material in your wet mount.
- 4) Review the sections in Direct Observations Of Eukaryotic Microbes From Field Material (pg. 127) appropriate for the substrate or media you sampled and use this as a guide for preparing samples for observation. You may need to try a few different methods.
- 5) With your group, draw and make notes on 10-20 different organisms present in your sample. Each group member must hand in a minimum of five drawings. Please refer to the section Biological Drawings (pg. 54) for instructions and tips on making biological drawings.

OBSERVATION OF ENRICHMENT CULTURES

- 1) Observe the enrichment culture flask and note any changes such as color, turbidity (cloudiness) from last week.
- 2) Take a sample and place in a watch glass or depression slide and observe under the stereomicroscope (Stereo- or Dissecting Microscopes, pg. 105). Are any organisms visible?
- 3) Make wet mounts of samples from your enrichment culture and observe using the compound microscope. Depending on the sample and the organisms present, you may want to try making a wet mount using methylcellulose to slow down organisms or try fixing and staining some samples. The fixatives and stains available for you to use are listed (Table 6, pg.130)
- 4) Draw 1-2 organisms from your enrichment culture viewed under the compound microscope.

WHAT TO DO NEXT WITH ENRICHMENT CULTURES?

- 1) Perform a bulk transfer of your enrichment culture to fresh media. See the Bulk Transfer of Enrichment Culture to Fresh Media section (pg. 143).
- 2) Keep your old enrichment culture as a backup.
- 3) Attempt to isolate a single organism either using a direct isolation method or serial dilution. See the Direct Isolation and Serial Dilution section (pg. 144). You may need to try a couple of isolations to ensure that you have successfully transferred an organism to fresh media.

FOR THIS WEEK

- 1) Hand in one drawing of an organism from the field material you collected.
- 2) Hand in two drawings of organisms present in the enrichment culture you started in Lab 3.

FOR NEXT WEEK

- 1) Read Labs 5&6.
- 2) Read the Sterile Technique & Practices For the Safe Handling of Microorganisms (pg. 139).
- 3) Read the Bulk Transfer of Enrichment Culture to Fresh Media (pg. 143).
- 4) Read the Direct Isolation and Serial Dilution section (pg. 144)
- 5) Watch the how to use a compound microscope videos on the course website.

LABS 5 & 6 - DISCOVERING AND CLASSIFYING EUKARYOTIC MICROBES

PART 3: OBSERVATION AND DESCRIPTION OF A NEW SPECIES

LEARNING OUTCOMES

By the end of this lab you should be able to:

Demonstrate observational and note-taking skills and can use lab equipment for making measurements and observations of protists.

- a) You can **use** modern compound and dissecting microscopes to make observations of protists and estimate organism size.
- b) You can make wet mount slides and apply simple fixation and staining techniques to environmental samples and lab cultures.
- c) You can effectively summarize microscopic observations in the form of drawings, tables, charts, written descriptions and photomicrographs.

Identify and **apply** basic lab techniques for the isolation, cultivation and identification of a protist from an environmental sample.

- a) Choose and apply appropriate methods for observing and identifying eukaryotic microbes from enrichment culture.
 - b) Use literature resources, including keys, field guides and primary literature to identify the family or genus for the species you isolated or enriched in culture
 - c) Write a formal description of the species you isolated or enriched
-

PRE-LAB PREPARATION

- 1) Read Labs 5&6
- 2) Read the "Sterile Technique & Practices For the Safe Handling of Microorganisms" (pg. 139).
- 3) Read the "Bulk Transfer of Enrichment Culture to Fresh Media" (pg. 143).
- 4) Read the "Direct Isolation and Serial Dilution" section (pg. 144)
- 5) Watch the how to use a compound microscope videos on the course website.

OBSERVATION OF ENRICHMENT CULTURES AND ISOLATION ATTEMPTS

- 1) Observe your enrichment culture flasks and isolation attempts
- 2) Note any changes such as color, turbidity (cloudiness).
- 3) Take individual samples from all enrichment and isolation attempts and observe under the stereomicroscope.
- 4) Make notes and sketches of what you see.
- 5) Make wet mounts of samples from your cultures and observe using the compound microscope. Depending on the sample and the organisms present, you may want to try making a wet mount using methylcellulose to slow down organisms or try fixing and staining some samples. The fixatives and stains available for you to use are listed (Table 6 pg. 130)
- 6) Are the organism(s) present in your enrichment culture the same as the ones you observed during Lab 4?
- 7) Prepare drawings and observations for the description of your species that you will present in your Species Description Assignment (pg. 8).
- 8) Each group member must make at least one drawing that will form one figure in the final report. Drawings will receive individual grades.
- 9) Attempt to identify your organism to genus level or higher using the literature resources available in the lab.
- 10) While attempting to identify your organism, make careful notes on additional observations you make and the choices you made while using dichotomous keys.
- 11) When you are completely finished with your materials, place the flasks in trays for autoclaving.
- 12) Attempt additional isolation or enrichments if you choose.

FOR THIS WEEK

- 1) Hand in two drawings of organisms present in the enrichment culture you started in Lab 3.

FOR NEXT WEEK

- 1) Following Lab 6 read Amoebozoa Isolations (pg. 148).

LAB 7 - DISCOVERING AND CLASSIFYING EUKARYOTIC MICROBES

PART 3 CONTINUED: OBSERVATION AND DESCRIPTION OF A NEW SPECIES AND CULTURING OF AMOEBAE

LEARNING OUTCOMES

By the end of this lab you should be able to:

Demonstrate observational and note-taking skills and can use lab equipment for making measurements and observations of protists.

- a) You can **use** modern compound and dissecting microscopes to make observations of protists and estimate organism size.
- b) You can make wet mount slides and apply simple fixation and staining techniques to environmental samples and lab cultures.
- c) You can effectively summarize microscopic observations in the form of drawings, tables, charts, written descriptions and photomicrographs.

Identify and **apply** basic lab techniques for the isolation, cultivation and identification of a protist from an environmental sample.

- a) Choose and apply appropriate methods for observing and identifying eukaryotic microbes from enrichment culture.
 - b) Use literature resources, including keys, field guides and primary literature to identify the family or genus for the species you isolated or enriched in culture
 - c) Write a formal description of the species you isolated or enriched
 - d) Apply a basic protocol for the isolation or enrichment of Amoebozoa from environmental samples.
-

PRE-LAB PREPARATION

- 1) Read the Amoebozoa Isolations section (pg. 148).

LAB OUTLINE

- 1) Continue to work on your Species Description Assignment (pg. 8).
- 2) Choose and execute two methods for isolation of Amoebozoa for next week (pg. 148).

FOR THIS WEEK

- 1) Hand in one drawing of the organism present in the enrichment culture you started in Lab 3.

FOR NEXT WEEK

- 1) Read Eukaryotic Microbes – Selected Lineages of Eukaryotic Microbes (pg. 68).
- 2) Read Lab 8 Unikonts (Amoebozoa) (pg. 69).

EUKARYOTIC MICROBES – SELECTED LINEAGES OF EUKARYOTIC MICROBES

DIVERSITY, LOCOMOTION, NUTRITION AND STRUCTURE

The second part of the course will be devoted to exploring some of the characteristics of the main lineages of eukaryotic microbes (Figure 34 pg. 73). These labs are organized according to phylogenetic relationships. As you will see, one of the major themes in the evolution of eukaryotic microbes is convergent evolution. Unrelated groups of organisms often have characteristics in common that evolved or were acquired independently and were not inherited from a common ancestor. For example, ameboid bodies and plastids. In addition to observing the structure of organisms you will relate structure to function by exploring how these organisms move, and acquire nutrients both through direct observation and experimentation.

A major skill you will continue to develop and practice through the next series of labs is three-dimensional (3D) thinking. Many eukaryotic microbes (especially members of the Ciliophora like *Paramecium*, *Stentor* and *Vorticella*) are structurally complex with a single cell often having multiple, highly differentiated, localized structures that perform a specific function such as attachment, locomotion or food acquisition. As a result, many single cells behave very much like multicellular organisms.

To understand the complex structure of these organisms, you will need to practice translating what you see under the microscope to a three dimensional picture of the organism's structure. This is a skill that requires considerable practice since you are only able to view a thin section of a large organism at a time due to the limited depth of field of microscope lenses (Figure 68 pg.120). The depth of field is often much less than the size of the organism. For even moderately large cells, it is generally not possible to have all parts of the cell in focus at the same time. This means that to view the entire cell, you will need to focus up and down through the cell and translate all of these views into a 3D composite image comprising all of the parts of the cell.

LAB 8 UNIKONTS (AMOEBOZOA)

LEARNING OUTCOMES

By the end of this lab you should be able to:

- 1) Recognize and apply morphological traits (where possible) to the identification, categorization and classification of members of the Amoebozoa.
- 2) Where characteristic traits are present, distinguish members of the Amoebozoa from other groups containing amoeboid organisms.
- 3) Observe and describe characteristics used to classify members of the Amoebozoa based on exemplars observed in the lab.
- 4) Observe, describe and discover how members of the Amoebozoa move, reproduce and obtain food and interact with other organisms.

Demonstrate observational and note-taking skills and can use lab equipment for making measurements and observations of protists.

- 1) Students can effectively summarize microscopic observations in the form of diagrams, tables, charts or written descriptions.
- 2) Make a wet mount slides of protists from environmental samples and lab cultures
- 3) Properly draw and label diagrams of protists.

PRE-LAB PREPARATION

- 1) Read Lab 8 Unikonts (Amoebozoa) (pg.69).
- 2) Review videos of amoeba morphology and types of locomotion on the course website.
- 3) Review the isolation or enrichment procedures you employed in Lab 7.

LAB OUTLINE

- 1) Observing (pg. 75).
- 2) Observing Demonstration Material (pg. 78).
- 3) Culturing of Amoeba (pg. 80).

INTRODUCTION

When thinking about eukaryotic microbes, one of the first kinds organisms many students think of are amoebae. For most biology students, amoebae are one of the first eukaryotic microbes they viewed under the microscope. These organisms can be large (e.g. *Chaos* sp. and *Amoeba* sp.), are easily observed with a basic compound microscope and are simply fascinating to watch crawling along the surface of a microscope slide. Amoeboid (amoeba-like) organisms are abundant in terrestrial, fresh water and marine environments. These organisms are important components of the detritus food web as predators and saprobes.

Generally, the term amoeba refers to single celled microorganisms that moves and engulfs food particles or prey by the formation of pseudopods (finger like projections of protoplasm). Amoeboid organisms actually occur within a number of lineages within the tree of life and do not form a monophyletic group (Figure 34, pg. 73). Therefore, the term amoeba does not refer to a taxonomic group but groups of distantly related organisms with a common morphology (Figure 34, pg. 73).

The presence of common morphological characteristics in unrelated organisms is a recurring theme within eukaryotic microorganisms. The occurrence of common features in unrelated organisms is due to convergent evolution. Convergent evolution is where organisms share morphological, biochemical or other characteristics that evolved independently rather than being inherited from a common ancestor. A classic example of convergent evolution is the evolution of flight independently in insects, reptiles and repeated evolution of flight or gliding in mammals (bats and flying squirrels). These organisms did not inherit the ability to fly from a single common ancestor, rather flight evolved multiple times in different groups. Examination of the mode of flight and wing morphology reveals different mechanisms of flight and different wing structure which points to multiple evolutionary origins rather than a single origin.

Convergent evolution has made classifying eukaryotic microbes difficult since unrelated organisms often appear very similar. Such similarities have resulted in distantly related organisms being grouped together. A major goal of taxonomy is to develop classification systems that reflect evolutionary relationships. Classification systems can be thought of as testable hypotheses for the relationship among organisms. For example, by placing organisms in a single genus the implicit hypothesis is that all of the organisms share a common ancestor and the genus should contain all of the descendants of the common ancestor. New data on biochemistry, DNA sequence, ultrastructure etc. can be used to test existing evolutionary hypotheses (classification systems) and can result in the development of new hypotheses that are then reflected in new classification systems.

The problems that convergent evolution has created for taxonomists trying to identify groups of related organisms is reflected in the changes in classification of amoeboid organisms. Amoeboid organisms characterized by the presence of pseudopodia were previously classified in the taxon Sarcodina (reviewed by (Pawlowski & Burki, 2009). Phylogenetic analysis using DNA sequences have resolved that the majority of these organisms actually belong to two separate lineages, Amoebozoa and Rhizaria (reviewed by (Pawlowski & Burki, 2009); (Keeling et al., 2005). The placement

of Amoebozoa, Rhizaria and other groups containing amoeboid organisms are shown in Figure 34 (pg. 73).

The Amoebozoa contain 3,000 + species and Rhizaria contain 11,500+ species (Adl et al., 2005). Four groups, formerly placed within the Sarcodina, are now placed within taxa other than Amoebozoa and Rhizaria (Figure 34, pg. 73). Of the two orders of Heliozoa that fall outside of the Amoebozoa and Rhizaria groups, one is placed within the Stramenopiles and the other sister to Haptophytes or Rhodophyta (Figure 34, pg. 73). Members of the class Heterolobosea are placed within the discicristates and the genus *Nuclearia* is placed sister to the fungi reviewed by (Pawlowski & Burki, 2009); (Keeling et al., 2005).

WHAT ARE THE AMOEBOZOA?

AMOEBOZOA Lühe, 1913, emend. Cavalier-Smith, 1998

Amoeboid locomotion generally with non-eruptive morphologically variable pseudopodia (lobopodia); sub-pseudopodia common in some groups; cells "naked" or testate; tubular cristae, often branched (ramicristate), secondarily lost in some; usually uninucleate, rarely binucleate, sometimes multinucleate; cysts common, morphologically variable; cell inclusions (parasomes and trichocysts) of diagnostic value in some; flagellate stages if present, rarely bikont, usually with one kinetid bearing a single flagellum.

(Adl et al., 2005)

The preceding quote from (Adl et al., 2005) lists the characteristics of the Amoebozoa as currently defined. The Amoebozoa are a group of organisms that share a common ancestor (they are monophyletic) (Figure 34, pg. 73; Figure 35, pg. 73). Phylogenies constructed based on DNA sequence data are further supported by morphological, biochemical and ultrastructural features. The description above consists of a set of characteristics that define the Amoebozoa although individual characteristics or even combinations of some characteristics may be present in other groups of organisms.

Before beginning your observations of amoebae, it is important to have an idea of the distinguishing characteristics of the major groups of amoeboid organisms since they often occur in the same habitats. The main morphological features visible under the light microscope that distinguish members of the Amoebozoa from other groups containing amoeboid organisms are i) the morphology of the pseudopods and ii) mode of locomotion.

Members of the Amoebozoa have lobopodia, pseudopodia that forms a broad and rounded to cylindrical lobe extending from the cell (Figure 36 pg. 74). Movement of amoeba is non-eruptive as in the Heterolobosea (see videos on the course website for examples of eruptive movement).

Lab 8 – Amoebozoa

Generally members of the Rhizaria have very fine pseudopods called filopodia (filose = terminating in a threadlike process) Figure 43 (pg. 87). The filopodia can be simple, branched, anastomosing or supported by ridged bundles of microtubules (Adl *et al.* 2005). Filopodia supported by microtubules are termed axiopodia. The Heliozoa are a polyphyletic group with some members grouped within the Rhizaria while others fall outside of the Amoebozoa and Rhizaria. These organisms have stiff axopodia that radiate from a round body giving the organisms a starburst or sun-like appearance from which the group derives its name (helio = sun; zoa = animals). Members of the Heterolobosea have an amoeboid morphology with eruptive pseudopodia and some have a flagellate forms. They also characterized by features of mitosis and ultrastructural characteristics (Adl *et al.*, 2005).

Within the Amoebozoa there is considerable morphological and phylogenetic diversity. The relationships among the major groups within Amoebozoa are given in Figure 35. The Amoebozoa contain “naked” amoeba, amoeba lacking shells, and amoeba with shells termed tests. Amoeba with shells are termed testate amoeba however, amoeba with shells also occur within the Rhizaria. The presence or absence of a test and the test morphology are important characteristics used to classify members of the Amoebozoa. As well, the mode of locomotion, type of pseudopodia and number of pseudopodia produced by the organisms are used in classification.

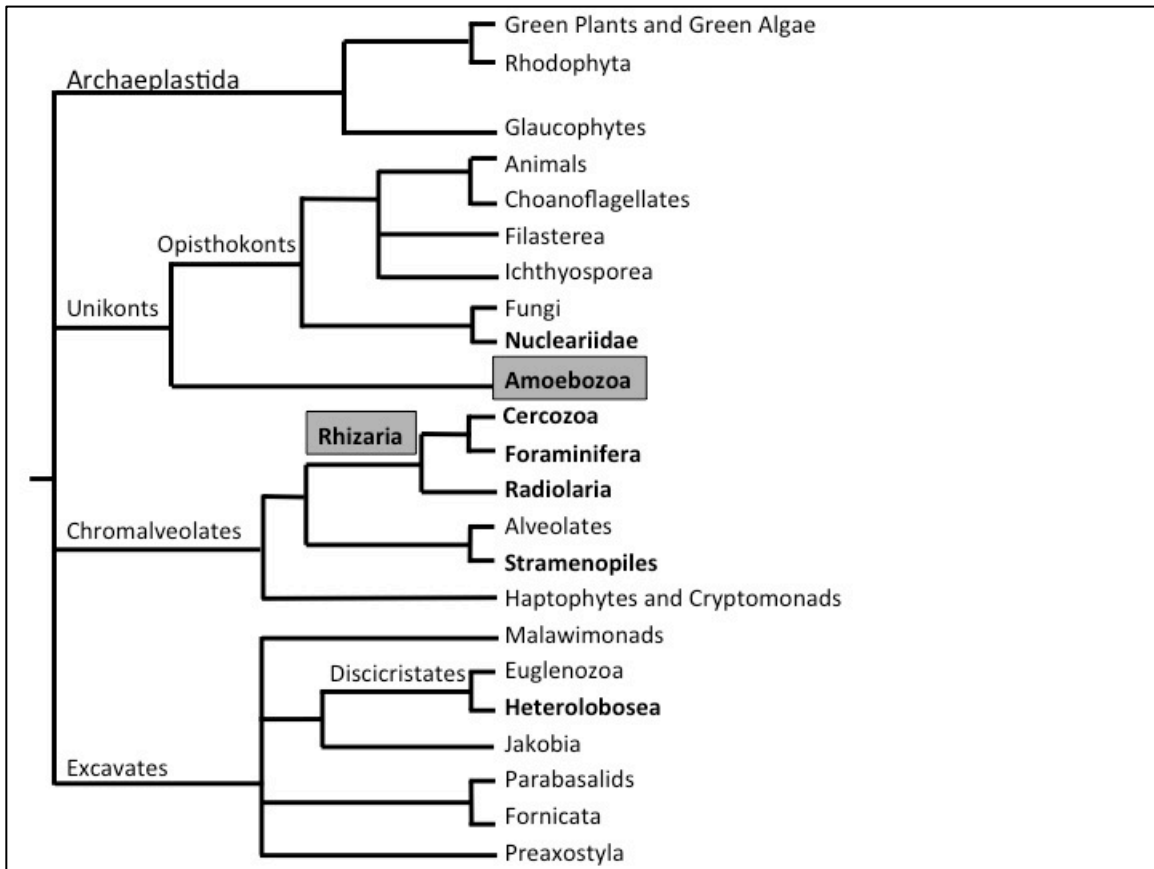


Figure 34 Phylogeny of the eukaryotes modified from Keeling, Leander, Simpson (Tree of Life Web Project; <http://tolweb.org/Eukaryotes>). The two main lineages, Amoebozoa and Rhizaria containing amoeboid organisms are indicated with gray boxes. Additional groups of known phylogenetic placement that contain amoeboid members are indicated in bold type.

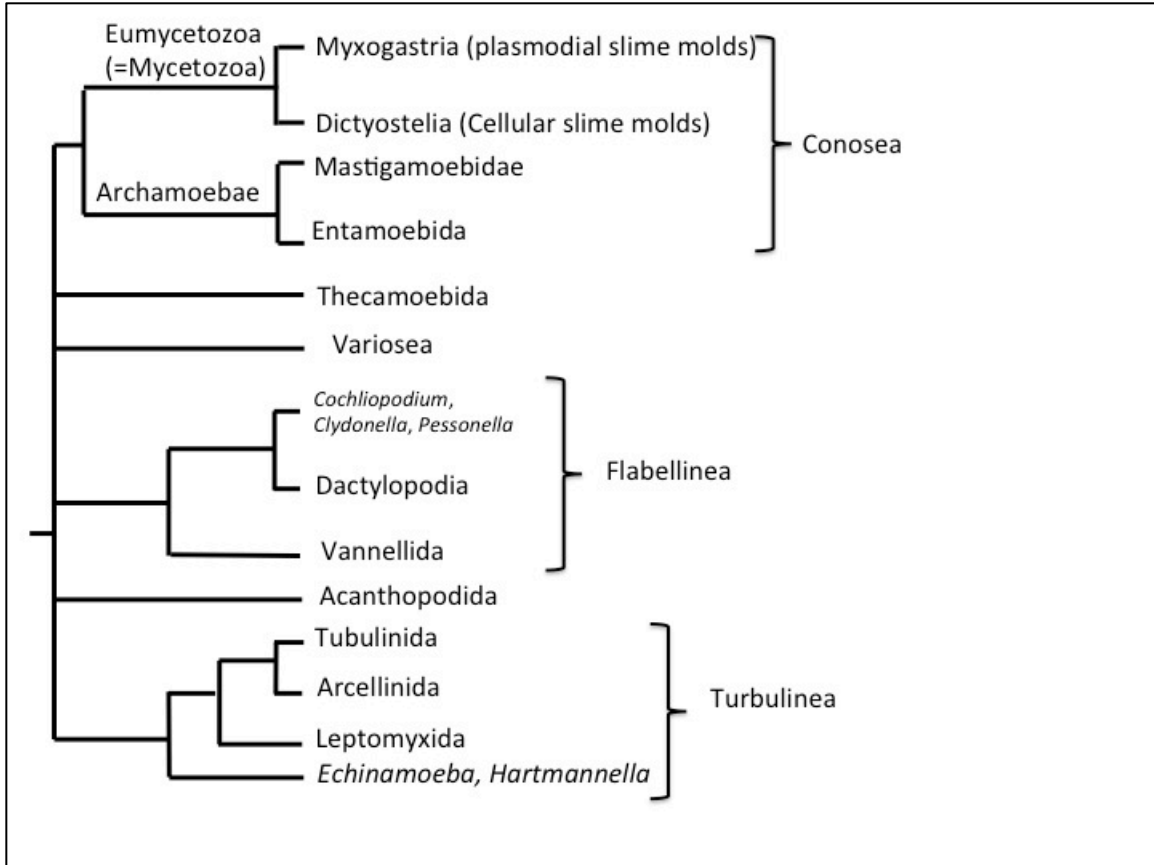


Figure 35 Amoebozoa phylogeny modified from Pawlowski and Burki (2009).

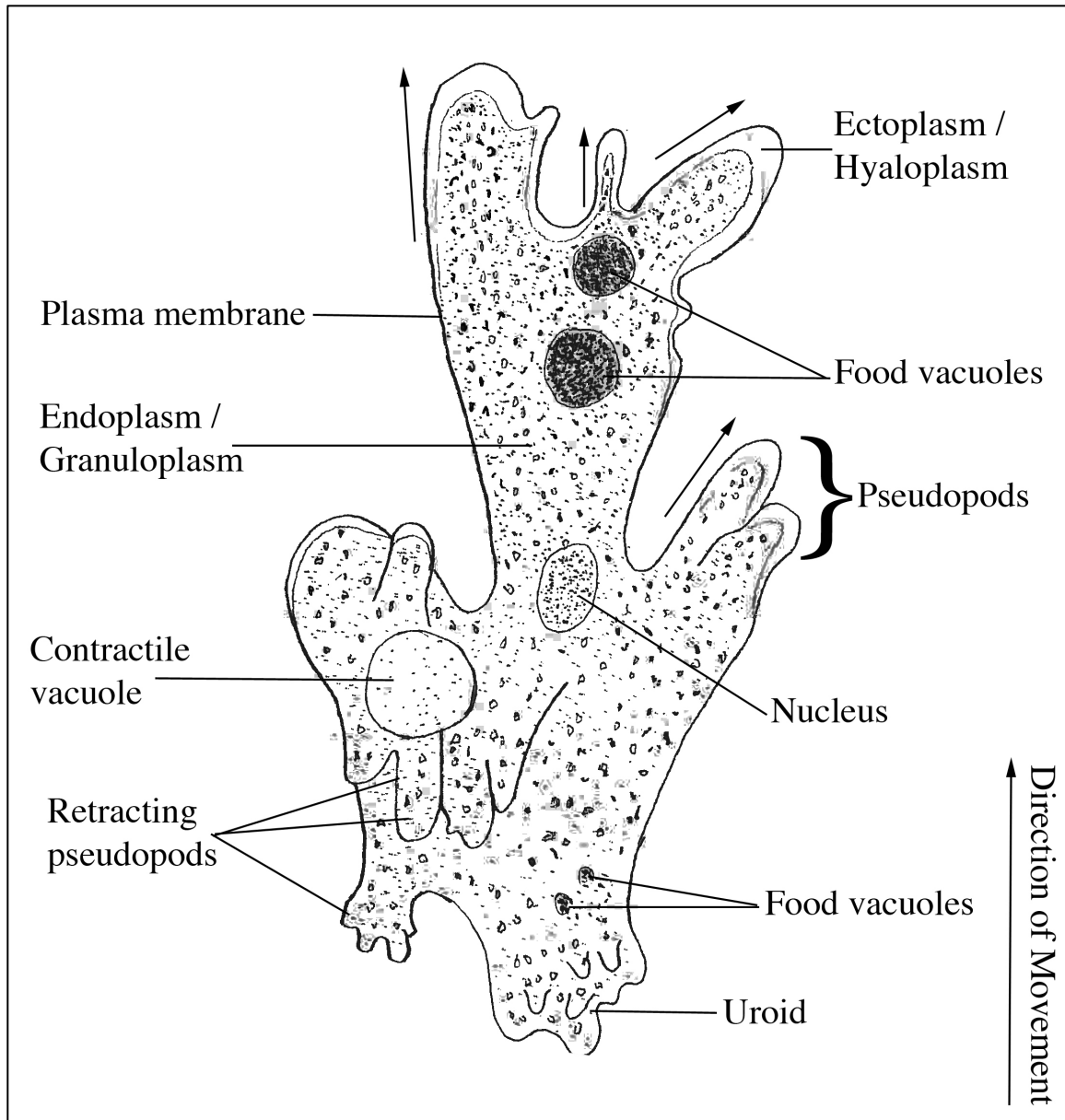


Figure 36 *Amoeba proteus* (redrawn from Radek *et al.* 2009) showing the basic features of a naked amoeba.

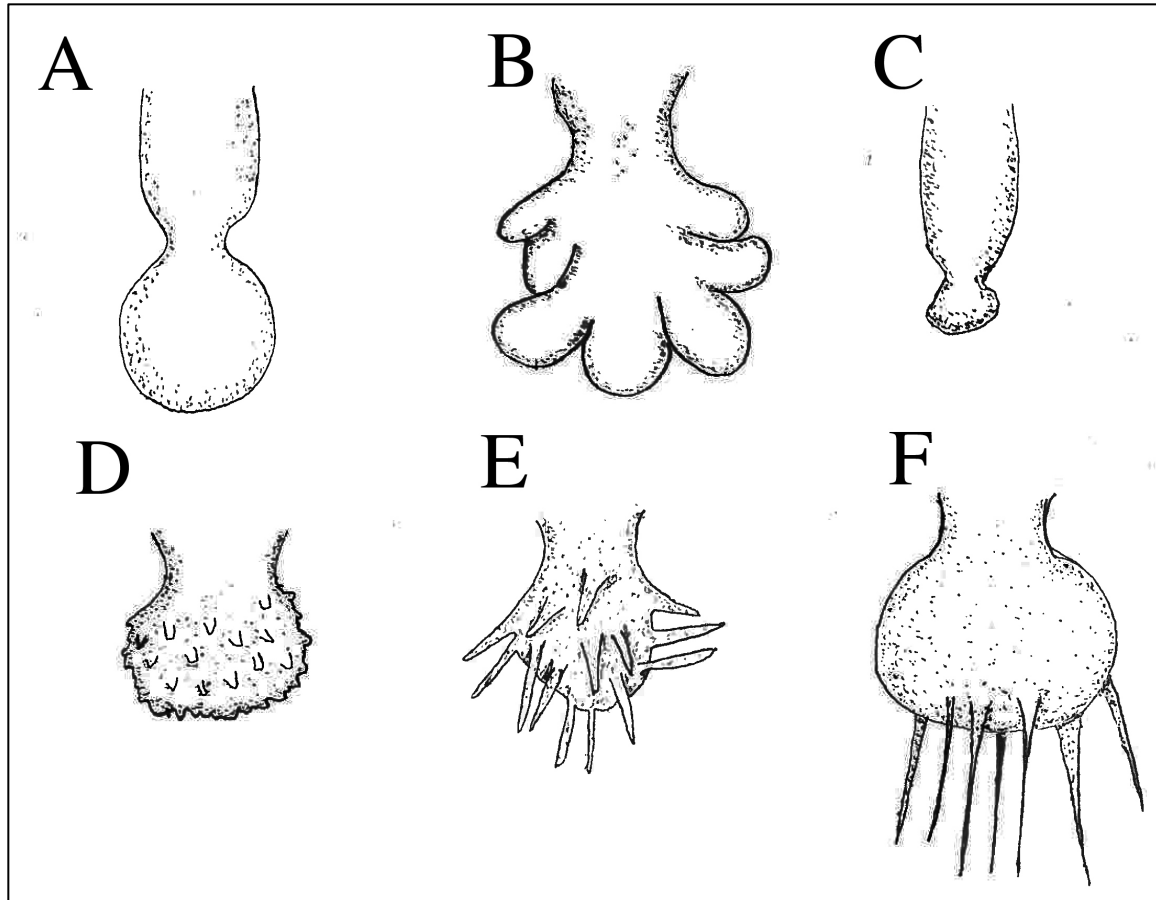


Figure 37 Uroid types (redrawn from Rogerson and Patterson 2000): A) bulbous, B) morulate (Latin *morum* = mulberry), C) globular, D) papillate (having papillae), E) villous F) knob-like with trailing filaments.

OBSERVING AMOEBEA

Plasmodial Slime Mold Fruiting Bodies [Myxogastria]

Plasmodial slime molds generally produce macroscopic fruiting bodies bearing haploid spores produced by meiotic divisions. Spores are produced in dry masses and are dispersed by wind.

- 1) Observe the various fruiting bodies on display.
- 2) Draw one of the fruiting bodies. Indicate the size of the fruiting body in the notes to your drawing.

***Fuligo* Swarm Cells Demonstration**

Here you will be observing a reproductive stage of a plasmodial slime mold. Haploid spores released from the fruiting body germinate to produce either amoeba or amoeba-flagellates. Amoebae are produced under dry conditions and flagellate cells are produced under wet conditions. Cells can switch between flagellate and amoeboid forms (Koevenig, 1964). Flagellate cells and amoeba act as gametes, fusing to form a diploid cell. Nuclear divisions without cytokinesis produce a multicellular plasmodium.

- 1) Take a small sample of liquid from flooded agar plate containing spores of *Fuligo* and make a wet mount (pg. 127).
- 2) Observe using the compound microscope and look for motile cells. Once you find a cell, increase the magnification to 40x and observe using phase contrast (pg. 121).
- 3) Draw the cells you observe and include notes on how the cells move.

Observation of Slime Mold Isolations

***Fuligo* Mating and Plasmodium Formation [Amoebozoa; Mycetozoa; Myxogastria]**

- 1) Observe the agar plates containing oat flakes with dissecting microscope scope and look for evidence of plasmodia. The plasmodia of *Fuligo* and *Physarum* are bright yellow and net-like.
- 2) Look for cytoplasmic streaming in the thickened cytoplasmic strands.
- 3) If visible, draw the plasmodium.

***Physarum* Plasmodium Demonstration**

- 1) Observe demonstration material of plasmodium of *Physarum* or *Fuligo*.
- 2) Using a dissecting microscope, look for cytoplasmic streaming in the thickened cytoplasmic strands.
- 3) Identify areas where the plasmodium is actively expanding over the substrate. A thin fan of cytoplasm is in close contact with the agar surface and is covered by a thin, gelatinous sheath.
- 4) Look for areas where the cytoplasm has retracted leaving behind a gelatinous or slimy layer on the surface of the agar.
- 5) Draw the plasmodium.

Induction of Fruiting Body Formation (Plasmodial or Cellular Slime Molds)

- 1) Observe material under the dissecting microscope and look for evidence of fruiting bodies.
- 2) Gently remove a fruiting body from the substrate with a dissecting needle and Make a wet mount.

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- 3) Observe using a compound microscope. Note that fungi in the Zygomycota produce fruiting bodies (sporangia) that are superficially similar to the fruiting bodies of slime molds. Zygomycetes produce filaments with or without regular cross walls and are generally ruderal organisms. If cross walls present, the cells are generally much longer than they are wide. You should compare your fruiting body to the drawings of zygomycete sporangia, slime mold sorocarps and slime mold sporangia.
- 4) Draw the structures you observe.

Isolation of Cellular Slime Molds From Soil Based on Raper (1951)

- 1) Observe the surface of the petri plate and look for fruiting bodies sticking up from the surface of the agar plate. Fruiting bodies will be tall (several millimeters), with a thin stalk with a bulbous top. Fruiting bodies are often clustered in a single region of the plate.
- 2) Gently remove a fruiting body from the substrate with a dissecting needle and Make a wet mount.
- 3) Observe using a compound microscope. Note that fungi in the Zygomycota produce fruiting bodies (sporangia) that are superficially similar to the fruiting bodies of slime molds. Zygomycetes produce filaments with or without regular cross walls. If cross walls present, the cells are generally much longer than they are wide. You should compare your fruiting body to the drawings of zygomycete sporangia, slime mold sorocarps and slime mold sporangia.
- 4) Draw the structures you observe.

Spot Plate Method For Isolation of Amoeba

This is a common method for enrichment and isolation of amoebae from soil and water samples. This method tends to select for smaller amoeba. The amoeba feed on the bacteria that are present in the sample, divide rapidly and begin to migrate out from the spot onto the surface of the agar where they can be easily viewed and isolated (Tips and Tricks for Viewing Amoeba Using a Compound Microscope, pg. 82).

- 1) Observe the area around the soil slurry or water spots with a dissecting microscope or invert the plate on the stage of your compound microscope (see Figure 38 pg. 82).
- 2) Amoebae appear as clear specks on the surface of the agar and will be clustered around the edge of the spots. Amoeba should be very abundant and clearly visible on the surface of the plate (Figure 39 pg. 83 and **Figure 40** pg. 83).
- 3) If your plate contains amoeba, suspend some of the cells in amoeba saline by pipetting a few drops onto the surface of the agar in an area with abundant amoebae.
- 4) Once the amoebae are suspended, draw some of the saline back into the pipette and make a wet mount of the cells.
- 5) For larger amoeba, it is advisable to support the cover slip with bits of broken cover glass, Vaseline or Plasticine (For Larger Organisms pg. 128) so as not to

squash the organisms you are trying to observe and so the organisms are free to move normally.

- 6) After making the wet mount, you will need to wait a few minutes for the cells to settle down and become active again. It may be worth making a couple of slides at a time so cells on the other slides have a chance to settle while you are observing another slide.
- 7) Observe the cells using both bright field and phase contrast. Amoebae are nearly clear and there is little contrast between the cell and the background. It will be easiest to find cells using phase contrast or bright field with the iris diaphragm closed down to increase contrast between the cells and the background.
- 8) Draw the cells and label all of the structures you observe (e.g. uroid, pseudopods, vacuoles, nuclei, granules etc.). Include notes on how the cells move.
- 9) Using the keys available in the lab, try to identify the amoeba you observed. List the characteristics you used in your identification.

OBSERVING DEMONSTRATION MATERIAL

Giant Amoebae (*Pleomyxa paulstris* or *Proteus-Chaos* group)

- 1) Obtain a petri dish containing a water sample that has been allowed to settle or starved amoeba from an enrichment culture. Handle the dish carefully so as not to disturb the sediment on the bottom.
- 2) Observe using a dissecting microscope and look for large amoeba on the bottom of the sample dish.
- 3) Carefully transfer the amoebae from the Petri dish to a slide that has Plasticine, or cover slip fragments to support the coverslip.
- 4) Place coverslip over the sample supported by the Plasticine or cover slip fragments.
- 5) Observe with a compound microscope. Since the organisms are large, you should begin with the low power objective. Once you find the organism, magnification can be adjusted as necessary.
- 6) Draw the cells and label all of the structures you observe (e.g. uroid, pseudopods, vacuoles, nuclei, granules etc.). Include notes on how the cells move.
- 7) Incubate amoeba in a water droplet with carmine red stained yeast cells on a slide and observe after ~1/2 hour. If the amoeba have ingested yeast cells the food vacuoles will appear red.

Thecamoeba

- 1) Obtain a petri dish containing an enrichment cultures supplemented with filamentous cyanobacteria to select for *Thecamoeba* species.
- 2) Make a wet mount and search for amoeba using a compound microscope. Sometimes searching using phase contrast (pg. 121) allows amoeba to be readily distinguished from the background.
- 3) Draw the cells and label all of the structures you observe (Figure 36 pg. 74; Figure 37 pg. 75). Include notes on how the cells move.

Smaller, *Limax*-Type Amoebae

Cultures to favor *limax*-type amoeba were generated by over-feeding cultures to favor the formation of a microbial scum on the surface (Hülsmann, 2009). The surface scum contains bacteria, nanoflagellates, ciliates and *limax*-type amoebae (Hülsmann (2009). Note that *limax*-type amoeba can be members of the Amoebozoa (Hartmannellidae; *Hartmannella*, *Glaeseria*, *Saccamoeba* and *Cashia*) or members of the Heterolobosea (Vahlkampfiidae; *Vahlkampfia*, *Naegleria*) (Figure 34).

The vegetative cells of Hartmannellidae and Vahlkampfiidae are differentiated mainly on the basis of type of movement. Members of the Vahlkampfiidae move in eruptive bursts (eruptive movement) while members of the Hartmannellidae move by a steady flow of cytoplasm (see videos on the course website for examples of types of amoeboid movement). Members of the genus *Naegleria* can switch between an amoeba-flagellate form and an amoeboid form lacking a flagellum (this morphological transition will be explored in a later lab).

- 1) Obtain an enrichment cultures set up to favor small amoeba that feed on bacteria. The amoeba should be abundant in the scum on the surface of the culture.
- 2) To make a wet mount of the scum for observation of amoebae, grasp a clean coverslip with a pair of forceps and touch one side of the coverslip against the scum. Gently lift the coverslip off the scum layer; some of the scum should stick to the surface of the coverslip.
- 3) Place the coverslip directly on a slide. Add a small drop of amoeba saline if necessary.

Observation of Testate (Shelled) Amoeba from a *Sphagnum* Bog

Testate amoebae are very abundant in soils, mosses and sphagnum bogs. Since tests are more resistant to degradation or weathering than their inhabitants, empty tests are also abundant in many environments. You likely saw testate amoeba or empty tests in your environmental samples, even if you did not recognize it as part of a living organism. Tests of *Euglypha* and *Trinema* [Rhizaria] are abundant in soils.

The testate amoebae are not monophyletic and belong to both the Amoebozoa and Rhizaria; within the Rhizaria tests have evolved multiple times (Pawlowski & Burki, 2009)(Pawlowski and Burki 2009). Testate amoebae in the Amoebozoa have lobopodia while members of the Rhizaria have filopodia. In *Sphagnum* bogs, the genera *Nebela* and *Hyalosphenia* (Amoebozoa) are the most common testate amoeba.

Tests come in a variety of shapes, sizes and are made from a variety of materials. As you might expect, structural characteristics of tests and the living organisms housed within the tests are used to classify these organisms. Except in a few cases, using test characters alone, even experts can't determine if a species belongs to the Rhizaria or Amoebozoa.

Extraction from *Sphagnum* (Meisterfeld, 2009)

- 1) Make wet mounts of sediment samples from *Sphagnum* washings and look for tests.
- 2) Find as many types of tests as possible.
- 3) Diagram the tests paying attention to:
 - a) the material(s) used to make the test
 - b) gross morphology of the test
 - c) fine structure or ornamentation of the test
 - d) location(s) of any opening(s) in the test

CULTURING OF AMOEBEA

Microorganisms grown in culture utilize resources from the culture environment and secrete waste products and other metabolites. Over time the available resources will become exhausted, and waste products or toxins will accumulate to levels that inhibit growth. Cultures will stop growing and eventually die. Storing cultures at lower temperatures often reduces the growth rate, extends the length of time before resources become exhausted and toxins become inhibitory. In order to maintain live cultures they must be periodically transferred to fresh media. Establishing a new culture by transferring a cell or cells to fresh media is termed subculturing. In this section you will be employing sterile technique to subculture an amoeba. The amoeba are maintained in mixed culture with the bacteria *Klebsiella pneumoniae*.

Subculturing Amoeba

Materials:

- Amoebae culture grown on streaks of *Klebsiella pneumoniae* on a ¼ strength hay infusion agar

Lab 8 – Amoebozoa

- Fresh *K. pneumoniae* "X" plate on ¼ hay infusion agar
- Alcohol lamp and matches
- Jar of 95% ethanol
- Paper towel
- Spray bottle 70% ethanol or 10% bleach
- Scalpel

Protocol for transferring an amoeba culture between agar plates:

- 1) Read the entire protocol before beginning!
- 2) Sterilize the surface of the bench by spraying with 70% ethanol or 10% bleach from a spray bottle and wipe with a paper towel. Note: 70% ethanol is flammable, light your Bunsen burner after the alcohol has evaporated from the bench surface.
- 3) Label the bottom of your (lids can be removed and switched by accident) Petri dish with the date, your name and the organism name using a permanent marker.
- 4) Organize your work area and petri dishes in a logical workflow (e.g. if you are right handed, you should place the destination dish to your left since you will be handling the metal loop with your right hand). Place your Bunsen burner in an accessible location where it will not easily be knocked over.
- 5) Locate active amoeba on your plate using a dissecting or compound microscope; mark the bottom of the Petri dish with a marker if necessary.
- 6) Light your Bunsen burner.
- 7) Dip your scalpel in 95% ethanol and pass through the flame of the alcohol lamp.
- 8) Allow alcohol on scalpel to burn off.
- 9) Allow scalpel to cool (10 s or so).
- 10) Lift lid of the source plate and carefully cut a square of agar ~ 5mm² (1/2 cm²).
- 11) Close the lid of the source dish.
- 12) Lift the square of agar out of the source plate and place at the apex of bacterial streaks on a fresh "X" plate.
- 13) Dip the scalpel in 95% alcohol and flame sterilize.
- 14) Seal plate with parafilm and place in the tray for cutlruers.

Don't panic if you accidentally light your jar of ethanol on fire. Cover the jar with a lid to smother the flame.

TIPS AND TRICKS FOR VIEWING AMOEBA USING A COMPOUND MICROSCOPE

Observing Organisms In Situ On The Surface of Agar Plates

- 1) Loosen the screws holding the mechanical stage in place and remove the mechanical stage (Figure 38).
- 2) Move the condenser front lens out of the light path.
- 3) Using the nosepiece, swing the 3.6 x objective into position.
- 4) Lower the stage to permit a petri dish to be placed on the stage.
- 5) Place a petri dish on the stage with the lid on and the agar side up.
- 6) Visually, identify an object (e.g. agar block, edge of a bacterial lawn) in a location on the plate that you want to observe and focus on it using the coarse focus knob. This ensures that you are focused through the agar onto the agar surface where organisms may be present (Figure 39).
- 7) Using the nosepiece, swing the 10X objective into position and adjust your focus.
- 8) Adjusting the light intensity and iris diaphragm can help somewhat in improving the quality of your image.



Figure 38 Observing organisms in situ on the surface of an agar plate using a compound microscope.

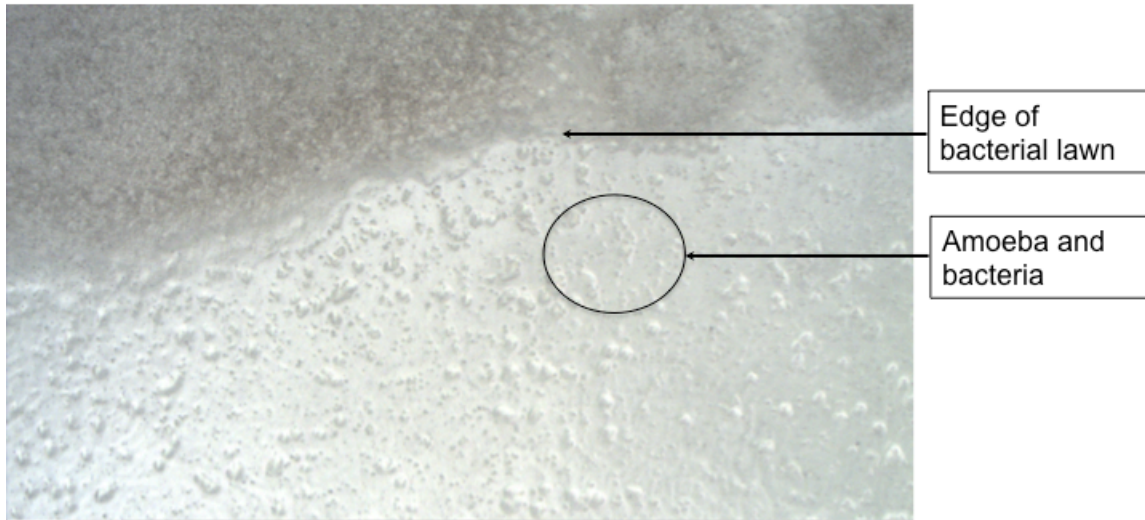


Figure 39 *In situ* observation of amoeba growing on a bacterial streak plate ("X" plate) with a 4X objective. The objective is focused on the edge of the bacterial streak as a reference point to ensure that the surface of the agar is in focus.

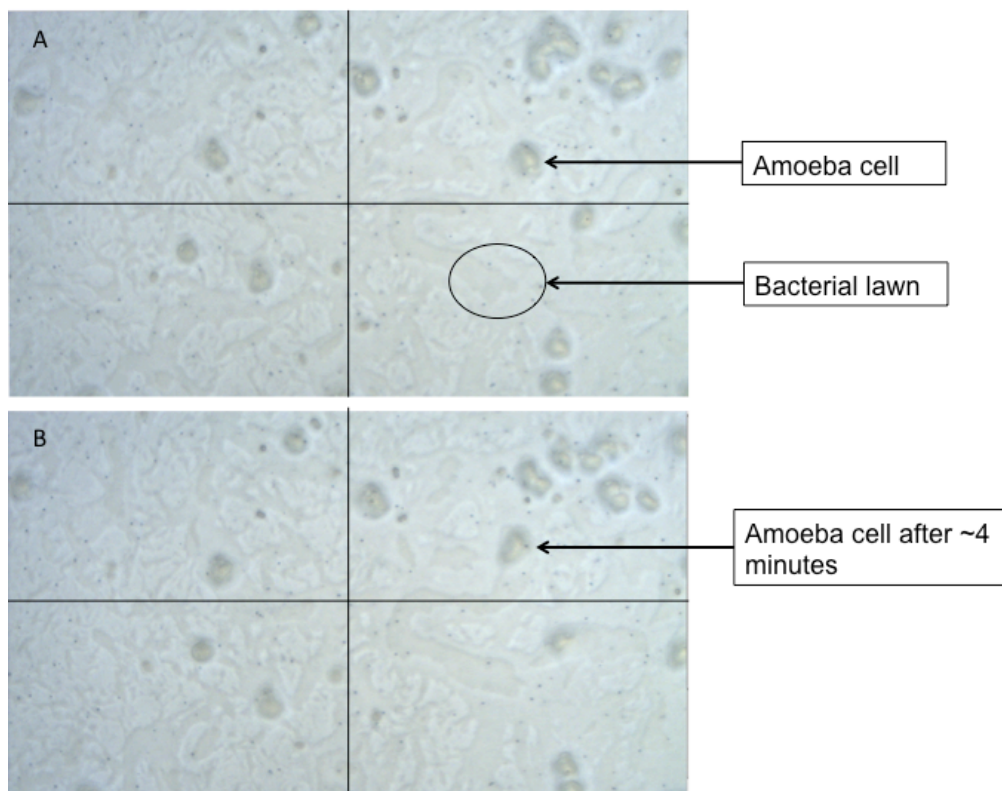


Figure 40 *In situ* observation of amoeba growing on a bacterial streak plate ("X" plate) with a 10X objective. Black lines have been overlaid on the image to provide visual reference points. Image "A" was the first image taken. Image "B" is the same field of

Lab 8 – Amoebozoa

view taken after 4 minutes. The amoeba have moved and changed shape over the period of four minutes.

Finding Cells

Finding amoebae can be challenging since the cells are clear and under high illumination there may be little contrast between the cell and the background. Recall from Lab 3 that contrast can be increased in two ways: 1) closing the iris diaphragm and 2) using phase contrast (Figure 41). The effects of closing the iris diaphragm or using phase contrast on increasing the contrast between an amoeba cell and the background are illustrated in Figure 41.

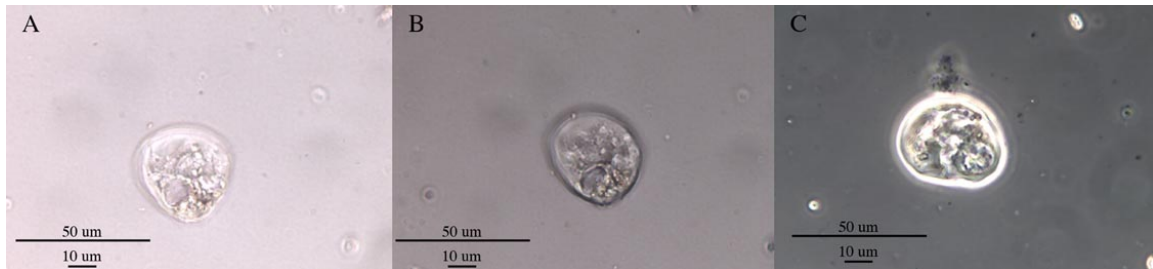


Figure 41 An amoeba from pond water viewed using a 40x objective showing the effects of microscope adjustments on the contrast between an amoeba cell and the background. A) Bright field illumination with high illumination and the iris diaphragm open. B) Bright field illumination with high illumination and the iris diaphragm closed. C) Phase contrast.

Stimulating Movement

For amoebae that use pseudopods for locomotion, observation of pseudopod morphology requires that the organism be moving while you are observing it. The level of activity of a given cell can vary. In the process of making a wet mount, many cells respond to the disturbance by retracting pseudopods and rounding-up. In order for you to view pseudopod structure, cells need to revert to their motile forms. Some species quickly revert to motile forms after a few minutes and some may not become motile. Therefore, all you may need to do is wait a minute or two before observing a wet mount to give the cells time to settle to the surface of the slide and become active. Starving cells for 24h prior to observation then providing them with prey immediately before making observations can also induce motility. Another approach to inducing motility is to take advantage of negative phototaxis in many species (Hülsmann, 2009). Partially illuminating the field-of-view as you would while setting up Köhler illumination will often cause amoeba to begin moving towards the dark periphery (Figure 42).

Procedure

- 1) Find an amoeba and bring into sharp focus with the objective of your choice.

Lab 8 – Amoebozoa

- 2) Centre amoeba in the field of view and close the closing the illuminator diaphragm to illuminate the cell while creating a dark periphery around the cell (Figure 42).
- 3) Wait until the amoeba begins to move and open the illuminator diaphragm to its normal position and observe the moving cell.

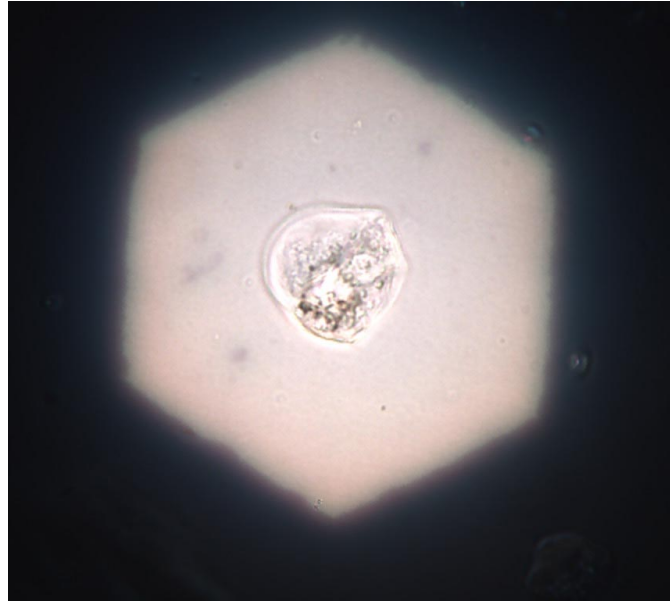


Figure 42 An amoeba centered in an illuminated area of the slide created by closing the illuminator diaphragm and focusing the condenser lens so the diaphragm is in focus (as for setting up Köhler illumination; Lab 3). Many amoebae are negatively phototactic (they move away from light) and will begin to move towards the darker periphery.

AMOEBIA TERMINOLOGY

Eruptive movement: This type of movement generally occurs in small monopodial, limax-type amoeba. The cytoplasmic movement occurs in bursts followed by breaks, when no activity occurs.

Granuloplasm: The interior, granular part of the cytoplasm of amoeba cells containing organelles.

Hyaloplasm: The hyaline (clear or glass-like) region at the periphery of some amoeba cells. This region appears clear when viewed under the microscope.

Limax-type: Slug-like, monopodial amoeba.

Monopodial: Amoeba that move as a single mass without multiple pseudopodia.

Plasmodium: A large mass of protoplasm containing many nuclei.

Polypoidal: Amoeba that move by producing multiple pseudopods.

Pseudopodia (pseudopod): (False-foot) a cytoplasmic protrusion that can be retracted or change shape and occur in protozoan cells lacking a rigid covering. Pseudopodia (or pseudopods) are used for feeding and locomotion. There are four types of pseudopodia.

Axiopodium: A straight, thin pseudopodium that extends from the surface of the body and possessing a rod formed of microtubules. Multiple pseudopods of this type extend radially from the cell and are characteristic of heliozoa and radiolarians. These pseudopods are used primarily for feeding by passive predation.

Filopodium: A long, slender, simple pseudopodium that is generally unbranched and does not form anastomoses. It is used for both food capture and locomotion.

Lobopodium: A pseudopodium that forms a lobe extending from the cell. The pseudopodium varies from broad and rounded to cylindrical. This type of pseudopodium often has a hyaline (clear; glassy; translucent) cap at the anterior edge. It is used for both locomotion and feeding

Reticulopodium: A fine, branched and anastomosing pseudoplasmodium.

Sorocarp: The term for the stalked, spore bearing structure produced by cellular slime molds such as *Dictyostelium* species (Amoebozoa; Mycetozoa; Dictyosteliida).

Sporangium (-a): The term for a stalked spore producing structure that is applied to morphologically similar but analogous structures from diverse organisms. For example, the term sporangium is used for spore producing structures from the Zygomycota (Fungi) and plasmodial slime molds (Amoebozoa; Mycetozoa; Myxogastria).

Test: A shell found in some amoeba. The shells can be formed from: particulate material from the environment (e.g. sand grains), secreted organic materials, silica or calcium carbonate.

Uroid: The posterior part of a moving “naked”, lobose amoeba (Figure 37 pg. 75). It often appears to have fine, fingerlike projections sticking to the substrate. It can be used to determine the anterior/posterior orientation of the organism. Often pinocytosis of vesicles can be observed in this area that could represent expulsion of water or waste.



Figure 43 Filopodia of a testate amoeba (Rhizaria)

FOR THIS WEEK

- 1) Hand in your drawings of amoeba, slime mold fruiting bodies, cellular slime molds ect. The specific drawings may vary depending on the quality and organisms present in the demonstration material.

FOR NEXT WEEK

- 1) Read Lab 9 Archaeplastida (Plantae) and handouts.

LAB 9 ARCHAEPLASTIDA (PLANTAE)

Learning Outcomes

By the end of this lab you should be able to:

- 1) Recognize and apply morphological traits to the identification, categorization and classification of members of the Archaeplastida.
- 2) Use published phylogenetic, ultrastructural and biochemical data to develop and evaluate hypotheses for the origin(s) and evolution of plastids in some major groups of photosynthetic eukaryotes.
- 3) Use data collected in lab to evaluate hypotheses for the origin(s) of blade-like photosynthetic organs.

ARCHAEPLASTIDA Adl et al., 2005

Photosynthetic plastid with chlorophyll a from an ancestral primary endosymbiosis with a cyanobacterium; plastid secondarily lost or reduced in some; usually with cellulose cell wall; flat cristae; starch storage product.

(Adl et al., 2005)

Lab Outline

- 1) Origin and evolution of plastids (handouts)
- 2) Evolution of blade-like photosynthetic organs.

EVOLUTION OF PLASTIDS

Photosynthetic organisms are scattered throughout the tree of life in both the Eubacteria and Eukaryota. Four lineages within the Eubacteria contain photosynthetic organisms (Heliobacteriaceae, green non-sulfur bacteria, Cyanobacteria, green sulfur bacteria and purple bacteria). Four out of the five major lineages within the eukaryotic tree of life contain organisms with plastids, organelles where photosynthesis and other biochemical pathways take place (Figure 44, pg. 93). The Archaeplastida (aka. Plantae) are only one of the lineages containing photosynthetic eukaryotes. As you will begin to discover through the course of this lab and in lecture, the Archaeplastida are central to the story of the evolution of photosynthesis in eukaryotes. In most lineages, plastids function in photosynthesis but can have other functions including storage of carbohydrates and pigments or provide essential biosynthetic functions (Waller & McFadden, 2005).

As the description from (Adl et al., 2005) suggests, biochemical characters such as the type of photosynthetic pigment or pigments (chlorophyll *a*) and cell wall components (cellulose), and the origin of the plastid are important characters for defining the members of the Archaeplastida and distinguishing them from other photosynthetic eukaryotes.

Four classes of pigments are involved in photosynthesis: bacteriochlorophylls, chlorophylls (Table 2, pg. 91), carotenoids and phycobilins (Table 3, pg. 92). The bacteriochlorophylls and chlorophylls are the primary photosynthetic pigments; carotenoids and phycobilins are termed accessory pigments. Within each pigment class there are numerous chemical variants on a common structural theme that alter their light absorbing properties. For example, there are over 50 different chemical variants of chlorophyll (Amerongen, Valkūnas, & Grondelle, 2000). The most common types are chlorophyll *a*, chlorophyll *b* and chlorophyll *c*. Chlorophylls and bacteriochlorophylls function both in the absorption of light energy and in photosynthetic reaction centers. All photosynthetic organisms possess carotenoids that function both in the absorption of light energy and in photoprotection; protecting the photosynthetic apparatus from the damaging effects of excess light energy (Cogdell, 2000; Siefermann-Harms, 1987). The energy absorbed by carotenoids is transferred to chlorophylls or bacteriophylls. Carotenoids are further subdivided into two classes: carotenes and xanthophylls. Xanthophylls are structurally similar to carotenes but possess an oxygen-containing functional group. Phycobilins function in absorption of light energy and transfer of energy to chlorophyll molecules in photosynthetic reaction centers (Biggins & Bruce, 1989).

Among photosynthetic organisms, the primary photosynthetic pigments and accessory pigments differ and are used as characters to group photosynthetic organisms. Members of the Eubacteria, except cyanobacteria, possess bacteriophylls. Cyanobacteria use chlorophyll *a* and phycobilins. Photosynthetic eukaryotes use either a single type of chlorophyll or combinations of chlorophylls. Some photosynthetic eukaryotes also use phycobilins.

The distribution of pigments among photosynthetic organisms has also been used along with ultrastructural, biochemical and genetic data to understand the origin and evolution of photosynthesis in Eubacteria and Eukaryota. Photosynthesis is hypothesized to have evolved first in bacteria. Based on the ability of chloroplasts to replicate within plant cells, their similarity in size to free living bacteria and the similarity of plastid division to bacterial cell division in 1905 the Russian botanist C. Mereschkowsky hypothesized that plastids were once free living cyanobacteria (reviewed by Kutschera and Niklas 2005). Empirical support for this hypothesis has accumulated over the last century; the origin of plastids from a free-living bacterium is now known as the endosymbiotic theory. The last century has also seen further refinement of Mereschkowsky's original hypothesis to account for the origin of plastids in all eukaryotic lineages (Keeling 2004).

Lab 9 – Archaeplastida

In this part of the lab you will be extracting pigments from photosynthetic organisms (one organism per group) and using the data you generate to classify the organisms and address questions regarding the evolution of plastids in eukaryotes.

The questions you will be addressing in this part of the lab are:

- 1) Did plastids in eukaryotes originate from cyanobacteria or another photosynthetic member of the Eubacteria?
- 2) Did plastids in eukaryotes originate from a single endosymbiotic event or multiple events?

Evolution of Leaf-Like Photosynthetic Organs

A major evolutionary development in the Archaeplastida is the organization of cells to produce more complex multicellular structures. These multicellular structures can vary in complexity from small colonies composed of a few cells to very complex organisms with highly differentiated tissue and organ systems. Blade-like structures (e.g. leaves) are an example of such a multicellular structure. Leaves or leaf-like photosynthetic organs are widely distributed within the Archaeplastida and are the major photosynthetic organs in many organisms. Blade-like structures occur in the red alga, green alga and green plants and (mosses, liverworts, gymnosperms [e.g. pines] and angiosperms [flowering plants]). Leaf-like structures also occur in the brown alga. Superficially, blades appear very similar but vary in how they develop and the number of different cell types comprising the blade.

In this section of the lab, you will be examining blade-like structures from the red alga, green algae, mosses, liverworts and flowering plants. The questions you will address in this part of the lab are:

- 1) What type(s) of cell division or organization are needed to form a blade?
- 2) Did blade-like structures evolve once in the Archaeplastida or multiple times? (i.e. did the multiple blade-like structures arise through convergent evolution)

Lab 9 – Archaeplastida

Pigment Class	Pigment	Color	Water Soluble	Major Absorption Peaks in nm (Solvent)*	Reference
Chlorophylls					
	chlorophyll <i>a</i>	bluish green	no	430, 662 (D) 430, 662 (A)	(Scheer, 2006)
	chlorophyll <i>b</i>	yellow green	no	454, 644 (D) 457, 646 (A)	(Scheer, 2006)
	chlorophyll <i>c</i>	light green	no	446, 579, 628 (D) 446, 578, 629 (AP)	(Scheer, 2006)
Bacteriochlorophyll	BChl <i>a</i>			357, 391, 573, 772 (D) 365, 608, 772 (M)	(Scheer, 2006)
	BChl <i>b</i>			368, 408, 578, 794 (D) 368, 407, 582, 795 (A)	(Scheer, 2006)

* The type of solvent can have a dramatic impact on the absorption spectrum of photosynthetic pigments from plants. Solvents: A = acetone, AP = acetone/1% pyridine, D= diethyl ether, E = ethanol, H=hexane, M = methanol, and PB = phosphate buffer

Table 2 The characteristics of some common photosynthetic pigments

Lab 9 – Archaplastida

Pigment Class	Pigment	Color	Water Soluble	Major Absorption Peaks in nm (Solvent)*	Reference
Phycobilins					
	allophycocyanin	Blue-green	yes	650 (PB)	Hattori and Fujita (1959)
	phycocyanin	blue	yes	615 (PB)	Hattori and Fujita (1959)
	phycoerythrin	red	yes	565 (PB)	Hattori and Fujita (1959)
Carotenoids					
Carotenes	α -carotene	orange	no	422,446,474 (H)	Zscheile <i>et al.</i> (1942)
	β -carotene			450, 478 (H)	Zscheile <i>et al.</i> (1942)
Xanthophylls		yellow, orange	no		
	Fucoxanthin	orange		453 (E)	Strain et al (1944)
	Peridinin	Red-orange		475 (E)	

* The type of solvent can have a dramatic impact on the absorption spectrum of photosynthetic pigments from plants. Solvents: A = acetone, AP = acetone/1% pyridine, D= diethyl ether, E = ethanol, H=hexane, M = methanol, and PB = phosphate buffer

Table 3 The characteristics of some common accessory pigments.

Lab 9 – Archaeplastida

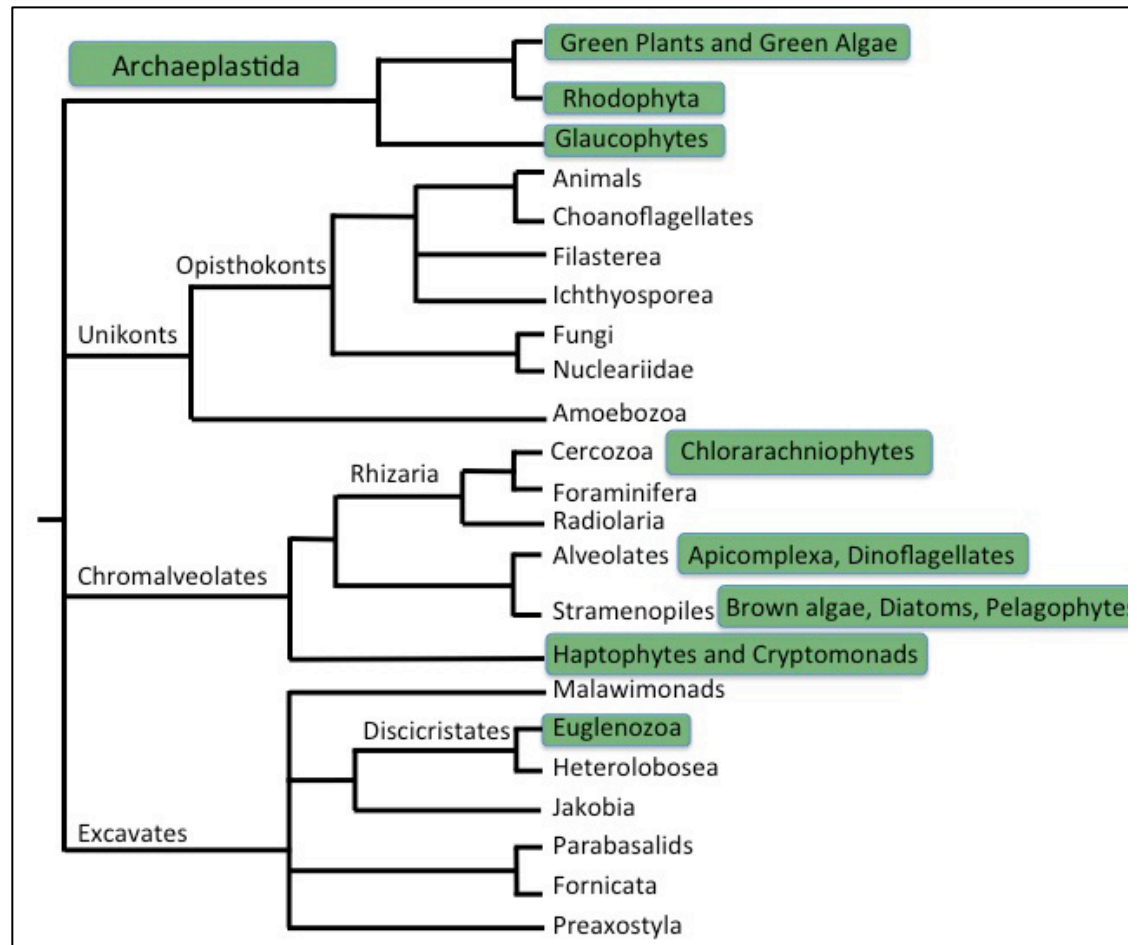


Figure 44 Phylogeny of the eukaryotes modified from Keeling (2009); Keeling, Leander, Simpson (Tree of Life Web Project; <http://tolweb.org/Eukaryotes>) showing the relationship between the Archaeplastida (Plantae) and other lineages of photosynthetic eukaryotes. Groups containing photosynthetic organisms are indicated with green boxes.

Lab 9 – Archaeplastida

Superkingdom	Supergroup	Phylum/Class	Pigments
Bacteria			
		Chloroflexi (Green nonsulfur)	Bact. chl <i>a</i> and <i>c</i> , carotenoids
		Proteobacteria (Purple bacteria)	Bact. chl <i>a</i> carotenoids
		Cyanobacteria	Chlorophyll <i>a</i> Carotenoids Phycobilins
Eukaryota	Archaeplastida	Glaucophyta	Chlorophyll <i>a</i> Phycobilins (water soluble) Carotenoids
		Rhodophyta (red alga)	Chlorophyll <i>a</i> phycobilins (water soluble) Carotenoids
		Chlorophyta	Chlorophyll <i>a</i> Chlorophyll <i>b</i> Carotenoids
		Streptophyta	Chlorophyll <i>a</i> Chlorophyll <i>b</i> Carotenoids
	Chromalveolata	Phaeophyceae	Chlorophyll <i>a</i> Chlorophyll <i>c</i> Carotenoids (fucoxanthin)
	Chromalveolata	Bacillariophyta	Chlorophyll <i>a</i> Chlorophyll <i>c</i> Carotenoids (fucoxanthin)
	Chromalveolata	Dinophyta	Chlorophyll <i>a</i> Chlorophyll <i>c</i> Carotenoids (peridinin)
	Excavates	Euglenophyta	Chlorophyll <i>a</i> Chlorophyll <i>b</i> Carotenoids

Table 4 Organisms for extraction of photosynthetic pigments and accessory pigments.

Evolution of Leaf-Like Photosynthetic Organs

Living and preserved samples of the members of the Archaeplastida that you used in your pigment extractions as well as additional species that have leaf-like structures are available for you to observe. For the unicellular organisms, make wet mounts of the materials provided.

For the multicellular leaf-like structures, your objective is to determine how cells are organized to make a blade. To understand the three-dimensional structure of the blades you will need use a number of approaches to observe the blade such as:

- 1) Wet mounts of entire leaf-like structures. This technique will allow you to observe the surface of some thin blades but is restricted to blades that are only one or two cells thick.
- 2) Cross-sections through the blade. By making a cross section and mounting it edge side up, you can determine the shape of cells forming the structure of the blade, determine the number of layers of cells comprising the blade and the organization of the cells. Your instructor will demonstrate techniques for making cross sections.
- 3) Longitudinal sections along the length of the blade.
Make as many mounts and sections as needed to determine the three dimensional structure of the blade and make drawings of the materials you observe. Include a drawing of the macroscopic structure of the blade as well.

Once you have finished with your drawings, use the Plasticine provided to model the shapes of the type(s) of cells that make up the blade. Make a number of cells to show how are the cells organized to make the blade-like macroscopic structure you observed.

- 1) Are all of the blades constructed in the same way?
- 2) Given the phylogeny of the Archaeplastida (Figure 45), and the structure of the various blades you observed, what can you conclude about the evolution of blade-like structures in the Archaeplastida?
- 3) What type(s) of cell division(s) or organization are needed to form a blades you observed?

REQUIRED METHODS

COLLECTION METHODS FOR EUKARYOTIC MICROBES AND SAMPLING METHODOLOGY

POINT SAMPLING

In this type of sampling you would choose one or a few types of substrates (soil, living organisms, sand) or media (fresh or salt water, muck from the bottom of a pond, air) that likely contain eukaryotic microbes. Basically, a substrate is a solid material that an organism can live on or in such as soil, rocks and plant surfaces. A medium (plural = media) is a liquid or gas that organisms are suspended in. You would collect a sample of the substrate or media along with the eukaryotic microbes it contains (e.g. a jar of water).

In the field, you can check potential substrates or media for the presence of organisms using the field microscope before sampling (Using a Field Microscope – Basic Setup, **pg. 103**; Making a Wet Mount **pg. 127**).

There are numerous substrates and media that contain eukaryotic microbes to be found in the terrestrial and marine sites. Even though the specific location of the sample may be small, you should still consider the spatial context of your sampling. Eukaryotic microbes are small so when you think of the particular habitat or niche occupied by an organism or group of organisms you need to think small, *smaller, smallest*. As a result what you might think of as a sample from a single habitat such as a scoop of soil may contain many microhabitats. For example, organisms associated with a decaying maple leaf may be very different than the organisms associated with bits of decaying wood even though both are present in a scoop of soil or dirt. Alternatively, what you might think of as a sample representing a single habitat may in fact represent only part of a habitat. In a soil sample, a sample from the upper soil layers will have very different physical and chemical properties from lower layers. Small differences in physical and chemical conditions from the soil surface to lower soil layers will again result in very different microhabitats that in turn will affect the organisms present in a given sample.

Point Sampling of Terrestrial Substrates and Media

1. **Soil:** The soil moisture content, amount of organic material, type of organic material, depth and time of year will all affect types of organisms you will find. Think about how you might sample to maximize the organismal diversity you will find.
2. **Plant Material:** Plants are the major primary producers in terrestrial environments. Plants may also provide substrates (surfaces) for attachment of

- eukaryotic microbes and biomass for the detritus food web. Consider the impact of this organic material on microbial populations. Decaying alga or plant material can serve as a major source of nutrients for saprobes and the saprobes can in turn support populations of predators. Moist, decaying plant material (e.g. rotten tree stumps and leaf litter) is likely to contain eukaryotic microbes.
- 3. Animals or Animal Parts:** Although vertebrates certainly harbor eukaryotic microbes, other animals such as arthropods (e.g. insects), worms and nematodes are much more abundant. Many contain parasitic or mutualistic eukaryotic microbes. Observation of eukaryotic microbes associated with animals generally requires dissection of the animal. As with many symbionts, the eukaryotic microbes occurring inside living hosts generally don't live very long outside of their host.
 - 4. Pond Water:** Microorganisms (both eukaryotic and prokaryotic) are extremely abundant in water from ponds and lakes that contain large amounts of detritus. When sampling this type of environment, think about the spatial dimensions of the environment. Even in a relatively shallow pool, there can be dramatic physical, chemical and biotic differences between the surface, the bottom and sediments. Most bodies of water have rocks, plant material (e.g. fallen trees or branches, and living plants) that can act as a substrate for eukaryotic microbes.

Point Sampling of Marine Intertidal Sites

- 1. Sand:** As with soil, the moisture content, depth, texture (grain size), position in intertidal (i.e. relative to high tide mark estimated by debris band) will affect the types of organisms you will find. Think about how you might sample to maximize the diversity of organisms you will find. In some cases, beach sand may be discolored indicating the presence of large numbers of organisms in a single location.
- 2. Plant Material or Algae:** In the intertidal region, organic material is continually washed up on the beach. Consider the impact of this organic material on microbial populations. Decaying alga or plant material can serve as a major source of nutrients for saprobes and the saprobes can in turn support populations of predators. Moist, decaying plant material, like organic material in soil is likely to contain eukaryotic microbes.
- 3. Animals or Animal Parts:** Mollusks and arthropods are common in the intertidal region. Eukaryotic microbes are abundant on or even inside these animals. The shells or exoskeletons of animals can serve as a solid substrate for eukaryotic microbes.
- 4. Water:** Microorganisms (both eukaryotic and prokaryotic) are extremely abundant in coastal marine environments. Coastal regions tend to be higher in nutrients and have abundant light both of which support the growth of photosynthetic primary producers, most of which are eukaryotic microbes. The photosynthetic organisms in turn support large food webs that include saprobes, mutualists, parasites and predators. When sampling this type of

environment, think about the spatial dimensions of the environment. There may be different organisms that occur in the water column (pelagic) and organisms that occur on the bottom or attached to a variety of surfaces (benthic). Even in a relatively shallow pool, there can be dramatic physical, chemical and biotic differences between the surface, the bottom and sediments. Most bodies of water have rocks, plant material (e.g. fallen trees or branches, and living plants) that can act as a substrate for eukaryotic microbes.

SYSTEMATIC SAMPLING METHODS

If you are interested in assessing diversity within a particular location (e.g. a forest or intertidal region) rather than at a single point (a rotten log or pond) you will need to employ a more systematic (structured or methodical) approach to sampling. One commonly used approach is transect sampling. A transect is simply a line. The orientation of the line will depend on the goal of sampling. For example in the intertidal region, the transect may run from the edge of the water up to the spray zone. Samples are made at intervals along the transect and contain whatever substrate or media is present at a particular location. Samples along a transect running from the water's edge at low tide to the high tide mark or spray zone would give you an idea of how diversity changes with across the intertidal zones (e.g. spray, high intertidal, mid intertidal, low intertidal). In contrast, a transect perpendicular to the water line at a particular height in the intertidal zone would give you an idea of the diversity within a particular zone.

A simple approach to sampling along a transect would be to:

1. Identify the objective of your sampling and choose the appropriate orientation of the transect.
2. Choose the sampling interval (e.g. 1m)
3. Choose the size of the sample from each point (e.g. 1g of surface sand from ~10 cm² from the top 1-2 cm)
4. Make a transect of a defined length and orientation, collect and label samples.

This type of sampling can be used to assess diversity since not every species present in a given habitat will be present in every sample and multiple microhabitats within a given habitat will be sampled. Don't restrict yourselves to thinking of transects as horizontal, if you are sampling from soil or sand you may consider a vertical transect down through the soil to a specific depth below the surface.

CONCENTRATION METHODS

In marine environments and large bodies of fresh water, microorganisms may occur at relatively low densities (e.g. only a few cells per liter of water or less).

Collection Methods for Eukaryotic Microbes

Collection of large amounts of water (100's of liters) and bringing them back to the lab only to recover a few cells may not be practical so methods are employed to concentrate organisms into a smaller volume of media. Three common methods are: filtration, netting and sedimentation. Filtration and netting are similar in principal: water is pumped through a mesh or a fine mesh net is dragged through the water and organisms are collected in the filter or a container at the bottom of the net. The filter or mesh size of the net will determine the lower limit of the size of organisms collected. Sedimentation is used to concentrate organisms that sink in a water column. A sample of media is placed in a column and organisms are allowed to sink to the bottom. After sitting for a period of time, organisms that have settled on the bottom are collected.

SAMPLING AND MEASUREMENT EQUIPMENT

- pH meter (e.g. Hanna Instruments Combination pH / EC / TDS / Temp.)
 - pH paper
 - thermometer (alcohol)+ poles
- Refractometer (for measuring the salinity (salt content) of water)
- Tape measure
- Flags / flagging tape
- Plastic sampling jars with lids (various sizes – source?)
- Small sampling tubes
- Plankton net
- Forceps / tweezers
- Measuring tape
- 1 Bucket
- Backpacks
- Masking tape / labels
- Waterproof pens (sharpie)
- Ziplock® bags
- String
- Magnifying glass / hand lenses
- Funnel and filter paper
 - Field vacuum filter
- Field Microscope
 - Slides
 - Coverslips
 - Batteries
 - Kimwipes
 - Eyedropper
 - Water Bottle for rinsing slides

Collection Methods for Eukaryotic Microbes

- Fresh water and dropper in screw top bottle for wet mounts (for both terrestrial and intertidal groups)
- Sea water and dropper in screw top bottle for wet mounts (for intertidal group)
- Methyl cellulose in a screw top dropper bottle

BASIC MICROSCOPY

FIELD OBSERVATION OF EUKARYOTIC MICROBES

Getting to a sampling site often requires considerable travel time and can be very expensive. You want to make the most of sampling opportunities. One way of evaluating potential samples is to observe material microscopically with a field microscope (Figure 47). Observing organisms in the freshest material possible will give you a better idea if a particular substrate or media is worth collecting or if it contains the type(s) of organisms you are trying to study.

Using a Field Microscope – Basic Setup

The microscope you will be using in the field is a compact, portable compound microscope. The objectives are below the stage and the light source is above the stage (Figure 46). The advantage of the inverse design is that the sample can be thick without worrying about damaging the objectives. Even though this microscope is tiny and portable, it is a big step forward in technology compared with the van Leeuwenhoek-style microscope you made in Lab 1.

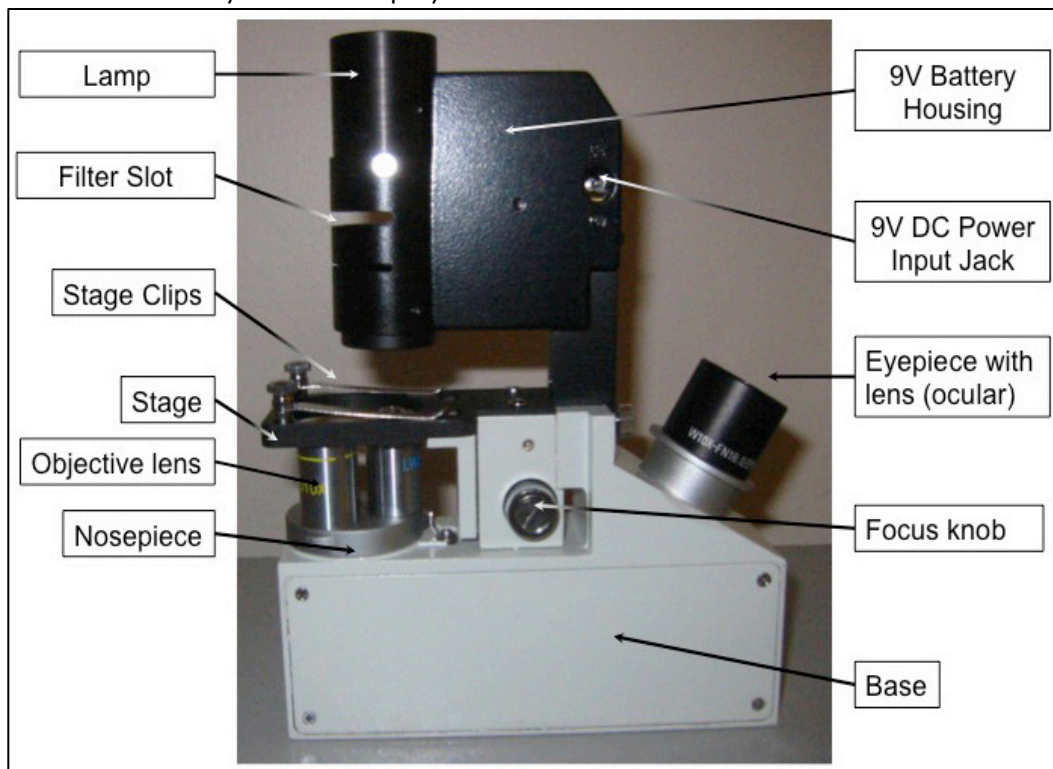


Figure 46 The parts of a basic field microscope. The dimensions of the microscope are 14cm x 18 cm x 4cm.

Basic Microscopy

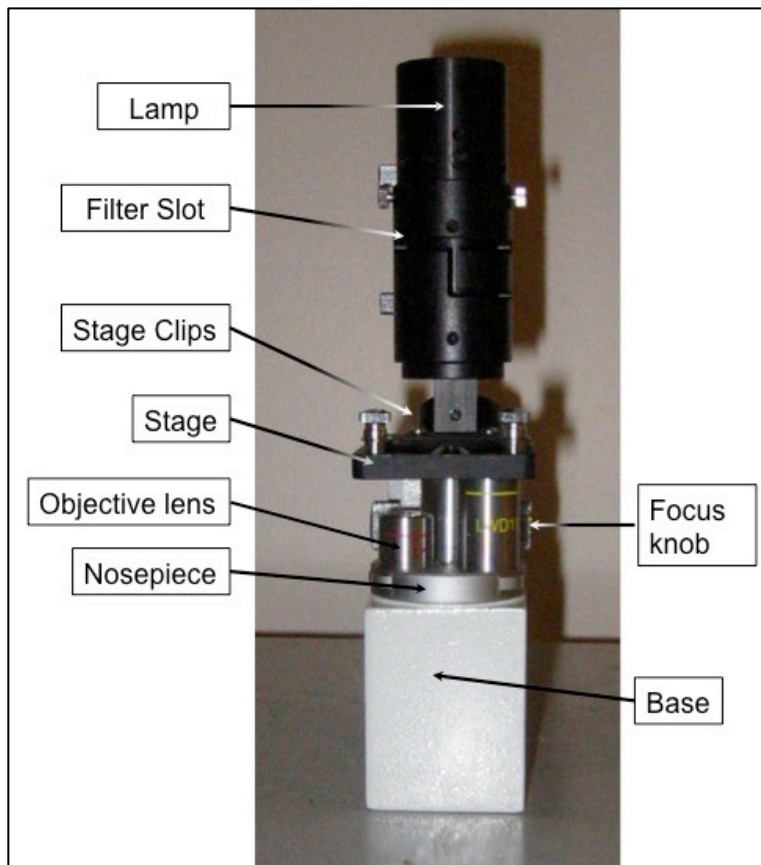


Figure 47 The parts of a basic field microscope. The dimensions of the microscope are 14cm X 18 cm x 4cm.

Mounting and Viewing a Sample Using a Field Microscope

1. Make a wet mount of the sample you wish to observe (Figure 82 pg. 127 to 128).
 - a. If you are sampling water, place a drop of water directly onto the slide and cover with a cover slip.
 - b. If you are sampling solid substrates (e.g. scraping material off rocks) mount a bit of the solid substrate in a small drop of water.
2. Turn on the lamp with the switch on the back above the ocular.
3. Rotate the nosepiece until the 4x objective is under the lamp (Figure 46)
4. Place the slide on the stage with the cover slip facing up (towards the lamp) as you would with a regular compound microscope.
5. Move the slide until the sample is over the objective lens.
6. Hold one edge of the slide and place a stage clip over the opposite edge of the slide. Make sure the clip is over top of the stage otherwise the slide will be catapulted into the light source.
7. Move the other stage clip into place.

Basic Microscopy

8. Using the focus knob, raise the stage to the maximum height (i.e. maximum distance above the sample).
9. Look through the eyepiece and gradually lower the stage toward the objective until the sample comes into focus.
10. Increase magnification as necessary to view your sample.

Using a Field Microscope – FAQ

1. I can't find anything moving, does it mean there is nothing in my sample?
 - a. Not all eukaryotic organisms are motile. Some are motile but do not move rapidly and movement may not be immediately apparent.
 - b. Depending on the medium or substrate, environmental conditions the abundance of organisms and many other factors, the organisms present in the sample may not be immediately obvious but will be easier to find with practice.
 - c. There may in fact be organisms present in the sample but they may not be active. Many organisms become dormant under stressful environmental conditions and may be present as cysts or spores.
 - d. Organisms may be hidden, for example, living within houses composed partially of sand grains or embedded in plant material.
2. What is the best way to view a thick sample?
 - a. Making the perfect slide may be difficult in the field. Try your best to use a small amount of material and avoid overly thick material.
 - b. Use a needle or probe to break up large bits of material.
 - c. If organisms are moving quickly and difficult to follow, try viewing a thin part of the mount near the edge. Organisms will have less room to move.
3. Help, the organisms are moving too quickly for me to view!
 - a. Great! Your sample has a lot of organisms that can be viewed in more detail when you get back to the lab.
 - b. Make a wet mount in a drop of methylcellulose to slow down fast moving organisms a bit. Methylcellulose forms a clear viscous solution or gel when dissolved in cold water.

USING LABORATORY MICROSCOPES

Stereo- or Dissecting Microscopes

Although the magnification is generally limited to approximately 40x, larger larger eukaryotic microbes can be readily observed using a stereomicroscope. The

Basic Microscopy

advantage of using the stereomicroscope is that the behavior and gross morphology of organisms can be easily observed, the field of view and depth of field are considerably larger than for a compound microscope even at comparable magnifications. The large field of view allows larger samples to be easily scanned for the presence of protists and for manipulation of individual organisms (e.g. picking up a single individual with a pipette).

Using The Stereomicroscope

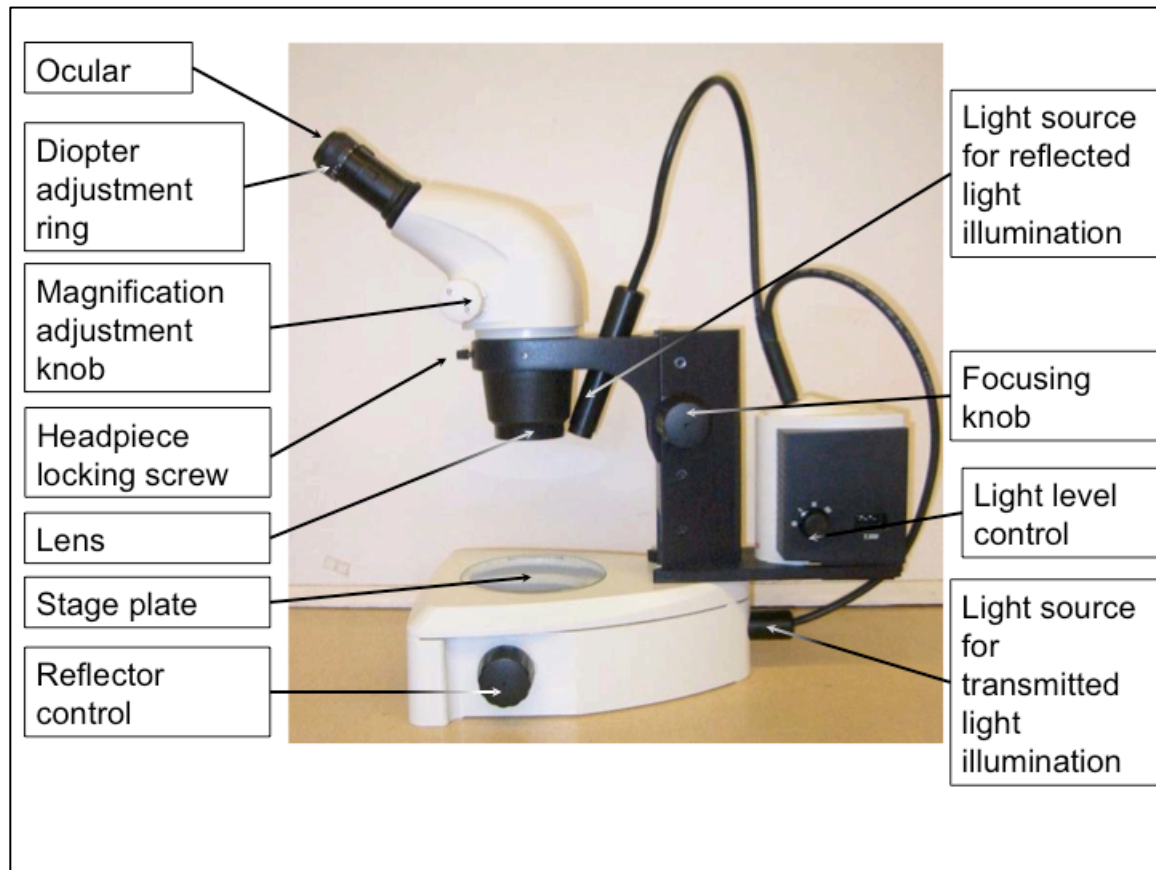


Figure 48 A stereomicroscope with parts labeled. The headpiece is shown in the "in-use" position.

- 1) Turn the focus knob to raise the headpiece assembly so the light source can be removed from the holder (Figure 48, page 106).
- 2) Remove the light source for reflected light.
- 3) Loosen the headpiece locking screw and rotate the headpiece from the "storage" position to the "in-use" position. Tighten the locking screw.
- 4) Return the light source for reflected light.
- 5) Plug in the power source.

Basic Microscopy

- 6) Adjust the magnification to low (0.63X) using the adjustment knob.
- 7) Roughly focus on a sample using the focusing knob.
- 8) Focus the eyepiece using the diopter adjustment.
- 9) Close or cover the eye looking through the ocular with the diopter adjustment. Look through the objective with the other eye and adjust the focus knobs to bring a selected object into clear focus.
- 10) Close or cover the eye looking through the ocular without the diopter adjustment. Look through the objective with the other eye and turn the diopter adjustment ring until the same object into clear focus.
- 11) Use the reflector control knob to switch between a mirror and white surface to adjust the type of transmitted light.

Using the Compound Microscope

In order to effectively use a microscope and follow the instructions to correctly adjust your microscope, you need know some terminology. The parts of the microscope are shown in Figure 53 (pg. 111) and Figure 54 (pg. 112). I can't emphasize strongly enough the importance of properly setting up your microscope, it is the difference between analog and HD, the difference between the Maserati across the street and my Chevrolet Cavalier, the difference between frustration and having fun in the lab looking at amazing stuff under the microscope!

The basic steps for using the compound microscopes in the lab are also presented in the videos on the course website.

- 1) Microscope setup and focusing
- 2) Setting up Köhler illumination
- 3) Phase contrast
- 4) Making a wet mount
- 5) Comparison of bright field and phase contrast

Basic Microscopy

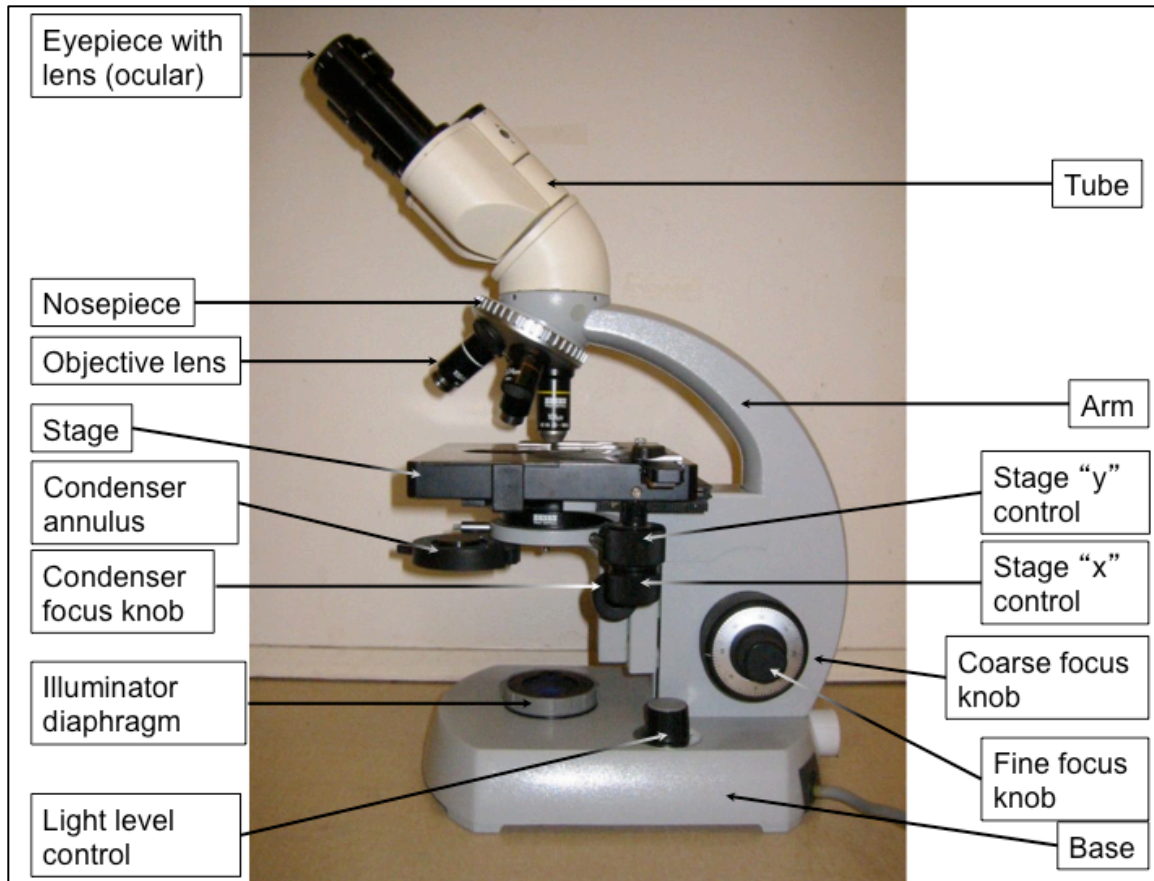


Figure 49 Parts of a compound microscope, side view.

Basic Microscopy

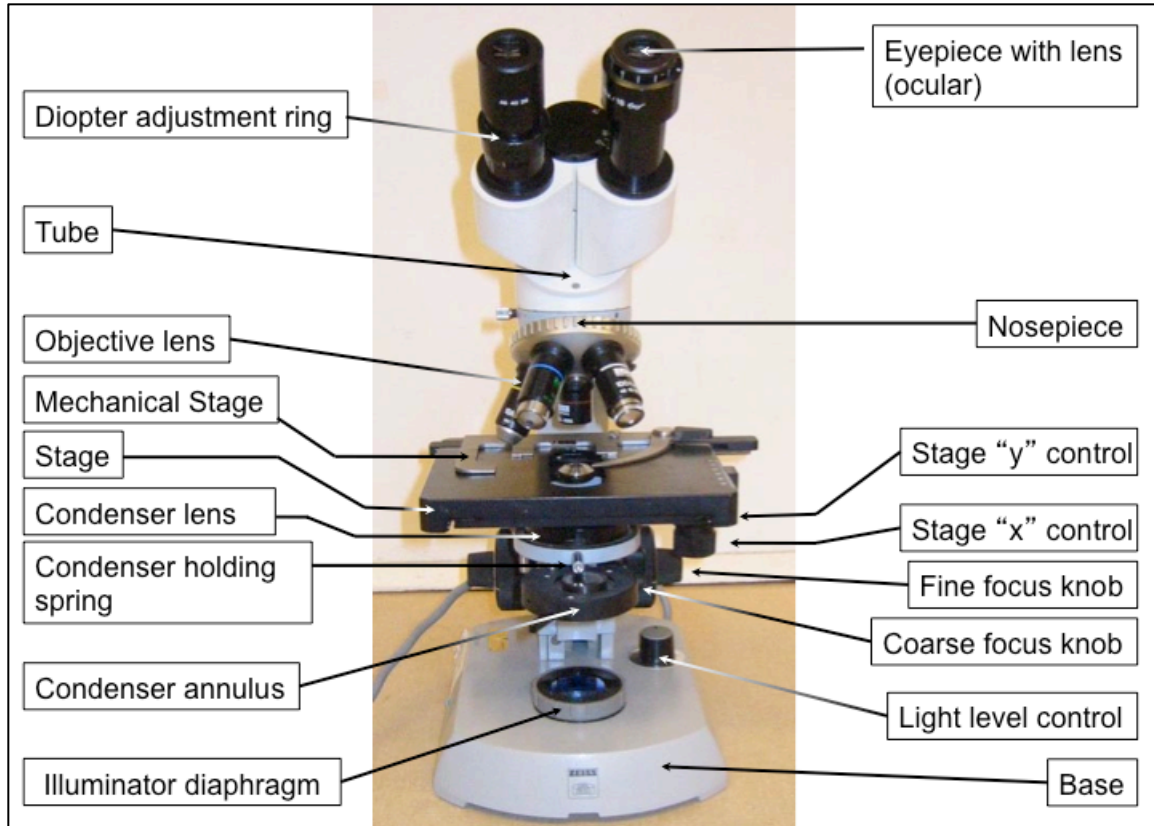


Figure 50 Parts of a microscope, front view.

In contrast to the simple microscopes you produced based on van Leeuwenhoek's design, compound microscopes have multiple lenses arranged in a series. The magnification of the lens system is the product of the magnification provided by each of the lenses in the system. For example, if a sample is viewed with the 40x objective and the ocular lens (Figure 49 and Figure 50) has a magnification of 10x the total magnification is $(40\times) \times (10\times) = 400\times$.

The microscopes you will be using in lab are much more advanced than van Leeuwenhoek's microscopes and many of the problems associated with early microscopes such as aberrations, small field of view difficulty focusing etc. have been ameliorated in the design of these microscopes. However, as with any optical system, proper adjustment of the microscope is essential for optimal image quality, viewing minute structures, reducing eyestrain and overall frustration.

Setting up the Microscope and Köhler Illumination

Initial Focusing

- 1) Unless you are using the 40× objective for phase contrast (pg. 121), the condenser annulus needs to be out of the light path (Figure 54 pg. 112). If the image appears very dark but the light source is on, make sure you have moved the condenser annulus out of the light path.

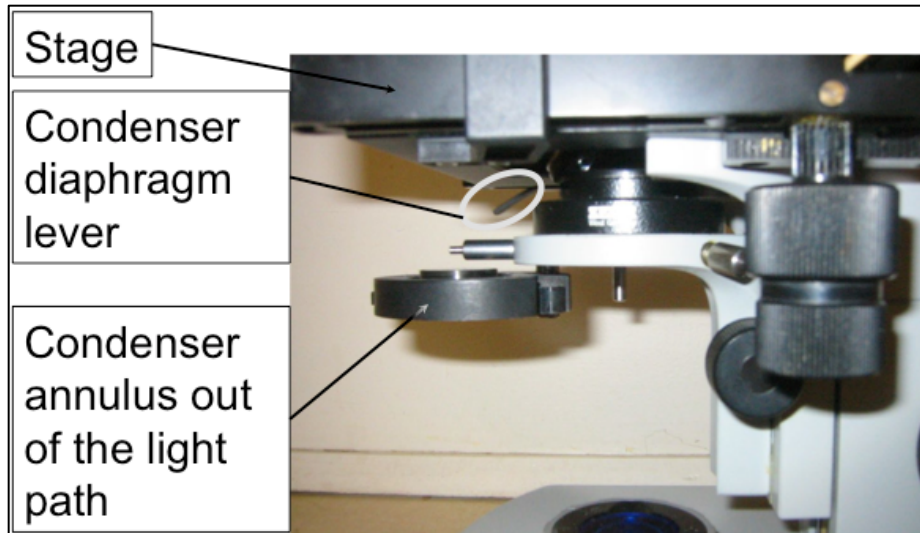


Figure 51 Left view of the condenser assembly showing the condenser annulus, the condenser diaphragm lever and the stage.

- 2) Make sure your slide is clean. Fingerprints, oil and dirt accumulate on prepared slides and re-usable glass slides. Dirt and oil can be removed using a couple of drops of lens cleaning solution and a KimWipe (Figure 52, pg. 111).

DO NOT USE KIMWIPES TO CLEAN MICROSCOPE OPTICS



Figure 52 Kimwipes and lens cleaner for cleaning slides. Kimwipes are not to be used to clean lenses.

- 3) Turn the nosepiece to rotate the 3.2 X objective into place (Figure 50, pg. 109).
- 4) Move the condenser front lens out of the path of the light (Figure 53 pg. 111).
- 5) Place a slide on the mechanical stage (Figure 53 pg. 111).
- 6) Adjust the stage with "x" and "y" control knobs to center specimen under the objective (Figure 61 pg. 116).

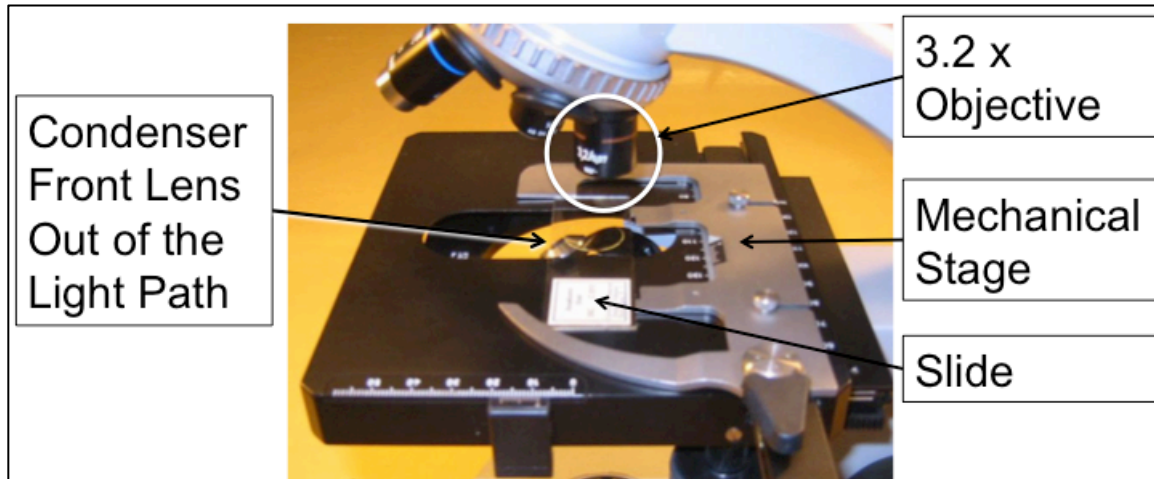


Figure 53 Left view of the stage of a compound microscope showing the position of the condenser front lens when using the 3.2x objective, the mechanical stage and the proper placement of a slide.

- 7) Focusing using the low power (3.5 x) objective.
- 8) Raise the stage to the maximum height using the coarse focus knob (Figure 61 pg. 116) .
- 9) Look through the eyepiece and lower the stage using the course focus knob until a part of the specimen comes into focus (Figure 59 pg. 115) .
- 10) Focusing first using the 3.2X objective allows the higher power objectives to be safely swung into place without hitting the slide.

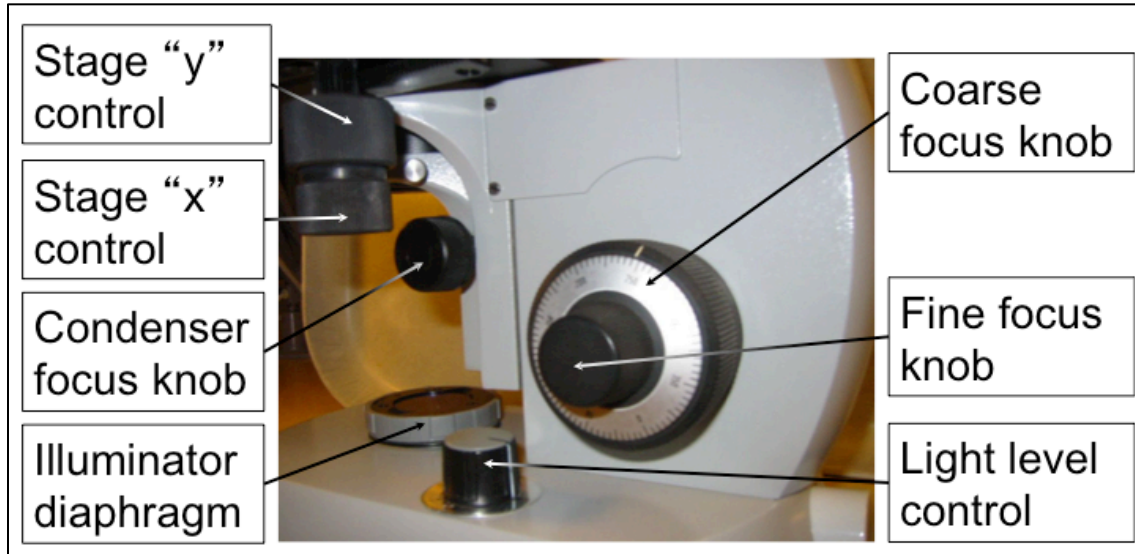


Figure 54 Left view of the compound microscope showing: i) the mechanical stage control knobs that move the slide left and right (Stage "x" control) and forward or backwards (stage "y" control), ii) the focusing knobs, iii) the light level control, iv) the condenser focus knob and v) the illuminator diaphragm.

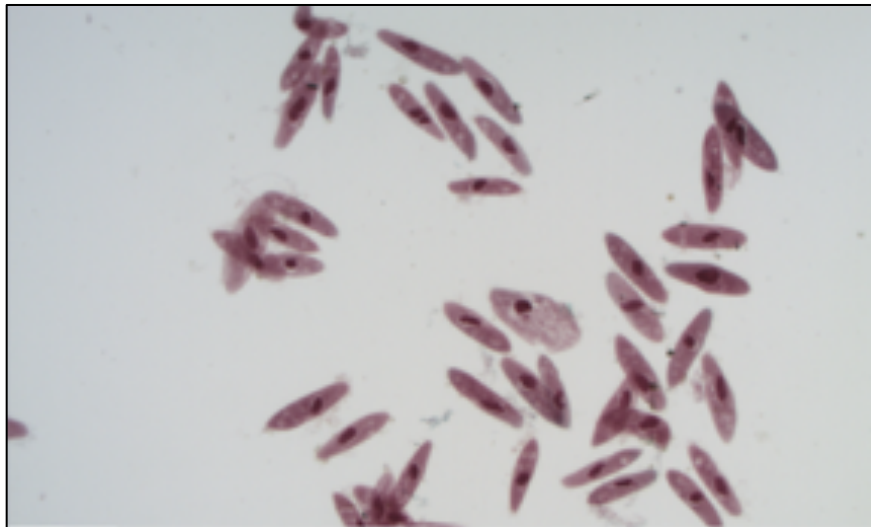


Figure 55 What you should expect to see under the microscope is shown to the right. A micrograph of a stained, prepared slide of *Paramecium caudatum*. In this preparation, the nuclei appear dark red.

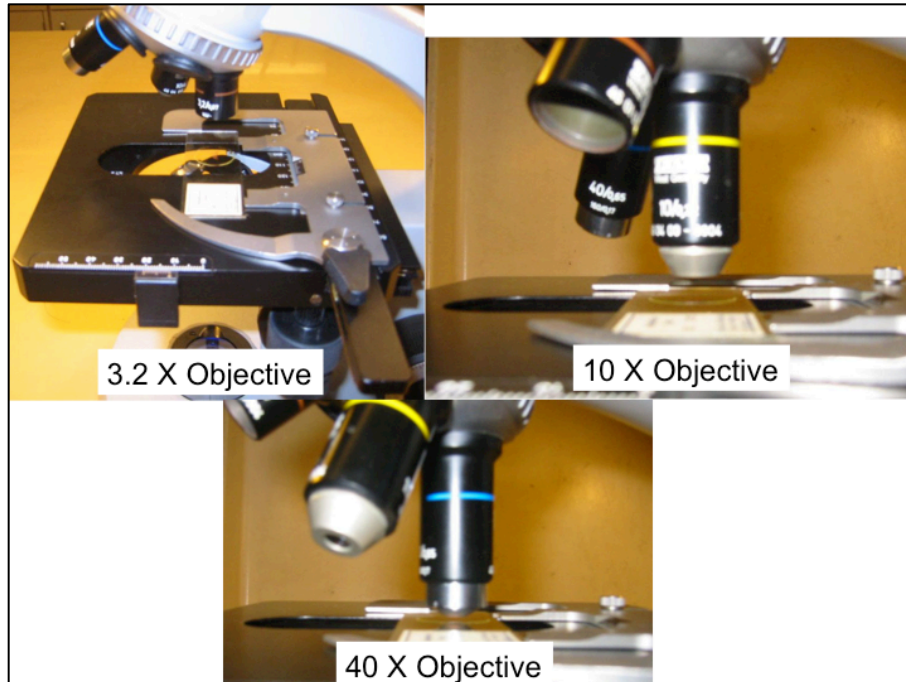


Figure 56 Right view of the nosepiece and objective lenses.

Focusing The Ocular Lens

This is an important step to reduce eyestrain by making sure both of your eyes are focused on the same object viewed under the microscope.

- 1) Close or cover the eye looking through the ocular with the diopter adjustment. Look through the objective with the other eye and adjust the stage using the coarse and fine focus knobs to bring a selected object into clear focus.
- 2) Close or cover the eye looking through the ocular without the diopter adjustment. Look through the objective with the other eye and turn the diopter adjustment ring until the same object into clear focus. Now when you look through the microscope, both eyes will be focused on the same object.

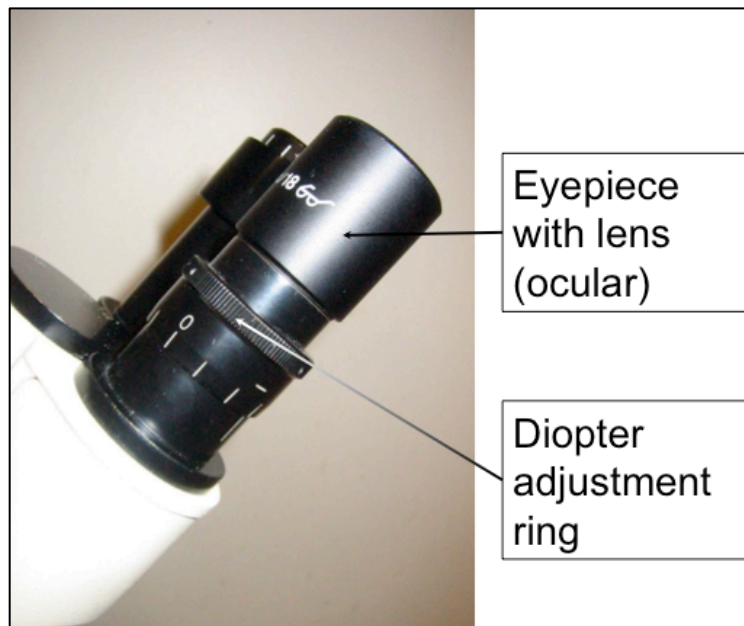


Figure 57 Left view of the eyepiece of a compound microscope showing diopter adjustment ring for focusing the ocular lens.

Setting Up Köhler Illumination

Proper adjustment of the light source is important for producing the best possible image of a sample. Again, it is the difference between analog and HD, the difference between the Maserati across the street and my Chevrolet Cavalier, the difference between frustration and having fun in the lab looking at amazing stuff under the microscope! Proper adjustment will be important when you are trying to view fine structures like flagella and cilia. When viewing organisms, adjustment of the light source can alter the contrast of a sample and the depth of field enabling you to better visualize some structures.

The light source is adjusted using the condenser lens, the condenser front lens, the illuminator (iris) diaphragm, the light level control knob, and the condenser (iris) diaphragm. Köhler illumination is the proper adjustment of lenses and irises to uniformly illuminate a sample and produce an image that is free from glare. Köhler illumination will allow you to adjust contrast using the iris diaphragm and will prevent your view from being obscured by focusing on dirt or other material in the light path.

- 1) The condenser front lens is not in the light path (Figure 58, pg. 115).
- 2) Using the nosepiece, swing the 10x objective into position over the sample.
- 3) Using the fine focus knob, focus on the specimen.
- 4) Move the condenser front lens into the light path using the condenser front lens knob.
- 5) Using the condenser focus knob, raise the condenser lens to its maximum height. Close the illuminator diaphragm.

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- 6) Lower the condenser lens using the condenser focus knob until the illuminator diaphragm comes into focus sharp.
- 7) Open the illuminator diaphragm until the entire field of view is illuminated and you can no longer see the edges of the diaphragm.
- 8) You will need to re-adjust Köhler illumination each time you change objectives. As you increase magnification, proper illumination becomes more critical to achieving a sharp image.

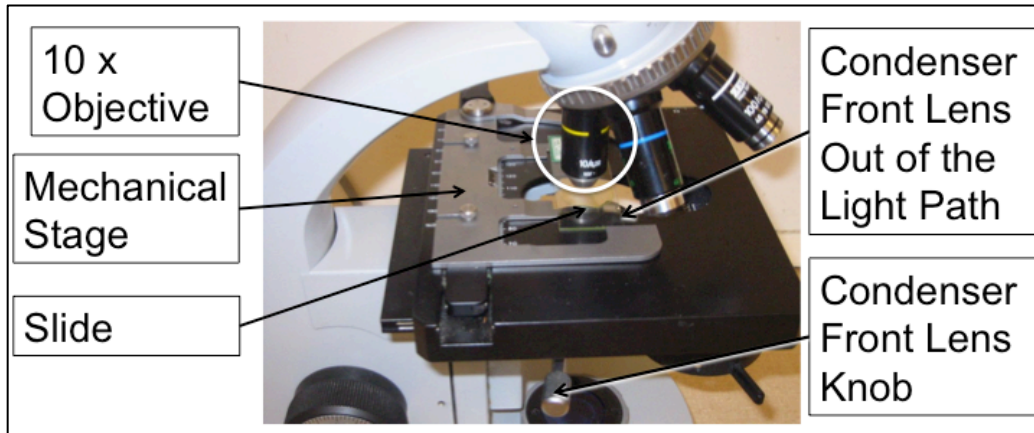


Figure 58 View of the right side of the compound microscope showing the position of the 10x objective when placed in the light path and the condenser front lens knob for moving the condenser front lens in and out of the light path. To move the condenser lens rotate the knob.

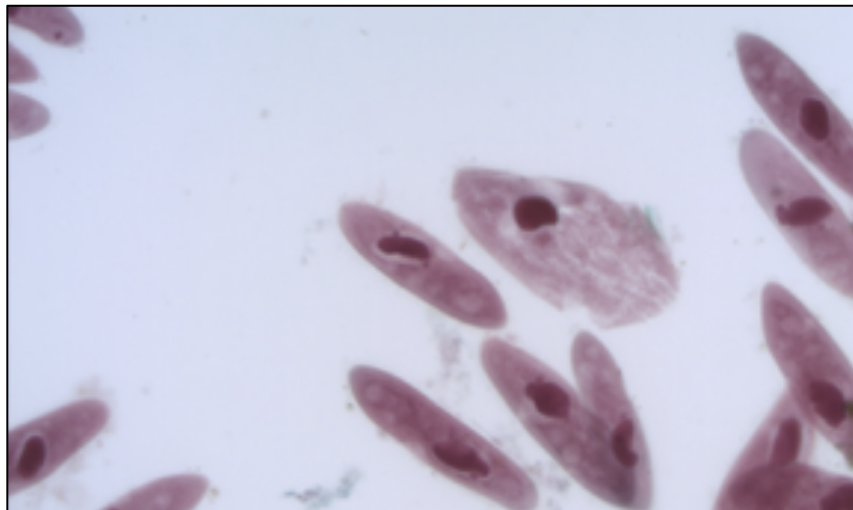


Figure 59 A micrograph of a stained, prepared slide of *Paramecium* sp. Note the reduced field of view relative to the image from the low power objective in step 4. (*Paramecium* sp. 100x)

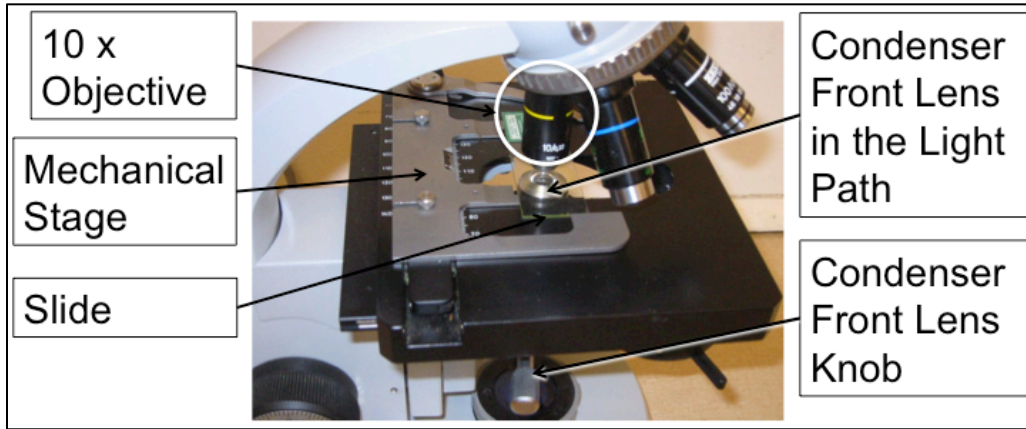


Figure 60 Right view of the compound microscope showing the condenser front lens in the light path.

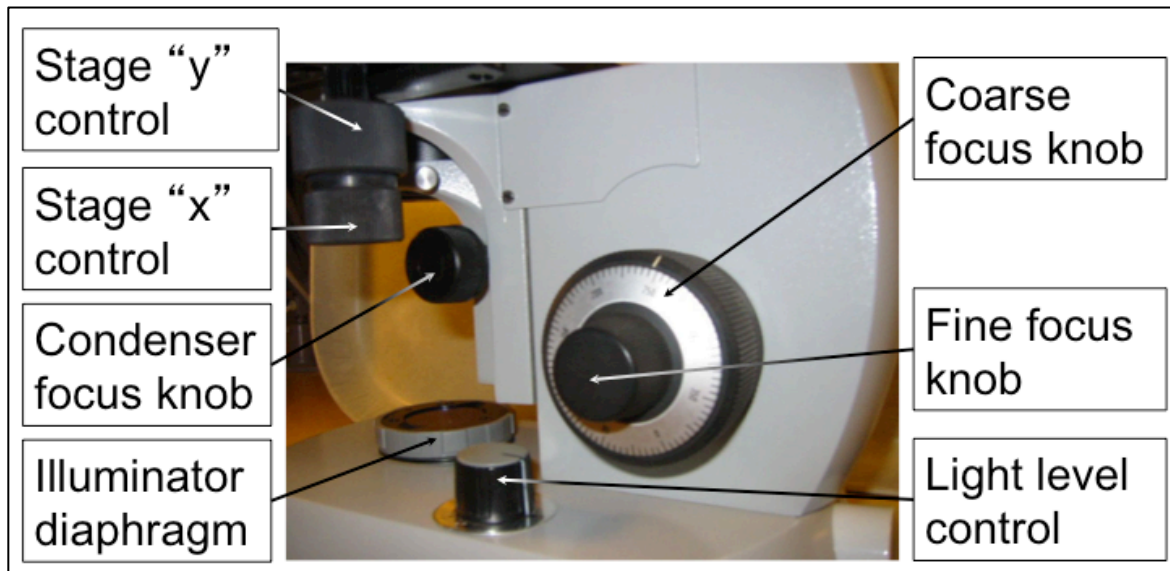


Figure 61 Left view of the compound microscope showing: i) the mechanical stage control knobs that move the slide left and right (Stage "x" control) and forward or backwards (stage "y" control), ii) the focusing knobs, iii) the light level control, iv) the condenser focus knob and v) the illuminator diaphragm.



Figure 62 View showing the view through the microscope with the condenser diaphragm completely closed. Note that the majority of the field of view will be dark. The small circle of light indicates the edges of the illuminator diaphragm.



Figure 63 Illuminator diaphragm in sharp focus.

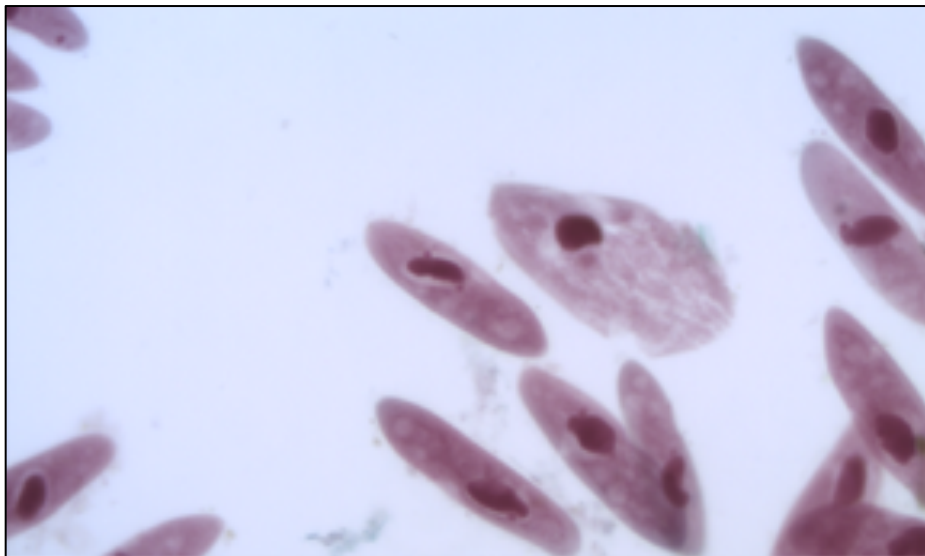


Figure 64 View of *Paramecium caudatum* with Köhler illumination.

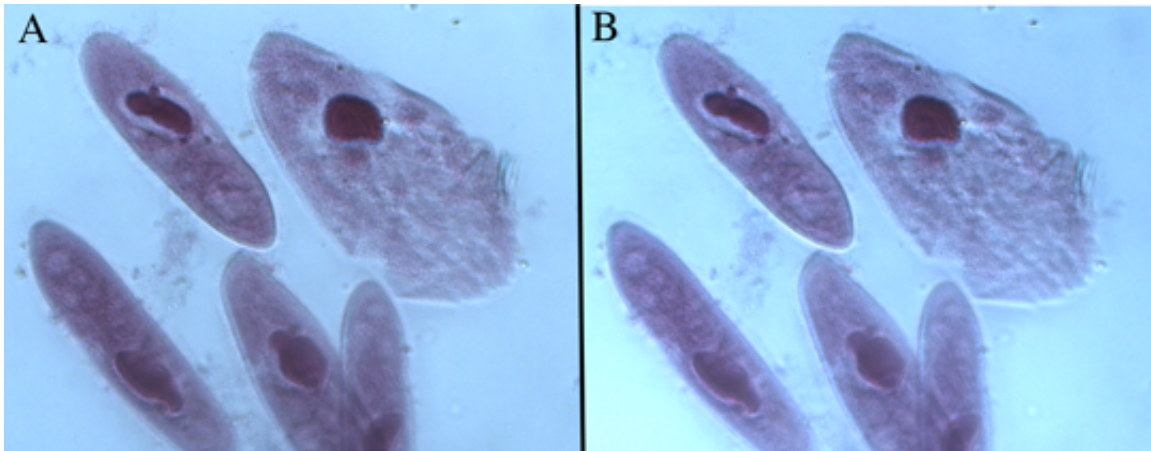


Figure 65 When Köhler illumination is not set-up and contrast can no longer be easily adjusted by opening or closing the iris diaphragm of the condenser; all images appear very high contrast and granular. For this example the condenser assembly was moved to the bottom end of the range as an extreme illustration of the effect of improper adjustment of illumination on image quality. Both images were made the same condenser focus and the iris diaphragm of the condenser A) open or B) closed.

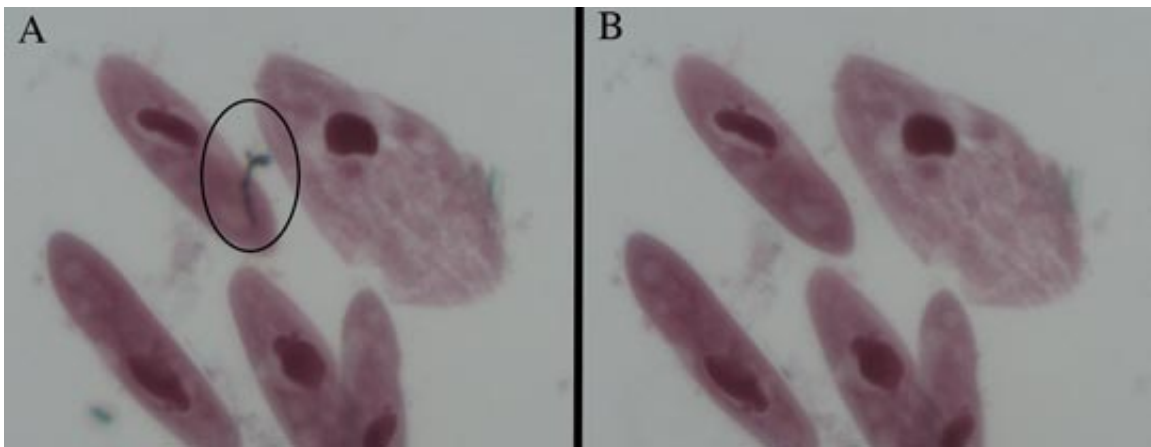


Figure 66 When Köhler illumination is not set-up properly, objects in the light path can appear in focus and obstruct the image of the sample. A) Köhler illumination is not set-up and dust in the light path appears in focus and obstructs the sample image. B) Köhler illumination is set-up. The dust was not removed but does not obstruct the image since it is not in focus.

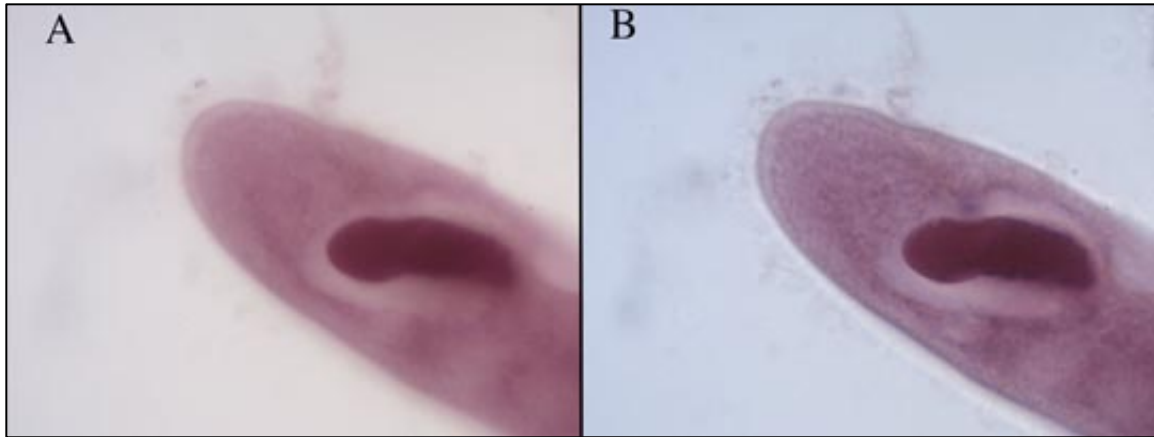


Figure 67 Images of *Paramecium* sp. showing the effect of Köhler illumination on image quality. A) *Paramecium* sp. viewed without Köhler illumination. The image appears slightly fuzzy, the edges are not well defined and cilia are not visible. B) *Paramecium* sp. viewed with Köhler illumination. The image appears crisp with well-defined edges and the fine cilia are visible surrounding the cell. Fine structures like cilia and flagella are still best viewed using phase contrast (pg. 121).

Adjusting Contrast and Depth of Field Using the Iris Diaphragm and the Light Level Control

As you view specimens under the microscope you will often need to make additional adjustments to illumination using the iris diaphragm and the light level control. Opening and closing the iris diaphragm has two effects: 1) altering the depth of field (Figure 68) and 2) altering the contrast between a specimen and the background. Closing the iris diaphragm reduces the amount of light passing through a sample and generally the light level needs to be increased as the iris diaphragm is closed. The light level often needs to be increased as the magnification is increased.

Depth of field is the distance between the top and bottom points in a sample that are in focus. Remember that although the organisms you are viewing are very thin they still have depth and in general, cells are large enough that not all parts of the cell will be in focus at the same time (i.e. if the top surface of the cell is in sharp focus, internal structures and the bottom surface of the cell will not be in focus). Viewing all parts of a cell generally requires focusing up and down through a sample using the fine focus knob on the microscope (Figure 49, Figure 50). Depending on the sample, you may want to adjust the depth of field to view specific structures. In some samples, increasing the depth of field can make distinguishing overlapping structures difficult and reducing the depth of field will create a crisper image but will require focusing up and down through the sample to view all structures.

Opening and closing the iris diaphragm is used to adjust the depth of field. Opening the iris diaphragm DECREASES the depth of field. Closing the iris diaphragm INCREASES the depth of field.

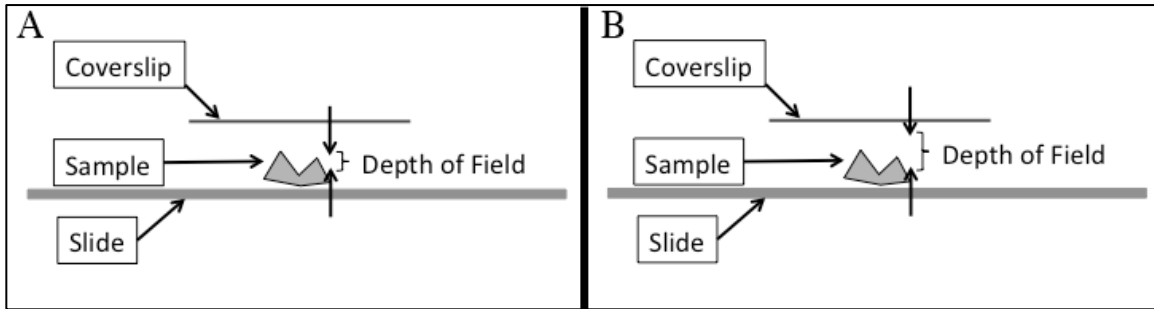


Figure 68 Illustration of the effect of opening and closing the iris diaphragm on the depth of field. A) Opening the iris diaphragm decreases the depth of field. B) Closing the iris diaphragm increases the depth of field.

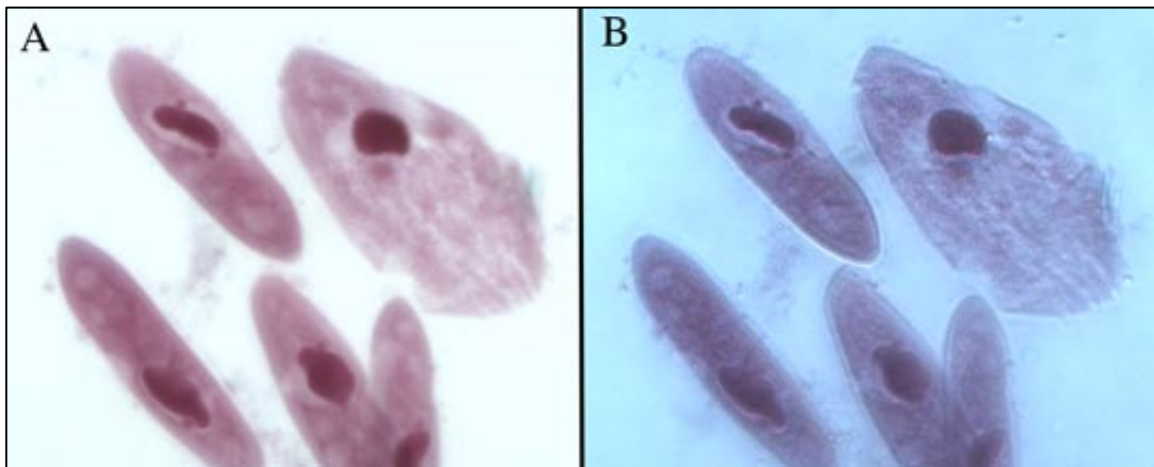
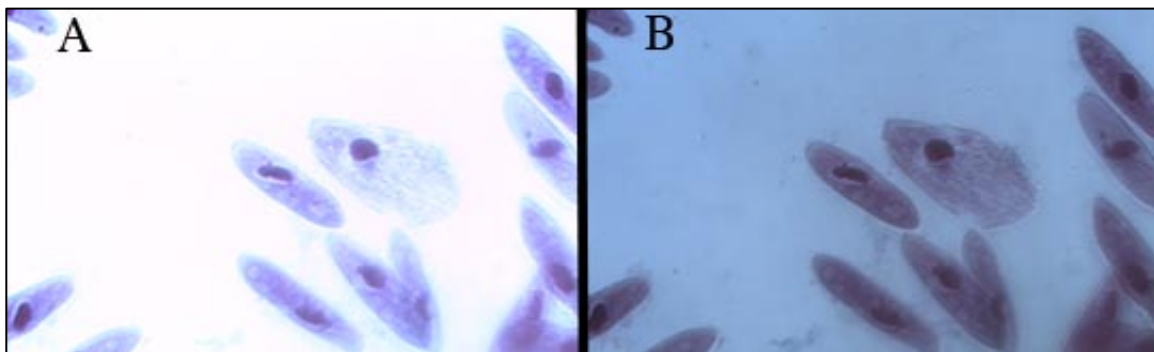


Figure 69 The effects of opening and closing the iris diaphragm on the contrast between a cell, cellular structures and the background. The light level was increased as when the iris diaphragm was closed. A) Opening the iris diaphragm decreases contrast between the specimen and the background and depth of field. B) Closing the iris diaphragm increases contrast between the cell and the background and increases the depth of field.



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Figure 70 The effect of adjusting the light level on contrast. The iris diaphragm is nearly fully closed and only the light level was adjusted. A) The light level is at the maximum setting. Contrast between the sample and the background is low; the sample appears “washed-out”. B) The light level is at a low-intermediate setting. The contrast between the sample and the background is high.

Phase Contrast

Your compound microscopes also come with phase contrast optics for the 40x objective lens. Phase contrast is an optical system that creates contrast between organisms and the background; the background appears dark but organisms are brightly illuminated. The effect is comparable to staining with the exception that living cells can be observed rather than fixed and stained (killed) cells

- 1) Focus on a specimen using the 40x objective.
- 2) Move the condenser annulus into the light path and make sure it clicks into place>
- 3) Open the iris diaphragm completely

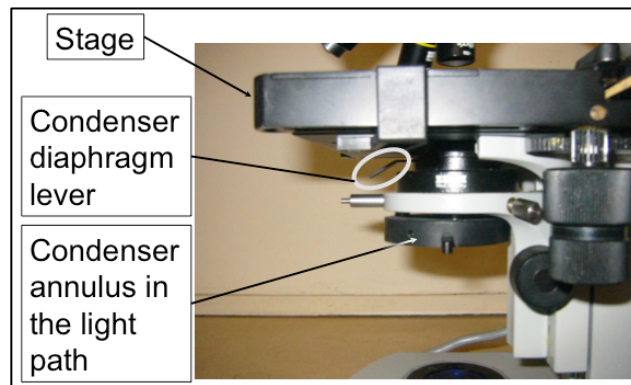


Figure 71 The condenser annulus must be in the light path to view a sample using phase contrast.

- 4) Move the condenser front lens out of the light path with the condenser front lens knob.

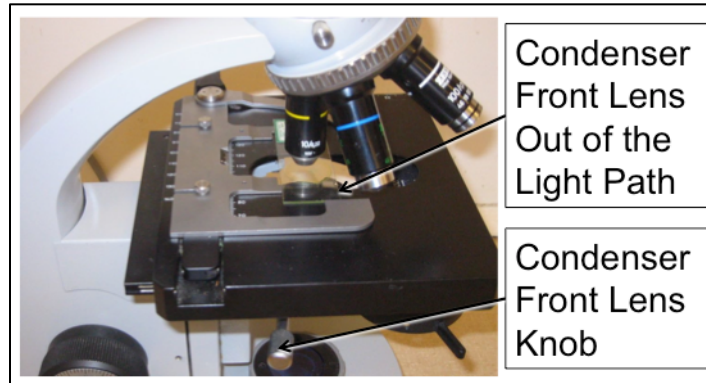


Figure 72 The condenser front lens must be out of the light path to view a sample using phase contrast.

- 5) Open the illuminator diaphragm all the way and adjust light level as necessary.

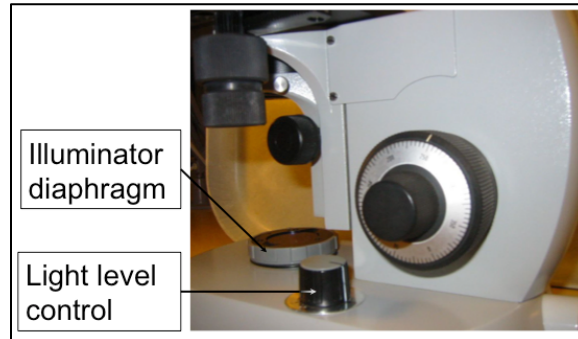


Figure 73 Increase light levels using the illuminator diaphragm and the light level control.

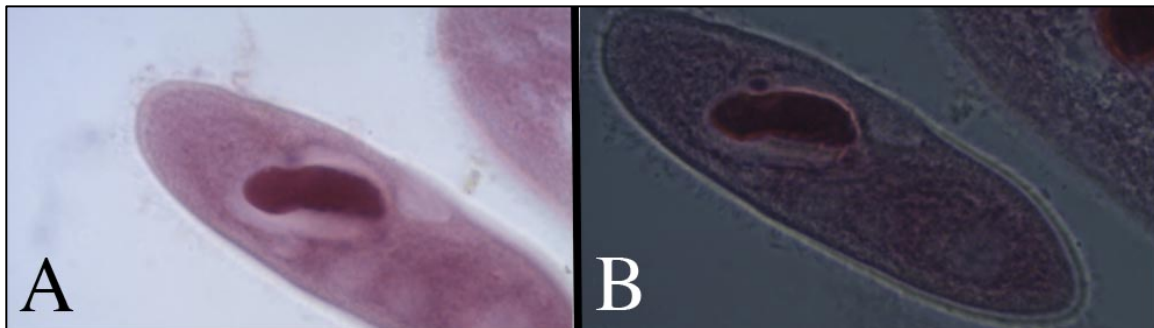


Figure 74 A comparison of bright field optics (A) with phase contrast (B). Notice the cilia on the surface of the cell are much more distinct when viewed using phase contrast than with bright field illumination.

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Cleaning the Microscope

Use lens paper and a drop of cleaning solution to clean objective lenses, oculars etc. Moisten the lens paper and gently wipe the objective. With a dry corner of the paper, gently blot the objective to remove any remaining cleaning solution. If you use oil immersion, clean the oil from the 100x objective after use. If you get oil on objective that are not oil immersion, they need to be cleaned immediately.

KimWipes are NOT to be used for cleaning lenses; they can scratch and destroy the coatings on the lenses.

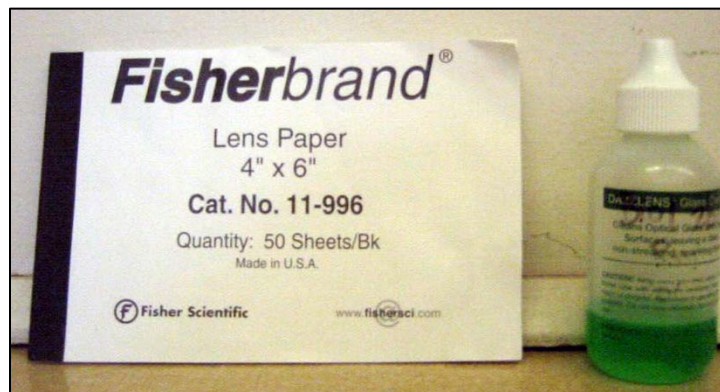


Figure 75 Lens paper and lens cleaning solution.

Estimating the Size of an Organism

A simple method for estimating the size of an organism viewed under the microscope is to estimate how many times the organism would fit across the field of view. The distance across the field of view has been measured for each objective of your microscopes using a tiny ruler mounted to a slide (a stage micrometer). By estimating the portion of the field of view taken up by an organism and multiplying by the known field diameter, it is possible to estimate the size of an organism.

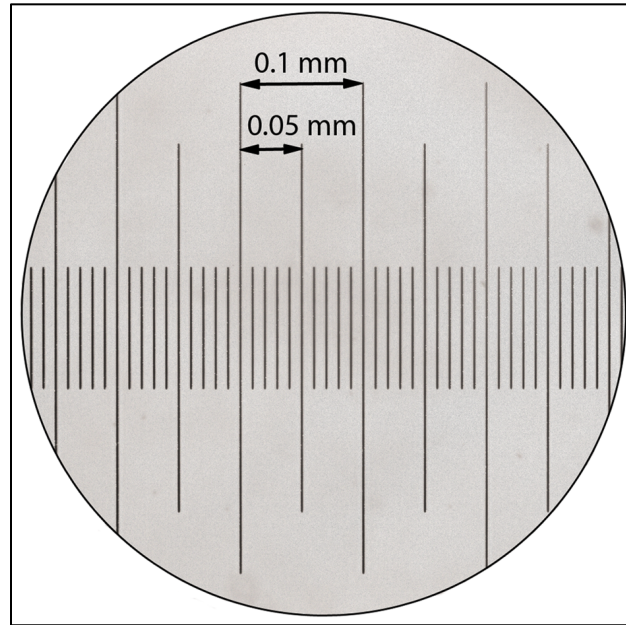


Figure 76 An example of measuring the field diameter of a 20 x objective using a stage micrometer. The circle represents the field of view. The distance between each of the small hatch marks is 0.01 mm (10 μ m). The field diameter is 0.94 mm (940 μ m; 940 micrometers)

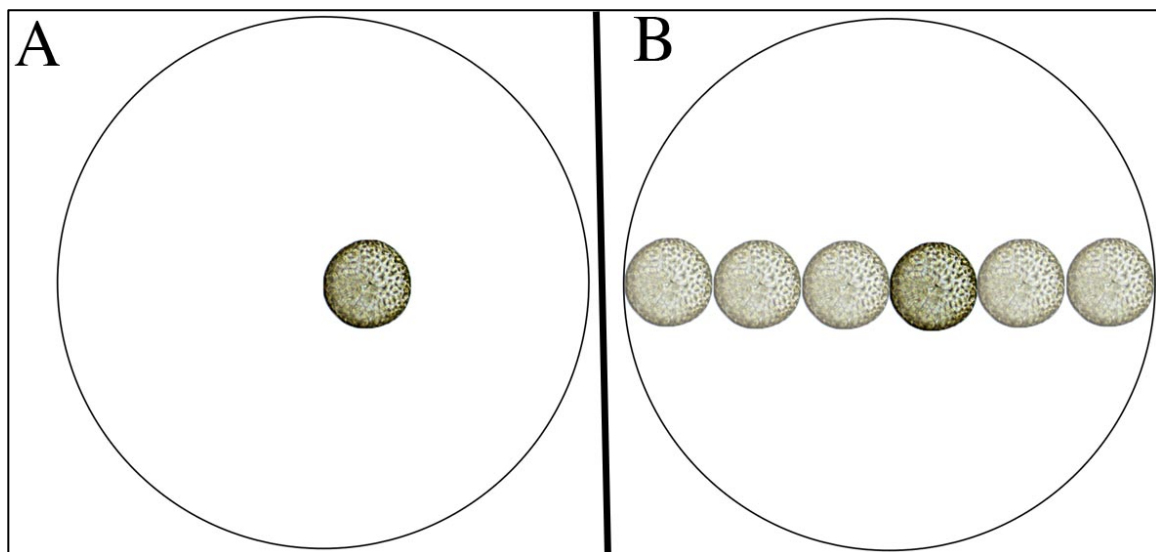


Figure 77 A micrograph of a marine diatom, *Coscinodiscus* sp. (Bacillariophyta; Coscinodiscophyceae). The circle represents the field of view that is 1.05 mm across. Approximately six individuals would fit across the field of view, therefore the size of the organism is: $1.05 \text{ mm} \div 6 = 0.175 \text{ mm}$ (175 μ m).

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Objective	Field Diameter
10 X	1.78 mm
40 X	0.45 mm (450 μm)
100 X	0.18 mm (180 μm)

Table 5 Table of approximate field diameters for objectives on the Zeiss microscopes.

Relative Sizes of Materials Compared to the Field Of View

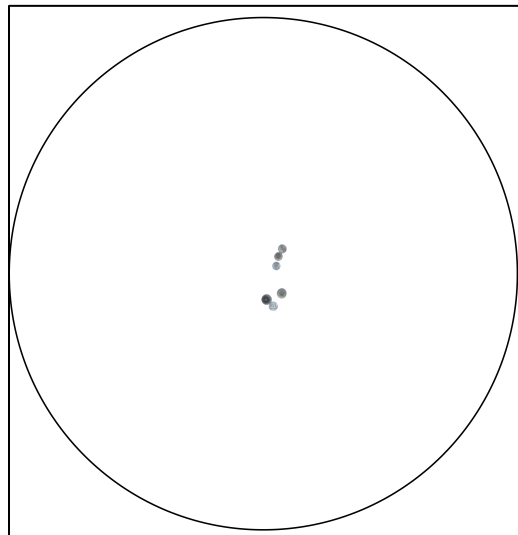


Figure 78 Grass pollen relative to the field diameter (1.78 mm) for the 10 X objective. The black circle represents the edge of the field of view. The pollen grains are 0.03 mm (30 μm) in diameter.

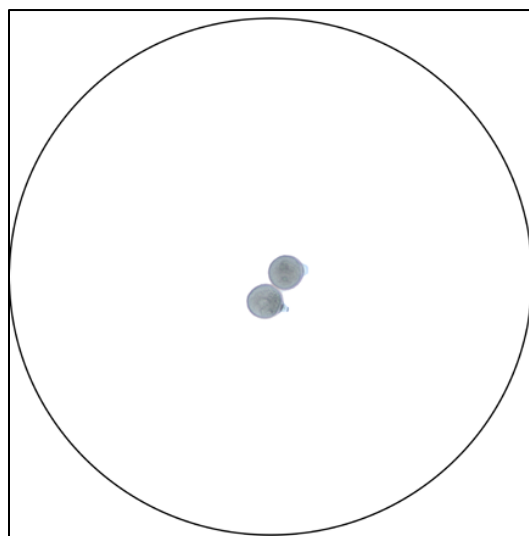


Figure 79 Grass pollen relative to the field diameter (0.45 mm) for the 40 X objective. The black circle represents the edge of the field of view. The pollen grains are 0.03 mm (30 μ m) in diameter.

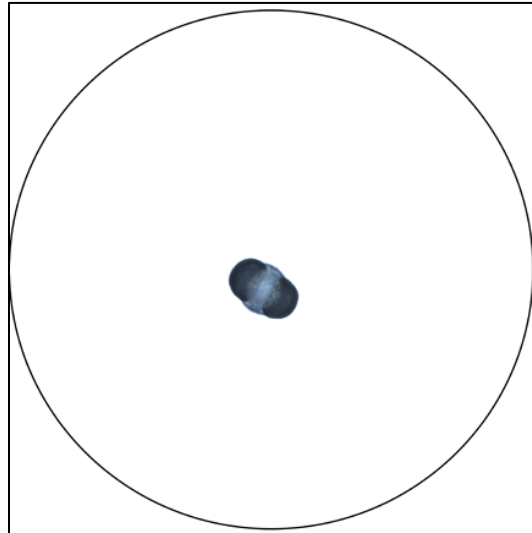


Figure 80 Pine pollen relative to the field diameter (0.45 mm) for the 40 X objective. The black circle represents the edge of the field of view. The pollen grain is 0.06 mm (60 μ m) across at the widest point.

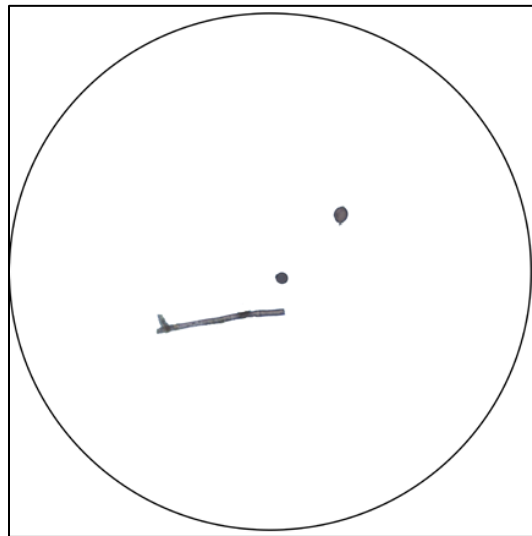


Figure 81 Spores and a fragment of fungal hyphae relative to the field diameter (0.45 mm) for the 40 X objective. The black circle represents the edge of the field of view. The spores are 0.01 mm (10 μ m) and 0.015 mm (15 μ m) at their widest points. The hyphal fragment is 0.11 mm (110 μ m) in length and 0.005 mm (5 μ m) in width.

OBSERVATION METHODS FOR EUKARYOTIC MICROBES

Direct Observations Of Eukaryotic Microbes From Field Material

There is no one “best” method of observing protists. Different methods and tools for observation of cells have their own advantages as well as limitations. For example, observing fresh material under a dissecting microscope will allow you to observe the behavior of large organisms in a more or less natural state. Wet mounts observed with a light microscope using bright field or phase contrast optics allows observation of the movement of smaller protists and observation of some fine structures (cilia and flagella) in living material. Internal structures such as nuclei and fine structures like cilia are often observed in fixed and stained samples. Diverse staining techniques have been developed for observation of specific structures. Rather than relying on a single method of observation, you will gain the most complete understanding of the by using multiple observation techniques.

- 1) Begin by placing a sample of field material in the lid of a Petri dish and observe under a dissecting microscope (Stereo- or Dissecting Microscopes, pg. 106).
- 2) If you observe any organisms, try transferring them to a slide to make a wet mount for microscopic observation (Making a Wet Mount, pg. 127). A drop of methylcellulose or relaxant may help slow down motile cells.
 - a. Vary the magnification, illumination, and iris diaphragm to resolve different structures using bright field microscopy.
 - b. Compare observations of a cell using bright field and phase contrast.
- 3) Mount samples directly on a microscope slide for observation under a compound microscope.
- 4) Fix and stain samples to stop movement and permit observation of internal structures and fine structures.

Methods for Observation of Protists Using a Compound Microscope

Making a Wet Mount

- 1) Place a very small drop of culture media, pond water, soil slurry etc. containing organisms in the center of a flat microscope slide with a pipette or eye dropper (Figure 82, pg. 128).

Observation Methods for Eukaryotic Microbes

- 2) For whole organisms (e.g. slime mold sporangia) or material from solid substrates such as agar plates, rotten leaves etc., place a drop of water in the center of the slide and place your sample in the water droplet.
- 3) Rest one edge of the cover slip on a dry section of the slide adjacent to the droplet.
- 4) Gently drop the coverslip onto the liquid droplet. The liquid should become completely dispersed under the coverslip.
 - a. If the liquid is not completely dispersed under the coverslip, another small droplet of media can be placed at the edge of the coverslip and will be drawn under the coverslip by capillary action.
 - b. For solid samples, you may need to tease them apart with dissecting needles prior to placing the cover slip on the sample or gently press the cover slip with the handle of a dissecting needle.
- 5) Following observation, place the slide and coverslip in a jar of 10% bleach to kill organisms.

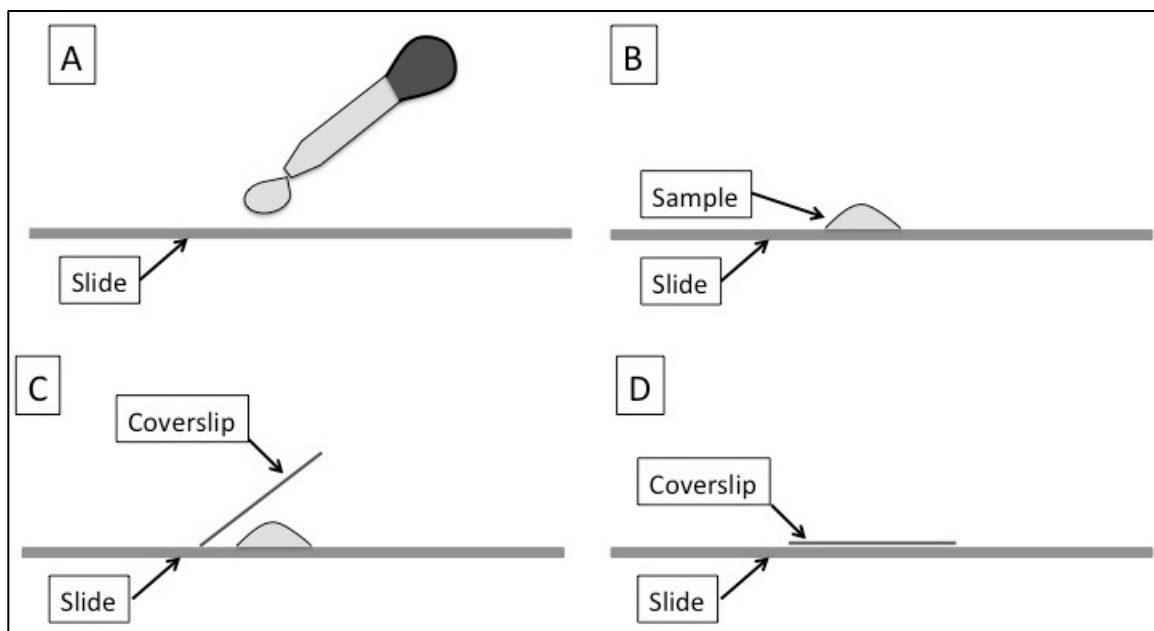


Figure 82 Making a wet mount. A and B) Place a drop of water containing organisms to be observed on the slide or place a drop of water for mounting a sample of dry material (e.g. sand or soil). C and D) Place one edge of the coverslip against the surface of the slide and gently lower onto the sample.

For Larger Organisms

- 1) Placing some shards of broken cover slips on either side of the media droplet to further raise the cover slip off the surface of the slide and create a small chamber.

Observation Methods for Eukaryotic Microbes

- 2) Following observation, place the slide and coverslip in a jar of 10% bleach to kill organisms.

To slow down fast moving protists

- 1) Place a drop of methylcellulose onto the surface of the slide and add the media containing your organisms. Methylcellulose is viscous and slows down the organisms.
- 2) Alternatively, organisms may be mounted in a solution containing magnesium chloride or cupric acetate that act as "relaxants" and reduce movement while keeping cells alive for a short period of time. Some experimentation may be required to find the right concentration of chemical relaxant that results in some movement without totally paralyzing or killing the cells.
- 3) Following observation, place the slide and coverslip in a jar of 10% bleach to kill organisms.

"Vital" Stains

"Vital" stains can be applied to living organisms and are of low enough toxicity that stained organisms will remain alive for a period of time. Organisms will eventually die but will remain alive an active long enough for observation. The type of stain, concentration of stain used and the carrier (e.g. ethanol) and type of organism will determine the amount of time organisms will remain alive when stained.

The amount of time required for effective staining of an organism will vary so it is important to keep good notes, be patient and make multiple observations of the organism over the course of the lab.

- a) to stain a wet mount:
 - i) apply a drop of stain to the edge of the cover slip and draw the stain under the coverslip with a KimWipe.
- b) to stain organisms in a freshly prepared wet mount:
 - i) place a drop of stain on a clean slide and allow the stain to air dry. Add a drop of the material to be observed to the dried stain.

Simple fixing and staining on a slide

- 1) Place a drop of fixative (Prefer, 1%-5% acetic acid), stain or fixative mixed with stain in the center of a glass slide.
- 2) Add a drop of media or water containing organisms.
- 3) Cover with a coverslip.
- 4) Stain if necessary.

Observation Methods for Eukaryotic Microbes

Stains

Stain	Stock Solution	Use / Structures Stained
Bismarck Brown ¹	0.1% in alcohol or 1.0% in alcohol diluted to 0.05-0.01% in water	Cytoplasmic inclusions
Brilliant Cresyl Blue ¹	0.1% in alcohol or 1.0% in alcohol diluted to 0.05-0.01% in water	Nucleolus
Carmine red stained yeast cells		Food vacuoles
Lugol's Iodine (neutral)	10% potassium iodide (KI), 5% g iodine (I ₂), in with distilled water	stains starch purple-black
Lugol's Iodine (neutral)	10% potassium iodide (KI), 5% iodine (I ₂) 10% acetic acid in distilled water	Fixes cells and stains starch purple-black
Janus Green B ¹	0.1% in absolute ethanol of diluted to 0.05% - 0.01% in ethanol	Mitochondria and golgi
Methylene Blue ¹	0.05% alcohol solution	nucleus, cytoplasmic granules, and cytoplasmic processes
Toluidine Blue ¹	0.1– 0.001% aqueous solution	cilia, cirri and flagella,

Table 6 Common stains for eukaryotic microbes.

Longer Term Observation

To observe organisms for longer periods of time (hours or days) you will need to make a sealed chamber under the cover slip.

- 1) Creating a chamber using Vaseline ¹

¹ Ward's Natural Science Teacher Resources, *Working with Protozoa* (<http://resources.wardsci.com/livecare/working-with-protozoa/>)

Observation Methods for Eukaryotic Microbes

- a. Trace the outline of a cover slip on the surface of a slide with a permanent marker.
- b. Remove the cover slip. With a toothpick, place a thin line of Vaseline on the coverslip outline.
- c. Place a drop of culture on the cover slip.
- d. Lower the slide onto the cover slip carefully aligning the cover slip edges with the Vaseline outline to make a seal.
- e. Invert the slide so the cover slip is on the top surface. Observe under a compound scope.
- f. Following observation, place the slide and coverslip in a jar of 10% bleach to kill organisms.

Do not get Vaseline on the microscope objectives.

- 2) Creating a chamber using silicone gum¹
 - a. Roll a small piece of silicone gum into a ball and place in the center of a slide
 - b. Wet another slide and press on the ball until it is the appropriate thickness.
 - c. Remove the wetted slide.
 - d. Using a scalpel or cork borer cut a chamber into the center of the gum.
 - e. Fill the chamber with culture media containing organisms to be observed and cover with a coverslip.
 - f. When not observing the material, store the slide in a moist chamber (a Petri dish containing a moist paper towel) to prevent the culture from drying out.
 - g. Following observation, place the slide and coverslip in a jar of 10% bleach to kill organisms.
- 3) The "hanging drop" method²
 - a. With a toothpick, place four very small drops of Vaseline on the corners of a coverslip.
 - b. Place a small drop of media or water containing organisms to be observed in the center of the coverslip.
 - c. Lower a depression slide over the cover slip, carefully aligning media droplet with the center of the depression.
 - d. Flip the slide over carefully to ensure the droplet does not move and observe organisms in the drop under the compound microscope.

² Western Michigan University, Biology 312 Microbiology Lab Procedures, *Hanging Drop*
(<http://homepages.wmich.edu/~rossbach/bios312/LabProcedures/Hanging%20drop%20procedure.html>)

Observation Methods for Eukaryotic Microbes

- e. Slides can be kept in a moist chamber to prevent drying if observations are to be conducted over hours or days.
- f. Following observation, place the slide and coverslip in a jar of 10% bleach to kill organisms.

Methods for Observation of Protists From Different Habitats

Observation of Organisms In Water Samples

Observation of organisms occurring in water samples is fairly straightforward. Many organisms in water are large enough to be easily observed with a dissecting microscope and can be very abundant; a few milliliters of pond water can contain hundreds of individual organisms. Living organisms are generally easily identified since they move. However, water samples often contain other material that can be confused with non-motile organisms (Figure 83). When observing organisms in water samples it to be sure to sample different microhabitats within the sample.

- 1) Motile organisms are not uniformly dispersed in a water sample, especially one that has been sitting for a few days. Some organisms are attracted to light and may aggregate at the surface; others will collect on the bottom. Take samples from the bottom, surface and mid-depth of your sample container.
- 2) Even non-motile organisms may not be uniformly distributed in a water sample. Some sink, others are associated with surfaces or have mechanisms to promote buoyancy.
- 3) Many types of protists are associated with surfaces, decaying or living plant material, rocks, and the interior of the container used to store the sample and aquatic animals. Observe small fragments of plant material, muck or sand settling to the bottom of a water sample. Scrape organisms off larger surfaces such as rocks or the interior of your container.

Observation of Organisms In Soil and Detritus

Observation of organisms occurring in soil or samples of organic material like leaf litter from terrestrial environments is more challenging than observation of organisms in water samples. The activity and diversity of organisms is strongly dependent on the availability of water. Although a particular organism may occur in a soil sample, if the sample is dry, the organism may be in an inactive form such as a spore or a cyst.

As well, soil is generally a complex mixture of organic and inorganic material living organisms. Spores from fungi, plants and protists as well as fungal hyphae are common in soil. For some examples of non-protist material you may find in your sample please refer to the section "Common, Non-protist Stuff You Might See Under the Microscope" (pg. 135). Direct observation of protists in soil samples using a dissecting microscope is often very difficult since the organisms are small, may not be very abundant and may occur within the sample in organic or inorganic material. Soil samples are generally too large to be mounted directly on a microscope slide.

Observation Methods for Eukaryotic Microbes

Generally soil samples are dispersed in water to release the organisms and droplets of soil slurry (pg. 134 - 134) can be observed under a dissecting or compound microscope. Initially, it is best to start with living (unfixed and stained) samples since it will be much easier to identify moving organisms against the background of soil particles if they are moving.

Preparation of Soil Slurry – Method I

- 1) Place a small amount of soil (the size of the tip of a pencil or pinhead) on a microscope slide
- 2) Add a drop of sterile distilled water.
- 3) Mix the soil and water with a dissecting needle.
- 4) Place a cover slip at the edge of the sample to draw some of the material under the cover slip.

If your sample contains a large amount of sand or other material you may not be able to find organisms easily or you may not be able to use the higher power objective² (40x and 100x) due to the thickness of the sample.

Preparation of Soil Slurry - Method II

- 1) Place ~ 10 g of soil in a 250 ml flask
- 2) Add 100 ml of sterile distilled water (1:10 dilution).
- 3) Shake or swirl for 2 minutes to remove organisms from soil particles and evenly disperse soil particles.
- 4) Pipette a sample into the lid of a Petri dish and observe under a dissecting microscope.
- 5) Make a wet mount of the soil slurry for observation under a compound microscope.
- 6) Serially dilute sample further if organisms or soil particles are too abundant (Figure 87; page 146)

Preparation of Soil Slurry - Method III

- 1) Cover the bottom of a glass dish with ~1cm of soil.
- 2) Add enough water to saturate the sample and let sit for 2 min.
- 3) Use the lid of a Petri dish to gently squeeze the water out of the soil sample.
- 4) Pipette a sample into the lid of a Petri dish and observe under a dissecting microscope.
- 5) Make a wet mount of the soil slurry for observation under a compound microscope.

Searching for Organisms in Soil Samples Using a Compound Microscope

- 1) Using the low power objectives (3.6× or 10×), scan a small part of the sample especially around bits of organic material. Look for movement or regular shapes.
- 2) If you don't see organisms, you may want to try a quick scan using phase contrast (Phase Contrast, pg. 121).
- 3) If you don't find something relatively quickly, don't waste your time with exhaustive searching just make another slide.
- 4) If you find a potential organism, observe using the higher power objectives and phase contrast.

Observation of Organisms From Surfaces (Plants, Rocks, Tree Bark etc.)

Small samples of material like rocks, decaying wood, tree bark; plants can be observed directly under a dissecting microscope. Often you can find evidence of protists (e.g. slimy films) or microhabitats that may harbor organisms (e.g. insect frass, mosses). You can scrape or remove a small sample material you think will contain protists and make a wet mount for observation using a compound microscope. Often plant material, especially aquatic plants or decaying plant material in ponds contain numerous protists. Small pieces of plant material can often be mounted on a slide for direct observation. You can search for organisms using the method outlined for soil samples (Observation of Organisms In Soil and Detritus, pg. 133).

Common, Non-protist Stuff You Might See Under the Microscope

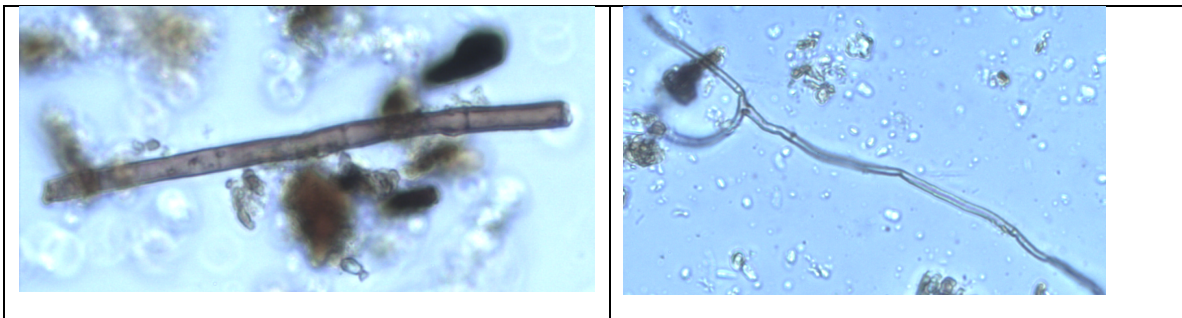


Figure 83 Fungal hyphae from soil.

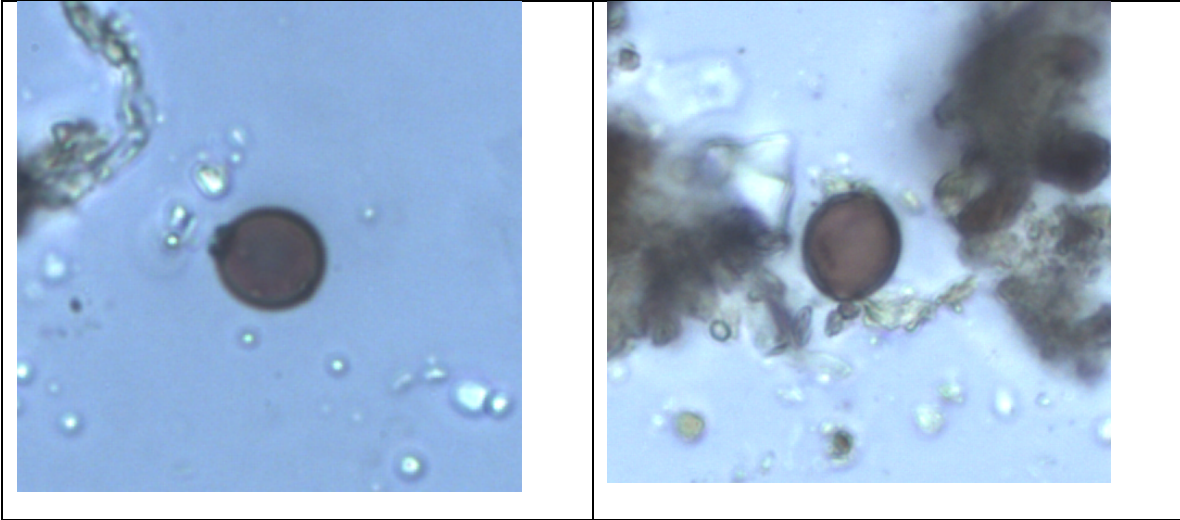


Figure 84 Spores. These are likely fungal spores but slime molds also produce small, dark-colored, smooth-walled spores. Spore walls can vary in pigmentation from clear to darkly pigmented and can also vary greatly in size and shape.

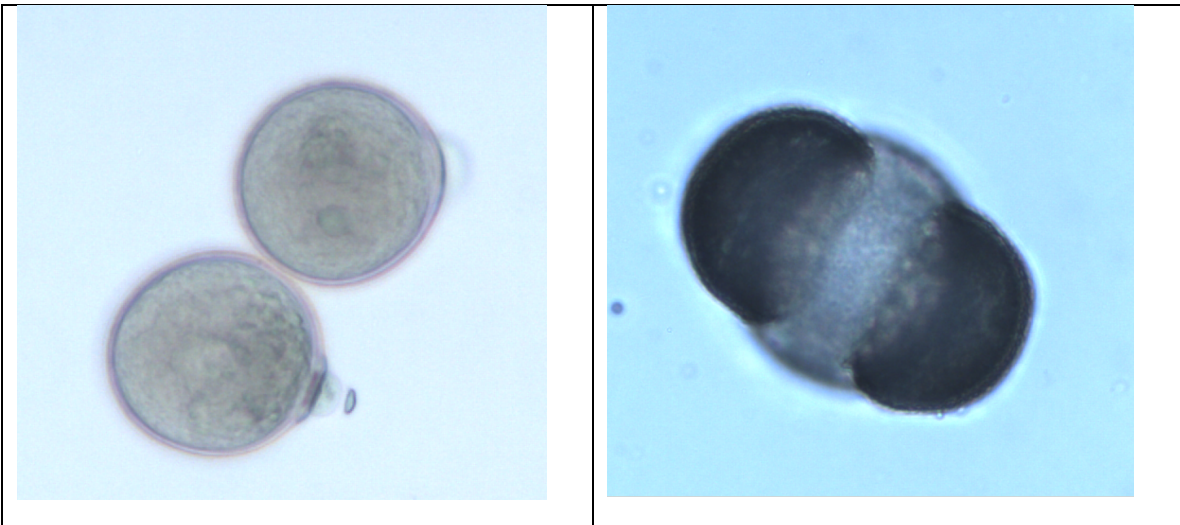


Figure 85 Grass pollen (left), pine pollen (right). Wind pollinated plants such as grasses, conifers and many trees (oaks and poplars) are wind pollinated. They produce large amounts of pollen, most of which ends up in soil and ponds.

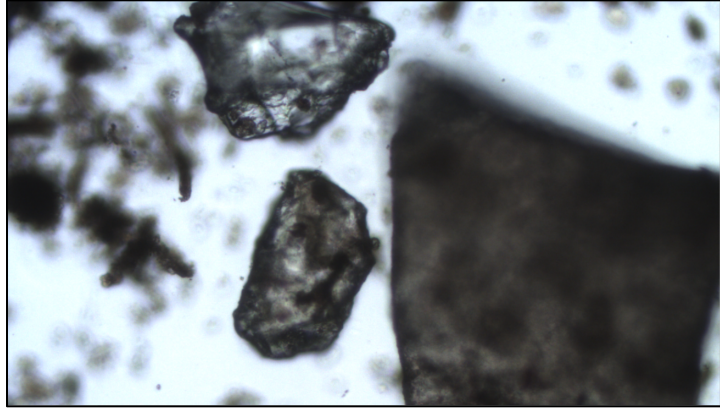


Figure 86 Sand grains.

MICROBIOLOGY METHODS

Isolation and Culturing of Eukaryotic Microbes From Environmental Samples

Culturing microorganisms is the cultivation or propagation of microorganisms in the laboratory. Culture media such as liquid broth or nutrient agar provides the environment for organisms to grow in the lab. What you will be doing is attempting to isolate a single eukaryotic organism from the mixture of organisms present in your enrichment cultures or environmental samples. Before discussing isolation methods, you need to be familiar with some basic concepts in microbiology, pure (axenic) and mixed culture.

Pure or Axenic Culture

A culture containing a single organism is known as an axenic culture. Growing an organism in axenic culture requires some knowledge of the nutritional requirements of an organism. Even with knowledge of an organism's nutritional requirements, it may not be possible to grow an organism in axenic culture. For example, many parasites need a suitable living host.

Some protozoa can be cultivated in axenic culture when supplied with the appropriate nutrients. For example, the ciliate, *Tetrahymena pyriformis* can be grown in a chemically defined media containing amino acids, nucleosides, sugar, salts, vitamins and trace metals. Photosynthetic protozoa can often be grown in axenic culture when provided with light and media containing salts and trace minerals.

Mixed Culture

Since many protozoa are predators or live in association with other organisms, they are often cultivated in a mixed culture containing one or more other organisms. For example, *Paramecium caudatum* is often grown in hay infusion media with the bacterium *Klebsiella pneumoniae*. The culture media provides nutrients for growth of the bacterium, the bacterium serve as a food source for the *P. caudatum* and also play a role in detoxifying components of the hay-infusion which would otherwise kill the Paramecium {3 Mizobuchi, N. 2003; 4 Tokusumi, Y. 2000;}. Amoeba are often grown in mixed, two-part, culture with bacteria or yeasts as food sources.

Mixed cultures may contain more complex mixtures of bacteria and protozoa. The ciliate, *Didinium nasutum*, is a predator of *Paramecium* species. Cultures could be composed of bacteria that serve as food for *P. caudatum* that in turn serves as food for *D. nasutum*.

STERILE TECHNIQUE & PRACTICES FOR THE SAFE HANDLING OF MICROORGANISMS

The material you collected in the second lab contains a complex mixture of microorganisms, both bacteria and eukaryotes. Most of these organisms are benign in terms of safety in the lab, however, a small portion can potentially pose a health risk if handled improperly. In your enrichment and isolation cultures, you are promoting the growth of a mixture of bacteria and eukaryotic microbes most of which are benign, however, since the composition of the mixture is unknown, it is possible that it contains organisms that can pose a health risk if released. Even if organisms are benign in nature, the presence of high concentrations of cells in culture can pose a health risk if released. The large number of cells present in a culture increases the chances an organism will be ingested by people in the lab or be introduced into a site on the body where they can cause an infection.

A good lab practice is to assume that all microbial cultures are potentially dangerous. Therefore, all microbial cultures are to be handled using proper sterile technique to reduce the chances of organisms being released. Basic lab safety practices should be followed to reduce the chances of infection in the event that organisms are released.

The goals of sterile technique are to 1) to keep cultures free of contamination, 2) prevent the accidental release of microorganisms and 3) isolate an organism of interest from other microbes. Sterile technique incorporates two basic principles: i) sterilization to kill contaminating organisms and ii) techniques to reduce or eliminate organisms entering or leaving a cultures or media.

Reducing or Eliminating Contaminants Entering Cultures or Media

For established cultures of microorganisms or sterile media, the main ways in which contaminants can enter cultures is airborne contaminants entering culture or media containers or having culture media or implements contact non-sterile surfaces.

Preventing Airborne Contamination

- 1) Keep media containers and culture dishes capped or closed until ready for use.
- 2) Keep media containers and culture dishes open for the minimum amount of time to reduce the chance of airborne contamination
- 3) Minimize the number of times you open the same Petri dish or culture tube

- 4) Work in an area with minimal air flow or in a transfer hood
- 5) Avoiding exhaling, coughing, sneezing etc. when culture dishes and media containers are open
- 6) Placing cotton wool in the mouths of pipettes to filter out contaminating organisms during pipetting
- 7) Sterilize the surface of the work area with 70% ethanol or 10% bleach. This reduces but does not completely eliminate organisms on your work surface.

Preventing Contact With Non-sterile Surfaces

- 1) Keep sterilized equipment (e.g. pipettes) wrapped until they are used
- 2) Do not touch the parts of equipment that contact media or cultures (e.g. pipette tips)
- 3) Keep parts of equipment that contact media or cultures from contacting non-sterile surfaces (e.g. the bench-tops, the outside of culture tubes, your hands etc.)
- 4) Gently flame the openings of glass flasks or culture tubes after opening and before closing
- 5) Assume an item or solution is sterile only if it is labeled as sterile and is unopened
- 6) Once a sterile object or solution comes in contact a non-sterile item; it is no longer sterile

Biosafety

One of the important goals of sterile technique is to prevent the accidental release of microorganisms. This has important implications for your safety. Accidental release of microorganisms could result in you becoming infected or injured by the microorganisms you are handling in the lab. You don't want to end up eating organisms you picked up in the lab along with your lunch.

- 1) Wash your hands thoroughly with soap before leaving the lab.
- 2) Use proper sterile technique when handling any microorganism
- 3) Dispose of microbial waste properly (see below)
- 4) Clean up spills properly.

Disposal of Microbial Waste

- 1) Used Microscope slides are sterilized in 10% bleach for at least 10 minutes prior to washing.
- 2) Plastic tips, tubes and pipettes used to handle cultures and media must be autoclaved before disposal. Place all contaminated tips and tubes in a designated solid waste container for autoclaving.

- 3) All microbial cultures must be autoclaved using a kill cycle before disposal.
 - a. Cultures on agar plates must be placed in the designated solid waste container for autoclaving
 - b. Place used liquid media in labeled waste containers for autoclaving
 - c. Place test tubes and flasks containing liquid cultures on labeled trays for autoclaving

Spills

If you spill small amounts (10 to 100 mL) of a liquid culture containing microorganisms:

- 1) Put on gloves
- 2) Carefully mop up the spilled culture with paper towels
- 3) Dispose of paper towels in the waste container for autoclaving
- 4) Spray 10% bleach disinfectant on and around the contaminated area
- 5) Wait 10 minutes for the disinfectant to kill the organisms and wipe up disinfectant with paper towels
- 6) Dispose of paper towels in the waste container for autoclaving

CULTURING OR ENRICHING EUKARYOTIC MICROBES FROM ENVIRONMENTAL SAMPLES

Enrichment Culture

One of the most basic methods for cultivation of microorganisms (eukaryotic and prokaryotic) is the enrichment culture method. A sample of water, soil etc. is collected and conditions are established and maintained long enough that one or a few types of organisms dominate the culture. The dominant organisms can be studied or isolated from the other organisms. A number of variations on the enrichment culture theme can be employed.

A standard method is to inoculate (introduce cells into) liquid media with an environmental sample (e.g. a few drops of pond water) and allow the culture to develop over a period of time (a few days to weeks). Regular transfer to fresh media will generally result in cultures containing one to a few species often with associated bacteria. Nutrients are often provided by infusions of plant or animal material. Infusions are made by boiling material such as hay and filtering out the material leaving behind a solution of soluble material from the hay. The nutrients can be used by organisms directly or can serve as a food source for prey such as bacteria that are then consumed by predators. Over time, the number of species present in the culture will generally decline. For photosynthetic organisms, enrichment may take the form of limiting the input of organic carbon but providing light for photosynthesis.

Microbiology Methods

You will have a choice of five possible enrichment media (75 mL of media in 150 mL flasks). With your group, you will inoculate two flasks of enrichment media. The media type and field sample you use as inoculum do not need to be the same.

- 1) Sterile, enriched artificial seawater medium (ESAW)³ or Sterile enriched seawater medium.
 - a. A seawater medium generally lacking organic carbon compound to select for autotrophs (i.e. photosynthetic organisms) made with either natural or artificial seawater.
- 2) Sterile enriched artificial seawater medium plus
 - a. To ~100ml of ESAW medium, add
 - i. 1-2 sterile hemp seeds or
 - ii. 1-2 sterile, cooked rice grains or
 - iii. 1-2 sterile oat flakes or
 - iv. 3-4 sterile sesame seeds.
- 3) Bacterized artificial seawater medium.
 - a. A seawater medium containing seaweed infusion that has been colonized by bacteria. The bacteria serve as a food source for predators such as ciliates.
- 4) Sterile Bristol medium (for cultivation of freshwater alga)
 - a. A simple freshwater medium lacking organic carbon compounds to select for autotrophs (i.e. photosynthetic organisms).
- 5) Sterile weak hay infusion (0.5%)
 - a. A simple freshwater medium where organic carbon compounds are limiting that could be used to cultivate either autotrophs or heterotrophs.
- 6) Bacterized hay infusion (1/4 strength hay infusion inoculated with *Klebsiella pneumoniae*)
 - a. A medium to select for predators that consume bacteria such as ciliates (e.g. *Paramecium*)

Inoculating The Enrichment Medium

³ Harrison, P.J., R.E. Waters and F.J.R. Taylor. 1980. A broad spectrum artificial medium for coastal and open ocean phytoplankton. J. Phycol. 16:28-35.

Microbiology Methods

- 1) Choose two flasks of media appropriate to the type of environment you sampled and the type(s) of organisms you would like to culture. In your notes indicate what media was chosen and why.
- 2) Choose one or two of your field samples for enrichment.
- 3) Label the flask containing the culture medium with:
 - a. The date
 - b. A unique sample number that corresponds to the number in your collection information.
 - c. Your name
- 4) For aqueous samples, add 1 mL of your sample to 75 mL of your chosen enrichment medium.
- 5) For solid substrates (shells, sand, soil etc.).
 - a. Suspend 1 g of material in 10 ml of sterile water (either seawater or distilled water).
 - b. Swirl for 2 minutes to suspend the organisms or spores.
 - c. Allow large particles to settle for 30 seconds.
 - d. Add 1 mL of your slurry to 75 mL of your chosen enrichment medium.
- 6) Choose incubation conditions and place your culture flask in the appropriate incubator.
 - a. To cultivate photosynthetic autotrophs, flasks must be incubated under lights.
 - b. To exclude organisms that are obligate photosynthetic autotrophs, flasks must be incubated in the dark or wrapped in foil to exclude light.
- 7) Next week you will observe your enrichment culture for evidence of eukaryotic microbes and transfer a sample of your enrichment culture to fresh media.

Bulk Transfer of Enrichment Culture to Fresh Media

- 1) Obtain a flask of the same type of media used in the first enrichment step last week (i.e. if you started your culture in enriched seawater medium then you will continue to use enriched seawater medium).
- 2) Label the flask containing the culture medium with:
 - a. The date
 - b. A unique sample number that corresponds to the number in your collection information and indicates that this is a subculture.
 - c. Your name
- 3) Swirl your first enrichment culture to evenly disperse organisms.
- 4) Loosen the foil caps on the enrichment culture and the new media you will be inoculating.
- 5) Using a sterile pipette, add 1 mL of your enrichment culture to 75 mL of fresh enrichment medium.
- 6) Place the foil caps back on both flasks.
- 7) Dispose of the pipette in the designated waste container.

- 8) Place both flasks back in the appropriate incubator.
 - a. To cultivate photosynthetic autotrophs, flasks must be incubated under lights.
 - b. To exclude organisms that are obligate photosynthetic autotrophs, flasks must be incubated in the dark or wrapped in foil to exclude light.
- 9) Next week you will observe your enrichment culture again.

Direct Isolation and Serial Dilution

Depending on the size of the organisms present in your enrichment culture you will choose use either the direct isolation or serial dilution methods for trying to establish a culture from a single cell. If organisms in your enrichment culture are large and are easily seen with a dissecting microscope, you can try the direct isolation procedure (page **Error! Bookmark not defined.**). If organisms are present but can only be viewed with your compound microscope, you will need to use a serial dilution method to establish a culture (page **Error! Bookmark not defined.**)

Direct Isolation

This technique involves directly removing a cell or spore from a mixture of organisms, rinsing it in sterile media or water to remove contaminants and inoculating fresh culture medium with the cell. Ideally, the cell will begin dividing and establish a population of cells that can be studied. The culture can then be propagated but regular transfer to fresh medium. For organisms in your enrichment culture, individual cells can be captured with a sterile Pasteur pipette and washed by transferring through small aliquots of sterile media in a sterile glass depression tray.

If large organisms are present in your original environmental samples, you could attempt direct isolation from your original sample following the procedure below.

Direct Isolation

- 1) View a sample of your enrichment culture or water samples from last week under the stereomicroscope (do not use the freshly inoculated culture). If cells are relatively large, you should be able to use the direct isolation method. With a lot of practice you should be able to isolate smaller cells.
- 2) Obtain two test tubes of sterile media of the same type used in your enrichment culture.
- 3) Label one test tube with:
 - a. The date
 - b. A unique sample number that corresponds to the number in your collection information and indicates that this is a subculture.

c. Your name

- 4) Obtain a sterile glass depression tray and transfer ~ 1mL of sterile medium from the unlabeled test tube to each well in the depression tray using a sterile Pasteur pipette.
- 5) With a fresh, sterile Pasteur pipette take a small sample of your enrichment culture and place in the well of a depression slide.
- 6) Discard the Pasteur pipette in the appropriate waste container.
- 7) Observe the organisms in the depression slide using your stereomicroscope.
- 8) With a fresh, sterile Pipette attempt to draw up media containing one or a few cells into the pipette.
- 9) Place the cell in the first well of the depression tray containing sterile media.
- 10) Discard the Pasteur pipette in the appropriate waste container.
- 11) With a fresh pipette, attempt to draw up a single cell and small amount of media and transfer to the second well in the depression tray.
- 12) Discard the Pasteur pipette in the appropriate waste container.
- 13) With a fresh pipette, attempt to draw up a single cell and small amount of media and transfer the cell to the third well in the depression tray.
- 14) Discard the Pasteur pipette in the appropriate waste container.
- 15) With a fresh pipette, attempt to draw up a single cell and small amount of media and transfer the cell to the labeled tube containing fresh media.

Dilution Methods

At this stage your enrichment culture likely contains a complex mixture of microorganisms, both eukaryotic and prokaryotic. If the organisms present in your sample are small, direct isolation may not be feasible until you have a lot more practice. The basis of dilution methods is to take a sample and perform a series of dilutions (serial dilutions) to reduce the concentration of organisms to a point where a sample used to inoculate a fresh culture contains only a single cell. For the purposes of this lab you will not be trying to eliminate bacterial contamination so ideally what you will really have is a single eukaryotic cell and lots of prokaryotic cells since they are generally more numerous.

Serial Dilution Method

- 1) View a sample of your enrichment culture from last week under the stereomicroscope (do not use the freshly inoculated culture).
 - a. If cells are relatively large, you should be able to use the direct isolation method (page **Error! Bookmark not defined.**).
 - b. If cells are not apparent, make a wet mount and view the sample using your compound microscope. For small, relatively transparent cells using phase contrast will help you pick out cells from the background material.

Microbiology Methods

- 2) Get a flask of sterile liquid medium of the same type you used for enrichment.
- 3) Choose a dilution factor. If organisms are not very abundant in your sample, you may use a $5\times$ dilution factor rather than a $10\times$.
- 4) Aliquot sterile media into a set of 4 sterile test tubes using a sterile pipette (4 mL for a $5\times$ dilution series and 4.5 mL for a $10\times$ dilution series). Label each tube with:
 - a. The date
 - b. A unique sample number that corresponds to the number in your collection information and indicates that this is a subculture.
 - c. Your name
 - d. The dilution factor
- 5) Swirl your enrichment culture to evenly distribute organisms.
- 6) For a $5\times$ dilution series use a sterile pipette to transfer 1 mL of your enrichment culture into the first test tube. For a $10\times$ dilution series, transfer 0.5 mL of enrichment culture.
- 7) Gently vortex the sample to ensure complete mixing.
- 8) For a $5\times$ dilution series use a sterile pipette to transfer 1 mL from the 1:5 dilution to the next tube (Figure 87, page 146). For a $10\times$ dilution series, transfer 0.5 mL of the 1:10 dilution.
- 9) Repeat steps 7 and 8 until you have used all 4 tubes.
- 10) Incubate the test tubes under the same conditions as the original enrichment culture.

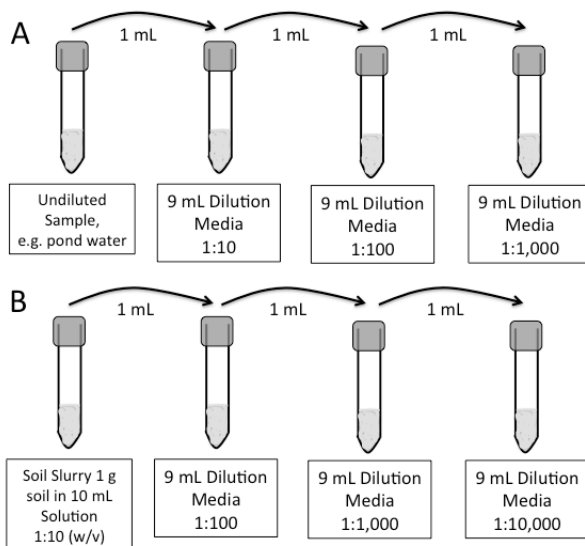


Figure 87 Preparing a 1:10 serial dilution of a water sample (A) or a soil suspension (B). Note that for a soil sample, suspending the soil in liquid constitutes the first dilution step. For less concentrated samples a dilution series of 1:5 or less may be appropriate.

AMOEBOZOA ISOLATIONS

Slime Mold Isolations

At the beginning of the lab, your group will be assigned one organism or method of isolation for amoeba or slime molds. You will be observing the results of these isolations next week.

Isolation From Spores

Some eukaryotic microbes, most notably plasmodial slime molds [Amoebozoa; Mycetozoa; Myxogastria], produce spores on macroscopic fruiting bodies. The fruiting bodies can be collected in the field and brought to the lab. The plasmodial slime molds, *Fuligo* and *Physarum*, produce spores that germinate to produce haploid flagellate cells or amoeba (refs XXX). These cells can act as gametes that fuse to produce a diploid cell which goes on to produce a large, multinucleate plasmodium.

Prior to lab, spores have been germinated on ½ strength cornmeal agar plates. In a wet mount, flagellate cells should be visible. You will be inoculating these flagellate cells onto an appropriate nutrient source so mating and plasmodium formation can take place. Next week you will be observing the resulting plasmodium.

Isolation of Slime Molds From Spores on Fruiting Bodies

Materials

- Sterile Pasteur pipette
- 4 Water agar plate
- Sterile oat flakes
- Fine forceps
- Alcohol lamp
- Jar of ethanol (95%)
- Lighter
- Spray bottle of ethanol (70%)
- Paper towel

Isolation From Spores - Method

- 1) Sterilize the surface of your bench by spraying a small amount of ethanol on the bench and wiping with a paper towel.
- 2) Allow remaining ethanol to evaporate.
- 3) Light your alcohol lamp.

Amoebozoa Isolations

- 4) Dip your forceps in the jar of 95% ethanol and pass through the flame of the alcohol lamp.
- 5) Wait until the alcohol burns off the forceps.
- 6) Transfer 4-5 oat flakes to the surface of the water agar plate.
- 7) With a sterile Pasteur pipette. Take up ~0.5 mL of water containing flagellate cells of *Fuligo* or *Physarum* and pipette a few drops onto each of the oat flakes until just wetted. Do not add so much liquid that the surface of the plate is flooded.
- 8) Discard the pipette in the appropriate waste container.
- 9) Seal the plate with Parafilm®.
- 10) Inoculate a total of 4 plates.

Induction of Fruiting Body Formation

If fruiting bodies (structures involved in spore production) are not present due to unfavorable environmental conditions, slime molds in environmental samples can sometimes be induced to produce fruiting bodies by incubation of material containing vegetative (assimilative) cells in a moist chamber.

Inducing Fruiting Body Production in a Moist Chamber

Materials

- Leaf litter, decaying wood, compost or organic soil (collected by instructor of TAs)
 - Forceps
 - Scoopula or spoon
 - 4 Sterile crystallizing dishes lined with filter paper disks
 - Alcohol lamp
 - Jar of ethanol (95%)
 - Lighter
 - Spray bottle of ethanol (70%)
 - Paper towel
 - Sterile distilled water
- 1) Sterilize the surface of your bench by spraying a small amount of ethanol on the bench and wiping with a paper towel.
 - 2) Allow remaining ethanol to evaporate.
 - 3) Obtain containers of organic material (leaf litter, decaying wood, compost or organic soil).
 - 4) Light your alcohol lamp.
 - 5) Dip your forceps or scoopula in the jar of 95% ethanol and pass through the flame of the alcohol lamp.
 - 6) Wait until the alcohol burns off the forceps or scoopula.

Amoebozoa Isolations

- 7) With the sterile forceps, fill the crystallizing dish $\frac{1}{3}$ to $\frac{1}{2}$ full with the organic material.
- 8) Add enough water to cover the material. If the material floats, use some sterile slides to weight it down.
- 9) Cover the dish.
- 10) Dip the forceps or scoopula in the jar of 95% ethanol and flame sterilize.
- 11) Repeat for the remaining dishes.
- 12) Your instructor will pour off the water after the sample has soaked overnight.

Isolation of Cellular Slime Molds [Amoebozoa; Mycetozoa; Dictyosteliida]

For some many organisms, specific isolation or culturing methods have been developed that select for the organism of interest. Kenneth Raper, a mycologist, microbiologist and botanist, developed a number of simple methods for isolating *Dictyostelium* and related slime molds from environmental samples.

Isolation of Cellular Slime Molds From Soil Based on Raper (1951)

Materials

- Soil, leaf litter compost or organic soil (collected by instructor of TAs)
- Forceps
- Scoopula or spoon
- Sterile 150 mL flask containing 50 mL of sterile distilled water
- 4 petri dishes containing $\frac{1}{4}$ strength hay infusion agar
- *Klebsiella pneumoniae* culture
- Alcohol lamp
- Jar of ethanol (95%)
- Lighter
- Spray bottle of ethanol (70%)
- Paper towel
- Sterile distilled water
- Electronic balance
- Weight boat
- Sterile distilled water
- Sterile test tubes
- Bent glass rod
- Pipettes

Amoebozoa Isolations

- 1) Obtain samples of soil or organic material.
- 2) Weight out 5 g of soil and place in a sterile 150 mL flask containing 50 mL of distilled water.
- 3) Swirl for 2-3 minutes to break up soil or organic particles and suspend spores or amoeba in the water.
- 4) Make 1:5, 1:10, 1:20 and 1:40 dilutions of the soil suspension in sterile distilled water. For instructions on making serial dilutions, please refer to the methods presented in Lab 4.
- 5) Light your alcohol lamp.
- 6) Label each of your Petri plates containing $\frac{1}{4}$ strength hay infusion with the date, your name and the dilution factor (1:5, 1:10, 1:20 and 1:40)
- 7) Using a sterile pipette, place 0.25 mL (250 μ L) of bacterial culture in the center of each $\frac{1}{4}$ hay infusion agar plate.
- 8) Dip the bent glass rod in 95% alcohol and pass through the flame of the alcohol lamp.
- 9) Wait until the alcohol burns off the glass rod and allow the rod to cool for 10 seconds.
- 10) Touch the rod to the surface of the agar and spread the bacterial culture over the surface of the plate
- 11) Pipette 0.75 mL of the diluted soil suspensions into the middle of the appropriate agar plate containing bacteria.
- 12) Flame sterilize the glass spreader and spread the soil slurry over the surface of the agar plate as in steps 8-10.
- 13) Place the lid on the Petri dish and seal with Parafilm.
- 14) Incubate agar side down for 1 week.

Spot Plate Method For Isolation of Amoeba

The ability to move can be used as a convenient method for isolation of organisms from complex mixtures. Amoeba in solid substrates like soil or sand samples can migrate out of a sample and onto the surface of an agar plate leaving behind non-motile organisms or slower moving organisms.

Spot Plate Method

Materials

- Soil, leaf litter compost or organic soil (collected by instructor of TAs)
- Forceps
- Scoopula or spoon
- Sterile 150 mL flask containing 50 mL of sterile distilled water
- 4 petri dishes containing water agar (non-nutrient agar)
- Alcohol lamp
- Jar of ethanol (95%)
- Lighter

Amoebozoa Isolations

- Spray bottle of ethanol (70%)
 - Paper towel
 - Electronic balance
 - Weight boat
 - Pipettes
- 1) Obtain samples of soil or organic material.
 - 2) Weight out 5 g of soil and place in a sterile 150 mL flask containing 50 mL of distilled water.
 - 3) Swirl for 2-3 minutes to break up soil or organic particles and suspend spores or amoeba in the water.
 - 4) Label each of your Petri plates containing water agar with the date and your name.
 - 5) Draw a grid on the bottom of the petri plate with a felt pen (Figure 88A).
 - 6) Take a sample with a sterile Pasteur pipette and place six small drops on the surface of the water agar as shown in Figure 88B.
 - 7) Handle the plate gently to avoid disturbing the drops.
 - 8) Allow liquid droplets to dry and carefully seal the plates with Parafilm.
 - 9) Incubate agar side down for 1 week.

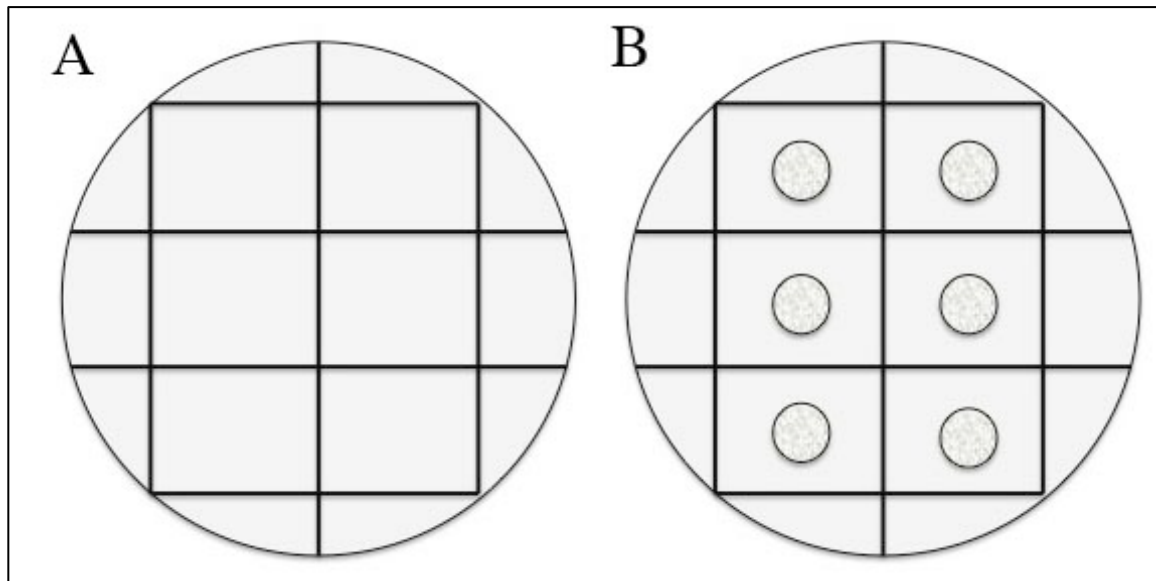


Figure 88 Drawing a grid and spotting soil slurry on a Petri plate with water agar.

MEDIA RECIPES

ARTIFICIAL AND ENRICHED SEAWATER MEDIUM

Artificial Seawater

- 1) Make up two separate salt solutions (I and II) following the instructions below.
- 2) Once all salts have dissolved add solution II to solution I with continuous stirring.
- 3) Transfer the solution to a graduated cylinder and bring volume up to 1 L with distilled deionized water.
- 4) This recipe results in a final salt concentration of 35.8316 parts per thousand (ppt. or ‰). To adjust the salinity to match sampling sites in estuaries or other coastal regions with lower salinities, dilute the stock solution to the desired salinity. For examples see Table 9 (pg. 154).

Solution I

- 1) Add 500 mL of distilled deionized water to a 1 L flask.
- 2) Place a stir bar in the bottom of the flask and set on medium speed.
- 3) Add each of the salts from Table 7 (pg. 153) in order and allow each of the salts to dissolve completely before adding the next salt.

Salt	Molecular Weight (g/mol)	g/L of Solution (Final Volume)	Final Concentration (mM)
NaCl	58.44	20.756	362.7
Na ₂ SO ₄	142.04	3.477	25.0
KCl	74.56	0.587	8.03
NaHCO ₃	84	0.17	2.067
KBr	119.01	0.0845	0.725
H ₃ BO ₃	61.83	0.022	0.372
NaF	41.99	0.0027	0.0657

Table 7 Solution I for making artificial seawater.

Solution II

- 1) Add 200 mL of distilled deionized water to a 500 mL flask.
- 2) Place a stir bar in the bottom of the flask and set on medium speed.
- 3) Add each of the salts from Table 8 (pg. 154) in order and allow each of the salts to dissolve completely before adding the next salt.

Media Recipes

Salt	Molecular Weight (g/mol)	g/L of Solution (Final Volume)	Final Concentration (mM)
MgCl ₂ · 6H ₂ O	203.33	9.395	47.18
CaCl ₂ · 2H ₂ O	147.03	1.316	9.134
SrCl ₂ · 6H ₂ O	266.64	0.0214	0.082

Table 8 Solution II for making artificial seawater.

Volume of Artificial Seawater (35.8316 ‰)	Final Volume	Final Salinity (‰, ppt)
837.2 mL	1 L	30‰
697.7 mL	1 L	25‰
558.2 mL	1 L	20‰
418.6 mL	1 L	15‰

Table 9 Dilution of artificial seawater stock to adjust salinity

METRIC UNITS AND CONVERSIONS

INTERNATIONAL SYSTEM (SI) UNITS AND CONVERSION FACTORS

Metric / SI Base Units

Unit	Abbreviation	Type of Measurement	Definition ⁴
Meter	M	Length	The distance light travels in 1/299,792,458 s.
Kilogram	Kg	Mass	The mass of a prototype kilogram; for the purpose of conversions and calculations the unit of grams is the base.
Mole	Mol	Amount	The amount of substance containing as many atoms, molecules, ions, electrons or other particles, as there are atoms in 0.012 kg of carbon 12.
Second	S	Time	The time it takes for 9,192,631,770 oscillations between the two ground state levels of a cesium 133 atom.
Kelvin	K	Absolute Temperature	1/273.16 of the temperature of the triple point of water.
Ampere	A	Electrical Current	The constant current required to maintain a force of 2×10^{-7} Newton per meter between two ideal, straight parallel conductors in a vacuum.
Candela	Cd	Luminosity	The light intensity from a source emitting a single wavelength of radiation of 540×10^{12} hertz of 1/683 watt per steradian (.

Table 10 Metric/SI base units, abbreviations and definitions from the Bureau International des Poids et Mesures (BIPM). http://www.bipm.org/en/si/base_units/

⁴ Definitions taken from the website for the Bureau International des Poids et Mesures (BIPM) website http://www.bipm.org/en/si/base_units/

Non-SI Units

Quantity	Name of Unit	Symbol	Value in SI Units
Time	Minute	min	1 min = 60s
	Hour	h	1 h = 60 min
	Day	d	1 d = 24 h
Plane angle	Degree	°	1° = (1/180) rad
	Minute	'	1' = (1/60)°
	Second	"	1" = (1/60)'
Area	Hectare	ha	1 ha = 10 ⁴ m ²
Volume	Liter	L	1 L = 10 ³ cm ³
Mass	Tonne (ton)	t	1 t = 10 ³ kg

Table 11 Non-SI units accepted for use with the international system of units.

Metric / SI Prefixes:

Abbreviation	Prefix	Conversion Factor
M	mega	10 ⁶
K	kilo	10 ³
Base Unit		1
d	deci	10 ⁻¹
c	centi	10 ⁻²
m	milli	10 ⁻³
μ	micro	10 ⁻⁶
n	nano	10 ⁻⁹
p	pico	10 ⁻¹²
f	femto	10 ⁻¹⁵

Table 12 Metric/SI prefixes and conversion factors.

Sequential Conversions

Abbreviation	Prefix	Conversion Factor	Sequential Conversions
Mg	mega	1 Mg = 1×10^6 g	1 Mg = 1000 Kg
Kg	kilo	1 Kg = 1×10^3 g	1 Kg = 1000 g
gram		Base Unit	
dg	deci	1 dg = 1×10^{-1} g	1 dg = 1/10 g = 0.1 g
cg	centi	1 cg = 1×10^{-2} g	1 cg = 1/10 dg
mg	milli	1 mg = 1×10^{-3} g	1 mg = 1/10 cg
μg	micro	1 μg = 1×10^{-6} g	1 μg = 1/1000 mg
ng	nano	1 ng = 1×10^{-9} g	1 ng = 1/1000 μg
pg	pico	1 pg = 1×10^{-12} g	1 pg = 1/1000 ng
fg	femto	1 fg = 1×10^{-15} g	1 fg = 1/1000 pg

Table 13 Example sequential conversions for weight measurements with metric/SI prefixes.

Performing Unit Conversions

When performing unit conversions it is best to write out your formula long hand and make sure all units cancel. If you do not write out complete formulas, you are more likely to make errors in your calculations, please see the examples "Equation 6 to Equation 8 (pg. 157).

$$\frac{11.8 \text{ ug}}{\text{mL}} \times \frac{1 \text{ mL}}{1000 \text{ uL}} \times \frac{1000 \text{ ng}}{1 \text{ ug}} = 11.8 \text{ ng/uL}$$

Equation 6 Example unit conversion (11.8 μg/mL to ng/μL) showing all units.

$$\frac{176.8 \text{ ng}}{\text{mL}} \times \frac{1 \text{ mL}}{1000 \text{ uL}} = 0.1768 \text{ ng/uL}$$

Equation 7 Example unit conversion (176.8 ng/mL to ng/μL) showing all units.

$$0.0003 \text{ M} \times \frac{1000 \text{ mM}}{1 \text{ M}} \times \frac{1000 \text{ uM}}{\text{mM}} = 300 \text{ uM}$$

Equation 8 Example unit conversion (0.0003 M to μM) showing all units.

Common Unit Conversions

Length

1 inch = 2.54 centimeters (cm)

1 foot = 0.3048 meters (m)

1 mile \approx 1.61 kilometers (km)

1 angstrom = 1×10^{-10} meters (m)

Volume

1 cubic centimeter (cc) = 1×10^{-6} cubic meter (cu m)

1 cubic centimeter (cc) = 1 milliliter (mL)

1 fluid ounce (oz) \approx 29.6 milliliters (mL)

Weight or Mass

1 metric ton (t) = 1000 kilograms (kg)

1 pound (lb) \approx 0.45 kilograms (kg)

Avogadro's number = 6.022×10^{23} = number of molecules of a substance that make up a mass in grams equal to its molecular mass

1 mole (mol) = mass of a substance in grams equal to its molecular mass

1 mole = 6.022×10^{23} molecules

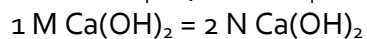
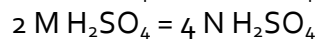
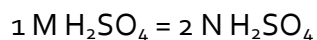
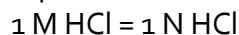
Concentration

Molarity (M) = moles of solute per liter of solvent (mol/L)

Molality (m) = moles of solute per kilogram of solvent (mol/kg)

Normality (N) = Molarity (M) \times number of protons or hydroxide ions per molecule

For example:



Time

1 year = 365 days (except during leap years)

1 day = 24 hours

1 hour = 60 minutes

1 minute = 60 seconds

Metric Units and Conversions

Temperature

$$^{\circ}\text{C} = (5/9) * (^{\circ}\text{F} - 32)$$

$$^{\circ}\text{F} = (1.8 * ^{\circ}\text{C}) + 32$$

$$\text{K} = ^{\circ}\text{C} + 273.15$$

$$^{\circ}\text{C} = \text{K} - 273.15$$

Water freezes at 0°C and boils at 100°C

Water freezes at 32°F and boils at 212°F

Water freezes at 273.15 K and boils at 373.15 K

BIOL 203 – Lab 2 – Finding a New Species Project Part 1: Sampling