

Phagocytosis in *Tetrahymena* as an Experimental System To Study the Toxic Effects of Cigarette Smoke

Preparation for Laboratory: Web Tutorial 6, *Tetrahymena* - submit questions

Additional Background: Freeman - sections on microtubules and cilia, pp 105-109.

Background

Tetrahymena are small unicellular organisms that can be found in pond water. They are eukaryotes and belong to a group of organisms known as the protista. They are characterized by thousands of hair-like structures known as cilia that cover the cell surface. Cilia allow the animal to move through its environment and create currents that move material into the oral cavity.

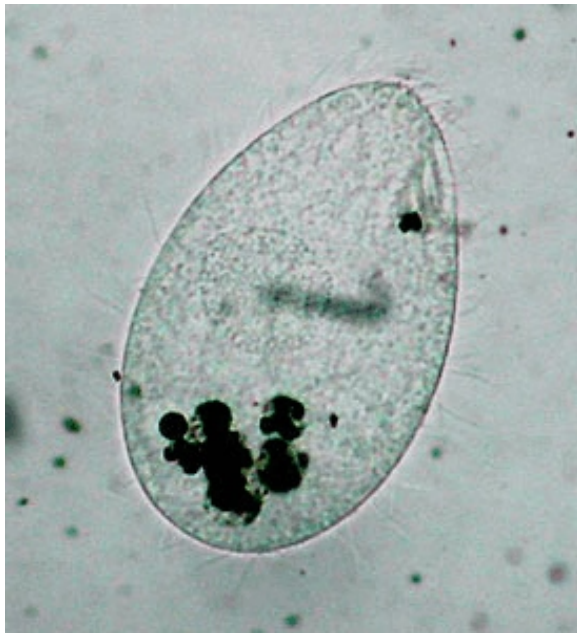


Figure 1. *Tetrahymena pyriformis* containing food vacuoles filled with India Ink. The oral cavity with its specialized cilia is visible at the top right of the organism. Photo courtesy of Emily Balf.

In ciliates the cilia near the oral cavity have become specialized for feeding. You can think of the oral cavity as a depression in the anterior surface of the cell. The right side of the oral cavity of *Tetrahymena* has a specialized feeding structure composed of cilia that adhere to each other, forming a curved structure known as the undulating membrane. On the left side of the oral cavity are three smaller ciliary membranellae that curve clockwise toward the cytostome (mouth) (Figure 1). The coordinated movement of these ciliary structures move food particles into the oral cavity and toward the cytostome. When food reaches the cytostome it is enclosed in a membrane bound food vacuole that pinches off and migrates through the cell. This type of vacuole formation is known as **phagocytosis** and is found in many unicellular organisms. In multicellular organisms only a few specialized cells such as the macrophages of the immune system are capable of phagocytosis. Food vacuoles fuse with lysosomes

and the food particles are digested. Undigested material is discharged from the cell at the cytoproct, which is a permanent region on the left, posterior surface of the cell. The time it takes for the processing of a food vacuole ranges from 20 minutes to 2 hours.

The movement of the cilia and the formation and movement of the food vacuoles depend on microtubules. In the cilia the microtubules are arranged in a 9+2 pattern. Nine pairs of tubules are spaced around the periphery and 2 single tubules lie in the center. Many protein bridges of dynein connect the peripheral pairs of microtubules and these are capable of altering their connections so the tubules slide past each other. If this happens on one side of the cilia the cilia can bend. Rows of cilia lie in integrated lines along the surface of the cell. The integrated alternate bending of the cilia creates the spiral swimming pattern seen when the animal is viewed under a microscope. It also creates currents that move food particles into the cytostome. Because cilia are responsible for both locomotion and moving food particles toward the

cytostome, factors that affect ciliary action should also affect the rate at which food vacuoles form. Emily Balf '05 conducted an independent project, "The short term effects of chemical components in cigarettes in both aqueous and ethanol extractions on the food vacuole formation in *Tetrahymena*." The idea for the project was based on knowledge that cigarette smoke can impair the function of the ciliated cells that line the lungs. Using techniques modified from Emily's project and Bozzone (2000) you will be have the opportunity to design a project to further investigate the relationship between smoke extracts and ciliary function.

Introduction to a Compound Microscope

In order to study phagocytosis in *Tetrahymena* it will be necessary to understand how to use a compound microscope. Rather than beginning with a wet mount prep containing *Tetrahymena* which are notorious for their rapid movement, we will use a prepared slide. The slide can be made by drawing a small letter "e" on one of the pieces of paper provided. Center the paper on a slide and gently lower a cover slip over the paper. You will use this slide to familiarize yourself with the major components of the Olympus CH2 microscope (Figure 2).

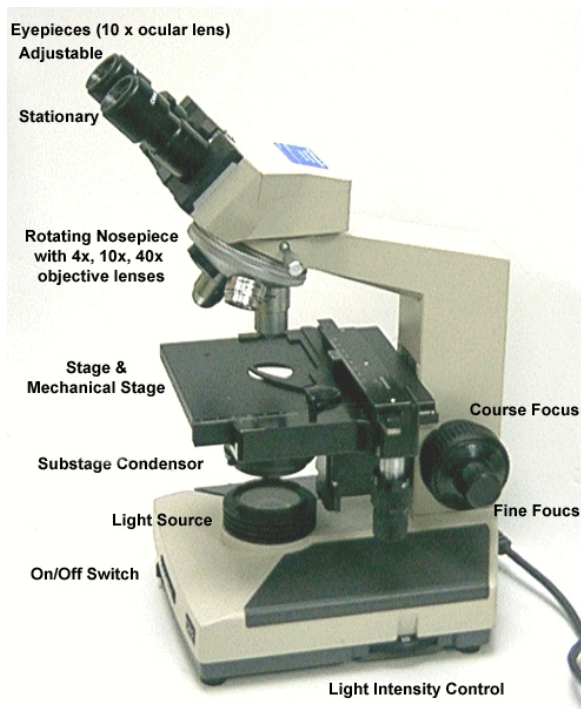


Figure 2. The major features of the Olympus CH2.

- Turn the scope on
- Always use the 4x objective when first examining material. Rotate the **nosepiece** until the 4x objective clicks into place directly above the hole in the stage.
- Rotate the **course focus knob** counter-clockwise to put the stage in its lowest position.
- Open the spring clip and place your slide in the **mechanical stage**.
- Raise the stage to its highest position.
- Use the stage control knob located to the right of the stage to center the letter "e" above the hole in the stage. The stage control knobs move the stage right/left or forward/backward.
- Adjust the distance between the eyepieces.
- Rotate the **course focus knob** counter-clockwise to lower the stage and bring the image into focus. If only part of the image is showing use the knobs under the stage to reposition the slide.
- With the left eye closed use the **fine focus knob** to sharpen the image. Then close the right eye and rotate the **adjustable eyepiece** to bring the image into focus for your left eye. You have now adjusted for differences in your right and left eye. You should not need to make this adjustment again.
- Note the orientation of the letter "e" on the stage of the microscope. Is it oriented in the same way when you view it through the microscope?
- There are three ways to adjust the light intensity. Experiment to see what each does.
 - ▶ the **light intensity control**
 - ▶ the **condensor** - Locate the knob under and to the left of the stage and note the effect of moving the condensor up and down. Then open and close the diaphragm

on the condensor.

- To increase magnification rotate the nosepiece until the 10 x objective clicks into place. The CH2 is a parfocal scope so your image should be almost in focus at 10x power. If not use *only* the **fine focus** to make adjustments. To make it easier to find an object as the magnification increases put the object you want to view in the center of the field before moving to a higher magnification.

Depth of field and magnification - When you begin working with the *Tetrahymena* they will be moving up and down as well as laterally. This will cause them to move in and out of focal plane. As you increase the magnification it will become more difficult to find them and to bring them into focus. You can briefly explore the relationship between depth of field and magnification by adding a hair to your slide. Remove the slide from the stage and take off the coverslip; place a hair across the letter “e” and replace the coverslip.

- Using 4x, focus on the letter “e” and then the hair. Notice that it is not possible to have both the hair and letter “e” in focus at the same time. The hair is lying on top of the paper so it is in a different plane. If the letter “e” is in focus, it will be necessary to move the stage away from the objective to bring the hair into focus. As the fine focus knob moves clockwise the stage moves down. Moving the fine focus counter-clockwise moves the stage up (Figure 3). When focusing make sure you know where you are beginning. A good rule of thumb is to position the stage in its highest position and gradually lower it. Continue to lower the stage to focus on successive planes. With this technique objects closest to the slide come into focus first. Make sure you understand this concept.

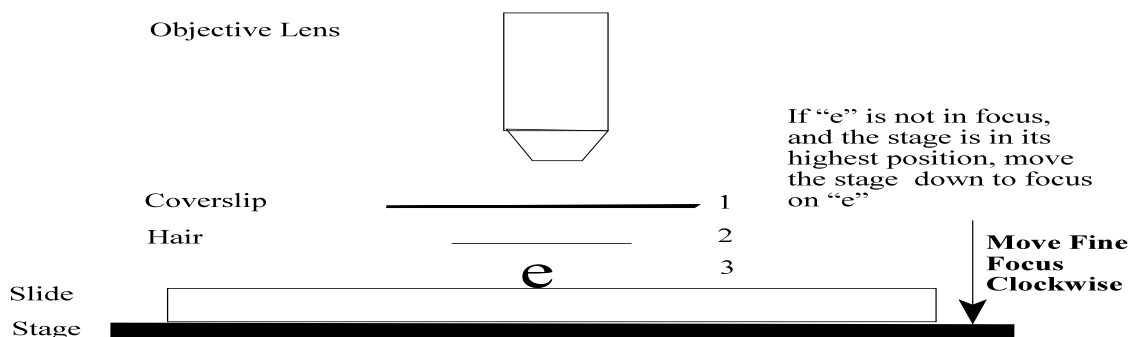


Figure 3. As the stage of the microscope moves from its lowest level toward the objective lens the first thing to come in focus is the coverslip (1) as the stage continues to move toward the objective the hair(2) then the “e” (3) comes into focus. If you continue to raise the stage you will eventually end up focusing on the slide. The reverse is true if the stage starts in the highest position.

- Move to the 10x objective. Carefully alter the **fine focus**. Notice how the depth of field has decreased and it is even harder to focus.
- Rule - always find your plane of reference using the 4x objective.**

Total Magnification = objective lens x ocular lens

Ocular Lens	Objective Lens	Total Magnification
10x	4x (scanning)	
10x	10x (low power)	
10x	40x (high power)	

Introduction to the Experimental System

You are now ready to examine your research organism. Wear gloves when working with *Tetrahymena*. The cultures you are working with were started 48-72 hours before laboratory by transferring 1ml of cell culture into 25ml of a sterile nutrient media containing 2% proteose-peptone. India ink has been added to a culture of *Tetrahymena* so you can study the feeding behavior. Cultures are located on the side bench.

Making a wet mount

- Place a drop of the *Tetrahymena* suspension in the center of a clean slide.
- Slowly lower the coverslip at an angle.
- Use Kimwipes to remove excess fluid. **Caution - avoid removing so much fluid you squash the organisms.**
- Examine living organisms under 4x, 10x, and 40x. Note the manner in which they move through their environment. By reducing the light intensity you should be able to see the movement of the undulating membrane as it directs particles of ink into the cytostome.

Preliminary investigation of the effect of time on food vacuole formation in *Tetrahymena*.

It takes about 30 minutes for a food vacuole to form and migrate through the cell to the ectoproct where its contents are expelled. Since you will be killing the cells by fixing them in 3% glutaraldehyde, they do not need to be examined immediately. Each table will have a stock culture to set up their experiment.

- Decide on a set of time intervals during which the animals will be allowed to feed. These should range from 0-20 minutes. Each person at the table can take responsibility for one or more of the time intervals.
- Place 100 microliters of 3% glutaraldehyde in a microfuge tube; one for each time interval.
- Mix 2ml *Tetrahymena* + 2ml 1% Hunt Speedball- ink diluted in deionized water in a glass test tube. This culture will be sampled at successive time intervals. For time 0 as soon as the ink is mixed into the culture withdraw 200 microliters and kill the cells.
- At the predetermined times kill the organisms by pipetting 200 microliters into one of the microfuge tubes containing 3% glutaraldehyde.
- The dead *Tetrahymena* will settle to the bottom of the microfuge tubes. Resuspend them before making slides. Examine the dead cells and count the number of vacuoles containing ink for 25-50 randomly selected organisms. To assure that group members are counting in the same manner you may want to practice by having everyone count the same cell. When a cell contains a large number of vacuoles they may overlap making it necessary to slowly focus up/down to get an accurate count.
- Record your group data in a table and do the appropriate statistical test to analyze the effect of time on the rate of food vacuole formation.

Ink Assay

As you have seen the *Tetrahymena* readily consume ink particles and the number of vacuoles present is related to the amount of time the animals are allowed to feed provided the time interval is less than 30 minutes. After 30 minutes vacuoles are expelled from the cell. This behavior can be used as a way of assaying the effect of various substances on the ciliary activity.

The Effects of Extracts from Cigarette Smoke on Food Vacuole Formation

From the web tutorial you learned that components of cigarette smoke can affect the function of cilia that line the lungs. Since cilia are also involved in channeling of food particles into the cytostome, extracts from cigarette smoke might also affect the formation of food vacuoles. Working in groups of four you will design an experiment to explore the relationship between smoke and ciliary function.

Factors that can be varied are:

- **extraction technique** (alcohol vs. water)
- **source of extracts** (smoke vs. filters)
- **concentration of extract** - the extract can be diluted
- **type of cigarette** - regular, menthol, light, ultra light, unfiltered
- **total exposure time** - Since food vacuoles can be voided after 30 minutes it is not advisable to have the ink and organism in contact for more than 20 minutes. If you want to explore the effect of exposure time it is possible to mix the *Tetrahymena* with the extract for x minutes before adding the ink. The total exposure time will be the sum of the pre-exposure time plus the assay time.
- **# cigarettes smoked or filters used** to make the extract
- **temperature** - (room vs a lower temperature will be available)

The above variables can be used alone or in combination, but you should not use more than two of them or your design will become too complex.

Experimental Design: In designing an experiment the first step is to clarify the question you are trying to answer. Then you determine type of design and statistical test that will allow you to answer the question.

1. What hypothesis will you test?
 - What variables will you use?
 - What controls are needed?
2. What type of data will be collected?
 - Number of vacuoles with ink?
 - Number of organisms with ink filled vacuoles?
 - Rate of vacuole formation?
3. How will the data be analyzed? The type of statistical test used will depend on the type of data collected and the nature of the design. There are several simple designs that lend themselves to this study.
 1. "With/without" Design - In this type of design you vary **one** factor and it is either present or absent. Use a Two Sample t-test to compare means.
 2. "Gradient" Design - in this type of design you vary **one** factor and use **several levels** of it. Use a One Way Analysis of Variance to determine if the treatment had an effect.

3. "Interaction" Design - in this type of design you vary **two** factors and may use **two** or **more** levels of each. In this design it is important to make sure that the treatments include all possible combinations of the factors being varied. The easiest way to make sure of this is to create a table that lists one variable across the top and the other down the side and fill in the boxes. Use a Two Way analysis of Variance (**GLM**) to determine if the factors independently or in combination had an effect.

Before leaving class outline your experimental protocol so you will be ready to go when you come to class next week.

Preparation for week 2

Have your experimental design approved by your instructor.

Obtain a vial(s) for making extracts.

Prepare extracts approximately 24-48 hours before coming to class. For:

- **Filters:** Soak 1-2 freshly smoked filters for 24-48 hours in either **10 ml** of alcohol or deionized water. Remove any tobacco that is adhering to the filter.
- **Smoke:** The day before lab meet with your instructor to prepare a smoke extract(s) in either alcohol or deionized water
- **Dilution Series:** If you plan to use several concentrations of smoke or filter extracts, review the web tutorial on serial dilution.

Literature Cited:

Bozzone, Donna M. 1999. Investigating Phagocytosis in *Tetrahymena*. Am. Biol. Teacher 62(2): 136-139.