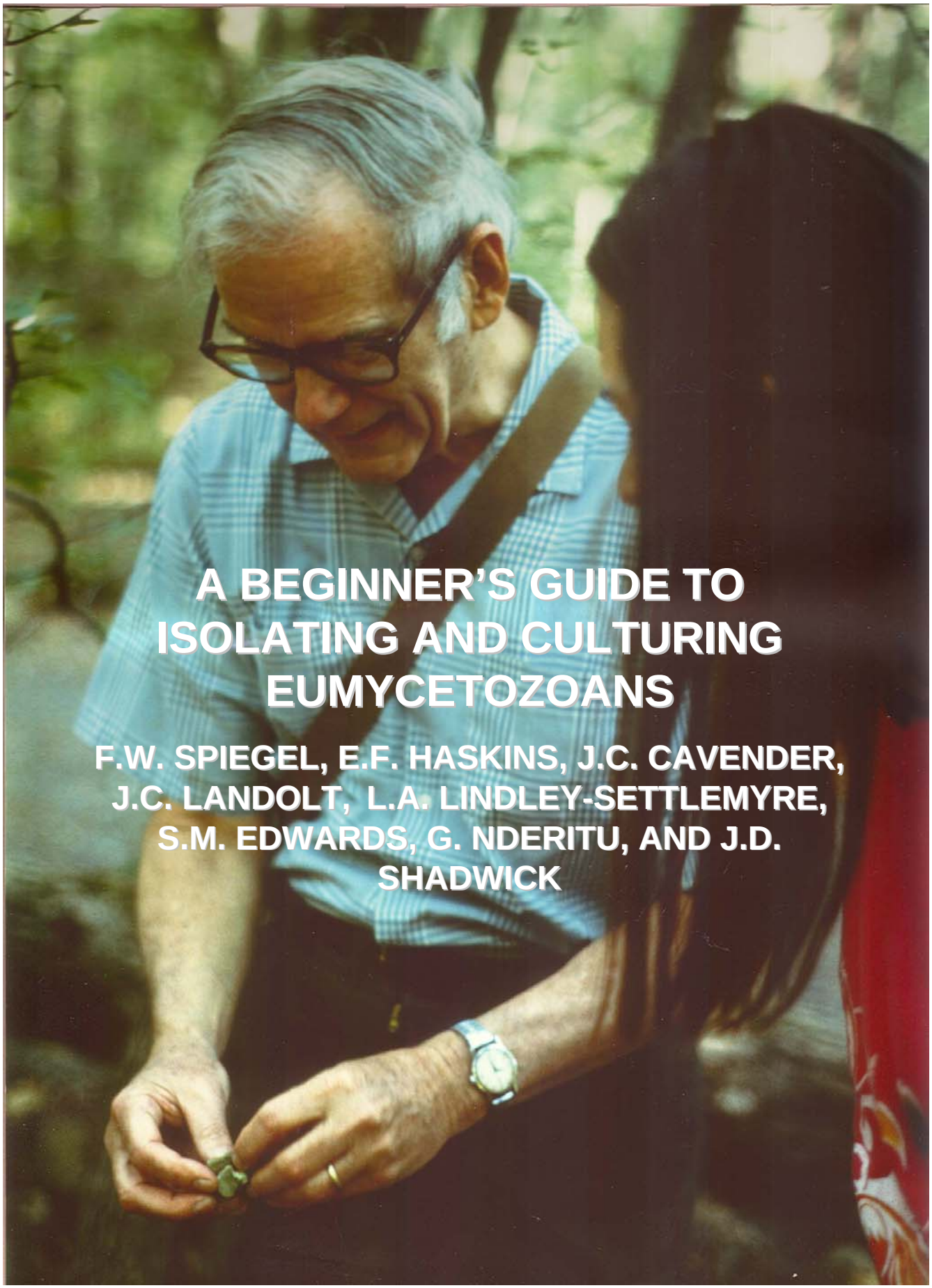


A BEGINNER'S GUIDE TO ISOLATING AND CULTURING EUMYCETOZOANS

F.W. SPIEGEL, E.F. HASKINS, J.C. CAVENDER, J.C. LANDOLT, L.A. LINDLEY-SETTLEMYRE, S.M. EDWARDS, G. NDERITU, AND J.D. SHADWICK

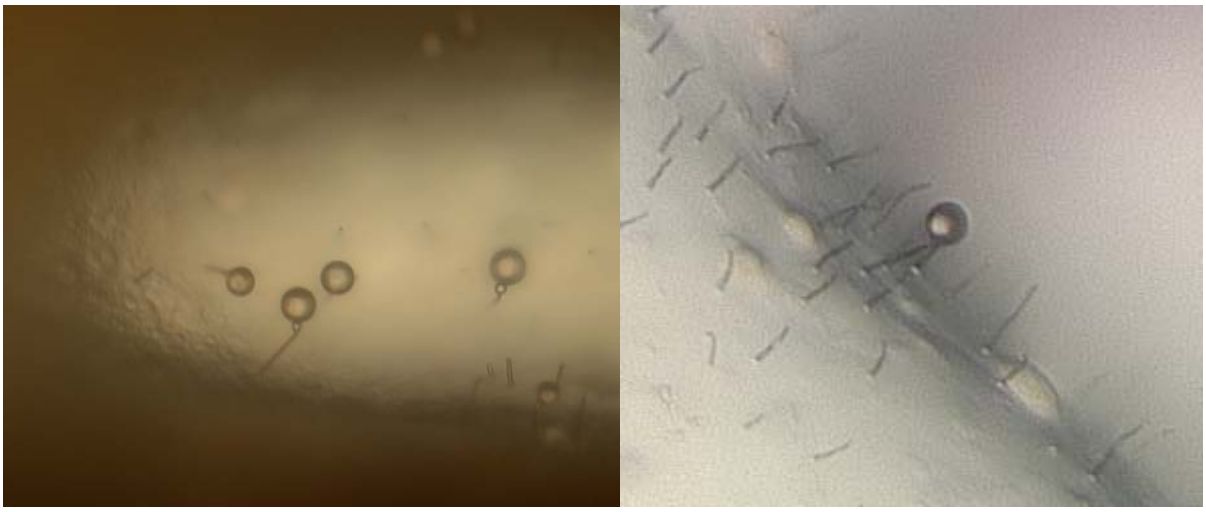
INTRODUCTION

When you went collecting for eumycetozoans with the late Lindsay Olive (cover photo), you often found interesting species that you wished to get into culture. Because his lab was one of the few in which all three groups of eumycetozoans - protostelids, dictyostelid cellular slime molds, and myxomycetes - were regularly isolated and cultured, you quickly learned there were a lot of different and effective ways to bring a slime mold into culture. In some cases many methods were equally effective. That should be the major take home lesson from this handbook.

We will show you some methods that work. Take them as starting points, and then modify them to suit you. Just be sure to keep good records.

Isolating and culturing eumycetozoans is, essentially, a number of variations on a basic theme. Therefore, Dr. Ed Haskins' *Useful Methods for Bringing Field Collections of Myxomycetes into Agar Culture* should be consulted for details regarding tools, media, etc. This handbook will focus on illustrating how you take protostelids, dictyostelids, and myxomycetes from substrates on which they occur and establish them in single slime mold cultures.

Although it is ideal if a species in culture will complete its life cycle repeatedly, it may be the case that you can only establish it in the trophic state. As long as you have followed careful procedures to validate that your culture is derived from what you were attempting to isolate, then the cultures are still useful for studies in such topics as molecular systematics, cell cycle control, comparative ultrastructure, etc. This handbook will give an example of one way to ensure the necessary quality control if the goal of complete life cycles cannot be attained.



The goal: getting a eumycetozoan into single slime mold culture. For example, a newly discovered species of *Protostelium* fruiting on a fragment of a leaf of *Pisonia* sp. in a primary isolation plate (left). The same species growing and fruiting in culture on a plate of wMY agar with *Cryptococcus laurentii* as its sole food source (right). This isolate has been deposited in the *Eumycetozoa Special Collection* at the American Type Culture Collection.

Myxomycetes

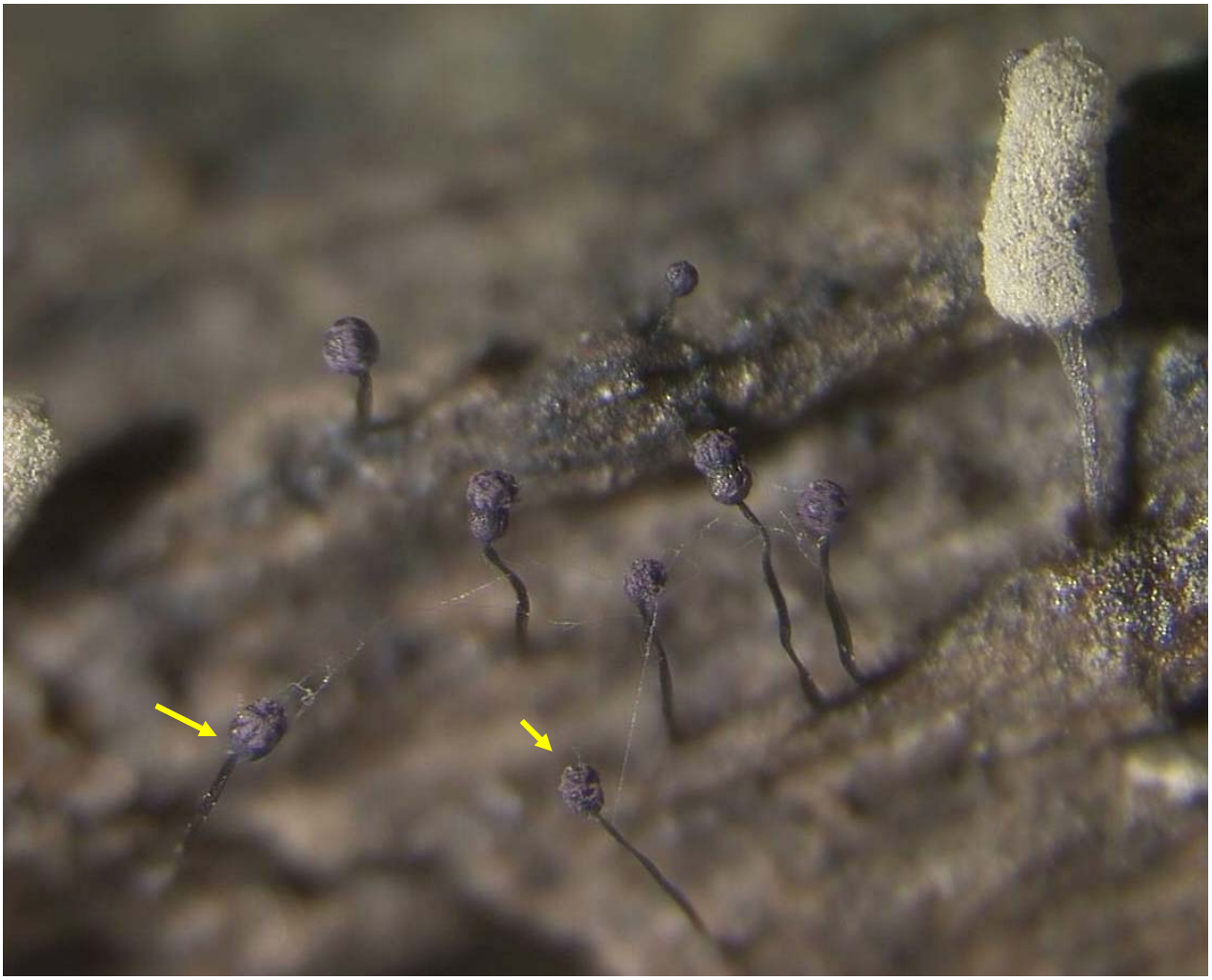
Myxomycetes are the most difficult of eumycetozoans to culture, especially if you are trying for a culture that grows spore to spore. In many cases, it may only be possible to get the amoeba state of the life cycle in culture. **Remember, there is no way to tell one species of myxomycete from another if all you have are amoebae!** Since, as the *Useful Methods…* states, "It is 'a dirty world' out there", you should do all you can to minimize the risk of cross contamination by multiple species of myxomycetes when you are attempting to establish cultures. Here are some handy tips toward getting clean cultures.

1. Keep field collections targeted for culturing separate from each other. Do not keep them in boxes that are used over and over for transporting specimens to a processing site. Put target specimens in fresh, clean containers and seal them against access by spores from other collections.

2. Transport collections to the lab in separate, sealed containers.

When to be careful. Diana Wrigley de Basanta and Arturo Estrada are placing collections of myxomycetes into a fishing tackle box that is used over and over to transport collections from the field. This increases the risk that spores from several collections can spread throughout the box to contaminate the new collections. Therefore, when they collect a speciment to culture, they place subsets of the collection into either clean herbarium boxes or, even better, fresh Eppendorf tubes.

3. When choosing a collection from which to attempt a culture, examine it carefully to see that only one species' fruiting bodies are present. Herbarium specimens with two or more species present can easily show cross contamination of spores.



What not to choose. This herbarium collection contains both *Cribraria violacea* and *Arcyria cinerea*. Light spots on the former (arrows) may be spores of the latter. This is an example of a case where a culture derived from this collection that consists of only amoebae could contain one or both species.

4. Another route to take is to set up moist chambers and to culture from fresh fruiting bodies as they appear.

It may be the case that you need to take some risks with collections that are not ideal in order to culture a desired species. This can happen if you are not able to collect fresh material of a target species. Before doing this, however, we suggest that you practice with species that are easier to work with to become familiar with the techniques. Also, if you follow the procedures that follow, you can insure good quality control.

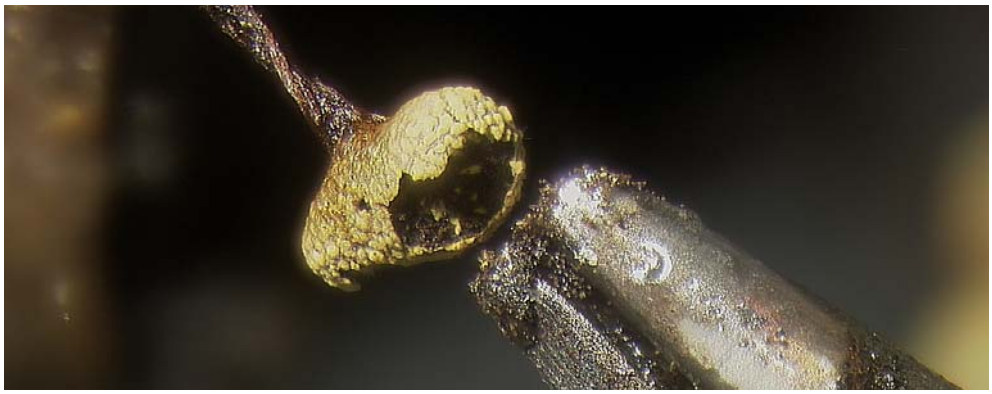
Step by Step Method for Isolating a Myxomycete into Culture

1. Identify and characterize the specimen from which the culture will be made. Identify the specimen to species, or if undescribed, to the most approptriate taxon. Record or reference the locale information. Record, preferably with photographs or *camera lucida* drawings, the morphology of the habitus of the fruiting bodies and the appropriate microscopic characters.

Habitus of fruiting body (left), spore and capillitial characters (center), and spores imaged with DIC (upper right) and bright field (lower right) of a myxomycete identified by Steve Stephenson as a yellow variant of *Physarum pusillum.*

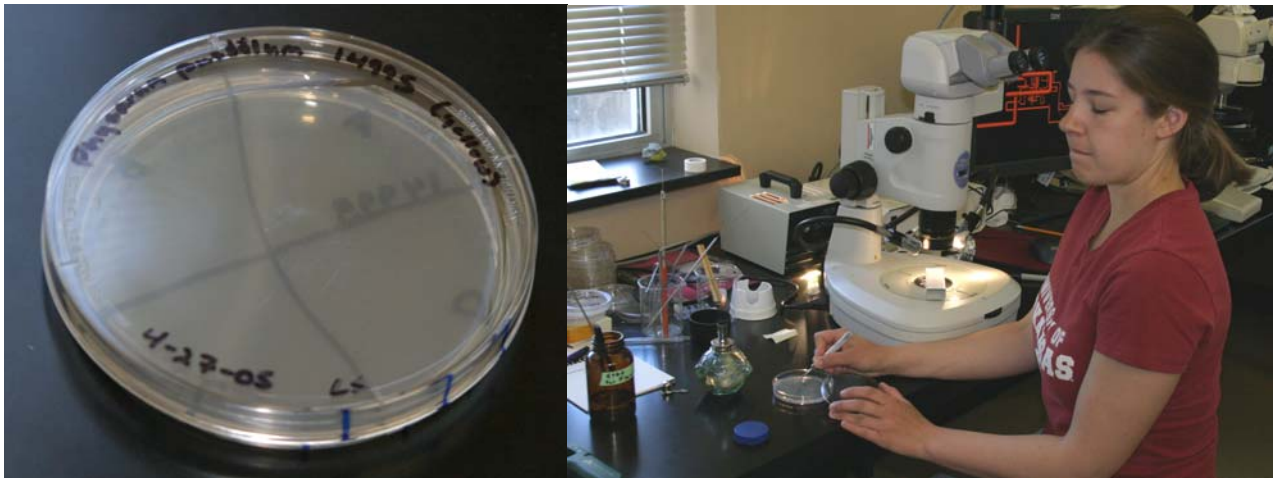
2. Give your culture a unique culture number. Remember to relate that culture number to collection from which it was taken.

3. Using a sterile needle or forceps (whatever you like), collect some spores to transfer.



Spores of fruiting body illustrated above being collected with fine tipped forceps that were flame sterilized. **A helpful hint**: Herbarium specimens are often so dry and brittle that the sporangia shatter when touched and the spores are lost. To soften the fruiting body, add a small drop of sterile, distilled water to the base of the sporocarp about 10 min before you try to collect the spores. That was done in this case.

4. Transfer the spores to a plate of the medium on which you want them to germinate. You may want to try several transfers, so you can divide your plate into sections on which to place each attempt. You may also want to try to transfer to more than one medium. A single spore touch often contains enough spores to inoculate two or three plates. It is best not to add any food organisms to the plate until after germination has occurred. This allows for better monitoring of the spores that germinate and, thus, better quality control. Check to see that all the spores you transferred have the morphology of the species you are isolating.

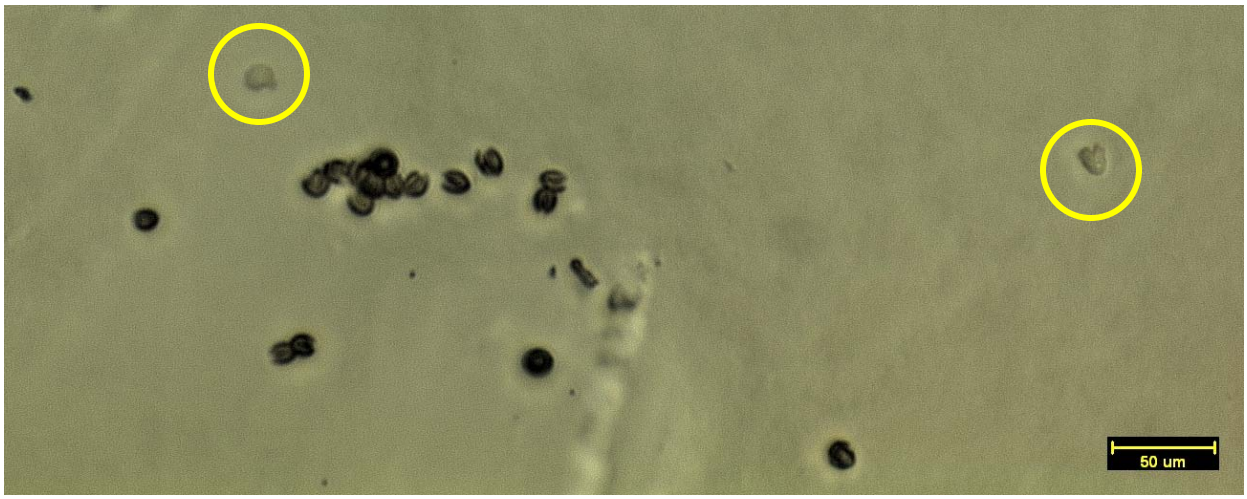


A primary culture plate set up to receive up to four transfers of the yellow variant of *P. pusillum* (right). Lora Lindley-Settlemyre transferring spores to the primary culture plate (left).

The successful transfer of several hundred spores to the primary culture plate (left). Examination of the spores on the surface of the plate with a compound microscope shows that all have the same morphology (right). There might be some fungal spores or some yeasts or bacteria or other protists with the spores, but as long as you can see only one kind of myxomycete spore, you are set to have a single myxomycete culture if germination occurs.

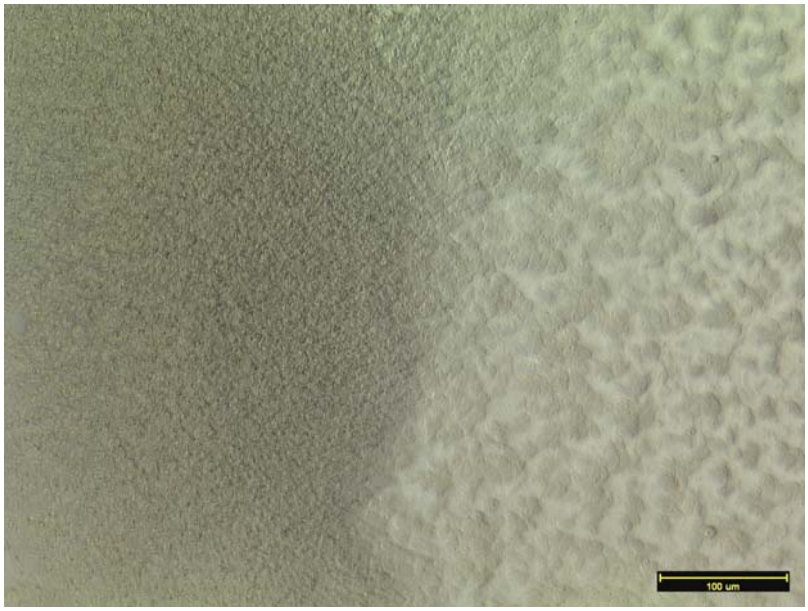
5. Monitor the spores for germination. This may occur in less than an hour or take up to several weeks. (In the case of long waits for germination you may want to make sure that the plate is not overrun with other microorganisms. This is where low nutrient agar or water agar is a big plus.)

Lora Lindley-Settlemyre monitoring for spore germination by looking through the bottom of the closed plate with a low power objective (left). Germinated spores and amoebae (arrows) as they appear with a 10x objective through the bottom of the plate. If the agar is too torn by the effect of transferring spores, you may want to monitor germination on the surface of the agar on an open plate. This does carry the risk of contamination by aerial contaminants.

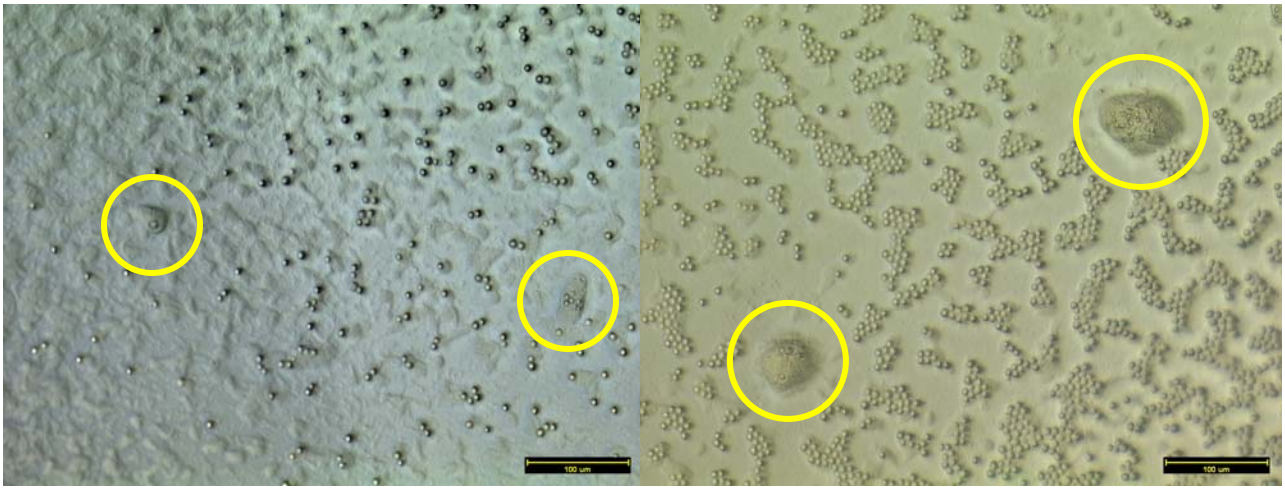


Amoebae (circled) on low nutrient agar or water agar migrate out in all directions and become well separated from the spore walls, each other, and from other microorganisms. Such amoebae are easy to see with high power on a good quality dissecting microscope with substage illumination, and they can easily be cut out singly to transfer to fresh plates if you desire to establish single cell cultures. Also, this behavior allows you to pick out amoebae for transfer that have “Outrun the Fungi” and other microorganisms if the plate has become contaminated. Finally, if you think a “blob” on the agar might be an amoeba, you can look for the track it might have eroded in the agar, and you can monitor the blob to see if it migrates; a small colony of bacteria or an imperfection in the agar will stay put.

6. Transfer some amoebae to a fresh plate and add food organisms. Label the new plate with the culture number. Streak an appropriate food organism, ideally one of the strains of *E. coli* mentioned in *Useful Methods*…, onto a plate of low nutrient agar or water agar, and then inoculate the streak with one or more amoebae. Monitor the culture for amoebal growth for several days and look for development of plasmodia.



A feeding front of amoebae of the yellow variant of *P. pusillum* moving from right to left along a streak of *E. coli*. The amoebae are densely packed in the bacteria and more spread out where they have already been ingested. It is a good idea to become familiar with the appearance of cultures at low power. It helps to monitor the success of the effort.



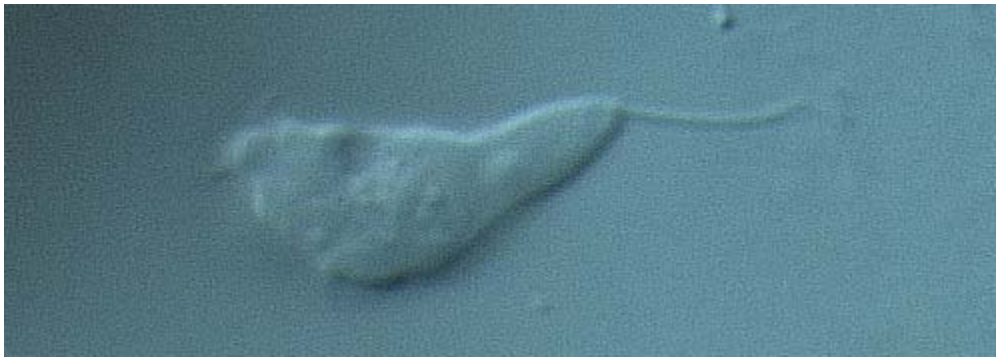
Very young plasmodia of the yellow variant of *P. pusillum* (circled) on the first day they appeared (left) and the next day (right). Learn to recognize young plasmodia by their slightly larger size and the presence of food vacuoles that contain ingested amoebae and microcysts. This is easy to observe by looking through the bottom of a plate with the low power (10x) objective. This is a good time to subculture and to place the cultures in stasis since many cultures with plasmodia only tend to die out. Another reason to learn to recognize young plasmodia in species that develop large plasmodia is that it helps to train your eye to see the plasmodia of species which have only protoplasmodia.

7. Some final hints on quality control for culturing myxomycetes. Your cultures may not proceed beyond having trophic cells, either amoebae or amoebae and plasmodia. (Even when some cultures fruit the sporocarps may be abnormal.) Therefore, there are some quality control steps you can take to insure that you have what you think you have.

A. Make more than one culture of a species. If you are doing molecular work, the appropriate genes should be the same from several cultures.

B. If you are concerned that you may have more than one myxomycete present, make several single cell isolates. If you are doing molecular work, you will be able to observe that the cultures will have different signatures. An added benefit of making single cell subcultures is that, for heterothallic isolates, you will be able to separate the mating types. If you are confident that the species you are targeting for culture will germinate well, you can spread the spores out by dilution methods or micromanipulation so that you can monitor the germination of the single spores.

C. If you are concerned that you may have isolated another amoeboid organism, flood the plate to stimulate the development of swarm cells. This type of contamination is most likely in cases where you have collected from small, stalkless fruiting bodies and may have picked up some other amoebae. Myxomycete swarm cells are unique and easy to recognize.



Swarm cell of the yellow variant of *P. pusillum.*

D. Do not be overly ambitious. Focus on establishing one culture at a time when you are starting out. As you get more practice, you will find that you can work on more than one species at a time.

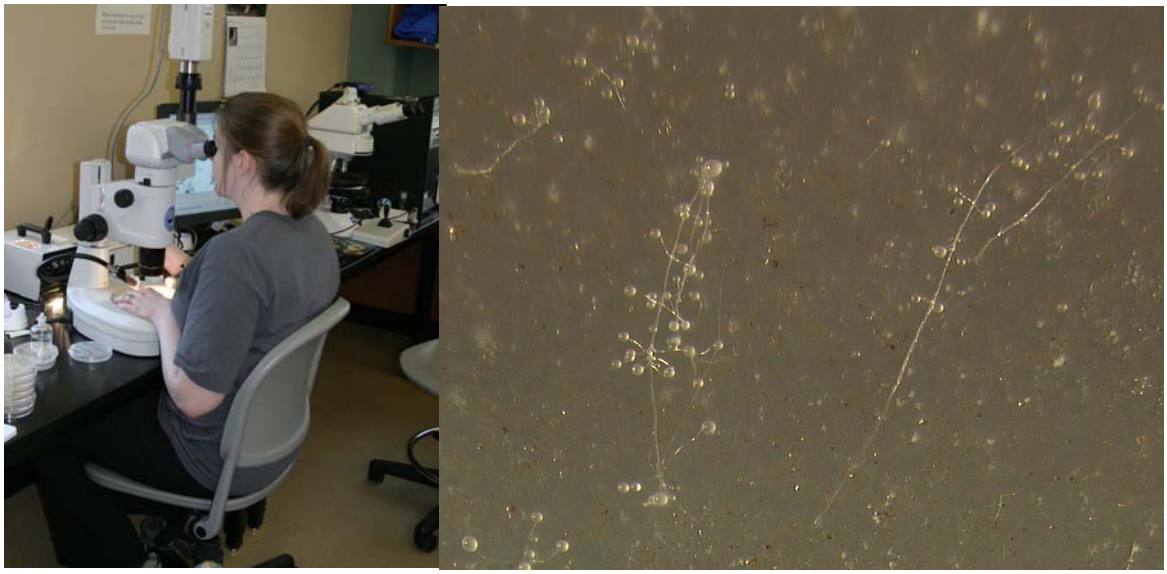
8. Maintain the culture. At this point, with a culture established, you should prepare several subcultures. Some should be placed in stasis (e.g. by drying or freezing in liquid nitrogen) and others can be maintained by serial transfers every month or so. Serially transferred cultures can be kept at temperatures from about 4C to room temperature.

Dictyostelids

Dictyostelids are, relatively speaking, the easiest of the eumycetozoans to isolate and culture, and it is often necessary to culture them from soil dilution plates in order to identify them accurately. Though we will be demonstrating isolation of dictyostelids from soil dilution plates produced by the Cavender method, it is important to remember that dictyostelids can show up in moist chambers of herbivore dung (the original substrate), in moist chambers of dead vegetation, bark, or wood set up for myxomycetes, and in primary isolation plates set up for protostelids. A recent examination of Lindsay Olive's collection notes showed dozens of cases over a collecting career of more than 20 years where dictyostelids were found on such "nontypical" substrates as tree bark and rotting logs.

Step by Step Method for Isolating a Dictyostelid into Culture

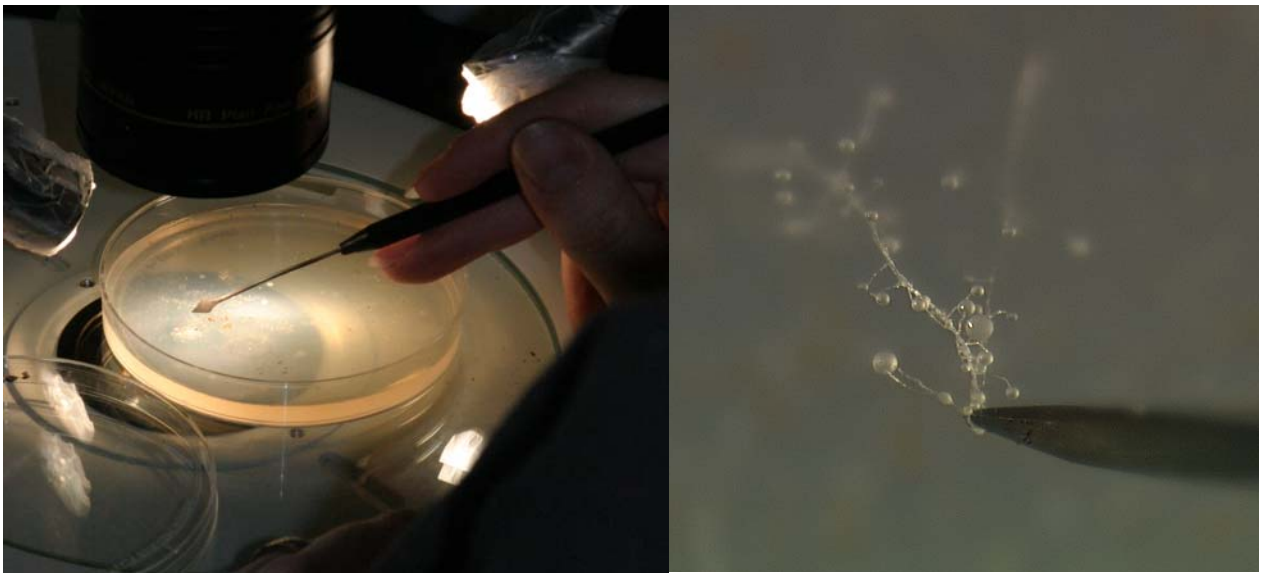
1. Locate a dictyostelid fruiting in a soil dilution plate. Scan a plate with the dissecting microscope to find patches of sorocarps.



Sally Edwards scanning a 3-10 day old soil dilution plate for dictyostelids with the dissecting microscope (left). Part of a patch of a white spored species of *Polysphondylium* fruiting on a soil dilution plate (right). This plate had at least three different species of dicytostelids fruiting on it and the patches of some overlapped with others. It is best to select a part of the patch where the species you are trying to isolate is clear of all other species.

2. Give your culture a unique culture number. Assign a number that relates to the designation of your collection. Label a plate of appropriate agar with the culture number. Media that work well for dictyostelid culture are those with very low concentrations of nutrients such as Hay Infusion Agar (HI) or wMY or water agar.

3. Using a sterile needle or forceps (whatever you like), pick some spores from a sorus on the aerial part of a sorocarp. You can dip a hot needle in agar to cool it and coat it with agar. Touching this to the sorus picks up spores that will not stay adhered to the needle when you transfer to the culture plate. Try not to pull up the whole sorocarp since this can bring along contaminating microorganisms from the surface of the soil dilution plate.



Picking spores from a sorocarp on a soil dilution plate under the dissecting microscope (left). Detail of needle making a spore touch on an aerial sorus of the white spored *Polysphondylium* (right). Note how the sorocarp has collapsed somewhat. This is typical. It is also likely that the sorocarp may wave about as the needle approaches.

4. Transfer the spores to the culture plate then add *E. coli* as a food organism.

Touching the spores to the surface of the culture plate (left). A few touches of the needle to the culture plate will leave hundreds of spores on the agar (center). Streak some *E. coli* (the tan, slimy stuff) on the agar adjacent to the spores (right). Spores can be placed directly into a streak of *E. coli*, but it is easier to monitor germination when the spores are in the open. Amoebae will quickly migrate into the bacteria and start feeding. If you have been careful to this point, you will have a monoxenic culture of your dicytostelid. **If you are concerned that the fruiting body you took spores from might be a genetic chimera (a possibliity if you are isolating from dung or a moist chamber), dilute the spores in a suspension of bacteria and spread thm on the plate such that you can isolate single spored clones.**

5. Monitor the culure for spore germination and subsequent development. Germination usually occurs within a few hours, and, if you inoculated spores at a distance from the bacterial streak, you may see aggregation and fruiting within 36 hours.

Amoebae (circled) and early aggregation behind feeding front in bacteria of the white spored *Polysphondylium* (left) at about 24 hours. Slug and sorcarp development at about 48 hours (right). It appears that this is *P. colligatum.*

6. If contaminating fungi, bacteria, or other protists appear in the culture, just repeat the spore touch and transfer process, or cut out amoebae using the "Outrun the Fungus" technique. With care, you will get to the point that you can establish a monoxenic culture of a dictyostelid with *E. coli* right from the soil dilution plate almost every time.

7. Once a culture is established, it should be prepared for stasis by drying, lyophilization, or freezing in liquid nitrogen. If you are going to maintain some subcultures by serial transfer, you should keep them on a low nutrient agar or water agar on streaks or lawns of pregrown *E. coli.*and transfer it every month or so. You can keep cultures at temperatures from 4C to room temperature. If a culture will not grow and fruit well, you may wish to add activated charcoal to the medium and/or play with the food organisms.

8. As with myxomycetes, do not try to establish too many cultures at once until you "get the hang of it."

Protostelids

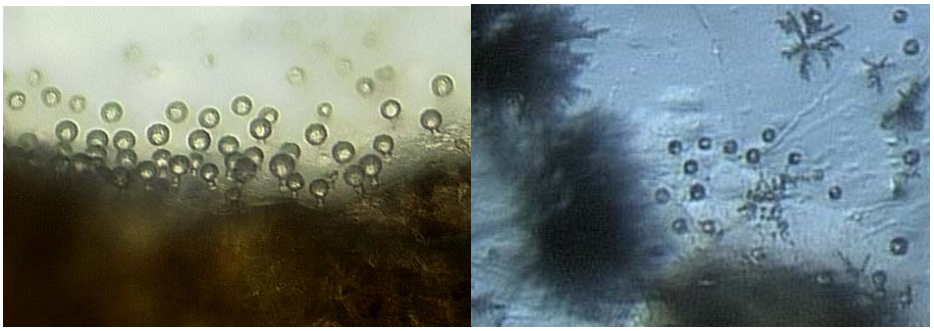
Because of their small size (12-200um) , protostelids offer some challenges if you try to culture them. However, most species take to culture relatively well once they are cleaned up. While most protostelids can be identified on the basis of sporocarp morphology, to classify an undescribed species of protostelid properly requires that its whole life cycle be observed; thus some culturing is necessary.

One of the major challenges with culturing protostelids that is associated with their small size is the likelihood of picking up a number of contaminant microorganisms, so the watch word for you if you want protostelid cultures is to be patient. It will usually take several passes before you achieve a single slime mold culture.

Step by Step Method for Isolating a Protostelid into Culture

1. Locate a patch of protostelid fruiting bodies on a Primary Isolation Plate (PIP).
First, find the protostelids you are interested in under the compound microscope at low power, mark their location on the plate, then find them again on the dissecting microscope. The better resolution of the compound scope allows you to identify the species, but you need the working distance of the dissecting scope to pick up the spores.

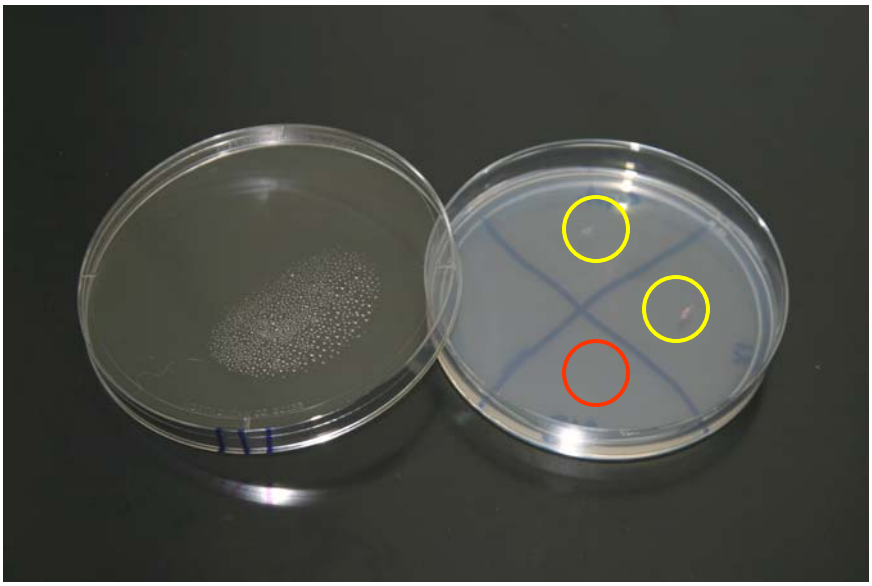
George Nderitu locating a patch of protostelids on a PIP with the compound microscope (left), marking their location on the plate (center), and finding them again on the compound microscope.



A patch of *Cavostelium apophysatum* sporocarps seen with the 10x objective on the compound microscope (left) and a patch of the same species seen with high power on the dissecting microscope (right). Note the much better resolution under the compound scope.

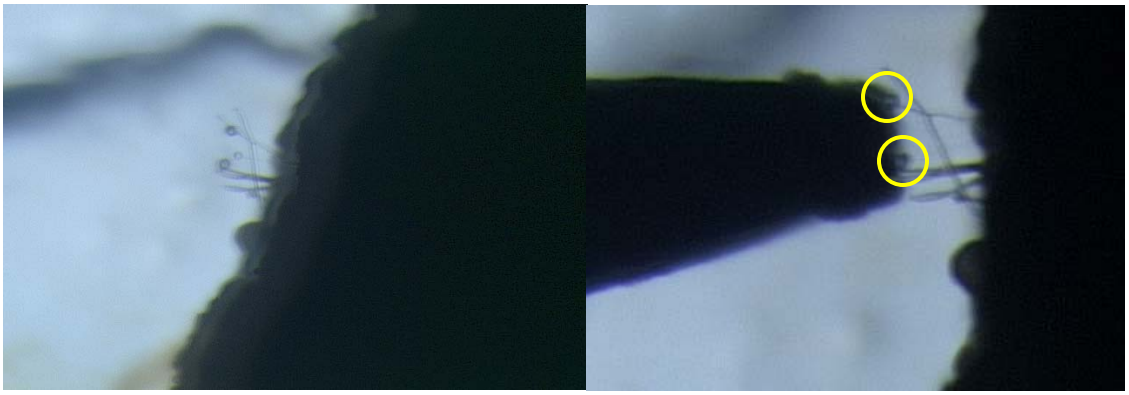
2. Give your culture a unique culture number. Assign a number that relates to the collection number of the PIP. Mark a plate of agar, preferably wMY in most cases, with the culture number.

3. Inoculate the culture plate with several small spots of food microorganisms. Protostelids do not all grow equally well on the limited set of foods that work for other eumycetozoans. Even different isolates of the same morphological species may have different food preferences; therefore it is preferable to try a lot of possibilities. Also, always mark one spot on the plate NB (natural bacteria). This will be a spot with no added food that will allow the microorganisms that always get picked up with the protostelids to grow since they may provide the right conditions for growth and fruiting.



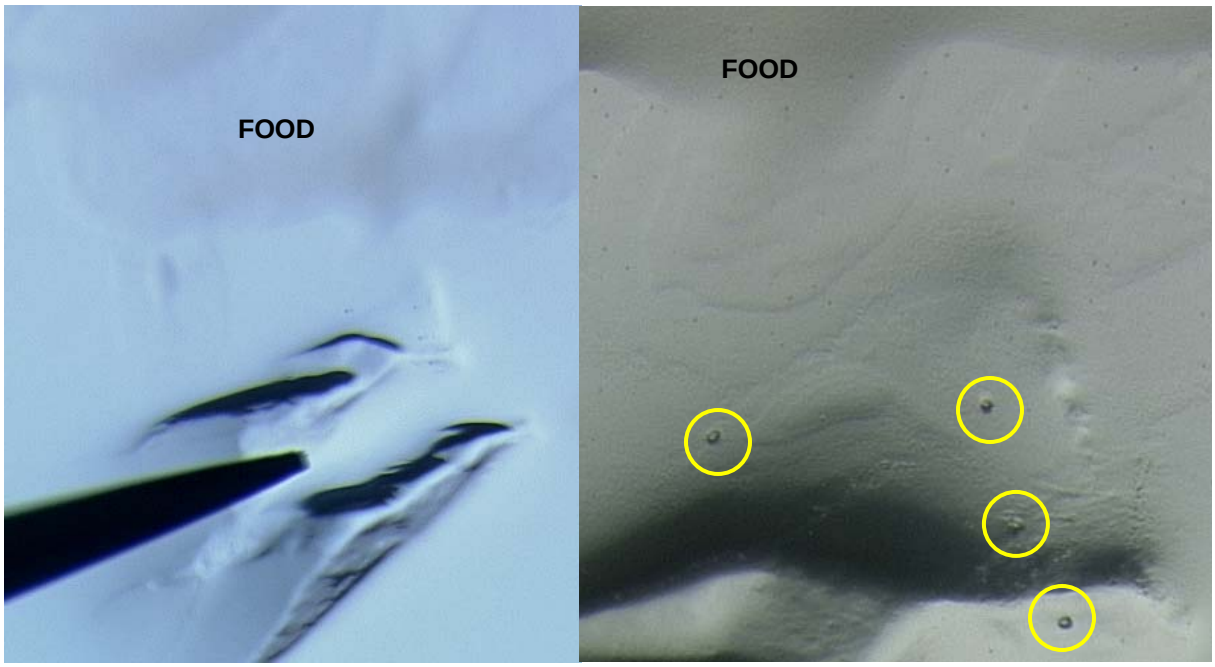
Protostelid culture plate inoculated with food microorganisms (yellow circles) and an NB spot for inoculating the protostelid with its accompanying microorganisms (red circle).

4. Using a sterile needle or forceps (whatever you like), pick some spores from sporocarps on the substrate in the PIP. Dipping the a hot needle in agar to coat it makes it easier to pick up spores and to inoculate them on the culture plate.



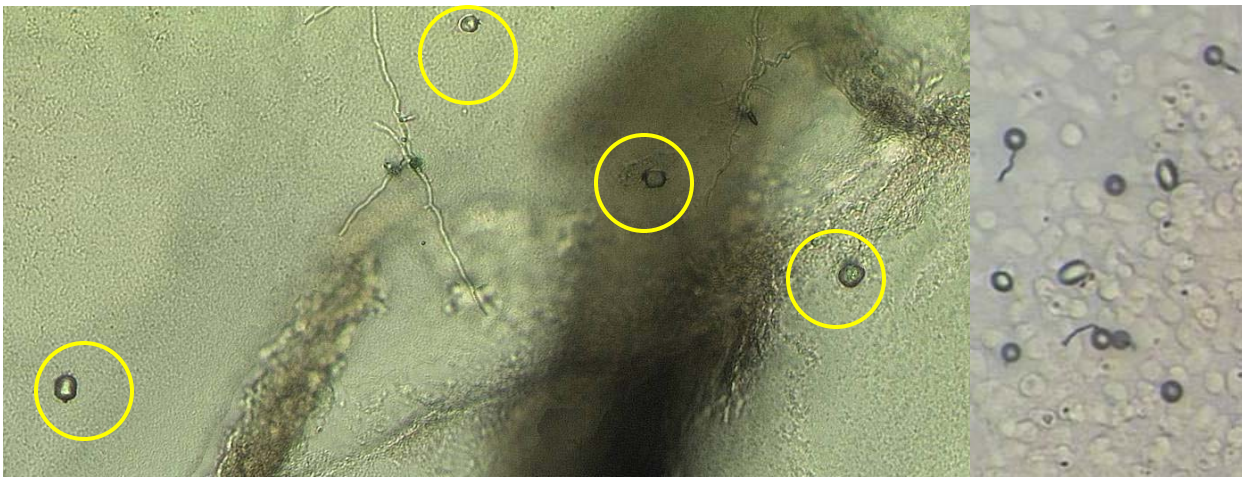
Cluster of sporocarps of *Nematostelium ovatum* on natural substrate (left). Spores (circled) on tip of sterile spear-point needle (left). Note that you can isolate spores either right or left handed ☺.

5. Gently inoculate spores on the culture plate just outside the edge of the patch of food organisms. This allows you to monitor spore germination, a process that can take from a few hours to several days.



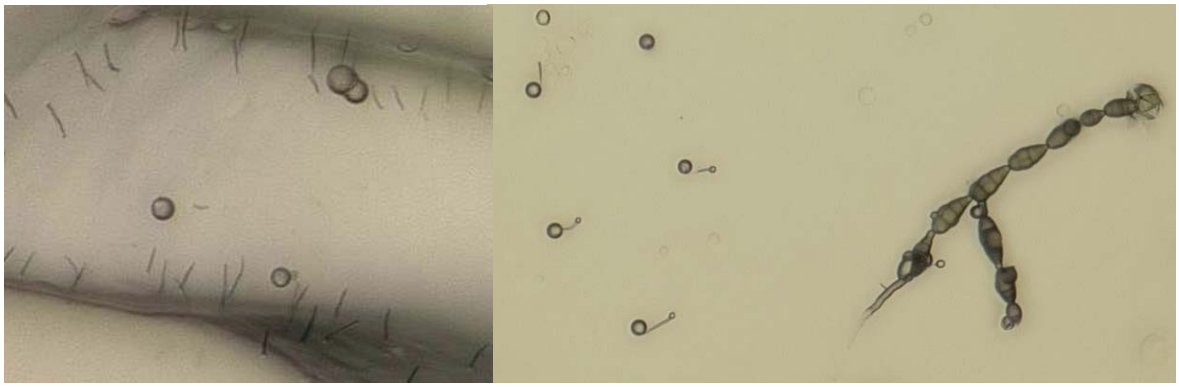
Lightly touching the needle to the agar in the culture plate (left) allows you to rub spores (circled) off onto the surface of the agar (right) without them getting buried in cuts in the agar. Although spores in cuts will germinate, it is hard to know if you successfully transferred them since they are difficult to see. Having the spores several hundred micrometers from the food also makes it easier to confirm transfer as well as to monitor germination.

6. Watch for germination and amoebae. Among protostelids amoebae are very variable in morphology, and there will usually be lots of contaminating fungi, bacteria, and other protists, so you might get fooled. You really have to wait for fruiting to be sure you have the species you are trying to culture.



The spores (circled) of *N. ovatum* from step 5 have germinated (left) but their amoebae do not show up well in the mix of microorganisms present. Do not assume the irregular blobs are amoebea until you can see more detail. Also not the germinating fungal spores. An example of amoebe (lighter patches) in the food bacteria can be seen in this early culture of *Protostelium nocturnum* that is beginning to fruit (right). The black spots in many amoebae are contractile vacoules, a good feature to look for to find amoebae.

7. Watch for fruiting to occur. This may take several days, so you must monitor the cultures frequently. Do not worry if there are contaminants; there usually are. If the species you are interested in is fruiting, you can reisolate by spore touch or, if you know its amoebae, by "Outrun the Fungus." If you have a species with deciduous spores invert a block of agar with fruiting bodies on it in the lid of a fresh plate and let the spores drop clear of the contaminants to the fresh agar surface.



Fruiting bodies of the newly discovered species of *Protostelium* occuring in a cut in the agar on a culture plate (left). This is clean enough to transfer without any additional effort to reisolate. Fruiting of *Protostelium mycophaga* (minute variant) on a culture plate where *Alternaria* sp. was transferred with it. This culture would need reisolation, but t is much cleaner than the example of *C. apophysatum* shown in Step 1.

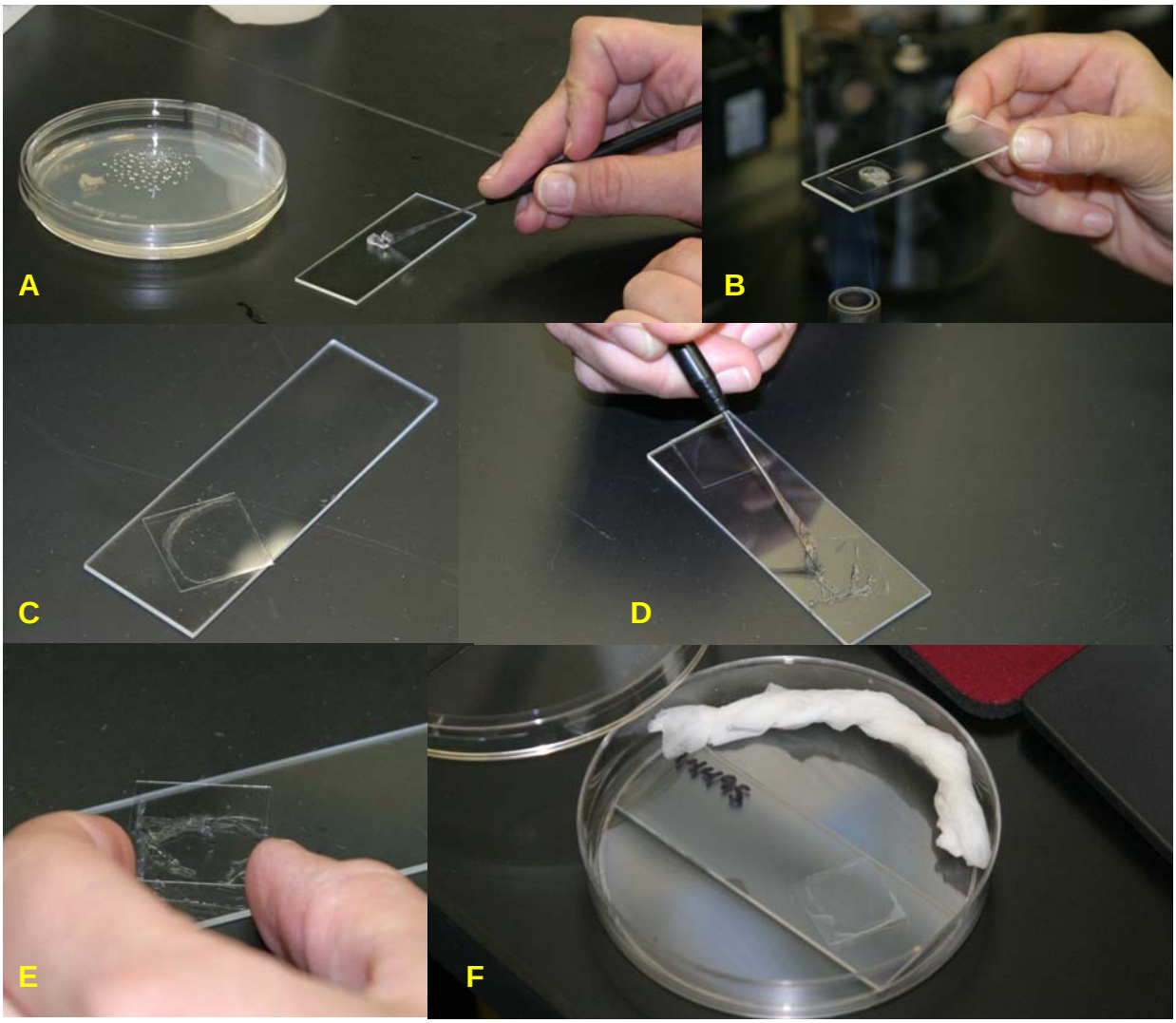
8. Once the culture is established, prepare it for stasis by drying or freezing in liquid nitrogen. Culture plates may be allowed to dry completely and stored for up to two years. The cultures can be revived by flooding the plates with sterile distilled water and a dilute suspension of the appropriate food organism(s). Subcultures that are kept for serial transfer should by kept on mWY and transferred to plates that have been streaked with the appropriate food organisms every 3 weeks to 2 months. Active cultures can be maintained at temperatures from 15C to room temperature. If you want good fruiting, it is best to keep the cultures on a light/dark cycle since many species will not fruit in constant light or constant darkness.

9. Again, be patient and do not try to do too many isolations at once.

Some alternative methods for isolating eumycetozoans. Instead of using hand held tools to inoculate spores onto agar plates consider trying some of the following.

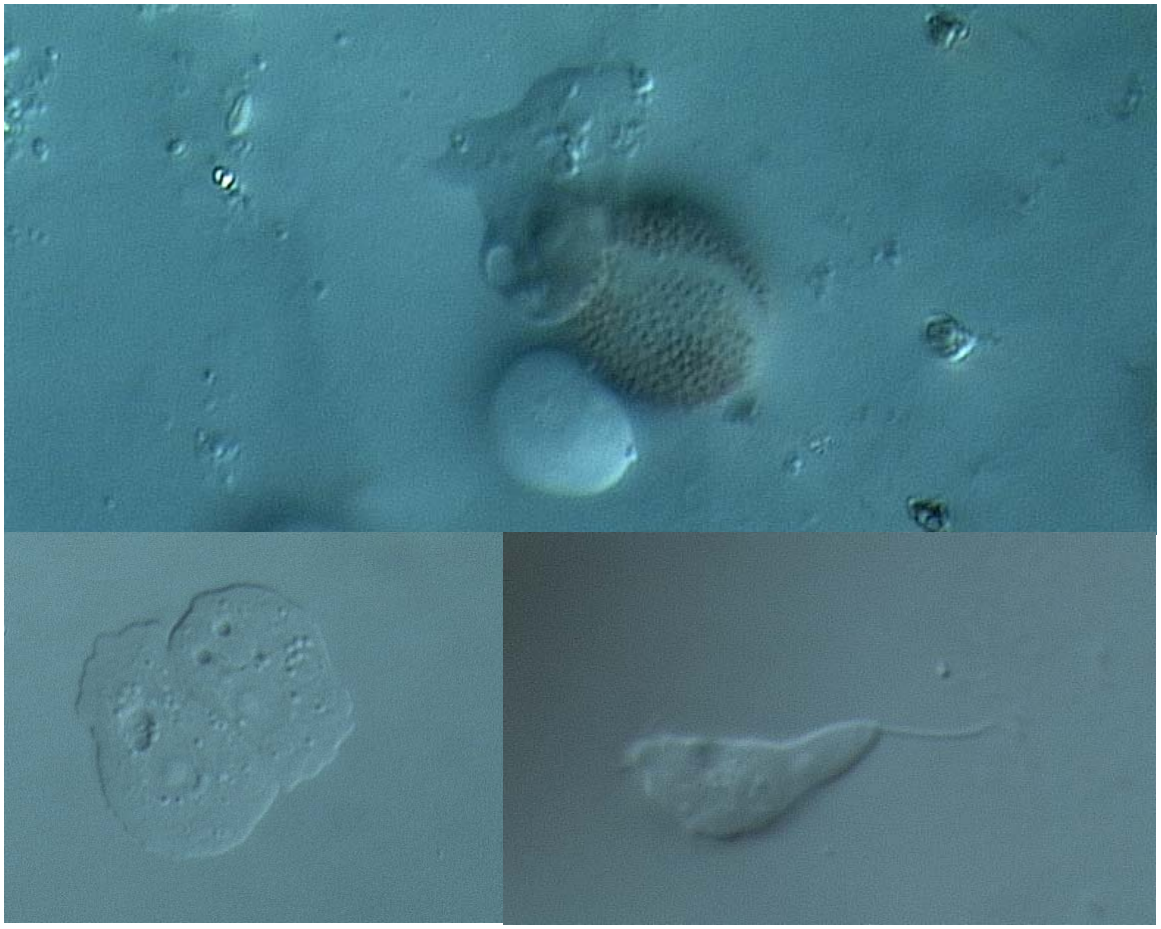
1. If you want to spread spores out to allow them to germinate singly, in isolation from each other, transfer them to a drop of sterile water on the surface of the plate then tip the plate such that the spores will be spread out as the drop runs down the slope. This works when you have lots of spores from myxomycetes or dictyostelids. If you have only a few spores, spread them out on the plate using a micromanipulator.

2. If you want to monitor spore germination in detail to watch exactly what the germling looks like, inoculate spores into a sterile hanging drop or onto a sterile culture slide. An example of the latter that we use frequently is illustrated below.



A. Chop a small piece of culturing agar onto clean slide. B. Cover the chopped agar with a clean coverslip and gently it. C. Allow the melted agar to cool under the coverslip. D. Remove the coverslip and inoculate the thin layer of agar with the mycetozoan and its food. Do not worry about the irregularity of the agar; it allows you to see lots of different amoebal morphologies. E. Add a drop of sterile water and cover with a sterile coverslip. F. Store the culture slide in a sterile petri dish with a moistend, twisted, sterile Kimwipe. This preparation will last for days if you just add sterile water to the edge of the cover slip. You can observe the culture with high quality optics such as DIC and transfer from the culture slide to a plate.

An example of a use for a culture slide culture. The images below were taken of germinating spores and amoeboflagellates of the yellow variant of *Physarum pusillum* shown in the section on Myxomycetes. The slide was between 24 and 48 hours old when these images were taken. As you can see, if you are careful, you can watch a single cell in detail and could easily isolate that cell to establish a culture. This would leave no doubt that you started a culture of a myxomycete from the source you intended.



Germinating spore (top), amoebae (lower left) and swarm cell (lower right) of the yellow variant of *P. pusillum.* All of these cells were followed from the spores from which they germinated. All the spores from the herbarium voucher specimen were the same. There can be no doubt as to the source of these trophic cells, and any culture started from this slide could reliably be linked to the voucher specimen. We suggest that as soon as such cultures are established that they be genetically bar coded so that later subcultures can be verified as derivatives of the original.

Summary

As you practice isolating and culturing eumycetozoans, you should be able to become quite adept at observing many aspects of their life cycles. Start with the techniques we illustrate here and go on to develop your own. As you produce cultures, we urge you to deposit them in the *Eumycetozoa Special Collection* at ATCC.

Suggested reading:

Spiegel, F.W., S.L. Stephenson, H.W. Keller, D.L. Moore, and J.C. Cavender. 2004. Mycetozoans. In G.M. Mueller, G.F. Bills, and M.S. Foster (eds.). *Biodiversity of Fungi, Inventory and Monitoring Methods.* Elsevier Academic Press, Burlington, MA. pp. 547-576.

The Eumycetozoan Project. http://slimemold.uark.edu