

Collection and cultivation of Dictyostelids from the wild

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Summary

Dictyostelium discoideum is a commonly used model organism for the study of biological processes such as chemotaxis, cell communication and development. While these studies primarily focus on a single clone, recent work has revealed a host of questions that can only be answered from studies of multiple genetically distinct clones. Understanding intraspecific clone conflict, kin recognition, differential adhesion, and other kinds of interactions likely to occur in the natural soil habitat can only come from studies of multiple clones. Studies of populations of wild isolates are also important for understanding the factors contributing to associations such as species co-occurrences and to observed inter- and intraspecific interactions such as those found between bacteria and *D. discoideum*. Natural isolates of *Dictyostelium* are easily found in soil and leaf litter in nearly all habitats. Here we describe a simple and successful method for isolating new wild clones from soil, then isolating single clonal strains, and storing them for future use.

Key Words: *Dictyostelium discoideum*; soil; natural isolate; wild population; strain preservation

1. Introduction

Social amoebae like *Dictyostelium discoideum* are commonly studied to address questions about social evolution, multicellularity, and cell biology (1-4). Though historically much of the work has focused on a single laboratory clone, in recent years studies of wild clones have become increasingly important. Studying natural populations allows researchers to ask questions about interactions like conflict, recognition, and differential adhesion between individuals that cannot be addressed using a single isolate. We know from recent studies that multiple lineages of *D. discoideum* can be found together on a small scale in about 0.2-g soil samples (5). The discovery that genetically distinct clones are found in close proximity provided evidence supporting the possibility that they could compete in nature. Specifically, any clone that could produce mainly spores in a mixture with another clone, and induce its partner clone to produce stalk, could gain an evolutionary advantage. Such cheaters have been found among wild *D. discoideum* clones and identified among genetically engineered knockouts (1,6). As recent studies have revealed, the availability of many genetically different natural isolates allows testing hypotheses on altruism, kin discrimination and cheating (7-10).

Other recent research emphasizes the importance of understanding where natural isolates come from and how they interact with other organisms in their environment. Brock *et al.* (11) found that about a third of wild *D. discoideum* clones now known as farmers have evolved to carry bacteria through the social stage. This primitive farming symbiosis includes dispersal and prudent harvesting of the crop, that provides a major advantage if edible bacteria are lacking at a new location. Douglas *et al.* observed phylogenetic structure by location, with more genetically similar clones occurring in soil collections from the same region (12). Since genetic relatedness among interactants plays a role in behaviors such as kin discrimination, isolates from the same

soil sample may interact differently as compared to interactions among isolates from more geographically distant soil samples, as shown by Ostrowski and colleagues (13). Finally, natural clones are essential for understanding the population genetics of *D. discoideum*. Little has yet been done on *Dictyostelium* in this area. An exception is a recent study on patterns of linkage disequilibrium in wild populations which support the hypothesis that sex is common in the wild (14), so with the right conditions it ought to be achievable in the laboratory. It is also possible to exploit the information present in wild populations to identify functional traits and genes under selection. This has been done successfully in other model systems, such as *Arabidopsis* (15,16) and *Drosophila* (17), so there is no reason it could not be done in *Dictyostelium*.

These studies reveal the importance of understanding more about where *D. discoideum* amoebae live and how to isolate them from their natural environments. Natural isolates of these amoebae can easily be identified and cultivated for use in laboratory studies and experiments from samples of soil and leaf litter. The methods in this chapter describe how to isolate these species in order to take advantage of this very useful organism. More than 70 years ago, Kenneth Raper first isolated and described *D. discoideum* from soil samples collected near a site off the Blue Ridge Parkway in North Carolina, USA (18). In general, *D. discoideum* can be found in soil and decaying vegetation (leaf litter) of temperate, deciduous forests in eastern United States and East Asia, as well as in parts of Mexico and Central America (19-21). *D. discoideum* is more prevalent in southwestern Virginia as compared to common Dictyostelid species such as *D. mucoroides*, *Polysphondylium pallidum*, and *P. violaceum* in other reported areas (22,23).

There are several important factors to consider before choosing a sampling site for isolating *D. discoideum* from the wild. Observations on soil pH from the Landolt and

Stephenson study (23) suggest that *D. discoideum* may have a greater tolerance for low soil pH than other Dictyostelid species. This tolerance may explain its abundance patterns. Food preferences may play a crucial role in coexistence and distribution of different Dictyostelid species because different species will selectively feed on different soil bacteria (24,25). Finally, it is important to collect from upper levels of the soil, where vegetative material is decaying. Cavender and Raper found that Dictyostelids primarily occupied the fermenting leaves and soil surface directly below the top layer of dry leaf litter (19). Once an appropriate sampling site has been chosen, it is easy to isolate *D. discoideum* from the soil. Our current methods for this are loosely derived from those reported by Eisenberg (26), though they differ in ways that make collection easier and more complete from a given soil sample.

2. Materials

2.1. Soil/Leaf Litter Collection and Culture

1. Straws, collection tubes and scissors: We recommend plastic drinking straws (6 mm in diameter) and 1.5mL microcentrifuge tubes for collecting soil samples.
2. Small sealable bags (Ziploc) and a spoon or small shovel: These tools are for an alternative collecting method to the one that uses straws and collection tubes.
3. Hay agar (used for isolating *Dictyostelium* from soil and litter samples): place 15 g of hay (dried cut grass) into 1.5 L deionized water (dH₂O) in a 4 L beaker. Cover with foil and leave overnight or boil to infuse. Filter infused water through a funnel lined with cheese cloth into a 2 L flask with a magnetic stirrer. Add, while stirring: 1.5 g of KH₂PO₄, 0.62 g of Na₂HPO₄, and 15 g of agar to 1 L of the hay infusion.

Sterilize by autoclaving, let cool slightly, and pipette 30 mL per 100-mm Petri dish.

Store the agar plates in a sealed bag or container to prevent drying.

4. Activated charcoal pieces: We use API activated filter carbon (Mars Fishcare Inc).
5. Luria broth Miller (LB): 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl in 1 L ddH₂O. Adjust final pH to 7.0 ± 0.2 . Autoclave and cool before use.
6. Food bacteria: We use the laboratory strain of *Klebsiella aerogenes* (KA) available from the *Dictyostelium* Stock Center (<http://dictybase.org/StockCenter/StockCenter.html>). We maintain KA suspensions in LB.

2.2. Detection, Isolation and Storage of Strains

1. Starving agar (used for low-nutrient growth of *Dictyostelium*): 0.3 g of Na₂HPO₄, 2 g of K₂HPO₄, 20 g of agar in 1 L ddH₂O. Sterilize by autoclaving and prepare and store agar plates as in Subheading 2.1 item 3 (*see Note 1*).
2. SM-medium agar (used for growth of *Dictyostelium* and food bacteria): 10 g of peptone, 1 g of yeast extract, 10 g of glucose, 1.9 g of KH₂PO₄, 1.3 g of K₂HPO₄, 0.49 g of MgSO₄ anhydrous, and 17 g of agar in 1 L ddH₂O. Sterilize by autoclaving and prepare and store agar plates as in Subheading 2.1 item 3 (*see Note 2*).
3. KK2 buffer: 16.5 mM KH₂PO₄, and 3.8 mM K₂HPO₄. Autoclave and cool before use.
4. Luria broth Miller (LB): same as in Subheading 2.1 item 5.
5. Food bacteria: same as in Subheading 2.1 item 6.

6. Sterile freezer vials with glycerol: we use 12 x 35mm glass freezer vials with screw tops, but any size glass or plastic freezer vial with a secure lid will suffice. Put 0.5 mL glycerol solution (60% glycerol (v/v) in ddH₂O) into each freezer vial. Screw the vial caps halfway on. Autoclave the vials on slow exhaust. After the vials have cooled, screw the tops on completely.

3. Methods

3.1. Field Collection

There are many techniques that can be used for collecting soil or leaf litter from the field, although only a few of these give optimal results. Because of this, there are a few important questions to ask before collecting: What *Dictyostelium* species are you looking for? How soon will you be able to plate out the soil? And, most importantly, what questions do you want to ask in your study? There are two techniques most commonly used to collect wild samples, a straw method and a bag method, both of which will be described here. We recommend collecting multiple small samples using a straw, since this method best preserves the structure of the soil, maximizing survivorship of the amoebae (5). The larger samples, collected using the bag method, provide a better buffer against temperature changes and desiccation if plating right away is not an option. The larger samples also provide excess soil for further analyses and allows for the collection of larger substrates such as leaf litter. Often, using a combination of both the straw and bag methods can be beneficial to a study (see **Note 3**).

3.1.1. *Collecting Soil Using the Straw Method*

1. Using your hands or a small shovel or spoon, remove the top layer of whole leaves and larger leaf fragments, exposing the layer of decaying leaf litter and soil below.
2. Press the straw into the soil, a centimeter or less up the straw (*see Fig. 1*).
3. Put the straw into a labeled 1.5 mL microcentrifuge tube (filled end first) and cut the straw short enough so that you can close the tube.
4. Continue collecting in this manner, sterilizing your equipment after each use with alcohol wipes, until you have collected an adequate sample size for your specific study questions (*see Note 4*).

3.1.2. *Collecting Soil Using the Bag Method*

1. Using your hands or a small shovel or spoon, remove the top layer of whole leaves and leaf fragments, exposing the layer of decaying leaf litter and soil below.
2. Using a small shovel or spoon, scoop 50 to 500 cm³ of soil (usually approximately 250 cm³) into a labeled Ziploc bag and seal the bag (*see Note 5*).
3. Continue collecting in this manner, sterilizing your equipment after each use with alcohol wipes, until you have collected an adequate sample size for your specific study questions.

3.2. *Plating Out Samples From the Field*

Plating out these samples is a compromise between providing nutrients dilute enough to discourage fungi and rogue bacteria, but concentrated enough to feed the bacteria *K.aerogenes* used as the food source for our social amoebae. We have found that weakly nutrient (hay) agar

plates combined with food bacteria work best. Diluting and then dividing each 0.2-g soil sample between four agar plates allows for maximum detection and isolation of strains. Adding activated charcoal lumps to the plates promotes *Dictyostelium* development both because it absorbs stray light and potentially inhibitive gases (27, 28). Be careful to avoid contamination during this process by using sterile pipetman tips (we recommend cutting the tips to make a larger opening) or single-use glass pipettes with rubber bulbs.

3.2.1. *Plating out Soil from Straw Method*

1. Add 1 mL KK2 to the tubes and vortex or shake vigorously.
2. Pipette 0.2 ml onto each of 4 hay agar plates, shaking out the remaining soil on the last plate (*see Fig. 2*).
3. Add 0.3 mL KA suspension to each plate.
4. Spread plates evenly with a bent glass rod that has been sterilized by being dipped in alcohol, flamed, and cooled on a corner of the petri plate or lid. You can spread the 4 plates from the same sample without sterilizing the spreader in between.
5. Sprinkle a few (5-10) pieces of activated charcoal on each plate (*see Fig. 2*).
6. Leave the plates in a laminar flow hood (lids slightly open) or on the bench (lids closed) until the liquid is absorbed, then store at room temperature (22°C) on a bench or in a drawer.

3.2.2. *Plating out Soil from Bag Method*

1. To prepare the soil solutions, measure out 0.2-0.5 g of soil and mix with 1 mL of KK2 in a 2 mL microcentrifuge tube.
2. Vortex or shake vigorously.
3. Continue with plating using the methods from **Subheading 3.2.1, steps 2-6**.

3.3. Detection and Isolation of Strains

The goal using the methods below is to visualize, identify, select, and clonally isolate as many clones as possible. The methods we describe are optimized for collecting a variety of social amoeba species. To select for a more specific subset, greater dilutions and longer wait periods may be optimal. We recommend the study by Schaap *et al.* for information on the different Dictyostelid groups (29). These methods only limit, but do not completely deter fungal growth therefore it is necessary to be able to differentiate social amoebae from other non-*Dictyostelium* species. There are many helpful keys and guides to help you with this. To identify Dictyostelid species, we recommend an online guide by Andrew Swanson (<http://slimemold.uark.edu/pdfs/GSMNPDicthyGuide.pdf>) or also two published guides (30,31). Ultimately, comparisons of 17S (18S) ribosomal DNA gene sequences are useful for identifying isolates to species (12,29).

3.3.1. Sample monitoring and isolate collection

1. Check samples two or three days after plating out and continue for 6 to 10 days.

2. When you check the samples, circle on the lid with a permanent marker any areas on the agar plate with social amoebae, and note what species they are if you can tell, in notes or on the plate (*see Note 6*).
3. Decide what samples you plan on isolating from each plate and prepare that number of labeled microcentrifuge tubes with 0.3-0.5 mL of KA bacterial suspension.
4. Pick up fruiting bodies or slugs with clean forceps or pipetman tips, preventing cross-contamination by sterilizing forceps with alcohol or changing tips between each collection.
5. Place the fruiting body or slug in the microcentrifuge tube with the bacterial suspension.
6. Vortex each tube gently and pipette in a single strip or cross onto a labeled starving plate (*see Fig. 3*).
7. When the starving plates have grown to yield fruiting bodies, check for contamination (mainly fungal hyphae).
 - a. If the plate is still contaminated, then pick up the cleanest looking fruiting bodies and plate them on a new starving plate with a 0.3 mL KA strip. Keep repeating this process until the fruiting bodies look clean.
 - b. If the fruiting bodies look clean, then move ahead to grow them clonally.

3.3.2. *Preparing clonal isolates (see Note 7)*

1. For each sample you wish to grow clonally, prepare a labeled SM agar plate and two 1.5 mL microcentrifuge tubes, the first (tube 1) with 1 mL of KK2 and the second (tube 2) with 0.99 mL of KK2.

2. From the clean starving plate, pick up a single fruiting body and place in the tube 1.
3. Vortex.
4. From that spore suspension take 10 μ L and place it into tube 2.
5. Vortex.
6. From that final spore suspension take 10 μ L and place on the SM agar plate from step 1 with 0.3 mL KA bacterial suspension and spread with a sterile glass rod (*see Subheading 3.2.1, step 4*).
7. Check clonal plates in 2-3 days for single clearings (*see Note 8*).
8. Collect a single colony with a sterile loop or pipetman tip and plate on a new SM agar plate with 0.3 mL KA bacterial suspension. Spread with a sterile glass bent rod.

3.4. Storage of Strains

Clones must be stored for two purposes, long-term archiving, and frequent access. The former is important to preserve the clone. The latter is important because experiments should be initiated from freezer stock. You should not keep clones growing on the bench for prolonged periods of time because they will accumulate mutations (32). It is therefore important to store the spores as soon as possible in an ultracold freezer (-80° C) after the clonal amoebae from the previous section have developed into fruiting bodies. The fruiting bodies produced by social amoebae isolated from soil and leaf litter samples can be a variety of sizes. Because of this, we will describe two methods for the collection and storage of spores, one for species with tall fruiting bodies such as *D. discoideum* or *D. purpureum*, and one for species with short fruiting bodies such as *D. rosarium*. The plates that you freeze away should be free of contamination

and have fully developed fruiting bodies. Both methods should be done under the hood to prevent contamination.

3.4.1. Storage of Spores from Tall Fruiting Bodies

1. For each clone, bang the agar plate upside down on the bench until a large amount of the spore mass has dropped on the lid.
2. Remove the agar plate and set it aside, leaving only the lid.
3. Pipette 3 mL of KK2 buffer into the lid.
4. Lift one side of the lid at a small angle (less than 45°) and wash the spores off the lid by pipetting the spore suspension in KK2 several times from the lid and back onto the lid and allowing it to pool.
5. Place approximately 1 mL each of the spore solution into two prepared freezer vials and label the vials.
6. Store the vials at -80° C.
7. To restart the population, use a sterile loop to collect a small amount of frozen stock and place on an SM agar plate with 0.3 mL of KA bacterial solution.

3.4.2. Storage of Spores from Short Fruiting Bodies

1. Using a spatula or other cell scraper, divide the plate into half, making sure that the agar is not disturbed.
2. Pipette 1 mL each of KK2 into two 1.5 mL microcentrifuge tubes.
3. Using the spatula, scrape up half of the plate and place it into one of the 1.5 mL tubes.
4. Vortex the tube.
5. Pipette it into a prepared freezer vial and label the vial.

6. Repeat steps 3-5 with the other half of the plate for a second vial.
7. Store the vials at -80°C .
8. To restart the population, use the method in **Subheading 3.4.1, step 7**.

4. Notes

1. We make a 50x starving buffer with only Na_2HPO_4 and K_2HPO_4 . 50x starving buffer: 125.4 mM KH_2PO_4 and 568.4 mM K_2HPO_4 . Use 20 mL starving buffer in 980 mL ddH₂O with 20 g of agar for 1 L starving agar. Autoclave and store remaining buffer.
2. For convenience and also to minimize variation, we order a pre-mixed powdered SM-medium broth from Formedium. For 1 L SM-medium agar, use 24.7 g of powdered medium, and 17 g of agar in 1 L of ddH₂O. All other steps remain the same.
3. The other two ways we take wild samples are to collect individual deer turds, or individual fruiting bodies found on turds. The turds need to go in a vial large enough to accommodate them and then should be placed on a hay agar plate without bacteria as soon as possible. Deer feces come in pellets about 1-2 centimeters in diameter. Feces of other animals might be subdivided, or even cored with straws. The fruiting bodies are placed in a microcentrifuge tube with water in the field and then plated on a low-nutrient agar plate with KA food bacteria as soon as possible.
4. We recommend sampling along a transect at collection points approximately one meter apart. Because each sample requires only a small portion of a standard drinking straw, you can continue to use what is left of the same straw for multiple

- samplings. It is important to remember, however, to sterilize the remaining straw with alcohol after each use so as not to contaminate any future samples. We also recommend keeping a record of each collection location by recording the GPS coordinates. If you need a more exact marker, use some type of flagging, and a rebar inserted into the ground in addition to the GPS coordinates.
5. Sample size, both quantity of soil/leaf litter per sample and total number of samples, should be decided before going out into the field. These will be determined by what other analyses are required for a particular study, i.e. soil water content, bacterial composition, mass spectrometry analyses, etc.
 6. You will take the lid off of the plate several times throughout this process, so it is important to make sure that the labels you make on the lid continue to correspond with the same regions of the agar plate. Do this by making a line on the plate edge so you can align the top and the bottom of the plate, or you can write on the bottom of the plate as well so you do not have to make the line.
 7. The isolated fruiting bodies are likely to be chimeric, possibly even between species. Any species identifications or experiments, by contrast, should be done on pure clones. At this stage, you can look for morphologically different clones and collect them separately. In the past, we have successfully isolated *D. discoideum* and *D. purpureum* from a single fruiting body at this stage.
 8. To pick up single clones, you are looking for a single clearing or plaque on the plates (not aggregations or fruiting bodies). Colony clearings should look like a circle and should have a different pigmentation than the bacterial lawn (usually a clear ring).

We usually find 2.5 days or around 60 hours optimal. If you see aggregation or fruiting bodies, you are too late and need to start the process again.

5. References

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Fig 1. Collecting soil using the straw method. Left panel: A demonstration of how to insert the straw into the dirt after removing the top layer of leaves. Gently push the straw into the dirt for about a centimeter. Right panel: A demonstration of how to cut the straw into the microcentrifuge tube. Cut the straw using sharp scissors about an inch from your soil sample. Make sure you have room to close the microcentrifuge tube.



Fig 2. Plating out soil. Left panel: A demonstration of how to divide the dirt dilution onto the hay plates using a glass Pasteur pipette to evenly distribute the dirt dilution over three separate plates. Right panel: Picture of a hay plate after the dirt dilution and *K. aerogenes* were spread. The charcoal has been sprinkled throughout the plate and not centered in one spot. The best way to get this type of pattern is by placing the sterilized charcoal in a sterilized 50ml Falcon tube and gently hitting the side of the Falcon tube to sprinkle the charcoal onto the plate.

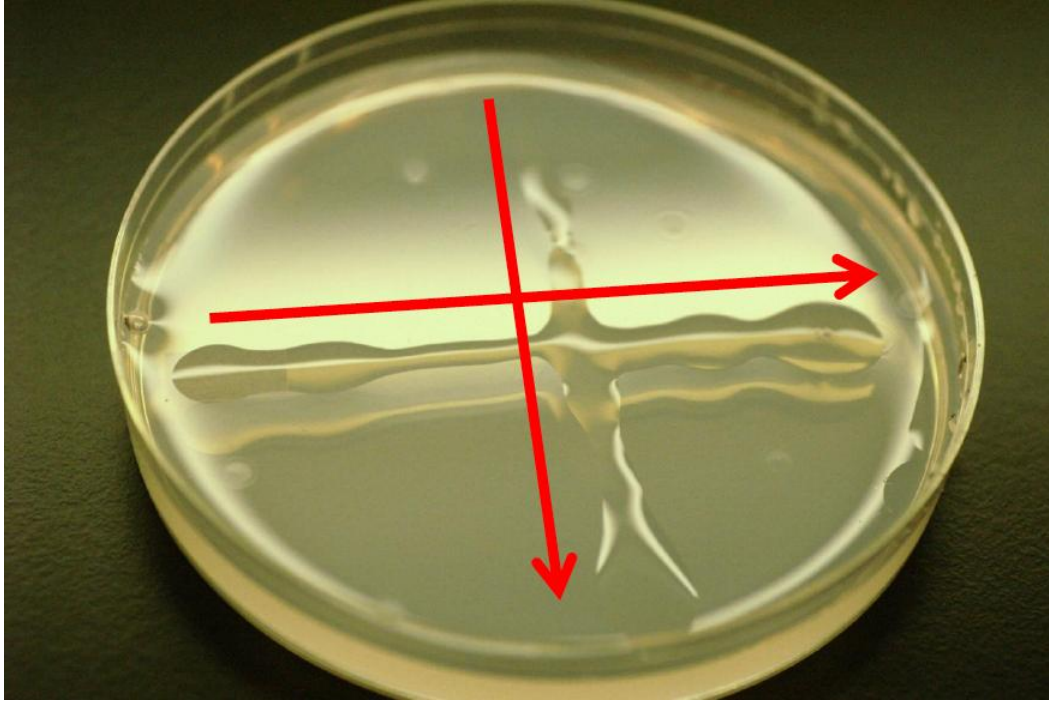


Fig 3. Checking samples. After placing the fruiting body or slug into the microcentrifuge tube, vortex and make a cross with the dilution onto the starving agar plate in the direction of the arrows. To make the cross, slowly dispense 0.1-0.2 mL of liquid onto the plate using a pipettman. Do not scrap the agar with the pipette tip. The liquid does not have to touch the sides of the petri dish. You can choose to do one strip rather than two.