Chapter 5

Microscopy of Natural and Experimental Biofilms in the Undergraduate Laboratory: A Simple Collection Kit with Multiple Applications

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Margaret (Cindy) Henk received a B.S. in Zoology in 1967 from the University of Georgia, where she also did graduate work and technical microscopy work in zoology and entomology, and managed an electron microscopy lab under the direction of Mel Fuller in the Department of Botany. In 1979, she relocated to Louisiana State University, where she assisted in the laboratory of Russell Chapman in the Department of Botany until 1986, when she assumed management of the electron microscopy lab in the Department of Microbiology. This lab recently became the Socolofsky Microscopy Center, a service center for multi-departmental use affiliated with the Department of Biological Sciences. She has been heavily involved in education and outreach since 1986, and has had joint projects with LSU's Department of Curriculum and Instruction, Louisiana Sea Grant, and a science education grant from the Howard Hughes Medical Institute to the Department of Biological Sciences. She has had a major interest in the development of the Scalar® Scope-On-A-Rope© and its application in education settings. She has submitted a manuscript to Applied and Environmental Microbiology outlining research and teaching applications involved in the lab activity presented here.

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Introduction

With the use of the simple field kit described here, students can easily collect intact air/water interface biofilms from nature or from laboratory aquatic systems. Because such naturally occurring two-dimensional consortia are well suited to microscopy examination, they make an excellent introduction to microbial microscopy for discovery and research applications at many levels in the undergraduate laboratory. Biofilm microbes are ubiquitous though varied in the environment, and unlike many routine undergraduate laboratory bacteria species, not only are they easy to view in the microscope, but they are also interesting!

This simple, inexpensive, and user-friendly field kit has been designed for the immobilization and collection of two-dimensional biofilms, particularly those of the air/water interface. A key feature of the kit is a thin film of collodion that overlies the examination substrate (e.g., microscope slide) and provides essential adhesion of the unaltered biofilm.

Undergraduate students can easily construct these kits during part of a lab period. After overnight drying, a biofilm can be collected in a few seconds in the lab, or from any desired environmental setting, and then viewed live or stained in the lab depending on the scope and goals of

the course and facilities. Instructions for routine brightfield and phase microscopy are included here, but the kit can also be used for advanced protocols using fluorescence LM or even transmission electron microscopy (TEM).

Microscopy of the collected biofilm reveals size, shape, and spatial arrangement differences among types of microorganisms living together in this microhabitat and can also help elucidate relationships among them. Biofilms from different sites or from different experimental conditions can be compared. The extent to which advanced correlative microscopy is pursued can be determined by class size and level.

With appropriate instruction and supervision undergraduate students can efficiently and successfully use instrumentation normally found in college microscopy labs to discover important concepts in microbial ecology. Use of such a simple but rewarding hands-on environmental activity inspires interest while encouraging development of technical and scientific skills.

The air/water interface

Bacterial ubiquity and diversity in the environment is an important concept for today's citizen. A powerful and reliable demonstration of this concept can be introduced to the undergraduate microbiology laboratory by using microscopy techniques to examine natural or experimental populations of bacteria sampled from air/water interface biofilm consortia. This recently developed collection technique enriches observational and microscopy skills while providing opportunities for original student research into an area which has received little attention to date.

The air/water interface provides unusual environmental conditions: an interface with both hydrophilic and hydrophobic properties, surface tension, high aeration, concentrations of specific kinds of nutrients, and often high levels of irradiation. It follows that the organisms that exist in this niche are particularly suited to such conditions and have characteristics that enable them to inhabit it. These features may include physical properties such as buoyancy and hydrophobicity, as well as visible structural adaptations such as flotation or attachment appendages.

Biofilm capture and observation

This lab protocol capitalizes on the hydrophobic tendencies that seem to be common to surface-dwelling microorganisms. The collecting substrates in the kit are coated with a transparent collodion film that is also very hydrophobic, and via its affinity with the hydrophobic surfaces of floating bacteria, it serves as the capture medium. Surface biofilm cells instantly adhere to the collodion as it makes contact, and the organisms remain in place, immobilized as they are arranged *in situ*. This monolayer biofilm is in fact the most nearly ideal type of specimen for light microscopy (LM), and as a result, mastery of the microscope is simplified for this laboratory activity.

Classroom considerations

Simple collecting kits can be constructed by students in the laboratory from common or easily-obtainable materials. In one 15-minute preparation activity, each student can produce a collodion-coated collection slide that allows examination of a sample by different types of microscopy including routine brightfield and phase contrast, as well as more advanced systems such as differential interference contrast and fluorescence. (If a suitable transmission electron microscope facility is available, copper TEM grids may be included on the collection slide for higher magnification observation as well.) The collection slide is dried overnight. Actual collection of the sample is easy and rapid, and if the sample is collected in the field, it can be stored for later

examination. However, if light microscope specimens are examined soon after collection, it is possible to observe bacterial movement, protozoan grazing, etc., while cells are alive and healthy. Dried samples are quite stable and can be stored for a year or more. Student protocols for live viewing as well as several staining procedures are included for brightfield and phase contrast microscopes.

Practical considerations and rationale

Access to fairly well maintained microscopes is important, and a phase contrast microscope with video or digital display is ideal for this activity, but most student microscopes can yield very satisfying results and interesting observations. Most undergraduates have never seen bacteria actively move, and even immobilized bacteria often can rotate, produce motile buds, or have motile microbial associates. This dimension of microbial existence is not well demonstrated in the typical undergraduate laboratory, where bacteria are experienced primarily as spots on a culture plate, cloudiness in a culture tube, or grainy debris on a Gram-stained slide.

Practical advantages for the student lab

The expense and culture facilities normally required for classroom observation of bacteria with the microscope are bypassed with this activity. After a small initial investment in reusable plastic petri dishes and specimen cups, the only cost associated with this laboratory is for slides, filter paper, the inexpensive collodion solution, and any optional stains, including Gram stains. No Bunsen burners are required, and the living organisms are those commonly encountered, though unobserved, in nature. It is not essential to use the highest magnification for this project, so immersion oil is not needed.

Laboratory goals and results

Although few of the organisms will be positively identifiable in this activity, the abundantly distributed air/water interface biofilms that can be collected intact with the collodion substrate kit demonstrate interesting and thought-provoking features when examined with microscopy. Assorted and often unusual morphologies, microcolonies, dividing cells, varying spatial arrays, flotation devices and appendages, grazing activities, and other intercellular relationships can all be observed and discussed. Comparisons may be made among different water samples or among various treatments of the same water samples.

Group projects

A student team can use several microscopy methods to examine the same sample, and report on the types of information each method provides. Alternatively, a class can be coordinated so that each team uses one method to examine several different samples. Observations and data can be pooled and analyzed by the class as a whole.

Research in this area is sparse to date. Perhaps introduction of this collection technique along with recent technology developments will stimulate investigation. At any rate, many student observations may never have been described in the literature, ideally an inspiring concept!

Advanced applications and technology

Other light microscopy techniques such as darkfield, differential interference and fluorescence optics can yield additional exciting information for advanced students or classroom demonstrations.

Chlorophyll-containing bacteria and algae may autofluoresce (red, with blue or green excitiation) without additional treatment, and a 30-second application of the UV fluorescent stain DAPI allows students to distinguish living or dead bacteria (nucleoids fluoresce blue with UV excitation) from nonliving particles. Other fluorescent stains and markers can be used to distinguish specific types of cells within the biofilm assemblage.

Note: When available as a class demonstration, transmission electron microscopy (TEM) can provide ultrastructural and sometimes physiological information as well. When preparing the collodion capture membrane, one or two copper TEM grids may be placed on the slide while it is immersed in water, and thus become sandwiched between the slide and collodion. After the biofilm has been collected and observed with light microscopy, the grid may be removed, negative stained (*e.g.,* with 2% uranyl acetate), and observed and photographed with TEM by a trained microscopist. A simple TEM demonstration of a biofilm previously examined by light microscopy (LM) gives the student exposure to a less familiar type of microscope technology, as well as a more sophisticated appreciation of visual data analysis while comparing images of the same biofilm viewed by two different media. In addition, TEM can display flagella, capsule, s-particles, appendages, predation, and other physiologically important features not visible by LM.

Conclusion

Undergraduates have repeatedly shown increased interest and appreciation of the microbial world after having the opportunity to view living bacteria from familiar surroundings. This simple and reliable collection kit offers an economical and efficient means to provide such a hands-on opportunity at many levels of sophistication.

Materials

The basic activity requires the items listed in the table below for brightfield microscopy of living or stained biofilms. The collected biofilm slides may also be stained with many additional prepared stains, including Gram stains. See Student Outline.

Table 1. Materials needed for preparing and using 100 air/water interface biofilm collecting kits with brightfield microscopy of living and stained bacteria.

Items needed (Starred items reusable.)	# /student	Approximate cost/100 students
*Modified plastic petri dish, 100 x 15mm, non-sterile	1	$15 US
Filter paper circle, 7 cm	1	$ 7 US
Microscope slide, with painted or frosted end!	1	$10 US
2% collodion solution in amyl acetate[1]	2 drops	$ 7 US
Lab wipes	2 sheets	$ 1 US
Coverslip	1	$ 5 US
Stain such as diluted gentian violet[2] or other regular lab and Gram stains	2 drops	$ 2 US
*Plastic 4 oz./120 ml specimen cup and lid, large enough to hold 2 microscope slides back to back[1]	1	(only if field kit used) $25 US
*Plastic resealable freezer bags, gallon size	1/ 2 students	(only if field kit used) $ 4 US

Lab supplies for 100 students cost a total of about $50 US the first time without the field kit and about $80 US with the field kit. By reuse of starred items, each additional time the cost will be about $35 US.

[1]May be obtained from Electron Microscopy Sciences at www.emsdiasum.com.

Specimen cup catalog # 64231-20, Collodion solution catalog # 12620-50. Label warnings refer to very large quantities. See Merck index entries in Appendix.

[2]Available at pharmacies. Dilute 1 drop in 10ml water immediately before use. Kills many bacteria.

For advance preparations the instructor will need to use a soldering iron or heated rod to make holes in the plastic petri dishes.

The student laboratory should be supplied with paper towels, pencils, a few clean disposable pipets, wooden applicator sticks or toothpicks, containers of clean tap water, and containers for waste water.

Student microscopes optimally should have good 10, 20, and 40X objectives. Oil 100X objectives are not required, but an instructor's demonstration with video camera can be an exciting addition, especially with phase contrast optics and living samples.

Notes for the Instructor

Day 1: Making the collecting slides.

This activity will take about 15 minutes in the student laboratory, but will require overnight drying of the collecting slide. **Advance preparations should be carried out by instructor.**

Advance preparation of reusable kit components and laboratory supplies.

1. Prepare a plastic petri dish strainer for each student/team kit by using a soldering iron or hot rod to melt about 10 drain holes through the petri dish bottom, being sure to make one hole right at the periphery. See Fig. 1A. This step should be done in a fume hood or other well-ventilated area. This strainer and its petri dish lid are reusable.

2. Prepare "environmental" biofilms in the lab 2 or 3 days in advance if you are not planning to have the students collect actual environmental samples. Use food storage or similar containers, as in Fig. 1B, that are at least 6 cm deep. Add water taken from aquatic settings. Experimental possibilities usually abound in the lab. Alternatively, 5-10 g of soil or other organic samples can be suspended in a container of water and allowed to settle. Allow biofilms to grow undisturbed and uncovered at least 2 days before use. A water surface of 100 cm^2 can be collected by 4-6 students on the same day. Alternatively, actual environmental samples may be collected by students, either individually or on group field trips.

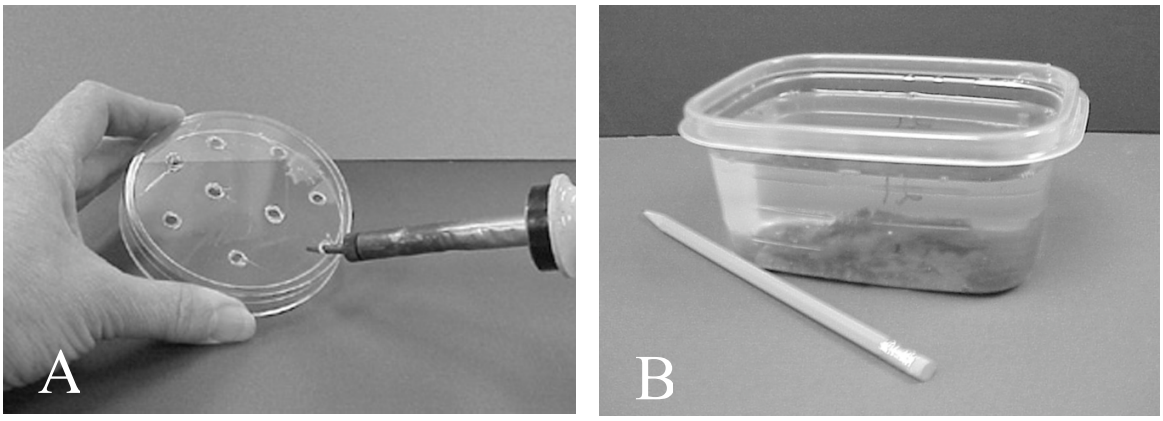

Figure 1. A. Melting drain holes in the strainer with a soldering tip. B. A laboratory "environmental" biofilm container with about 100 cm^2 of surface area.

Preparations in the teaching laboratory

1. In the teaching laboratory make sure that the lighting is sufficient for seeing the floating collodion film and that the ventilation is good.
2. Assemble the kit components at workstations. Cafeteria-style trays are useful setup areas for about four kits each.
3. It is convenient to dispense collodion solution from a common pipet to avoid contamination of solution and excessive bubble formation. Use a dry pipet and draw in about 1ml to provide for about 30 kits. Most dropper bottles are not suitable.

Day 2: Collecting an environmental biofilm (field trip or in lab)

Field trip vs. in lab collection

You have several choices of how best to utilize the collection slides for your purposes. Actual environmental samples may be collected on day 2 and utilized as part of a larger study, or laboratory-grown biofilms may be used if available lab time is short.

(Optional day 2 field kits.)

Assembling field kits will take about 10 minutes in the lab if students or student groups will be collecting environmental biofilms on their own or with a field trip that you plan. Two students will work together to make one kit containing both of their slides. Each pair will remove their collecting slides from the filter paper, put them back to back in a lidded specimen cup, fill a second lidded cup with water, and pack the cups and several lab wipes into a plastic bag. The collections will be made, rinsed and replaced in the cup. The rinse water may be discarded and a bulk water sample taken from the site for microscopic comparison with the biofilm if you wish. The biofilm will often stay alive for 24 hours or more in the cup if well sealed, but will be perfectly usable if dried. Long storage in this manner will usually result in overgrowth of fungi and other microbes, obscuring the original biofilm, however.

Day 2 (or 3): Observation of biofilms with the microscope

Students may collect laboratory biofilms and observe them on the same day if you have prepared lab-grown "environmental" biofilms as outlined previously. For best results, have several different aquatic sources represented in your lab biofilms so that students can compare samples. As collections are made, the biofilm will be removed, so plan to have no more than 4-6 students use a $100cm^2$ biofilm source per day. The biofilms usually will regrow in a day if left undisturbed.

Observation of freshly prepared or stored biofilms with the microscope is fully explained in the student outline. You have a choice of viewing living or stained preparations. For most brightfield student microscopes, stained samples work best. Staining can be done with a common drugstore or lab stain (Choices 1 and 2), or with a modified Gram stain that does not require good smear technique or heat fixation (Choice 3). All of the staining procedures usually kill the microorganisms so that you can't see them moving around, but they are easier to see with most student microscopes.

If the microscopes are in good shape, or if phase contrast scopes are available try to use Choice 4 with living cells! It is very common to see moving protozoans associated with (sometimes feeding on) the biofilm, bright appearing bacterial spores, or twirling vibrio bacteria attached to the film by their flagella. Algae such as diatoms may be seen gliding along on the biofilm, and cyanobacteria such as LEGO®-like *Merismopedia* often float at the surface. Darkfield optics can also be spectacular if aligned correctly.

Almost any prepared stain will work with either wet or dry biofilms. If you do use Gram stains, you will be able to see various organism-specific hues of pink and purple, but remember that assignment of Gram stainability to a genus of bacteria is reliable only with log phase cells, and that you will not know for sure what developmental stage your biofilm bacteria were experiencing when they were collected.

Try to make sure that the microscope objectives are clean beforehand. Since no oil objectives are required for this activity, your maintenance worries are reduced. Some rudimentary instructions for microscope adjustment are included in the student outline. If you do not wish students to make these adjustments, the instructor or TA should check scopes in advance and be prepared to help.

Student outline

In this lab you will be collecting a very thin biofilm that is almost always present at the air/water interface of any sort of body of water in nature and many interior environments. The organisms you will see are specially adapted for living at the water surface. Many of them have specific appendages to help them float, or have very hydrophobic surfaces that keep them suspended at the air/water interface. These characteristics increase the likelihood that an assemblage of microbes will be organized into a coherent sheet, or thin biofilm, consisting of the organisms and their products.

Air/water interface organisms have not been well described, so most of what you see may not be identifiable by consulting the best textbook! Their arrangement on the water surface, which you will capture just as they are arranged in life, can help identify some of these organisms. If it is possible to photograph your slides and save them digitally for inclusion in a database, your work in this lab may help identify and describe microbes that have never been cultured in the lab before.

The first step is to make an adhesive-coated slide with which to collect the biofilm. The adhesive coating, a collodion polymer, is very thin and transparent so that you will be able to see through it when you look at the collection with your microscope. You will make the adhesive collodion membrane on a clean water surface and bring your slide up through the water to contact it. Once it has dried as a thin sheet on the slide, it will be an attractant for the hydrophobic microorganisms in air/water interface biofilms.

Day 1. Preparing the collecting slide.

A. The Slide.

Use a pencil to label a frosted-end microscope slide with your name or other ID, lay it on a paper towel and thoroughly scrub the "good" side (label up) with a dry lab wipe. Blow off any dust, and avoid touching the good side with your fingers.

B. The "Strainer"

1. Invert the lid of the petri dish on a paper towel and place the "strainer" (perforated dish) into it.
2. Center a filter paper disk in the dish and place the clean slide on top of the filter paper, good side up, as in Fig. 2A.
3. Add water to the dish, filling it about to the level of the lid. Make sure the slide is completely immersed.

C. The Adhesive Coating

1. From about 2 inches above the dish, drop two drops of 2% collodion solution onto the center of the water surface, as in Fig. 2B. You will soon see rainbow colors form as the liquid evaporates. It smells something like bananas.
2. Allow collodion solution to dry completely. The resulting collodion membrane should be silver/gold colored and maybe a bit wrinkly at the edges. It may take up to 5 minutes.
3. Nudge the edge of the collodion membrane with a wooden stick if necessary, so that it is centered over the slide.

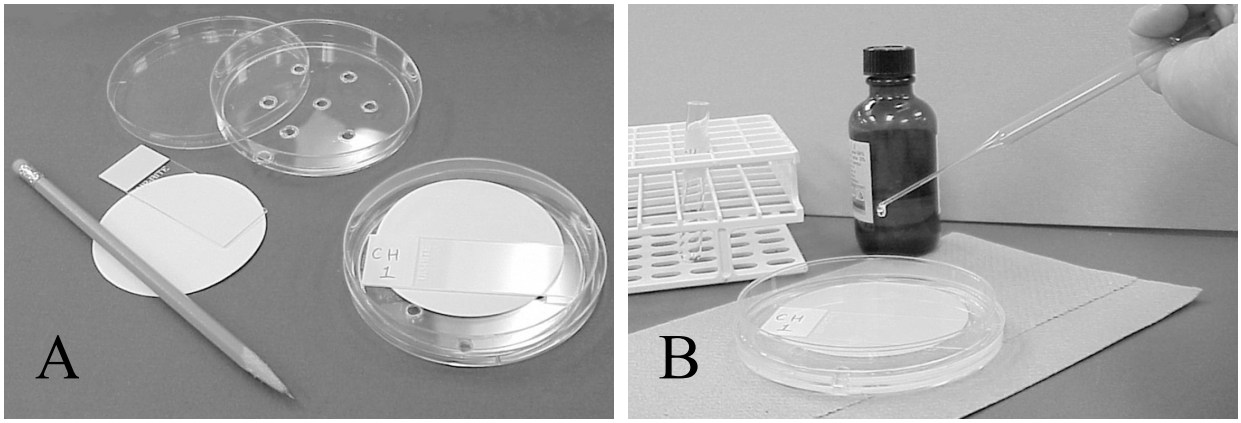

Figure 2. A. The petri dish lid and strainer, and the arrangement of filter paper and slide before adding water to the dish. B. Dropping collodion solution onto the water surface above the immersed slide.

D. Draining and Drying

1. Gently lift the strainer dish (with holes) straight up, so that the membrane stays smooth and unwrinkled while the water drains out into the lid.
2. Lay the strainer on a paper towel, being careful not to disturb the slide. The collodion membrane should cover most of the slide and filter paper (Fig. 3A).
3. Empty the water from the petri dish lid and lay the lid face down on a dry paper towel. Then carefully pick up the strainer with its slide still in position, locate the peripheral hole in the strainer, and prop the assembly against the lid so that water can drain out the hole onto the paper towel, as in Fig. 3B.
4. Dry overnight. The filter paper and slide must be completely dry before a biofilm can be collected.

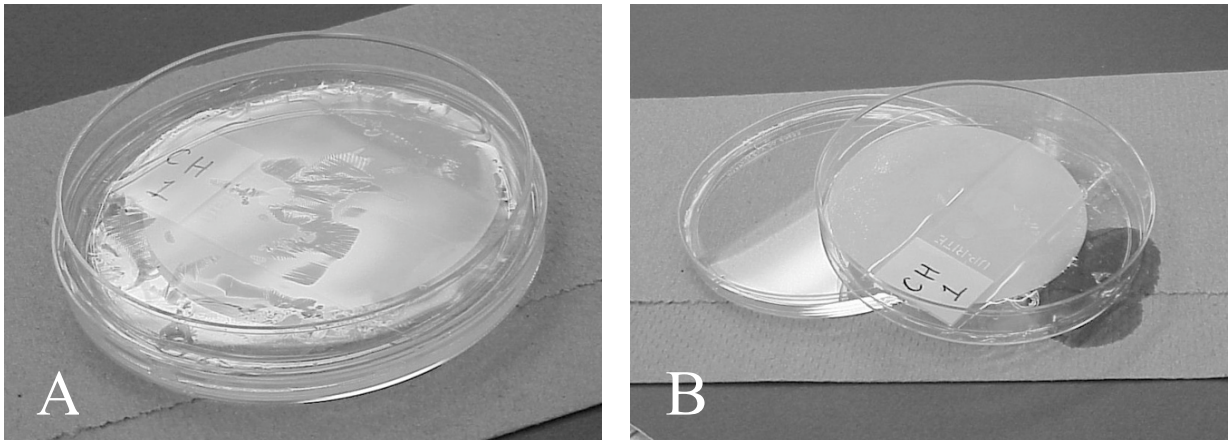

Figure 3. A. The dry collodion membrane floating on the water surface above the slide. B. The collodion membrane membrane spread out on the slide and the damp filter paper. It should dry overnight in this position.

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Day 2. Removing and preparing the membrane-coated collecting slide for use.

A. Removing the dried collecting slide.

When the filter paper under the collecting slide looks completely dry, carefully run a wooden stick along the edge of the slide so that you can remove it from the filter paper without tearing the thin collodion membrane (Fig. 4A). Avoid touching the collodion surface.

(B. The optional field kit.)

If you are going to collect an environmental biofilm, you and a partner can make a field kit (Fig. 4B). Hold your two slides by the labeled end and put them back-to-back into a dry plastic specimen cup. When you put the lid on, the collodion membrane surfaces will not be able to be scratched or to scratch each other.

Fill a second cup with clean water and put the lid on. Pack the 2 cups and a couple of paper towels or lab wipes into a plastic bag and you are ready to go collecting! (A pair of long barbecue tongs that securely grip the slide can extend your reach in many settings.)

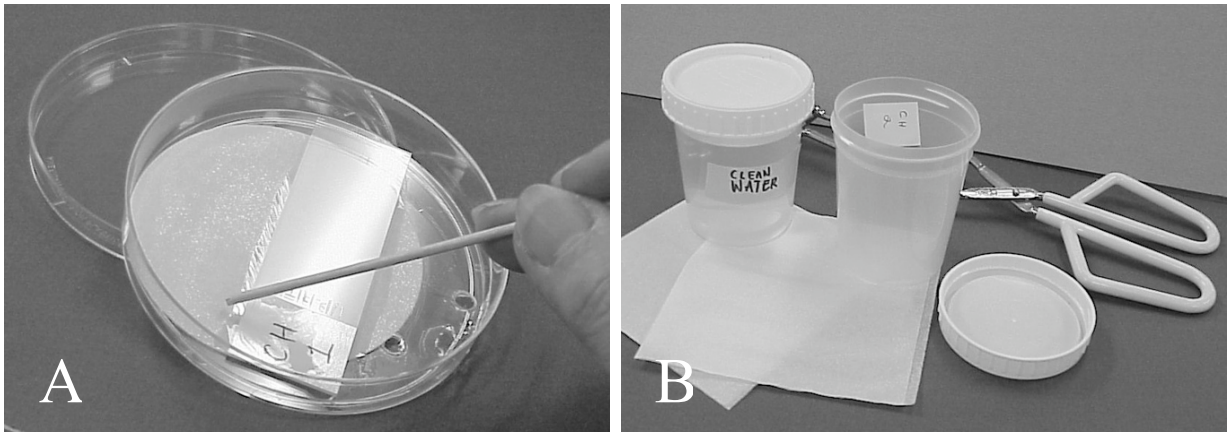

Figure 4. A. Scoring along the edge of the membrane-coated collection slide. B. Items to assemble for the field kit. Barbecue tongs may extend your reach in difficult areas!

Day 2 (or Days 2 and 3 if field collections are made)

A. Collecting a floating biofilm

The ideal biofilm for light microscopy observation is almost invisible on the water surface. It may look like a thin oil film if the lighting angle is perfect. Thick films, although easily visible to the naked eye, will be difficult to analyze because there will be too many organisms.

1. Write down your slide ID and the collection site so you'll know where the biofilm came from.
2. Holding a slide by the label end, in one smooth movement, touch the "good side" down to the biofilm as horizontally as you can, immerse the slide completely, then draw it out of the water vertically. See Fig. 5 A and B.

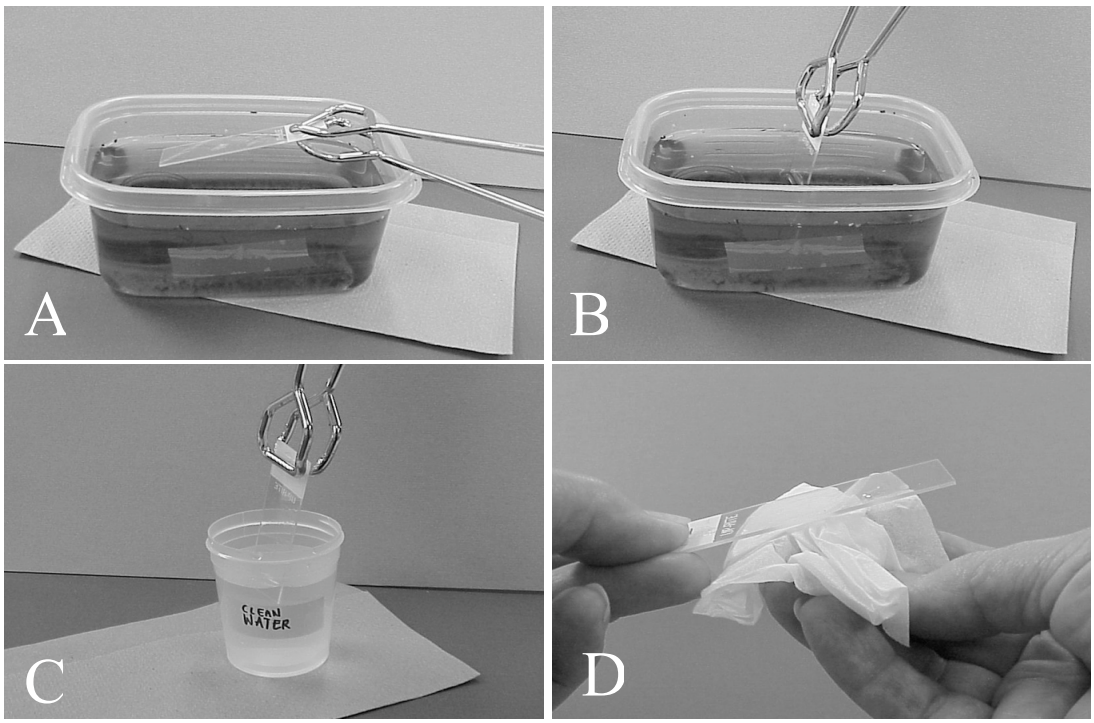

Figure 5. A. Horizontal contact of the collodion membrane with the floating biofilm. B. Vertical withdrawal of the slide from the water. C. Vertical immersion and withdrawal of the slide in the rinse water. D. Drying off the back of the slide. Avoid touching the biofilmed side.

B. Removing other particles

1. Rinse the slide by completely immersing it into a cup of clean water, and immediately draw it out vertically as in Fig. 5 C.
2. Holding it by the edges, wipe off ONLY the back of the slide (the non-labeled side) with a lab wipe as in Fig. 5 D. Don't wipe off your biofilm!

(C. The optional field trip.)

Collect and rinse the biofilm as described in sections A and B above, but follow these additional instructions:

1. Put the slides back into the dry cup, back-to-back as before, and fasten the lid well. The good sides of your slides will stay wet and alive for at least 24 hours if you don't let them get too hot.
2. If you want to collect some of the subsurface water that bathed the underside of your biofilm, just pour out the "clean" water cup and fill it about halfway up with sample water. You need to leave some air inside, and keep the sample cool but not cold if you want to see protozoans. Some of them eat or otherwise interact with organisms residing in floating biofilms.

Make notes about your location. Date, GPS data, temperature, illumination, photographs, time of day, etc., can provide useful data for analysis, especially if multiple collections are being taken

from this or other sites. After you have rinsed your biofilm in the clean water you can discard it and collect a water sample from the water beneath where your biofilm was. This water can be observed microscopically or analyzed in other ways.

D. Preparing the slides for the microscope

Your instructor will let you know which of the four choices below you should follow:

Choice 1. Staining with Gentian Violet

1. Place a clean coverslip on a paper towel. Make sure you do not have two coverslips stuck together.
2. Put a drop of prepared stain about the diameter of a pencil eraser on it, then carefully lower the good side of the biofilm slide down onto it. When the water drop makes contact with the slide it will automatically attach the coverslip to the slide with very few air bubbles.
3. Hold the edge of the slide vertically against the paper towel to drain off excess water. Make sure it's dry on the back.

Choice 2. Staining with Other Common Lab Stains

Note: Avoid getting these concentrated stains on your skin or clothing! Alcohol will usually remove them from your skin, but many fabrics stain permanently.

1. Place the slide, biofilm up, on a paper towel.
2. Place a flat drop of stain about 1 cm in diameter on the biofilm.
3. Stain for about 15 seconds.
4. Dip the slide up and down into a cup of clean water for about 10 seconds.
5. Remove it, blot it vertically on the paper towel, then dry off the back with a lab wipe.
6. Let it dry on the paper towel, biofilm side up for a minute, and make sure it's dry on the back.

Choice 3. Gram Staining

1. Place the slide, biofilm up, on a paper towel.
2. Place a flat drop of Crystal Violet Stain about 1 cm in diameter onto the biofilm.
3. Stain for about 30 seconds.
4. Dip the slide up and down into a cup of clean water for about 10 seconds to wash out the excess stain.
5. Place a similar drop of Gram's Iodine on the same spot as before.
6. Stain for about 60 seconds.
7. Dip the slide up and down in a cup of 95% ethyl alcohol for about 10 seconds to decolorize it. The same alcohol can be used for several slides.
8. Then dip the slide into a cup of clean water again to rinse off the alcohol.
9. Place a flat drop of Safranin Stain on the same spot as before.
10. Stain for about 60 seconds.
11. Then dip the slide into a cup of clean water again to rinse off the alcohol.
12. Remove it, blot it vertically on the paper towel, then dry off the back with a lab wipe.
13. Let it dry on the paper towel, biofilm side up, for a minute or so. Make sure it's dry on the back.

P.S. Gram positive bacteria usually stain purple, gram negative ones red - BUT NOT ALWAYS!

Choice 4. Live Biofilms.

Use collections that are no more than 24 hours old if you want to see what they looked like *in situ*. Some stains kill microorganisms, so if you want to watch them swim or glide around, it's best to avoid stains, but you will need to use a coverslip. (Phase contrast microscopes are best for this kind of observation.)

1. Place a clean coverslip on a paper towel. Make sure two coverslips are not stuck together.
2. Put a drop of water about the diameter of a pencil eraser on it, then carefully lower the biofilm side of the slide down onto it. When the water drop makes contact with the slide it will automatically attach itself to the slide with very few air bubbles
3. Hold the edge of the slide vertically against the paper towel to drain off excess water. Make sure it's dry on the back.

Using the microscope

No matter which preparation Choice you used, set up your microscope this way for the quickest and best view.

1. Rotate the lowest magnification objective lens (usually 4X or 10X) into position and make sure light is coming up through the hole in the stage.
2. Put the slide on the stage, label on top and good side up, and move the label end over the hole.
3. Focus on the edge of the label closest to the center of the slide.
4. Then, without changing the focus, rotate the next higher objective into place, and slightly adjust the focus again.
5. Repeat step 4 again until you get to the 40 X objective, then move the slide over so you can see the edge of the coverslip. Adjust the focus a little as you move along the slide if necessary.

If you used Choices 1, 2, or 3 for staining the biofilm, you should be able to see the biofilm well at this magnification if the lens is clean. You should not need to use any immersion oil on this slide unless specifically instructed how to do so in lab.

If you have difficulty seeing well, look at the instructions for microscope adjustment below. Otherwise ask for help or proceed with your worksheet or teacher's instructions. See Figure 6 below for views of biofilms prepared by each of the four choices.

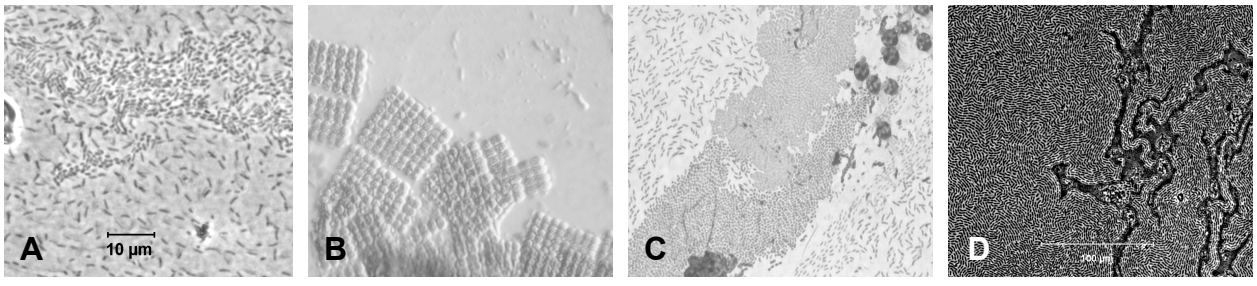

Figure 6. Low magnification views you might expect to see. A. Choice 1: A biofilm stained with Gentian violet and observed with the 20X objective. B. Choice 2: Floating rafts of the cyanobacterium *Merismopedia,* stained for a few seconds with the prepared stain Malachite Green and viewed with a 40X objective. C. Choice 3: A Gram stain preparation showing various shadings of both red (Gram -) and purple (Gram +). D. Choice 4: A live biofilm viewed with 20X objective phase contrast optics.

Microscope adjustment instructions.

Adjusting the microscope: Brightfield

If you are looking at a living biofilm, your microscope may have to be adjusted for optimum viewing. If *"Ph"* is not written anywhere on your 40X microscope lens, you probably have a regular brightfield microscope and you must optimize the light. The light must be turned on brightly at the controls, but you have to adjust it just beneath the slide so that the spot of light stays bright but becomes smaller, so that contrast improves. Most microscopes have one of two ways to do this. There can be either a set of different sized **apertures** that rotate just under the slide, or a single **diaphragm** that can change sizes by moving a lever or little wheel, also just under the slide.

To optimize the contrast do the following:

1. Focus on the edge of the coverslip, and rotate the apertures or adjust the iris diaphragm while you are watching through the oculars. When it looks quite dark against a bright background, stop!
2. Now adjust the focus again, looking for dark bacteria and other objects under the coverslip, remembering that the image through the scope looks like it's moving backwards!
3. Move the slide a bit and look at the microscope from the side just to make sure the light is coming up through the coverslipped area.
4. You now should be able to see organisms going about their normal business. If you can't, get some help.
5. Then proceed to the worksheet or follow instructions from your teacher.

Adjusting the microscope: Phase contrast.

Does your 40X objective lens have the letters *"Ph"* on it? If so, you should have a phase contrast microscope, the ideal kind for this lab. It should already be in alignment so that all you have to do is rotate the proper phase ring into place just under the stage. The letters you see at the front of the stage should match what the objective lens says, usually *"Ph2"* or *"Ph3"* for the 40X objective.

1. Match up the letters by rotating the wheel.
2. You should be able to focus slightly and see plenty of objects moving around under the coverslip, remembering that the view is backwards as you move the slide along.
3. If you can't see anything, get help.
4. Then proceed to the worksheet or follow instructions from your teacher.

Adjusting the microscope: Dim Lighting?

If the light is dim on your microscope, you may be able to improve it. Many microscopes have a focusable condenser lens that optimizes the light spot for different specimens. The small focusing knob under the stage controls this feature. It does not focus your slide! If the condenser is far below the slide, the light will be very dim. While the 10X objective is in place, try adjusting the condenser focus knob to move the condenser lens upwards under the slide and almost touching it. This should improve the lighting for all objectives. If the lighting is still too dim, ask for help.

STUDENT WORKSHEET

NAME_____ DATE_____

1. Where was the biofilm collected?

2. Are any crystals or other apparently nonliving particles abundant?

3. What colors do you see, and do the colors have any known significance?

4. Can you identify any larger organisms in a general way by shape or size? Diatoms, amoebae, and filamentous algae are some of the common larger microbes.

5. Some protozoans identify and eat specific types of bacteria from the biofilm. Can you identify any bacterial types by shape in this community? Rods, cocci, spirals, and comma shapes are some possibilities.

6. Do you see any associations between two or more different kinds of organisms based on attachment or proximity? Describe.

7. Try to fill out the Community Census table below for one field of view. Do all fields of view on your slide look similar to each other as far as the Census information goes?

Air/Water Interface Biofilm Community Census Slide ID _____

Organism #	Shape (Draw it.)	Relative Size	Relative Number	Color	Arranged in Microcolonies? Draw, if so.	Attached to Another?
1						
2						
3						
4						
5						

8. What observations can you make about the general appearance of the biofilm?

9. Do you have any additional comments about the biofilm slides in this class?

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Appendix

The warning on the collodion bottle is rather daunting. The amount handled in this exercise, however, is very small and does not even require special shipping.

The odor produced by amyl acetate is quite noticeable (bananas!) but this substance has been used in the food industry as a flavoring agent and it is not harmful in laboratory concentrations.

Below are entries from *The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals*. 10th Edition. Martha Windholz, ed. Merck & Co., Inc. Rahway, N.J., USA. 1983.

2447. Collodion. A soln of 4 g pyroxylin (chiefly nitrocellulose) in 100 ml of a mixture of 25 ml alcohol and 75 ml ether. Contains about 70% ether and 24% abs alc by vol.

Colorless, or slightly yellow, clear or slightly opalescent, syrupy liquid. Has the odor of ether. d_{25}^{25} 0.765-0.775. Exposed in thin layers, it evaporates leaving a tough, colorless film. On the addition of water the pyroxylin ppts. *Caution: Highly flammable! Keep tightly closed in a cool place and away from flame!*

USE: In photography; manuf lacquers, patent and artificial leathers, artificial pearls; process engraving; in cements.

THERAP CAT: Topical protectant.

THERAP CAT (VET): Skin protectant.

4957. Isoamyl Acetate. Amylacetic ester. $C_7H_{14}O_2$; mol wt 130.18. C 64.58%, H 10.84%, O 24.58%. CH_3COOCH_2-$CH_2CH(CH_3)_2$. The technical product is also known as ***pear oil*** or ***banana oil.***

Colorless, neutral liq; pear-like odor and taste. d_4^{15} 0.876. Pure isoamyl acetate bp 142°. n_D^{21} 1.400; the ordinary grade of commerce boils between 120-145°. Flash pt, closed cup: 92° F (33° C); open cup: 100° F (38° C). Sol in 400 parts water; miscible with alcohol, ether, ethyl acetate, amyl alcohol. Soly of water in isoamyl acetate (25°) 1.6% by volume.

USE: In alcohol solution as a pear flavor in mineral waters and syrups; as solvent for old oil colors, for tannins, nitrocellulose, lacquers, celluloid, and camphor; swelling bath sponges; covering unpleasant odors, perfuming shoe polish; manuf artificial silk, leather or pearls, photographic films, celluloid cements, waterproof varnishes, bronzing liquids, and metallic paints; dyeing and finishing textiles. A special grade of the amyl acetate has been used for burning in the Hefner lamp serving as a photometric standard.