Working with Bacteria and Fungi

5100 West Henrietta Rd • PO Box 92912 • Rochester, NY 14692-9012 • p: 800 962-2660 • f: 800 635-8439 • wardsci.com

ward's science

Working with Bacteria and Fungi

Introduction

Bacteria

Bacteria are the most numerous and, arguably, the most successful organisms on the planet. They are found virtually everywhere. Their diverse sources of nutrition, rapid rates of reproduction, and adaptive abilities have enabled them to survive successfully in almost every habitat. From the arctic tundra to the near-boiling hot springs, from the depths of the ocean to the internal organs of other living things, bacteria have thrived since the very earliest beginnings of life on Earth.

Bacteria are in the Kingdom Monera. Monerans are prokaryotes–organisms that have a nucleoid and mitochondria. They have DNA chromosomes and are very rarely multi-cellular. Most bacteria are very small, ranging in size from 1 – 10 microns, and their metabolism is highly variable, unlike the "standard" metabolic oxidation modes of higher organisms.

There are two major groups in the Kingdom Monera: the Archaebacteria and the Eubacteria. As the name suggests, the Archaebacteria are an "ancient" form that evolved from the bacteria that first existed several billion years ago, when conditions were not conducive to any other type of life. Archaebacteria are unique in their chemical makeup and the structure of their DNA and include the methane-producers that break down organic matter and produce methane gas. "Salt-loving", or halophilic, bacteria that inhabit the Great Salt Lake and the Dead Sea are another group of Archaebacteria. A third group of Archaebacteria is adapted to thrive in the waters of hot springs at temperatures up to 90°C. This group has also been found in the depths of the oceans in areas around thermal plumes.

Eubacteria include both aerobic and anaerobic forms. Two of the major groups are the Cyanobacteria and the Schizophyta. The Cyanobacteria are the "blue-green algae". They are autotrophic and photosynthetic, but unlike plants, their chlorophyll is in the cytoplasm, not in discreet chloroplasts. Cyanobacteria contain chlorophyll *a* and a blue pigment, phycocyanin, which together give them their namesake color. They also contain other pigments, and when abundant, they can color the water in which they grow. The Red Sea and the Black Sea owe their names to "blooms" of Cyanobacteria.

Schizophyta are the largest groups of heterotrophic Eubacteria. Their classification is based on the Gram test, a staining method developed by a Danish doctor. Crystal violet stain is retained by the cell walls of certain bacteria. The cell walls of other bacteria lose the crystal violet complex by decolorization and are then counter stained. Bacteria that are stained appear purple under the microscope, and are termed Gram-positive. Those that do not take up the stain appear light pink and are termed Gram-negative.

Bacteria are also identified on the basis of their shape. Positive determination must be made by biochemical analysis. The three major bacterial forms are readily identified under the microscope. Spherical bacteria (cocci) may grow close together but do not link. Some species are causative agents of diseases such as strep throat, pneumonia, and gonorrhea. Rod-shaped bacteria (bacilli) includes species that cause anthrax and tetanus, while the spiral-shaped bacteria (spirilli) include species causing cholera and syphilis. However, very few of the large numbers of bacterial species are pathogenic, since the majority are beneficial members of Earth's community, breaking down wastes, recycling nutrients, and enabling higher organisms to digest their food.

Fungi

The Fungi Kingdom includes spore-forming eukaryotes that lack undulipodia (amastigote) at all stages of their life cycle, but some taxonomists include chytrids and oomycetes, which are undulipodia, in the Kingdom.

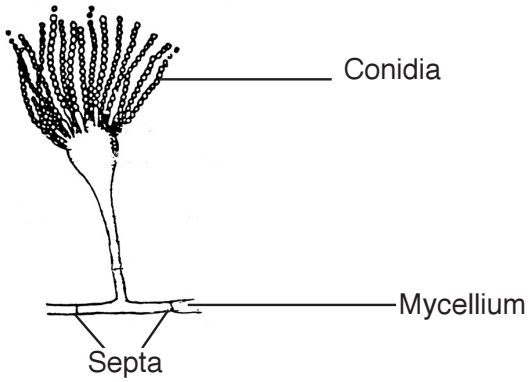
Almost all fungi are aerobic, and all fungi are heterotrophs, which means that they absorb their food without ingesting it. Fungi secrete powerful digestive enzymes that break down the food material, usually decaying plant or animal matter, into compounds that can be absorbed through the fungal membrane.

5100 West Henrietta Rd • PO Box 92912 • Rochester, NY 14692-9012 • p: 800 962-2660 • f: 800 635-8439 • wardsci.com

Fungi are abundant in most terrestrial habitats. There are about 100,000 known species, a few of which are marine. Because of their abundance and diversity, there are likely many more species to be discovered and described.

Reproduction in fungi is generally asexual and occurs through vegetative spores (conidia). These spores are distributed by wind and water, and are highly resistant to adverse environmental conditions. Under favorable conditions they will germinate and initiate the growth of a new organism. The germinating spore produces a thin, tubular structure called a hypha. Cross walls called septa divide the hypha into "cells", though the septa rarely completely enclose an entire portion of the hypha. Some hyphae lack septa entirely. Hyphae grow in large masses called mycelia, which constitutes the vegetative body of the fungus. The cell walls of most fungi are composed of chitin, similar to that found in insects, which is very impervious to desiccation, thus enabling the fungi to survive under harsh conditions (fig. 1).

Figure 1



Sexual reproduction can also occur in fungi. This occurs when hyphae of opposite mating types grow together and fuse. The haploid hyphal nuclei grow and undergo subsequent division, but they remain in pairs, one nucleus from each of the two parental hyphae. Such hyphae are called dikaryotic. Nuclear fusion occurs in time, forming diploid zygotes. The zygote then immediately undergoes meiosis to form haploid spores that are distributed in the same manner as the vegetative spores.

Sexual reproduction modes and structures form the basis for classifying the major groups of fungi. There are four phyla of fungi.

1. **Zygomycota.** These are the "algae-like" fungi. There are no septa in the hyphae, though the reproductive structures are separated from the rest of the mycelium. Genetic material is exchanged in thick-walled zygospores, which are formed by the conjugation of opposite mating types, and then the haploid spores are released from the zygospores. Asexual reproduction is by conidia, resistant spores that develop within the sporangium. Some examples are common bread molds and parasites of protists, nematodes, insects, and small animals (fig. 2).

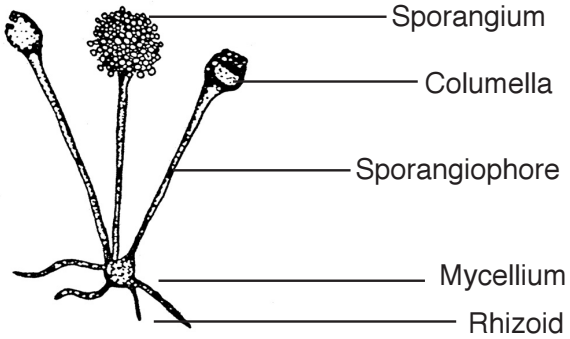


Figure 2

5100 West Henrietta Rd • PO Box 92912 • Rochester, NY 14692-9012 • p: 800 962-2660 • f: 800 635-8439 • wardsci.com

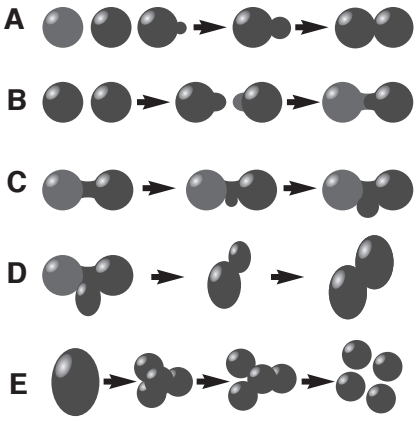
2. **Ascomycota.** The mycelium of Ascomycetes form a cottony mass of multi- branched hyphae. This phylum is characterized by sac-like reproductive structures called asci, which result from the conjugation of two compatible mating strains. Each ascus typically contains eight ascospores, which form when the short-lived diploid nuclei undergo meiosis. Many serious plant pathogens are in this phylum, including Dutch Elm Disease and apple scab. The group also includes beneficial yeasts (vital to baking and brewing industries) and highly prized edible fungi, including the morels and truffles (fig. 3).

3. **Basidiomycota.** The phylum is distinguished by its reproductive structure, the club-like basidium, which contains the products of sexual reproduction called basidiospores. Germinating basidiospores produce a mycelium that develops septa as it grows. Hyphae of compatible mating types conjugate to form a secondary mycelium. A tertiary mycelium develops to form the familiar reproductive structures of mushrooms, puffballs, and bracket fungi. Some basidiomycetes, the rusts and smuts, are serious crop pests, while others can be used for food or medical products. Some members of this phyla can also be deadly because of their poison. (fig. 4)

4. **Deuteromycota.** These fungi are often termed "fungi imperfecti" because they lack structures for sexual reproduction. Only asexual reproduction is known to occur naturally, although some genetic recombination has occurred under laboratory conditions. Germinating spores produce mycelia with septate hyphae. The phylum includes economically important species like the pathogenic Penicillium (fig. 5), source of the powerful antibiotic Penicillin. It also includes an interesting genus of predaceous fungi, Arthrobotrys, which actively captures and feeds upon nematodes.

Biotechnology has enabled us to use the unique biochemical makeup of these organisms to our advantage. Fungi are important in the processing of many foods, including tea, coffee, cheese, vinegar, beverages, and breads. They are also influential in the manufacture of industrial chemicals such as acetone, alcohols, and citric acid, as well as in the production of antibiotics and vitamins. Last, but by no means least, is the recycling of organic matter. Fungi are vitally important in initiating the breakdown of matter through decay, helping to return vital nutrients to the soil.

Figure 3



A. Haploid yeast cells budding.

B.Haploid cells forming smoos & zygotes.

C. Zygote budding off diploid.

D. Diploid budding.

E. Diploid forming asci with ascospores, freed spores which are haploid.

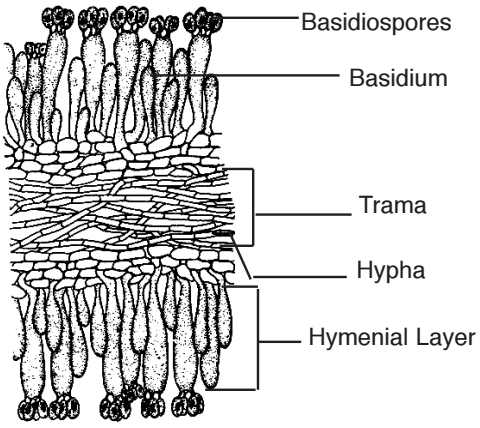


Figure 4

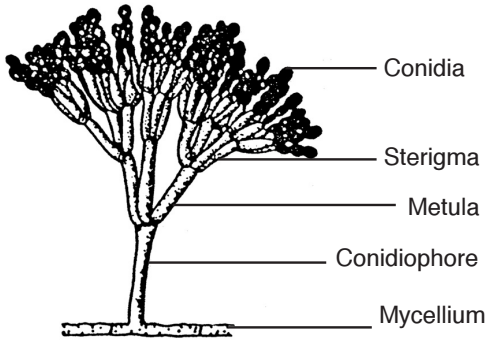


Figure 5

General Technique for Bacteria and Fungi Cultures

Caution: *Do not work with pathogenic (disease-causing) organisms unless you have had sufficient training and experience in their handling. Treat all bacteria as being pathogenic, both to establish proper work habits and because of the chance (however small) of a culture are inadvertently becoming contaminated by a pathogen. Even non-pathogens are pure cultures and are heavily concentrated, and, as such, should be handled with care.*

5100 West Henrietta Rd • PO Box 92912 • Rochester, NY 14692-9012 • p: 800 962-2660 • f: 800 635-8439 • wardsci.com

1. Sterilization
    a. Media and glassware: Use an autoclave or pressure cooker at 15 lbs. for 15 min. at 121°C.
    b. Glassware: Dry heat in oven at 160°C for at least two hours.
2. Wear a clean lab coat, smock, or apron to protect clothing and to reduce possible contamination of cultures.
3. While in the lab, avoid any hand to mouth operations, such as eating, smoking, or licking adhesive labels.
4. Wash hands thoroughly with soap and water, both before and after working with cultures.
5. Keep work surface clear of any unnecessary objects (e.g., books, purses, etc.)
6. Wash work surface with a capable disinfectant, such as 10% Lysol, 70% alcohol, or household bleach both before and after working with cultures.
7. Culture transfers

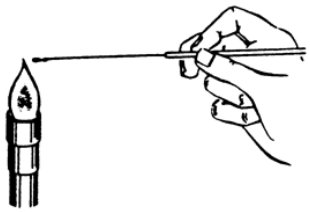
The only articles of equipment needed to make a transfer from the initial culture to a tube of sterile medium are a Bunsen burner or similar heat source, and an inoculating loop, needle, or sterile swab.

a. Hold both tubes in the left hand (Fig. 1).

Figure 1

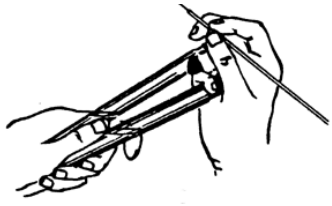
b. With the needle in the right hand, pass the entire length of the wire through the flame until it has all been red hot (Fig. 2).

Figure 2



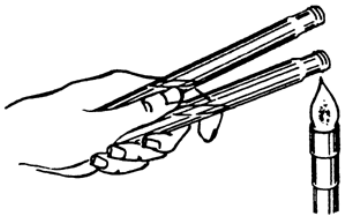
c. While still holding the needle, QUICKLY remove the caps or plugs from the tubes, holding them between the fingers of the right hand (Fig. 3). Flame the mouths by passing them two or three times through the burner flames (Fig. 4). Hold these tubes almost parallel to the table top if they contain a broth. This will reduce the possibility of air-borne contaminants.

Figure 3



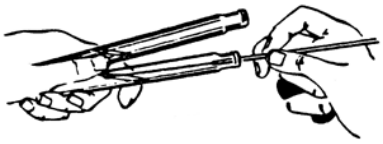
5100 West Henrietta Rd • PO Box 92912 • Rochester, NY 14692-9012 • p: 800 962-2660 • f: 800 635-8439 • wardsci.com

Figure 4



d. Touch the needle to the medium in the culture tube to be sure it is cooled, and then to the culture mass. Apply the needle to the sterile medium in the other tube (Fig. 5). (This may be done either by streaking the surface of a slant, making a stab into a semi-solid media, or swirling the needle in a broth.) If Petri plates are used, place them on the table and lift the cover only enough to maneuver the needle when inoculating. It should be noted that it is not necessary to attempt to remove a large volume of the culture mass with the needle. A slight touch will place more than enough on the needle to make the inoculation.

Figure 5



e. Flame the mouths of the tubes and cap them.

f. Flame the inoculating needle until it is "red hot".

8. If screw cap culture tubes are used, the cap should be kept loose to allow the aerobic cultures to get oxygen. (Most common bacteria and molds fall into this category.)
9. If cultures are to be kept for an extended period, however, they should be sealed tightly to prevent dehydration of the media. Then refrigerate to slow the metabolic processes of the organisms, unless the label states no refrigeration.

Procedures to follow in a biological spill:

Materials on hand at all times:

Bio-hazard bag (autoclavable)
Paper towels
Gloves
Tongs or similar instrument
Disinfectant solution — 70% isopropyl alcohol, 10% Lysol, or household bleach in a squeeze bottle.

Procedure:

1. Pour disinfectant on all broken glass and contaminated surfaces. Extend coverage over 3" around original (contaminated) area.
2. Cover the spill area with paper towels. Add additional disinfectant to fully saturate them. Wait 30 minutes.
3. Wearing rubber gloves and using tongs, pick up all glass or residue along with paper towels and place in Bio-hazard bag.
4. Disinfect the area again by following steps 1–3.
5. Seal the Bio-hazard bag and autoclave the contents. Dispose.
6. Wash hands thoroughly.

5100 West Henrietta Rd • PO Box 92912 • Rochester, NY 14692-9012 • p: 800 962-2660 • f: 800 635-8439 • wardsci.com

Discarding of Microbiological Cultures

All cultures must be autoclaved prior to disposal.

1. Autoclave at 121°C, 15 psi for 15 minutes.
2. Contents of containers may be discarded.

   Note: *Do not dump melted agar down the drain as it will later solidify and block flow.*

3. Wash glassware in hot water and rinse well.

If an autoclave is unavailable, soak in bleach or incinerate.

Care of Cultures

When you receive your live cultures, they should be refrigerated to slow metabolic rates. The recommended medium and the optimum temperature for each bacterium is given on the tubes of the cultures supplied. The above technique is used for all but the following organisms:

1. *Vibrio fischeri*
   a. *V. fischeri*, a marine bacterium, is one of the simplest light producing organisms and also one of the easiest to work with.
   b. Since *V. fischeri* will only produce light in fresh cultures, it is necessary to subculture it in a dark environment (you can use a cardboard box for this purpose) in order to observe its luminescent characteristics. This should be done 18–48 hours before the luminescence is to be observed.
   c. For best results, the room in which the observations are to occur should be completely darkened, and the eyes of the people who observe the experiments should be allowed to acclimate to the darkness. If this is not feasible, the room should be made as dark as possible and the experiments may be viewed in the bottom of a double paper bag with the opening of the bag held tightly about the viewer's eyes.
2. *Halobacterium salinarium* requires a 25-35% salt medium for growth. It is a slow growing organism, taking 5 - 7 days at 37°C for agar slants. Twelve drops of Halobacterium solution (in 25% salt water) added to the tube and incubated horizontally in a slant rack will provide ample bacteria. Halobacterium can be stored at room temperature and can survive at temperatures up to 40°C.
3. *Chromobacterium violaceum* (pathogen) should not be refrigerated or subjected to cold temperatures as the culture will quickly die. Chromobacterium prefers to stay at 30°C and will keep for months this way.
4. Aquaspirillum prefers room temperature storage, although refrigeration will not hurt the culture. *Spirillum volutans* prefers to be held at 30°C. Rhodospirillum rubrum is photosynthetic and needs room temperature and a light source.

Lyophilized (freeze-dried) Bacteria or Fungi Cultures

Under this mode of culture preservation, bacteria cultures will remain true-to-type for at least two years. It is recommended that these cultures be stored at 5–6°C, which is normal refrigeration temperature.

Culture re-establishment requires no sophisticated equipment or special technical experience. A serological pipet, along with general sterile technique, is all that is required. Please follow the instructions to be assured of success.

1. Using a sterile serological pipet, aseptically add to the lyophilized material no more than 0.5 ml of the appropriate sterile liquid transfer medium.
2. Mix well by drawing the hydrated cell suspension up and down through the pipet at least ten times.
3. Using a sterile swab, inoculate the agar.
4. Put remaining culture in broth tube. Re-established culture may then be sub-cultured onto growth medium.
5. All materials used (shell vial, pipet, etc.) should be autoclaved prior to disposal.
6. Incubate agar tubes horizontally and agar plates agar side up.

Given proper treatment and conditions, freeze-dried bacterial cultures will grow out in 24–48 hours, and fungal cultures will grow in 3-5 days. Some strains may exhibit a prolonged lag phase and should be given twice the normal incubation period before discarding as unviable.

5100 West Henrietta Rd • PO Box 92912 • Rochester, NY 14692-9012 • p: 800 962-2660 • f: 800 635-8439 • wardsci.com

BACTERIA

Observing Bacteria under the Microscope

1. Spread a thin film from the culture on one half of a microscope slide, using the inoculating loop or needle.
2. Let the film dry.
3. Flame-fix the film by passing the smeared end of the slide through a Bunsen flame several times.
4. Flood the slide with 1% Methylene Blue stain (several drops will do).
5. Let stand for several minutes, then dip the slide into a container of water to remove the excess stain.
6. Blot dry. Do not rub, as this may remove the prepared culture from the slide. Add a drop of immersion oil and examine under high power (100X).

This procedure gives a quick look at bacteria and will easily demonstrate bacteria shape.

Gram Staining Procedure for Bacteria

1. Flame-fix a bacterial smear as described above.
2. Add several drops of Gram's Crystal Violet stain.
3. Let stand for one minute, then rinse by dipping in water.
4. Add several drops of Gram's Iodine Solution to "set" the stain.
5. Let stand one minute, then rinse by dipping in water.
6. Tilt the slide, then add 95% alcohol as a destain. Add drop by drop, letting the alcohol flow over the stained smear. Do this until there is no more color in the runoff. This step is to remove the color from gram-negative bacteria. The gram-positive organisms will retain the purple color.
7. Counterstain with Gram's Safranin stain. Allow to stand for about 30 seconds, then rinse as before. Blot away excess moisture and allow to dry.
8. Add a drop of immersion oil and examine under high power (100x).

The cell walls of gram-negative bacteria are chemically different. They have a higher lipid content, which allows the alcohol destain to wash the stain out of the cell. The most accurate determinations using the Gram technique are done with newer cultures, preferably less than 24 hours old, although to view spores in *Bacillus* sp. or *Clortridium* sp. older cultures are necessary.

FUNGI

Media Preparation for Fungi Cultures (Pouring Plates from Bottled Media)

Materials needed for plates: Sterile Petri plates, bottled media, large beaker, hot plate, thermometer, inoculating needle or loop, Bunsen or similar flame source

Plates: Use sterile technique as outlined above.

To make plates using bottled solid media:

1. Place the bottle of media in a large beaker. Add water to the beaker until the water level is just above the level of the medium in the bottle.
2. Loosen the cap on the bottle.
3. Put a thermometer in the beaker; set it on a hot plate and heat the water to boiling.
4. Meanwhile, set the Petri plates (each bottle of media will supply 5 – 7 plates, depending on the thickness poured) on the work surface.

5100 West Henrietta Rd • PO Box 92912 • Rochester, NY 14692-9012 • p: 800 962-2660 • f: 800 635-8439 • wardsci.com

5. Boil the water gently for several minutes until the medium is completely liquefied. Swirl the bottle gently to be sure all is melted.
6. Turn off the heat. Allow the water temperature to cool to between 45°C and 50°C.
7. Using an insulated glove, pick up the bottle, remove the cap, and flame the bottle mouth.
8. Lift the lid of a Petri plate just enough to admit the neck of the bottle, and pour the first plate, using just enough medium to slightly more than cover the bottom of the plate. Replace the lid.
9. Swirl the plate gently to distribute the medium.
10. Proceed to pour the rest of the plates.

To transfer cultures to plates:

1. Follow aseptic technique as described for slants
2. Lift the lid of the Petri dish just enough to introduce the inoculating loop. Gently wipe the needle or loop across the culture (you only need a small amount).
3. Replace the lid, and then lift the lid of the sterile petri dish containing fresh medium just enough to introduce the needle or loop. Gently wipe the needle or loop across the surface of the media, then replace the lid and sterilize the needle or loop as before. You have now transferred the fungal culture to the plate.

Special Techniques for Some Fungi Cultures

General: The techniques previously outlined apply to most fungal cultures. Some special techniques apply to specific fungi:

Saprolegnia ferax, (water mold). This species is best grown on cornmeal agar. It can be grown in liquid medium by adding sterile rice grains or cucumber seed to distilled water and inoculating it with Saprolegnia.

Demonstration of sexual reproduction in fungi can easily be done using *Rhizopus stolonifer, Phycomyces blakesleeanus,* or *Mucor hiemalis.*

Inoculate plus and minus strains on opposite sides of a Petri plate containing sabouraud dextrose agar or potato dextrose agar. Be careful not to mix the strains when plating them.

When hyphae of the opposite strains grow to meet in the center of the Petri plate, a line of mature zygospores will develop where the strains meet. Have students observe these under a dissecting microscope and sketch what they see.

Macrofungi Cultures: Corprinus and Schizophyllum cultures are shipped in jars. They can be subcultured, using sterile technique, by excising a bit of the media upon which they are growing, and transferring it to fresh medium in another jar. The jar should be large enough to allow the reproductive structures to develop. Other larger fungi may be kept in jars to display the morphology of the reproductive structures and in some cases part of the mycelium. Larger fungi may also be maintained in a terrarium. They should be collected with a large portion of the substrate to be sure that the mycelium is included. Mushrooms, bracket fungi, coral fungi, and others are easily collected and can demonstrate diversity within the fungi kingdom. They can also be used to illustrate the structural differences between the phyla.

Making wet mount preparations:

Observe the mold colony under a dissecting microscope. Following sterile technique, use an inoculating needle or loop to remove a small amount of mycelium bearing conidia or sporangia. Place this in a drop of water on a clean microscope slide. Tease it apart with the needle, if necessary. If staining is desired, add one or two drops of methylene blue. Cover with a coverslip and observe under low (40X) then high (400X) power of a compound scope. Draw what you see.

5100 West Henrietta Rd • PO Box 92912 • Rochester, NY 14692-9012 • p: 800 962-2660 • f: 800 635-8439 • wardsci.com

Constructing a moist chamber:

Use our Silicone Culture Gum to construct a moist chamber, so that you can grow a fungus as a slide culture and observe the entire life cycle. This simple yet beautiful preparation will let you see all phases in the growth of the fungus. The fungus is grown on a block of agar under a coverslip on a glass slide.

1. Roll a marble-sized ball of culture gum and flatten it to form a disc about ¼" thick.
2. Press the disc to a clean microscope slide. Use a cork borer to cut and remove the center of the disc.
3. Immerse the slide into 70% alcohol to sterilize and let dry. Cover with a sterile coverslip.
4. Use a sterile scalpel to cut a small block of agar. Remove the coverslip from the slide.
5. Place the agar block in the center of the slide. Using a sterile needle, inoculate the center of each of the four sides of the agar block with mycelia or spores. Replace the coverslip. You can observe the growth of the fungus over a number of days. If necessary, remove the coverslip and add a drop of distilled water to keep the chamber moist.

5100 West Henrietta Rd • PO Box 92912 • Rochester, NY 14692-9012 • p: 800 962-2660 • f: 800 635-8439 • wardsci.com