

Experimental evolution of multicellularity

Teacher's guide

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Time required: 30 minutes a day for 3 weeks

Overview. The evolution of multicellularity was one of a few events in the history of life that allowed for increases in biological complexity. All known multicellular organisms evolved from single-celled ancestors, most notably in the animals, land plants, and fungi. Take a moment to imagine the world without multicellular organisms. The most vibrant tropical rainforest would be reduced to little more than a barren open landscape encrusted with a slimy layer of photosynthetic bacteria and algae. The evolution of multicellularity resulted in radical changes in organismal size and complexity. Single cells, which for billions of years were organisms in their own right, give up this autonomy and become parts of new, more complex, higher-level organisms. They evolve cellular differentiation, allowing the multicellular organism to do things that were never possible before. And perhaps most remarkably of all, multicellularity has evolved not just once, or twice, but more than 25 times in different lineages (Figure 1)¹.

All known transitions to multicellularity are ancient. Even the most recent transitions (brown algae, such as kelp, and the volvocine algae) occurred more than 200 million years ago. Because of their ancient origin, early multicellular forms have largely been lost to extinction, making it hard for scientists to study the first steps in the evolution of multicellularity. Until now.

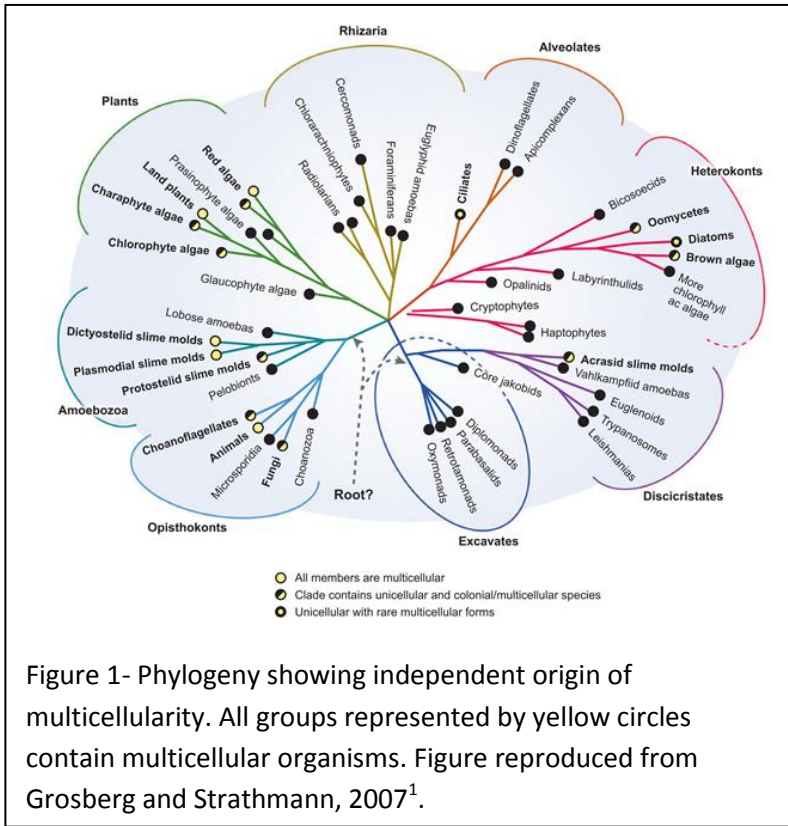


Figure 1- Phylogeny showing independent origin of multicellularity. All groups represented by yellow circles contain multicellular organisms. Figure reproduced from Grosberg and Strathmann, 2007¹.

In this lab we will examine the very first step in the evolutionary transition to multicellularity- the evolution of cellular clusters. We will use the method of *experimental evolution* to select for cluster formation in large populations (roughly a billion yeast cells per tube) of yeast. We will use gravity as our selective agent. Clusters of cells fall through liquid media faster than single cells, allowing us to quickly and easily separate single-celled yeast and any multicellular mutants that arise during the experiment. As a result, if a random mutation arises that results in cluster formation, these will have a *huge* competitive

advantage over the wild-type unicellular yeast, ultimately driving them to extinction.

In addition, students will perform a *divergent selection experiment* on a multicellular ‘snowflake’ yeast isolated from 3 weeks of experimental evolution using the same protocol. The goal here is to select for either faster or slower settling on a multicellular yeast, and examine multicellular adaptation to these different environments. Every day that you evolve your yeast, they will go through about 7 generations. Over the course of the experiment, you’re going to watch them go through 70-80 generations. The short generation time of microbes is a key reason that scientists use them in experimental evolution studies. How long would it take for us to do an 80-generation experiment with another organism, such as mice, dogs, chimpanzees or oak trees?

The evolution of multicellularity is a two-step process. First, clusters of cells must evolve from single-celled ancestors. In nature, this may be due to predation (predators can eat single cells, but not clusters), or benefits clusters gain over isolated cells (e.g., UV resistance, metabolic efficiency, etc.). Next, clusters of cells must possess the key ingredients required for Darwinian evolution: clusters must vary from one another, they must be capable of passing on this variation to their offspring, and this variation needs to affect fitness. If this is true, then mutations that affect the phenotype of clusters can be acted upon by natural selection, and clusters that possess beneficial mutations will outcompete those that do not. This process is known as adaptation (process illustrated in Figure 2, as well as in a short video on our website, <http://www.snowflakeyeastlab.com>). Over time, this can result in the evolution of multicellular complexity, such as cellular division of labor.

Objectives. Experimentally evolve single-celled yeast into multicellular ‘snowflake’ yeast. Examine multicellular adaptation through divergent selection for either faster or slower settling.

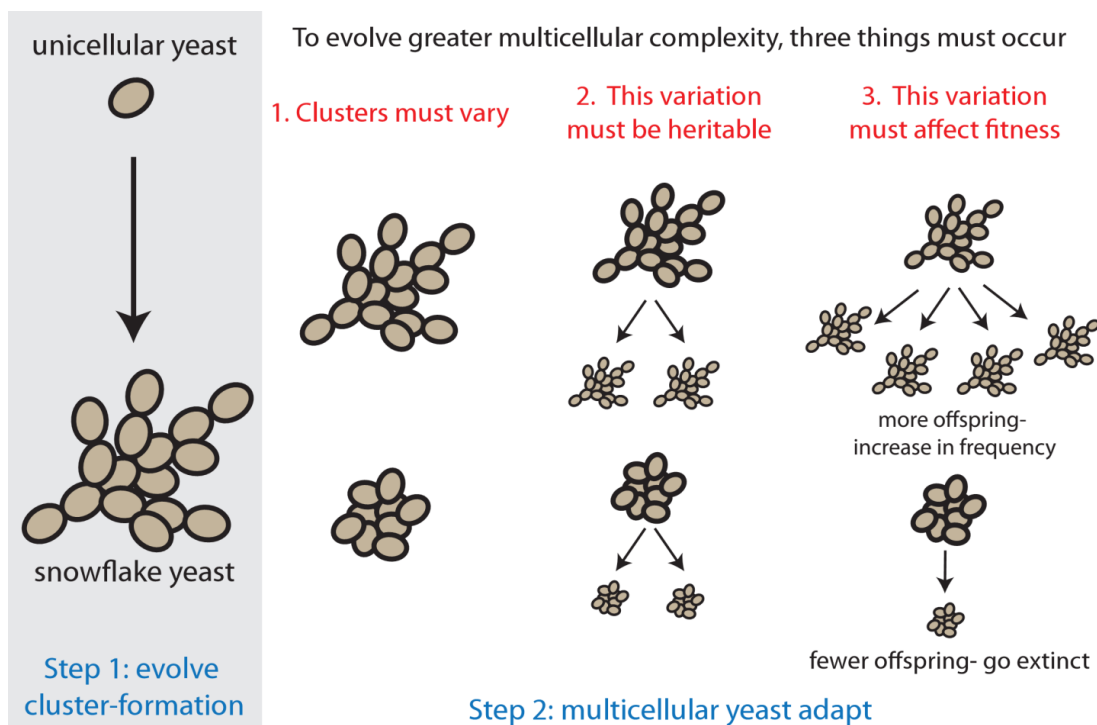


Figure 2- The evolutionary transition to multicellularity. First, clusters must evolve from single-celled ancestors. Next, clusters must adapt. To do this, there things must be true. First, clusters must vary from one another (shown

are two different ways of forming a cluster, snowflake and clumpy). Second, this variation must be heritable. And finally, these cluster-level traits must affect fitness. If, for example, snowflake shaped clusters settle faster than clumpy clusters, then snowflake clusters will outcompete clumpy clusters and will take over the population. Similarly, if cellular division of labor evolved and improved a snowflake's ability to produce offspring, then natural selection would result in an increase the frequency of this trait in the population. Like a ratchet, multicellular complexity can increase through time.

Instructor preparation

Step 1: Thoroughly read this teacher guide and make copies of student labs.

Step 2: Two weeks before start of lab, order needed supplies.

Step 3: Five days before start of lab, culture yeast from spores onto YPD agar plates.

Step 4: Two days before start of lab gather materials and organize student workstations

Timeline

Monday	Tuesday	Wednesday	Thursday	Friday
	10 working days prior to day 1: Assess and order materials			
In the week before the lab: Prepare YPD media for 120 test tubes. Sterilize.			Four days prior to day 1: streak out yeast spores onto YPD agar, incubate.	
Day 1: Introduce yeast experiment Give pretest	Day 2: <i>Inoculate YPD with yeast</i>	Day 3: <i>Yeast selection</i>	Day 4: <i>Yeast selection</i>	Day 5: Due: Draft of methods sections of lab report <i>Yeast selection</i>
Day 6: Annotated bibliography lesson <i>Yeast selection</i>	Day 7: Lab report lesson <i>Yeast selection</i>	Day 8: <i>Yeast selection</i>	Day 9: <i>Yeast selection</i>	Day 10: Due: Annotated bibliography with 5 citations <i>Yeast selection</i>
Day 11; <i>Yeast selection</i>	Day 12: <i>Yeast selection</i>	Day 13: <i>Yeast selection</i>	Day 14: <i>Yeast selection</i>	Day 15: Due: Final draft of introduction and methods of yeast lab <i>Yeast selection</i>
Day 16: Final observations, completion of experiment	Day 17: Lab report lesson on results and discussion			Due: Experimental evolution of multicellularity lab report

Student work station checklists

Student work stations

The list below is a list of materials that should be present at each student work station prior to the beginning of the lab.

Common work station

The list below is a list of materials, supplies, and equipment that should be present at a common location and accessible by all. It is up to the discretion of the instructor as to whether or not students should access and mix staining solutions and plate, suspend, or isolate yeast, or whether the teacher should aliquot solutions in microtubes and include at student work stations.

<u>Student work stations</u>	<u>Number needed</u>	
Test tube rack	1 per group	<input type="checkbox"/>
1.5 mL microcentrifuge tubes	2 per group	<input type="checkbox"/>
Serological pipettes (5 mL)	3 per group	<input type="checkbox"/>
Serological pipette pump or bulb	1 per group	<input type="checkbox"/>
Compound microscope	1 – 2 per group	<input type="checkbox"/>
Rulers	1 – 2 per group	<input type="checkbox"/>
Permanent marker	1 per group	<input type="checkbox"/>

<u>Common work station</u>	<u>Number needed</u>	
Y55 yeast in liquid YPD	1	<input type="checkbox"/>
C1W3 yeast in liquid YPD	1	<input type="checkbox"/>
YPD media in test tubes	3 test tubes per lab group	<input type="checkbox"/>
100µl micropipettes	1	<input type="checkbox"/>
Micropipette tips	2 boxes	<input type="checkbox"/>
22 x 22 mm coverslips	1 Box	<input type="checkbox"/>
Microscope slides (plain)	1 Box	<input type="checkbox"/>
Microscope slides (depression)	2 per group	<input type="checkbox"/>
Parafilm	1 Box	<input type="checkbox"/>

Baker's yeast are harmless, but in our experience it is difficult to maintain sterility in a student lab. Therefore, it is hard to know if something else besides yeast is growing in the media. *As a precaution, gloves and eyewear should be worn during the lab.*

How to proceed with the lab

After ordering required materials, the first thing to do is streak out Y55 and C1W3 spore onto solid agar media. Protocols for how to do this are found in Appendix 1. Streak your yeast out at least 4 days before the start of the experiment. Once the plates are growing well, use them to inoculate several tubes (of each strain) of liquid media. Students will use this yeast to found their replicate populations. The rest of the lab is methodologically quite straightforward.

Student directions for the selection experiment:

Day 1: Initial tube inoculation

1. Obtain three test tubes containing 5 mL YPD.
2. Label each tube using a permanent marker (tip: write on tape, then put tape on the tube) with the inoculation date, strain (Y55 or C1W3), and selection scheme (i.e., faster or slower settling).
3. Inoculate one tube with 100 µl of the Y55 stock culture, and two tubes with 100 µl of C1W3.

4. Incubate overnight (30°C, shaking).

Day 2 (and beyond): Settling selection (see Figure 3 for an illustration).

Selection for faster settling

1. Obtain three test tubes containing 5 mL YPD. Label tubes with transfer number, date, strain, and selection scheme.
2. Obtain a 5 mL serological pipette with bulb.
3. Extract 5 mL of the turbid yeast culture from incubated tube into a sterile 5 mL serological pipette using a bulb or pipette.
4. Seal the bottom of the pipette with a piece of parafilm or hold firmly with the flat part of your thumb.
5. Allow the pipette to stand upright in a glass beaker or pipette rack for 10 minutes. Make sure the media is not leaking out.
6. Transfer the bottom 0.5 mL to sterile YPD.
7. Place all three tubes in the test tubes racks in either a dry shaking incubator or shaking water bath. Incubate 24 hours at 30°C.

Selection for slower settling

1. Obtain three test tubes containing 5 mL YPD. Label tubes with transfer number, date, strain, and selection scheme.
2. Obtain a 5 mL serological pipette with bulb.
3. Extract 5 mL of the turbid yeast culture from incubated tube into a sterile 5 mL serological pipette with a bulb or pipette.
4. Seal the bottom of the pipette with a piece of parafilm or hold firmly with the flat part of your thumb.
5. Allow the pipette to stand upright in a glass beaker or pipette rack for 10 minutes. Make sure the media is not leaking out.
6. Discard the bottom 4.5 mL of media in the pipette into waste beaker, transferring the top 0.5 mL to sterile YPD.
7. Place all three tubes in the test tubes racks in either a dry shaking incubator or shaking water bath. Incubate 24 hours at 30°C.

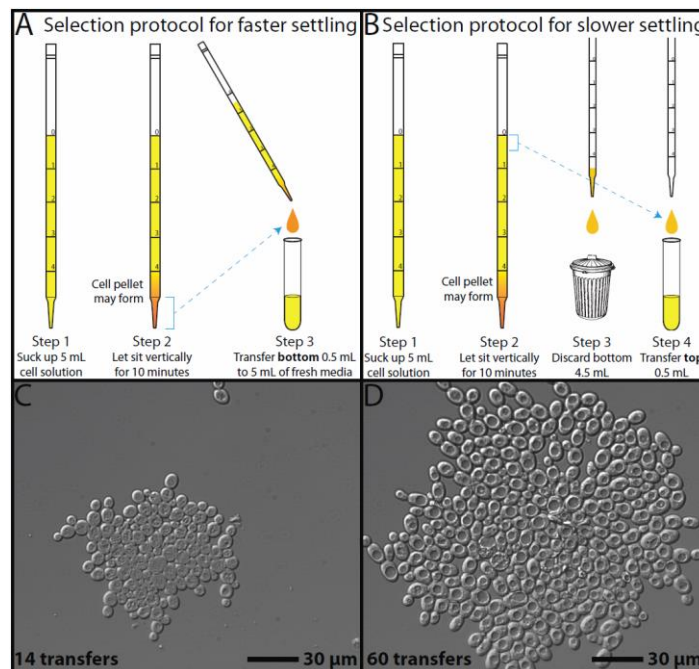


Figure 3. Settling selection protocols illustrated. Selection regime for faster settling (A), or slower settling (B). Snowflake yeast evolve faster settling by evolving larger size. Shown in (C) is an isolate taken from 14 transfers, and in (D) 60 transfers. The cluster in (D) is larger both because there are more cells per cluster, but also because the size of individual cells has increased. Figure reproduced from Ratcliff et al (in press).²

Data Collection

1. Have students record their observations daily. This should include what the test tube looks like (cloudy, presence/absence of a pellet, are tiny specks visible within the tube, or is it a homogenous haze?). Students should record what they did daily (which transfers they conducted, if they made any mistakes, etc).
2. Every second or third transfer, students should examine their populations under the microscope. They should draw a representative field of view at 10, 40 and 100x for each of the three populations they are transferring. A guide for organization is suggested below.

	10x	40x	100x
Date			
Strain			

3. At the end of the experiment, students should see what they've got.

- Did any clustering evolve in the Y55 unicellular populations?
- Is it a snowflake yeast? The way to tell is through a characteristic branching pattern associated with its growth form: only snowflake yeast form clusters that are made up of long branches of cells.
- Did the snowflake yeast evolve to settle at different speeds in the divergent selection experiment? To measure settling speed, suck up 4 mL (make sure to leave some for microscopy!) of each of the C1W3 populations into two separate 5 mL transfer pipettes. Be sure to mix them well before pipetting. Allow them to stand upright for 5 minutes. Using a ruler, measure the height of the cloudy cell haze from the base of the pipette; faster-settling yeast will leave a smaller cell cloud.



How large was the cell cloud (in mm) for-

C1W3 faster settling:

C1W3 slower settling:

- Did the snowflake yeast evolve different sizes in the divergent selection experiment? Unfortunately, accurate analysis of cluster size is beyond the scope of this lab. However, there are some simple ways that students can examine cluster size.

First, have students view the yeast on both the depression slide and on regular slides. Low power objectives on the microscope will give them the ability to view more clusters. They should switch back and forth between the C1W3 faster and C1W3 slower selection lines. If a microscope camera is available, we recommend that the student take pictures of each line. These images should be labeled with the strain and selection line. Then through a single-blinded poll, they can see if their peers can tell which one was selected for faster settling, and which one for slower settling. The student (or teacher) should gather several other students, then he/she should show them the images they recorded *without telling them which treatments they came from*. After several viewings, he/she should ask their peers to guess, based on size, which image is from the faster-selection line, and which is from the slower-selection line.

- Were there any changes in cell size in the C1W3 selected to settle faster in comparison to the C1W3 selected to settle slower (e.g., Figure 3)? Why might larger cells be adaptive under selection for faster settling?

Discussion Questions

The duration of this lab spans three weeks. To help students get and keep their bearings, we provide suggested daily discussion questions.

Day 0 (the day before the lab)

Give the pre-lab quiz. Do this before handing out the lab packet to the students.

Day 1 (given first day of lab)

1. Of the following groups, which are exclusively multicellular (circle any/all that apply)?

- a) Animals
- b) Land plants
- c) Fungi
- d) Bacteria
- e) Algae

2. What is yeast? Describe some of its characteristics. Using taxonomy, what is the hierarchical structural organization of baker's yeast, *Saccharomyces cerevisiae*?

Ex. Silver Back Gorilla

Kingdom: Animalia

Phylum: Chordata

Class: Mammalia

Order: Primates

Family: Hominidae

Genus: Gorilla

Species: Gorilla

3. How do the two strains of *S. cerevisiae* (Y55 and C1W3) differ? How are they similar?
4. What do you hypothesize will happen in each of your three selection lines? Why?
5. Make an illustrated flow chart in your journal of the selection scheme for this lab. This illustrated procedure is an introduction to the lab but should also be an aid in the lab so be sure to include units and amounts.

Day 2 and 3

Using the document called “Reading and Analyzing a Scientific Article,” please read the paper “Experimental Evolution of Multicellularity” from the journal *Proceedings of the National Academy of Sciences* by Ratcliff et al., 2012.

Day 4

1. What are the three criteria that Ratcliff et al. describe as key steps in the transition from unicellularity to multicellularity?
2. What is cellular differentiation?
3. What is apoptosis?
4. Why is apoptosis beneficial to snowflake yeast?
5. Are you surprised that multicellularity can evolve so rapidly?

Day 5

1. What purpose does settling the yeast and taking either the top 0.5 mL or the bottom 0.5 mL serve? What are you selecting for?
2. What do you think would happen if you changed the viscosity of the media so that it was like honey? Would the selection experiment still work?

Day 6

1. In this lab, settling selection favors the evolution of clusters because they get to the bottom of the test tube and survive. Researchers (including Ratcliff et al.) don’t think this is what happens in nature, but use the method because it is experimentally practical. Name some ecologically-realistic agents that could select for cluster formation.
2. Do you think snowflake yeast would do better than unicellular yeast in these environments?

Day 7

1. What have you observed thus far in the lab?
2. Describe the two different environments you have created for the yeast with the methods described in Figure 3.
3. How do the yeast populations respond to these different environments?
3. Place 10 μ L of the discarded Y55 yeast on a slide with a coverslip and observe under a microscope. Can you observe budding? What is this type of cellular reproduction called (hint: they are reproducing asexually)?

Day 8

1. List three advantages of multicellularity.
2. List three disadvantages of multicellularity.
3. What is natural selection? Is natural selection acting upon the yeast? Explain your answer.

Day 9

1. What is Darwinian evolution? What three critical ingredients are required for Darwinian evolution to occur?
2. Are the Y55 yeast evolving to form clusters? Are they snowflake shaped-clusters, or some other type of cluster?

Day 10

1. Explain what you have been seeing in the yeast these past few weeks. Are the yeast evolving? What data do you have to support your claim?
2. Could you, as the researcher, infer that the genome of your yeast *S. cerevisiae* has changed? What data or evidence would you need to support this? How could you test this idea?

Day 11

1. Why is this research approach called “experimental evolution”?
2. What kinds of questions in evolutionary biology is experimental evolution good at answering? What kinds of questions is it bad at answering?
3. Does this work show a plausible mechanism for the evolution of unicellular organisms into multicellular organisms? Use evidence from the lab in your answer.
4. What are some limitations of this lab? Where there any sources of error?

Day 12

1. What is an individual?
2. Are you an individual? Is one of your skin cells an individual?
3. Are single-celled yeast (like Y55) individual? Are cells within a C1W3 cluster individual?
4. Isn't it kind of mind blowing to think that evolution can both endow and rob an organism of its individuality? About 750 million years ago, you had a single-celled ancestor that was clearly an organism in its own right. Now, each of your cells is a part of a more complex whole. This isn't a question, but just think about this for a minute.

Day 13

1. Think about another major evolutionary transition- the evolution of ‘superorganisms’. Just like with multicellularity, where single cells evolve to be parts of a new higher-level individual, multicellular individuals can evolve to become parts of a new-higher-level ‘superorganism’.
2. Read "The honey bee colony as a superorganism." By Thomas Seeley. *American Scientist* 77.6 (1989): 546-553.

Day 14

1. Do you agree that a honey-bee hive is best thought about as an organism, with individual bees as parts analogous to cells in your body? Why or why not?
2. What arguments does Seeley make in favor of honey bee superorganismality?

Day 15

- 1) What is a mutation? Do snowflake yeast mutate to better fit their environment, or are the mutations random and then those that provide a benefit filtered by natural selection?
- 2) What is experimental evolution?
- 3) Why is a short generation time beneficial to experimental evolution studies?

Wrap up questions

Review

What is experimental evolution?

What does ‘settling selection’ actually select for?

What did your experiment show? How did this compare with Dr. Ratcliff’s experiment?

Why did we use both unicellular and multicellular yeast in this experiment?

Describe a plausible scenario through which multicellular organisms could evolve in nature. Pictures are encouraged.

What distinguishes a multicellular organism from a clump of cells or a colony of unicellular organisms?

What is an individual organism? How does an individual organism evolve into a ‘part’ of a new, larger organism?

Which of these is an individual organism, which are ‘parts’ of an organism (circle your answer):

- | | |
|---|--|
| 1. You. | (whole individual / part of an individual) |
| 2. A single cell in your body. | (whole individual / part of an individual) |
| 3. A single honeybee. | (whole individual / part of an individual) |
| 4. A honeybee hive. | (whole individual / part of an individual) |
| 5. A Y55 (unicellular strain) yeast cell. | (whole individual / part of an individual) |
| 6. A single cell in C1W3 (snowflake yeast). | (whole individual / part of an individual) |

Big picture

Is multicellularity ‘easy’ to evolve? Why or why not?

Multicellular life has evolved at least 25 times in different groups over the last 2 billion years on Earth.

Are you surprised by this number? Before the lab did you expect there to be more, or less than 25 origins of multicellularity? What about now?

Why haven’t there been 1,000 origins of multicellularity?

If NASA said that it found single-celled organisms on a planet, would you expect there to be multicellular life as well? Why or why not?

See our paper “A novel laboratory activity for teaching about the evolution of multicellularity” in *The American Biology Teacher* (found on our [website](#)) for an in-depth description of these and other discussion topics.

Final day of lab- Give post-lab quiz. By returning the pre- and post-lab quizzes to [William Ratcliff](#), you will be participating in active pedagogical research, and will help us improve the labs. With your permission, your name will be included in the acknowledgements section of the paper describing our results.

Appendix 1. Protocols

Here you will find detailed procedures for all the techniques needed for these labs. List of materials and notes are also given. Videos of some procedures can be found on our website (<http://www.snowflakeyeastlab.com>).

Mixing and pouring YPD agar

Note: You will find a premixed bag containing YPD agar in your kit. This can be used to make a few Petri dishes, or pour solid media in a few culture dishes. This is enough media to streak out and grow spores, but not enough to do experimental evolution.

1. Add 150 mL distilled water to 250 mL or larger Erlenmeyer flask. Place on heated stir plate on medium.
2. Add packet of YPD agar (6.5g) to flask.
3. Autoclave for 20 minutes at 121degrees C.
4. Cool to the touch on stir plate.
5. On a clean bench (wipe down with ethanol prior to use), pour plates. You should add just enough agar to cover the bottom of the dish. Plates should set within 5 – 10 minutes. You can cover them immediately after the have been poured.

*Alternatively, if an autoclave is not available, sterile YPD media can be made using the same ingredients but following the directions below.

1. Add 150 mL distilled water to 250 mL or larger Erlenmeyer flask. Place on heated stir plate on medium.
2. Add packet of YPD agar (6.5g) to flask.
3. Swirl the flask to dissolve the media, and heat to **vigorous** boiling in the microwave or hot plate. (If using a hot plate be careful not to burn your media, swirl until dissolved.) Boil for 20 minutes.
4. Pour into plates as above.

Plating Yeast from Spores

Notes:

- The incubation of the yeast will take 2-4 days in an incubator set to 30°C, one week without.
- Enclosed in your reagent bag are dried spores of the yeast *Saccharomyces cerevisiae*, strain Y55 (unicellular) and strain C1W3 (multicellular). The latter is a ‘snowflake’ yeast, derived from Y55 with 3 weeks of settling selection.
- The yeast spores are dried onto filter paper. Use a different agar plate for each strain of yeast.

Materials

(2) 1.5mL microcentrifuge tube

Alternative: covered/capped test tube

(2) Sterile loop/stick

Alternative: pipette tip or sterilized toothpicks

Sterile water

Sterile forceps

Flame

70% ethanol

Saccharomyces cerevisiae unicellular yeast spores (strain Y55)

Saccharomyces cerevisiae multicellular yeast spores (strain C1W3)

Procedure

1. Carefully open the foil package labeled Y55.
2. Using flame-sterilized forceps, remove paper from foil package.
3. Unfold and place the filter paper onto a culture plate of YPD medium with sterile forceps. Label plate.
4. Moisten with 50 μ L or so of sterile water or liquid YPD. Streak the paper around on the agar surface using sterile forceps. Leave the filter paper on the culture plate.
5. Incubate at 30°C. You should see colonies in a few days.
6. Repeat process with C1W3.

*When plating more than one yeast culture, be sure to flame sterilize forceps between each plate.

As an alternative, transfer the filter paper directly into a 10 mL tube of liquid Yeast Peptone Dextrose (YPD) medium with sterile forceps. Incubate these tubes at 30°C with vigorous shaking (requires shaking incubator). Within two days there should be plenty of growth. One can then extract 100 μ L from these stock tubes for initial tube inoculation.

Suspending yeast into liquid (*in preparation for inoculating liquid YPD*)

Procedure

1. Fill 1.5 mL microcentrifuge tube with 1 mL sterile water
2. Scrape off a small clump of Y55 yeast with sterile loop or sterile “stick.”
3. Insert sterile loop into microcentrifuge tube containing the water and twirl to release yeast. Label tube *Y55 Stock*.
4. Shake tube to suspend yeast.
5. Repeat steps 1-3 with C1W3 yeast. Label tube *C1W3 Stock*.

YPD liquid media preparation

Three test tubes of YPD media are needed for each group for every day of transfer (15 test tubes are needed for each group each week). This amount will make approximately 200 tubes of media. If you have 6 lab groups you will need approximately 270 tubes, make 300 as there will be mistakes and spills.

Ingredients:

- 20 g dextrose
- 20 g peptone
- 10 g yeast extract

1. Dissolve the above ingredients into 1 L distilled water in a 2 L or larger Erlenmeyer flask.
2. Bring the final volume up to 2 L.
3. Using the 25 mL serological pipette and pump, aliquot 5 mL YPD into each 25 mm X 150 mm test tube, and place into test tube racks. Test tubes of smaller diameter can be used, but you should increase the shaking speed of narrower test tubes to ensure adequate mixing during incubation.

4. Cap or cover tubes with foil. If using foil, make sure it covers the top 2" of the tube and is well-crimped.
5. Autoclave to sterilize at 123°F for 15 minutes.

*Alternatively, if an autoclave is not available, sterile YPD media can be made using the same ingredients but following the directions below.

1. Dissolve the above ingredients into 1L distilled water in a 2L or larger Erlenmeyer flask.
2. Bring the final volume up to 2 L.
3. Swirl the flask to dissolve the media, and heat to **vigorously** boiling in the microwave or hot plate. (If using a hot plate be careful not to burn your media, swirl constantly.)
4. Repeat heating and swirling about three times until all the media is dissolved (no more specks swirl around), but use caution not to burn yourself. Boil 20 minutes.
5. When media is dissolved, cover flask with foil and store the media in the refrigerator for up to 7 days.
6. Using a **sterile** 25 mL serological pipette and pump, aliquot 5 mL YPD into **sterile** 25 mm X 150 mm test tubes, and place into test tube racks.
7. Cap with **sterile caps** or cover tubes with **sterile** foil.

* Non-liquid items (e.g., caps) can be sterilized by baking in a standard oven for 45 minutes at 300°C. Small items like caps can be sterilized inside a large beaker covered in foil. Test a small number of items the first time you do this to make sure they don't melt.