Amoeba Isolation Techniques

This document describes techniques that have been successfully utilized at the LCTS for culturing and isolating amoeba. The same techniques may also be applied to other organisms that consume or infect algae, especially if they are motile.

Techniques for amoeba isolation/cultivation:

1. **Crawl/spot plates**
2. **Plug plates**
3. **Liquid cultivation of amoeba**
4. **Plaque plates (See SOP LC-0x-00x-0xx)**

Plaque plating techniques are described in SOP LC-0x-00x-0xx. The other techniques listed above are described below.

Since Las Cruces has a warm climate it is possible that pathogenic organisms including amoeba are present in the environment. It is recommended that users wear gloves and work in the flow hood with environmental samples. Avoid procedures that may create aerosols such as vortexing samples without lids. It is also recommended that incubation at 37°C be avoided since this may encourage growth of organisms that are human pathogens (i.e. grow at body temperature).

1. Crawl/spot plates

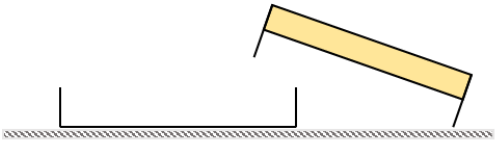
This procedure describes initial steps that can be taken when attempting to isolate amoeba from samples. Samples containing amoeba are spotted onto agar plates nearby to a food source (typically bacteria or algae). The amoeba will then move from the sample towards the food source. Amoeba progress can be observed as a clearing of the food source and can also be tracked microscopically.

This procedure assumes that you have the appropriate media for cultivating the amoeba and its food source, and that you have a healthy source of organism for the amoeba food source. The most typical media/food source combination used at the time of writing was PYE media and *E. coli* bacteria and thus the protocol is written with this in mind. The techniques described should work for any solid media and microorganism food source (e.g. algae on algae media). It is suggested to attempt cultivation of amoeba pests on bacteria since after several passages of cultivation, laboratory bacteria (e.g. *E. coli*) can easily be killed off with antibiotics aiding in the isolation of the amoeba from other field organisms and crop algae.

If you wish to use a media that resembles algal field cultivation media and are concerned that it does not contain sufficient food source for growth of *E. coli*, try adding 0.05%w/v peptone and yeast extract to your media recipe.

A recipe sheet for PYE is saved in the recipe folder. This procedure begins with the assumption that you have already poured the plates.

1.1. Clean and wipe your work area with 70% Ethanol. If using the flow hood, refer to SOP LC-01-001-018.
1.2. Gather supplies required to complete task: PYE plate, fresh bacterial source (either a recently struck plate or an actively growing liquid culture; preferably wild type strain with no plasmids / antibiotic resistance), inoculating loops, pipette and tips (up to 20ul), gloves, pen or label maker, parafilm.
1.3. Collect PYE plate/s. Dry if necessary. Plates need to be dry before use. If you observe any moisture on the plate surface, they are not dry enough.



Plates can be dried by placing them at an angle on their lids in an incubator or flow hood for 30-60 minutes. Alternatively, in dry climates they can be left closed on the bench overnight.

1.4. Label plate with at least the date, sample name, food source, initials, media type. Label plates on the underside and close to the edge. Never label lids in case they become separated from the plate.
1.5. Remove loop from package [insert section on loop use from other SOP] and
1.6. Remove an inoculating loop from its package. Open package and remove inoculating loop touching only the upper end of the loop and in such a way as to prevent anything contacting the lower end of the loop (that will be used to transfer the biomass). If at any point in this process the end of the loop that will be used for biomass comes into contact with anything other than biomass or the plate you are streaking, discard the loop and get a fresh one (do this even if you are think it may have touched something, but are unsure).
    1.6.1. If using bags containing multiple loops this can be accomplished by:
        1.6.1.1. Hold the bag horizontally
        1.6.1.2. Gently squeeze the opening of the bag from each side to create a wide opening.
        1.6.1.3. Gently motivate the package so that the loops protrude from the opening.
        1.6.1.4. Remove loop without putting fingers into the bag and pull out the loop while keeping the mouth of the package open and without letting the loop touch the sides of the package opening as it is removed.
        1.6.1.5. Leave the ends of the remaining loops protruding from the package and lay flat on the work surface (to aid in their use in future steps).
    1.6.2. If using individually wrapped loops:
        1.6.2.1. Peel back the two sides of the package towards the bottom end and clasp with one hand at the bottom of the package.
        1.6.2.2. Remove loop with other hand and discard packaging.
1.7. Insert loop into liquid food source and then remove or touch a colony if food source is bacteria on solid media. If food source organism is algae, scrape a small amount of biomass onto the loop similar to as depicted in diagram below.

A small amount of algae biomass for streaking: 

1.8. Streak using a back and forth and side to side motion pattern across the center of the plate similar to shown in the diagram below.
1.9. If using a liquid food source, allow to dry before continuing.
1.10. Place the plate on the work surface with the media facing up.
1.11. Pipette a 20ul sample of the amoeba source. To aid in isolation a serial dilution of the sample may be made and each dilution plated.
1.12. Remove the lid far enough to allow you to move the pipette above the media.
1.13. Pipette the sample onto a spot nearby to the food source as shown in the diagram below.

1.14. Replace the lid.

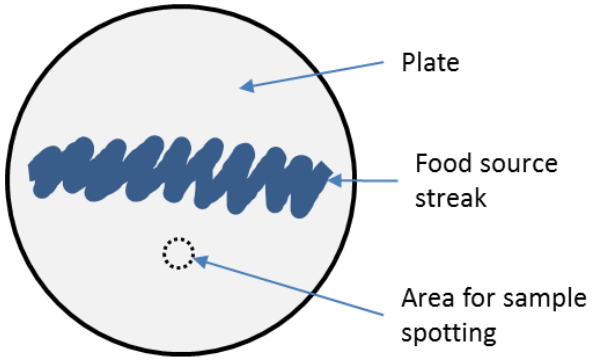


Diagram of a plate streaked with food source and indicating area for spotting sample.

1.15. Allow the sample to dry to the plate by leaving plate in this orientation until sample is dry/adsorbed into media.
1.16. Once dry, invert plate so that media is facing down and plate rests on its lid.
1.17. Wrap plates with parafilm and poke at least two holes with a pipette tip to allow air flow.
1.18. Store plates and frequently observe for amoeba growth. Plates are typically placed in CO2 boxes or at the bench. Prior to knowing the ideal temperature or conditions for any amoeba it is wise to make multiple plates and place in different environments.
1.19. If amoeba are present you should observe a clearing of the food source. Microscopic examination of the plate prior to this observation may show amoeba cysts or individuals moving across the surface of the plate towards the food source. Some example pictures characteristic of amoeba isolated at the LCTS are shown in the appendix below.

2. Plug plates

Plug plates are a step sometimes used as part of amoeba isolation. A plug of an agar plate containing a putative amoeba source is removed and placed face down onto a lawn of food organism (e.g. bacteria or algae). If amoeba are present in the plug they will move from under the plug consuming the food source as they go. Potential sources for plugs might be plaques or areas of crawl/spot plates that are hypothesized to contain amoeba.

As with the previous procedure it is assumed that you have a suitable media plate and food source for culturing the target amoeba. This procedure has been successfully used with PYE media and *E. coli* as a food source but should work equally well with any microorganism and its respective media.

2.1. Clean and wipe your work area with 70% Ethanol. If using the flow hood, refer to SOP LC-01-001-018.
2.2. Gather supplies required to complete task: PYE/other plate, fresh bacterial/other liquid food source (preferably wild type strains with no plasmids / antibiotic resistance), centrifuge tubes, liquid media, spreader or tranny balls, pipette and tips, gloves, pen or label maker, parafilm.
2.3. Plate a lawn of food source (aim to plate approximately 10^8 cells per plate but this step may be optimized depending on food source / media / amoeba / etc.)
    2.3.1. Concentrate cells from an actively growing culture by centrifugation.
    2.3.2. Re-suspend cell pellet at a concentration of 10^8 cells per 200ul.
    2.3.3. Plate 200ul of food source per plate and allow to dry/absorb into the plate.

2.4. Label plates with at least sample ID, media type, initials, date.
2.5. Locate areas of source plate that will be plugged.
2.6. Using a sterile scalpel or equivalent implement cut a small section of the plate and carefully remove the "plug".
I have not really optimized this step. Probably my favorite thing that I have tried is wooden sticks with wire attached to the end that Sal used with Pete for the chytrid work. He had a box of sticks and wires that can be inserted into the end of the stick using pliers. These can be autoclaved. Pete also gave Sal some metal implements that also work reasonably well and can also be autoclaved. You can also use pipette tips that have the end cut off (trim with sterile scissors). The plug can then be removed from the tip with a wire tool or pushed out with an inoculating loop or by cupping your finger over the other end of the tip and pushing to expel the air and push out the plug which would then have to be oriented with some other sterile tool.
2.7. Invert plug and place face down onto the lawn of food organism.
2.8. Incubate plates appropriately and observe frequently for the presence of amoeba. Amoeba should crawl out from under the plug and an area of clearing should appear around the plug. Microscopic observation of the plate may aid in phenotypic identification.

3. Liquid culture of amoeba

Amoeba may be cultivated much like other microorganisms in liquid media. Some amoeba may be cultivated axenically while others will require a live food source in co-culture e.g. bacteria. At the LCTS we have had success only once with an axenic culture of amoeba, using Pages amoeba saline (PAS) media. The other successes have been using co-cultivation of amoeba with a bacterial food source in liquid PYE media. This can be useful for generating a liquid source for infections. Also, it allows for the easy addition of antibiotics to clean out the *E. coli*. We have relatively little experience at doing this but it is best to keep the OD of the culture low. If the bacteria get too concentrated they may inhibit amoeba growth. If this continues to be an issue consider lowering the amount of Peptone and Yeast extract in the media (this can be achieved by dilution with sterile water since the media only contains peptone and yeast extract). Other parameters that can be adjusted are growth conditions (shaking, temperature, etc.).

3.1. If using a food source organism, cultivate food source in media that will be used for amoeba cultivation.
3.2. Clean and wipe your work area with 70% Ethanol. If using the flow hood, refer to SOP LC-01-001-018.
3.3. Gather supplies required to complete task: PYE/other liquid media, fresh bacterial/other liquid food source (preferably wild type strains with no plasmids / antibiotic resistance), pipette and tips, gloves, pen and tape or label maker, culture vessel(s), amoeba source, 96-well plate for OD measurement.
3.4. Dilute food source to 0.05-0.1 OD in appropriate growth vessel (e.g. 5ml tube, 50ml flask etc.)
3.5. Inoculate with amoeba source (e.g. liquid transfer by pipette, insert plug, inoculate from plate with loop).
3.6. Set up a similar culture with no amoeba for a control.
3.7. Incubate on shaker. Until ideal conditions have been established for target amoeba it is a good idea to make multiple cultures and place at various growth conditions.
3.8. Clean up the work area and wipe down with 70% Ethanol.
3.9. Observe periodically for OD change.

3.9.1. If OD drops below 0.05, add more food source and consider starting culture at higher OD of food source or lower amoeba inoculum in future.

3.9.2. If OD rises quickly, dilute with fresh media and consider starting future cultures at lower OD or using alternative media or growth conditions, or adding a larger amoeba inoculum.

3.10. Microscopic observation should allow determination of presence or absence of amoeba.

4. Plaque plates

Plaque plating

There are two methods that have been routinely employed at Sapphire to make plaque plates. One method simply plates a mixture of cells and infection directly onto the agar plate. The second method uses a soft agar overlay for the plating where a mixture of cells and infection source is added to molten soft agar (media with a low % of agar) and then poured onto a plate, forming a thin layer on top of the agar media below. The media used for the overlay is typically the standard laboratory media for the host algae in question.

Direct vs. agar overlay plating

The advantage of the direct plating method is that it is easier since it requires less steps and equipment and as long as you have plates in the deli it can be done at any time. The advantage of the agar overlay method is that it yields more consistent plaque size and morphology as well as generating a more homogenous lawn of the host organism. These observations are from literature and personal communications with phage biologists and algal (chlorella) virologists as well as some experience. The advantages may not hold true for other host/pest combinations and equally there may be other advantages for either technique in these novel situations. Some pests may be so temperature sensitive that the soft agar overlay method could be detrimental. When discussing plaque plates with Dave Dunigan for example he said that the chlorella viruses would be inhibited at 55°C and so it was very important to have the water bath no higher than 50°C. The method should work fine as low as 45°C but at this lower temperature you may need to work more quickly or with fewer samples at a time since the media will solidify faster.

Plates

Agar plates may need to be poured slightly thinner than normal for the soft agar overlay technique. This will depend somewhat on the plates and how thick you normally pour the plates. When using the Fisher plates (cat#FB0875712) pour approximately 25ml for the standard/underlay plate. This should allow sufficient room to pour the 4-5ml of soft agar/sample on top. Plates from VWR are not as deep or wide so care should be taken when pouring plates to leave enough room for the overlay layer.

Soft agar is typically made with 0.4-0.6% w/v agar and at Sapphire we have used 0.5%w/v. Soft agar can be made at the time of the procedure and left in the 55C incubator until ready for use as with other solid media recipes. It can also be made ahead of time in small bottles (e.g. 100-200ml) and then stored as solid media. When needed it can be melted in the microwave (with caution!) or a boiling water bath before use. Soft agar can be re-melted for use multiple time.

When we worked with Dave Dunigan on the Chlorella viruses he suggested that plates should be fresh (up to 3 days old) for good viral plaque formation and that more erratic results were seen with older plates. It is unclear where this information comes from or if it translates to other organisms.

Optimization of conditions for plaque formation

For any system with a host/pest interaction there are many opportunities for optimization. It is important that conditions for growth and infection are balanced so that one is not overwhelming the other. In the extreme cases this could mean no plaques at all and only a lawn of the host survives, or the opposite

situation where the host is completely decimated very early on. Adjusting the amount of host cells plated per plate will also have an effect in a similar way.
Some work was done in San Diego trying different light and CO2 levels (+/-) with SE00004 and various fungal pests. I do not have the data for these studies but what I saw suggested that slower growth of the algae in this case (lower light, no CO2) produced better plaques. However, without knowing all the details this information should be taken lightly and it is likely that the ideal conditions will be different for different pests/hosts.
Certain media additions may be required for infection. For example, the level of Ca or Mg is important for some bacteriophage infections. If infections do not occur in standard lab media then trying a media that more closely resembles the environment where the pest was found may improve success.

Safety:
Wear gloves and work in the flow hood with unknown organisms and/or environmental samples. Avoid steps that generate aerosols in the lab such as vortexing without lids. Regularly clean pipettes to prevent cross contamination.

Materials required:
1. Agar plates of appropriate media
2. Soft agar of appropriate media (if using agar overlay method)
3. Tranny balls or hockey stick (is using direct plating method)
4. Liquid media for dilution of infection
5. Vessel for dilution (e.g. 96-well plate or microfuge tubes)
6. “preview” tubes, capped and sterile (plastic test tubes large enough to hold 10+ml)
7. Pipettes and tips
8. Pipette filler and serological pipettes
9. 45-50°C water bath (if using agar overlay method)
10. Host strain in log phase growth
11. Pen or label maker

Procedure for agar overlay:
1. Gather supplies, clean and wipe work area with 70% Ethanol. Refer to Laminar flow hood use SOP LC-01-001-018 for proper hood use.
2. Turn on water bath and allow to heat up to 50°C.
3. Melt the soft agar and hold in the 50°C water bath.
4. Dispense 2.5 ml of soft agar to sterile “preview tubes” using a serological pipette and filler and place in a test tube rack in the water bath at 50°C. Do this for the number of plates that will be poured plus a few spares.
5. Concentrate the host strain to 4.0 X 10^8 cells/ml by centrifugation and re-suspension with fresh media. Concentrate enough so that 0.3 ml can be used for each plate plus some extra for backup.
6. Dilute the pest sample in fresh media by 1/10 serial dilutions in a volume sufficient to allow 0.1ml of each dilution to be plated on the number of plates you intend to use. The volume needed will determine the best vessel for this but sterile 96-well plates work well and allow the use of multichannel pipettes which can be useful if using multiple pest sources. How far to dilute the pest will depend on the host/pest system. For an unknown sample/pest dilute source to at least 10^{-6}.
7. Label the plates with at least date, initials, host strain, pest sample, dilution. Use pen to write on the edge of the base of the plate or attach a label towards the edge of the plate.
8. Remove 13 x 100 mm tubes of soft agar from the water bath to a test tube rack as needed. Start by doing only 1-2 at a time until you are familiar with how long it takes to complete the task before the media solidifies. Once you are comfortable with the procedure the number of tubes handled at one time can be increased.

9. To each tube, add by pipette 0.3 ml of the concentrated host and 0.1 ml of the diluted pest.
10. Mix briefly (by rolling the tubes between the palms of the hands) and pour the contents of the tube onto the corresponding plate.
11. Tilt the plate gently until the entire surface of the plate is covered with soft agar (this needs to be done quickly, as the soft agar will start to solidify as soon as it has been poured onto the plate).
12. Allow the agar to solidify (a few minutes).
13. Invert the plates with the lid down (so moisture condensation stays in the lid and off the agar surface)
14. Turn off the water bath, tidy up and clean and wipe down work area with 70% Ethanol.
15. Incubate the plates at appropriate conditions. If ideal conditions for the target pest have not yet been established, make duplicates plates and place in multiple conditions to compare results.
16. Expect plaques to appear in 5-15 days.

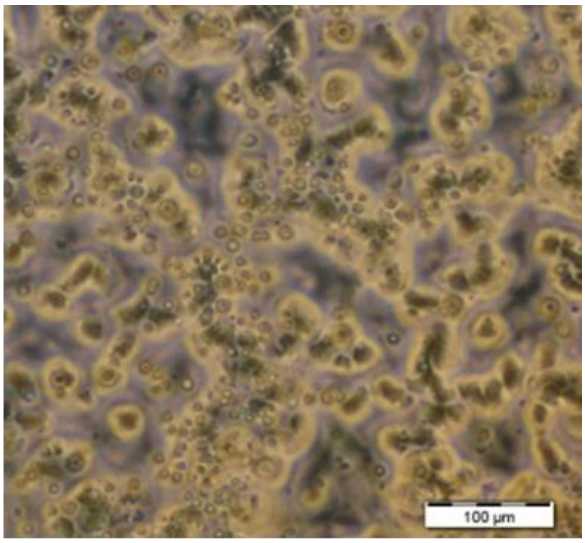
Procedure for direct plating without agar overlay:

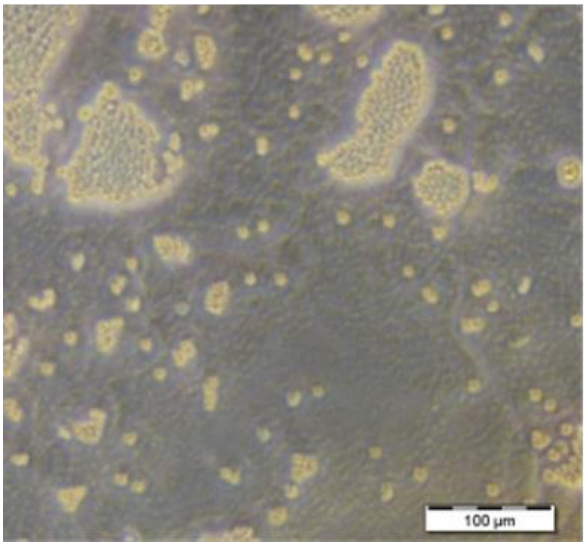
1. Gather supplies, clean and wipe work area with 70% Ethanol. Refer to Laminar flow hood use SOP LC-01-001-018 for proper hood use.
2. Concentrate the host strain to 8.0 X 10^8 cells/ml by centrifugation and re-suspension with fresh media. Concentrate enough so that 0.15 ml can be used for each plate plus some extra for backup.
3. Dilute the pest sample in fresh media by 1/10 serial dilutions in a volume sufficient to allow 0.05ml of each dilution to be plated on the number of plates you intend to use. The volume needed will determine the best vessel for this but sterile 96-well plates work well and allow the use of multichannel pipettes which can be useful if using multiple pest sources. How far to dilute the pest will depend on the host/pest system. For an unknown sample/pest dilute source to at least 10^{-6}.
4. Label the plates with at least date, initials, host strain, pest sample, dilution. Use pen to write on the edge of the base of the plate or attach a label towards the edge of the plate.
5. If using plating balls for plating add them to each plate.
6. Collect and label sample vessels. Any suitable sterile vessel can be used for this step. If multiple plates of the same dilution are to be plated then a master mix can be made.
7. To each sample vessel, add by pipette 150ul of the concentrated host and 50ul of the diluted pest.
8. Mix briefly by pipetting up and down and pipette 200ul of the mixture onto the corresponding plate.
9. Spread sample over plate with desired technique.
10. Allow sample to dry/adsorb with the plate facing up.
11. Once dry, invert the plates with the lid down (so moisture condensation stays in the lid and off the agar surface)
12. Wrap plates if desired. If using parafilm, poke 1-2 holes to allow air flow and to reduce moisture build up.
13. Tidy up and clean and wipe down work area with 70% Ethanol.
14. Incubate the plates at appropriate conditions. If ideal conditions for the target pest have not yet been established, make duplicates plates and place in multiple conditions to compare results.
15. Expect plaques to appear in 5-15 days.

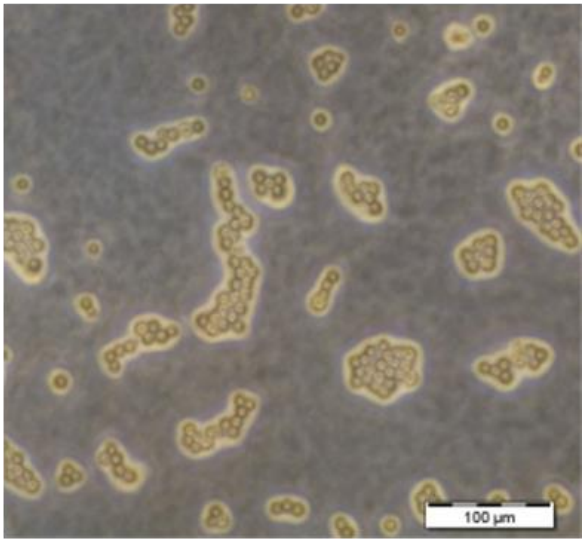
5. Appendix:

Examples of amoeba phenotypes when cultivated on solid and liquid media at the LCTS:

Amoeba phenotypes consuming *E. coli* on PYE plates

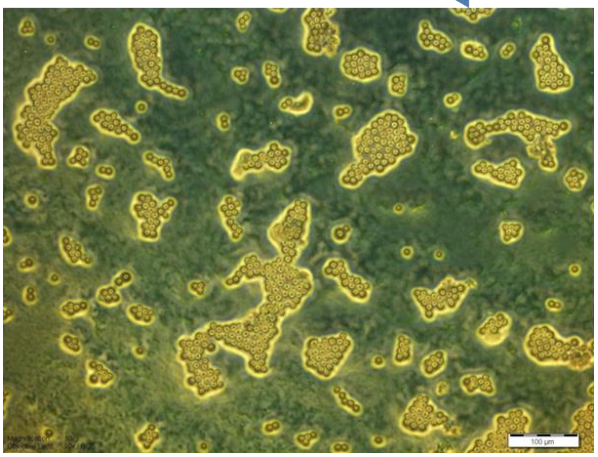


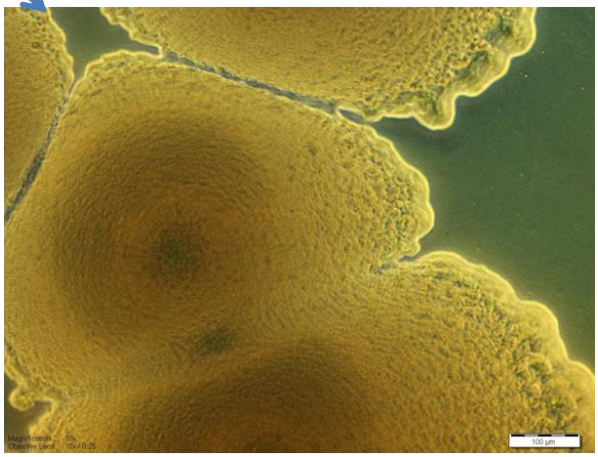


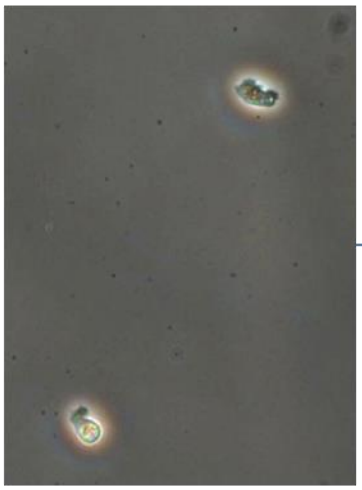


Example of amoeba growing on E.coli food source on PYE plates

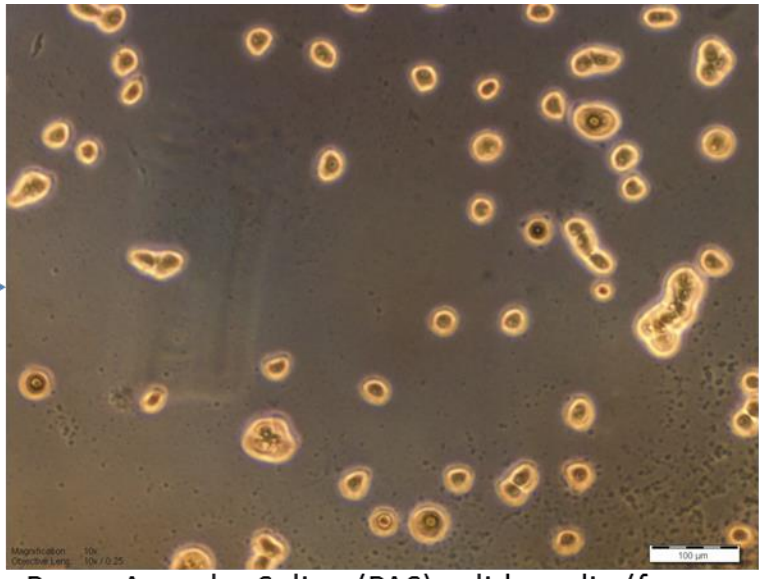
Media = PYE (peptone and Yeast Extract 0.05%w/v each)
Food = *E. coli* (TOP10)
Upper: PYE plate showing area where bacterial food source was initially struck. To the right hand side (black arrow) the boundary between the area cleared by amoeba and the pure *E. coli* can be observed. The red circular area is where the original sample was spotted. A red bacteria has grown here whereas the amoeba have moved from this location.
Lower: microscope pictures taken at 10X objective with phase contrast of the cleared (left) and uncleared (right) areas.



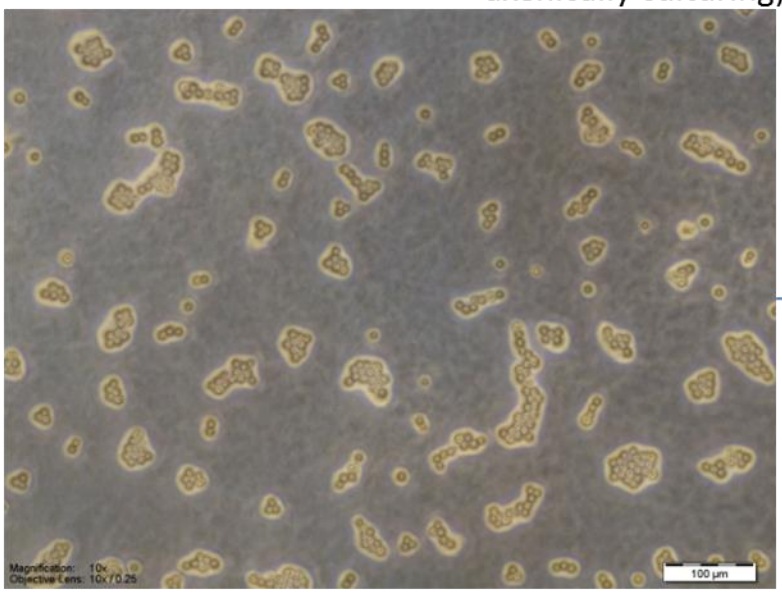




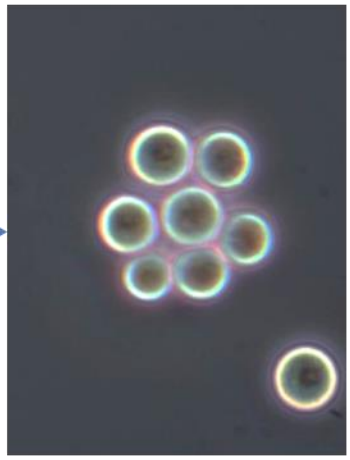
PYE liquid culture
(E. coli as food)



Pages Amoeba Saline (PAS) solid media (for axenically culturing)



PYE solid media with *E. coli* as food viewed on plate



Sample viewed on microscope slide in buffer