Chapter 20

An Experimental System to Study Phagocytosis

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Introduction

In many eukaryotic cells, substances taken into the cell start their journey at the cell surface when the material being ingested is enclosed by a membrane invagination. The invagination pinches off to form a membrane vesicle containing the ingested material. This process is called endocytosis. The membrane vesicle travels within the cell and ultimately fuses with a lysosome. There, the ingested substance can be digested. One type of endocytosis is phagocytosis where large (>250 nm) particles are "eaten" by cells. This dramatic cell behavior is characteristic of many protozoa whereas in multicellular organisms, it is a behavior seen only in certain specialized cells (for example, macrophages). In protozoa, phagocytosis is a feeding mechanism. Particles are brought into the cell in large endocytic vesicles called phagosomes. The phagosomes fuse with lysosomes and digestion of the ingested particles occurs (Alberts, *et al.* 1994).

Since phagocytosis is such an interesting cell behavior, it is an excellent topic for student exploration. However, one of the challenges in laboratory teaching is to choose an experimental organism that is suitable for studying a particular question. Ideally, it would be good if the organism is easy to grow and maintain, relatively inexpensive, and amenable to experimental work by undergraduates both at the introductory and advanced levels. In this chapter, we present the advantages of the freshwater, ciliated protozoan, *Tetrahymena* for 1) introducing students to microscope use, and 2) investigating phagocytosis and vacuole formation. We also discuss other possible experiments and student projects using *Tetrahymena*. The experiments described here are organized as modules so they can be adapted for use in introductory or advance courses. There are more experiments here than could be completed in a three-hour lab period.

Materials Required

[Equipment and supplies (per student pair)

- Compound microscope
- Ten microscope slides and coverslips
- Four or five 5-ml pipettes (these don't have to be sterile) and pipette bulb
- Six Pasteur pipettes and bulb
- Twelve 15-ml disposable centrifuge tubes with screw caps and test tube rack
- Forty 1.5-ml microcentrifuge tubes in rack
- Lens paper
- Sharpie or other marking pen
- One vortexer (can be shared with other groups)
- Automatic pipettor that can measure 10 and 20 microliters
- Rack of tips for automatic pipettors

Equipment for entire lab for starvation study

- Two tabletop centrifuges with swinging bucket rotors that can hold 15-ml centrifuge tubes

Reagents and culture (/student pair) for *all* feeding studies

- 15 ml 1% ink
- One culture of fed *Tetrahymena* in 2% protease peptone (30 ml of cell suspension in 125-ml flask)
- 5 ml 3% glutaraldehyde

Additional reagents and cultures

For observations on phagocytosis and swimming, and to test whether the concentration of ink affects the rate of vacuole formation

- 5 ml 5% ink (/student pair)
- 10 ml 10% ink (/student par)

To test whether nutritional state affects the rate of vacuole formation

- One culture of starved *Tetrahymena* in starvation buffer (25-30 ml of cell suspension in a 125-ml flask per student pair)
- One liter of starvation buffer (for preparation)

To test whether the cytoskeleton is involved in the formation of vacuoles

- 1 ml cytochalasin B stock (for the entire class)
- 1 ml colchicine stock (for the entire class)
- 1 ml 70% ethanol (for the entire class)

Laboratory Exercises

Introduction to the Microscope

Because phagocytosis in *Tetrahymena* is such a dramatic cell behavior, it is a good exercise for introducing students to microscope use. When hungry *Tetrahymena* encounter food, they use their cilia to sweep it into each cell's oral groove. This process can be visualized by feeding stained yeast cells, India Ink (Keenan, 1984; Bozzone, 1998; Bozzone, in press), or *Chlorella* to *Tetrahymena*. Phagocytosis is most readily observed using ink as the "food". The vacuoles that form are full of ink and therefore are easy to identify. Students can also see the movement of the vacuoles inside the cell as new ones form. If they are sufficiently patient, sometimes they can see vacuoles "void" their contents into the extracellular space. Besides phagocytosis, students can also see ciliary motion and swimming behaviors when *Tetrahymena* are suspended in a dilute ink suspension.

Student Instructions- Observations of Phagocytosis and Swimming

1. *Tetrahymena* grown in 2% proteose-peptone for 48-72 h will be provided.
2. Pipette 1 ml of *Tetrahymena* and 1 ml of 1% ink into a test tube. Mix gently.
3. Using a compound microscope at 100-200X total magnification, observe the behaviors of the cells that have not been exposed to ink as well as those that have. Pay attention to how the cells swim and eat. Record your observations as diagrams and verbal descriptions.
4. Ten minutes after the cells have been exposed to ink, pipette 20 microliters of cell and ink suspension into a microfuge tube containing 10 microliters of 3% glutaraldehyde. Exposing cells to glutaraldehyde, a fixative, will kill the cells. Consequently, you will be able to observe the appearance and location of food vacuoles, and cilia in a stationary cell. Describe and diagram what you see.
5. Repeat steps 1-4 using 5% and/or 10% ink instead of 1%. Do you see any changes in swimming or feeding behaviors in the higher ink concentrations?

Exploration of Phagocytosis

Using modifications of the basic protocol described above, students have designed and implemented many experiments that investigated some aspect of phagocytosis. The three projects outlined below are examples of questions students asked and answered experimentally. We have presented student instructions for each experiment as if they were independent units so it will be easier to adapt them for instructional use.

Student Instructions

A. Does the Concentration of Ink Affect the Rate of Vacuole Formation?

1. *Tetrahymena* grown in 2% proteose-peptone for 48-72 h will be provided.
2. Pipette 2 ml of *Tetrahymena* and 2 ml of 1% ink into a test tube.
3. Sample cells at 0, 2, 5, 10, 20, and 30 minutes. To sample, pipette 20 microliters of cell plus ink suspension into a microfuge tube containing 10 microliters of 3% glutaraldehyde. Since

glutaraldehyde is a fixative, it will kill the cells. Consequently, you can work at your own pace once the cells have been sampled. Note that the "0" minute time point refers to the first sample you are able to collect right after the combination of the cells and ink. In real time, it will be less than one minute.

4. Place a drop of sampled cells on a slide and using a compound microscope at 100-200X total magnification. Count the number of vacuoles containing ink per cell for at least 20 cells for each time point. Calculate the average number of vacuoles containing ink/cell for each time point.
5. Repeat steps 2-4 using 5% ink instead of 1%.
6. Repeat steps 2-4 using 10% ink instead of 1%.
7. Prepare a table and graph to display your results.

B. Does the Nutritional State of *Tetrahymena* Affect the Rate at Which Food Vacuoles Form?

1. You will be provided with *Tetrahymena* grown in 2% proteose-peptone for 48 h and with *Tetrahymena* starved for 48 h in starvation buffer.
2. Pipette 2 ml of non-starving *Tetrahymena* cell suspension into a test tube. Pipette 2 ml of the starving *Tetrahymena* cell suspension into another test tube.
3. To each of these test tubes, add 2 ml 1% ink. Mix gently.
4. Sample cells from each test tube at 0, 2, 5, 10, 20, and 30 minutes. To sample, pipette 20 microliters of cell plus ink suspension into a microfuge tube containing 3% glutaraldehyde. Glutaraldehyde is a fixative which will kill the cells.
5. Place a drop of sample cells on a slide. Using a compound microscope at 100-200X total magnification, count the number of vacuoles containing ink per cell for at least 20 cells for each time point. Calculate the average number of vacuoles containing ink per cell for each time point.
6. Prepare a table and graph to display your results.

C. Is the Cytoskeleton Involved in the Formation of Vacuoles?

1. To determine whether microfilaments are essential for phagocytosis and vacuole formation in *Tetrahymena*, we will test the effect of the microfilament inhibitor, cytochalasin B, on these processes. (Caution: hazardous chemical. Gloves should be worn for handling these chemicals. According to the MSDS Sheet for cytochalasin B, the chemical should be dissolved with a combustible solvent and burned in a chemical incinerator equipped with an afterburner and scrubber. Be sure to observe all Federal, State, and local environmental regulations.)
2. Pipette 2 ml of a 48-72 h *Tetrahymena* culture into each of two test tubes.

3. To one tube, add 20 microliters of cytochalasin B (5mg/ml stock). To the other tube add 20 microliters of 70 % ethanol. Mix each tube gently. Incubate at room temperature for 10 minutes. The incubation is necessary to allow the cytochalasin to enter the cells. The 70% ethanol treatment serves as the control because this is the solvent into which the cytochalasin B is dissolved.
4. After the 10-minute incubation has been completed, add 2 ml of 1% ink to each tube. Mix gently.

5. Sample cells at 0, 10, 20, and 30 minutes (0 minutes means right after addition of the ink). To sample, pipette 20 microliters of cell plus ink suspension into a microfuge tube containing 10 microliters of 3% glutaraldehyde. Glutaraldehyde is a fixative which will kill the cells.
6. Place a drop of sampled cells on a slide. Using a compound microscope at 100-200X total magnification, count the number of vacuoles containing ink per cell for at least 20 cells for each time point. Calculate the average number of vacuoles containing ink per cell for each time point.
7. To determine whether microtubules are essential for phagocytosis and vacuole formation in *Tetrahymena*, we will test the effect of the microtubule inhibitor, colchicine, on these processes. (Caution: hazardous chemical. Gloves should be used for handling these chemicals. Instructions for disposal are the same as those described above in No. 1 for cytochalasin B.) To do this, repeat steps 2-6 substituting colchicine (400mg/ml stock) for the experimental treatment and distilled or deionized water for the control.

Sample Results

We have done these experiments many times and so can predict the results of the phagocytosis explorations. In addition, we present data collected by workshop participants.

Effect of Ink Concentration. The rate and degree of phagocytosis will be inversely proportional to the concentration of ink (Table 20.1). This observation is usually a surprise to students. While they have not determined the specific mechanism accounting for this phenomenon, they have articulated additional testable questions to explore this result further. For example, they wondered whether the high concentration of particulates in the ink might have damaged the cilia around the oral groove so that material could not be swept in efficiently. To test this they proposed exposing cells to 5% and 10% ink for 10 minutes and then washing the cells to see if they would now eat 1% ink at the control rate.

Table 20.1. Vacuole Formation in *Tetrahymena* at Different Concentrations of Ink

Time (minutes)	Average number of vacuoles/cell		
	1% Ink	*5% Ink*	*10% Ink*
2	1.8	2.3	2.0
5	3.2	2.9	2.8
10	4.8	3.5	2.9
20	6.0	3.1	2.3
30	9.9	6.2	3.0

Effect of Nutritional State. Starved cells usually exhibit a reduced rate of vacuole formation as compared to the well-fed control (Table 20.2). At first, this result seemed counterintuitive to students but after additional research they reasoned that perhaps starved cells will only eat actual food while well-fed cells will ingest anything that will fit into the oral groove, even ink particles. Again, students were able to design a logical experiment to test this hypothesis: would starved and well-fed *Tetrahymena* eat *Chlorella* or yeast at similar rates?

Table 20.2. Vacuole Fromation in Well-fed and Starving *Tetrahymena*

Time (minutes)	Average number of vacuoles/cell	
	Well-fed	*Starving*
0	0.5	0.05
2	3.1	1.4
10	5.1	3.5
30	8.1	6.2

Effect of Cytoskeletal Inhibitors. Cytochalasin B dramatically inhibits vacuole formation (Table 20.3). The cells ingest ink but the vacuoles that form never pinch off and do not move into the cells. Colchicine also decreases the rate of vacuole formation (Table 20.3; Results collected by the group that contributed these data did not exhibit an effect of colchicine at 25 minutes. However, only ten cells were counted). Students hypothesized that microfilaments are

necessary as part of the motor machinery that pinches off vesicles and that microtubules function as "tracks" for the intracellular transport of vesicles. Questions posed by students included: Are the effects of the drugs reversible? Are the drug effects dose-dependent?

Table 20.3. Vacuole Formation in *Tetrahymena* After Exposure to Cytoskeletal Inhibitors

Time (minutes)	Average number of vacuoles/cell		
	Control	*Cytochalasin B*	*Colchicine*
0	0	0	0
15	5.8	0.2	3.9
25	9.0	0.7	9.0

Because this experiment uses cytochalasin B and colchicine which are potentially hazardous materials, students should be carefully supervised during the experiment and appropriate cautions should be taken. Students should wear gloves while handling these chemicals. As described above, cytochalasin B and colchicine should be disposed of in accordance with Federal, State, and local regulations.

Suggestions for Other Experiments and Projects

Using the simple procedure of measuring phagocytosis and vacuole formation by feeding India ink to *Tetrahymena*, students can design and implement experiments of varying complexity. In addition to the ideas already described above, examples of other testable questions generated by students include:

1. Does the rate of phagocytosis vary with temperature?
2. Does the velocity with which *Tetrahymena* are agitated affect the rate of phagocytosis?
3. Will *Tetrahymena* select specific items for ingestion when presented with mixtures of food?
4. Does the duration of starvation influence the rate of phagocytosis?

Invariably, an experiment done to answer a particular question leads to more questions and ideas for experiments. Note that many of the papers that describe basic studies of phagocytosis and vacuole formation are available to provide context for the work that students do (Ricketts, 1972; Nilsson, *et al*., 1973; Hoffmann, *et al*., 1974). Also, phagocytosis in *Tetrahymena* has, in recent years, become a model for learning about pharmacology and cell membrane function (Stefanidou, *et al*., 1990; Csaba & Darvas, 1992; Renaud, *et al*., 1995). Consequently, the projects students do have a connection to current research problems. The ease with which the procedure can be done, along with the wide array of potential testable questions that can be

pursued, make studying phagocytosis in *Tetrahymena* an experimental system rich with possibilities.

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Suggested Readings

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Appendix: Instructions for preparation

- 2% proteose–peptone: 2 g proteose-peptone (Difco) per 100 ml distilled or deionized water. Autoclave 20 minutes slow exhaust. This medium does not store well. Cells do not grow well in old media. Refrigerate the media and use it within a month.

- To culture *Tetrahymena* (24-72 h before lab): Use aseptic technique to inoculate 1 ml of cell suspension into 25-30 ml of proteose-peptone in a 125-ml Erlenmeyer flask (the ratio of medium volume to total flask volume should be 1:4 or lower). Incubation at room temperature, 20-22^{o} C is best. Tetrahymena can be maintained over long periods by subculturing weekly in 2% proteose-peptone.

- *Tetrahymena* can be purchased from many suppliers, such as Ward's, Carolina Biological, and possibly Connecticut Valley. (We usually use Carolina Biological Supply Company). Be sure to order the "bacteria-free" culture and loosen lids upon arrival. Check to see if the cultures are viable. Cultures will be cloudy if the cells are alive. Stock cultures may be kept at room temperature for several days before subculturing.

- Salt solution for starving *Tetrahymena*: 6 mg KCl, 4 mg $CaHPO_4$ (or $CaCl_2$), 2 mg $MgSO_4$-$7H_2O$ per liter of distilled or deionized water. Autoclave 20 minutes, slow exhaust.

- To starve *Tetrahymena*, spin down cells from a 24-72 h culture in a centrifuge tube with a swinging bucket rotor (1000-rpm for 4 minutes), and wash the cells by resuspending the pellet in the dilute salt solution. Centrifuge and wash the cells two more times. Resuspend the final pellet in a volume of salt solution equal to the volume of medium initially removed. Incubate the resuspended cells at room temperature.

- Ink: As Keenan (1984) described, the type of ink used matters a great deal. Inks which contain detergents will not work. We use Hunt-Speedball. All dilutions are made in distilled or deionized water.

- 3% glutaraldehyde: Sigma sells 25% aqueous stock solution. For one lab section, mix 5-ml stock solution with 35 ml distilled or deionized water. Distribute the solution among screwcap bottles or vials. Refrigerate.

- Cytochalasin B: Make a stock solution of 5 mg/ml in 70% ethanol. Use it at a final concentration of 50 ug/ml. Lower concentrations might work, but we have not tested this.

- Ethanol: 70 % in distilled or deionized water.

- Colchicine: Make a stock solution of 400 mg/ml in distilled or deionized water. Use it at a final concentration of 4 mg/ml. As with cytochalasin, we have not tested the use of lower concentrations.