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Experimental Design Using India Ink to Study the Effects of Phagocytosis in *Tetrahymena*

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Tetrahymena is a freshwater, ciliated protozoan that is cheap, easy to maintain and well suited for students of all academic levels. It is an excellent model to gain experience with experimental design and to learn various laboratory techniques. The *Tetrahymena* lab has been used and modified in a core Cell & Molecular 2000 level course (~100 students) at Acadia University for the past 2 years. Every summer, Acadia runs a free, week-long science summer camp to girls in Grades 7 and 8. This laboratory will be one of the workshops offered in August 2019 and will include data analysis. ABLE Participants will study the rate of vacuole formation in *Tetrahymena* using a 5% India Ink solution and compound microscopes (100X or 400X). Participants will discuss the experimental setup based on variables to be tested, controls, limitations and how the lab can be adapted to address student outcomes in different courses. Discussion may also include maintenance of the *Tetrahymena* culture, creation of rubrics with an array of designs, student collaboration, and management of large data sets and how to make sense of it all.

Keywords: *Tetrahymena*, phagocytosis, experimental design, India Ink

Introduction

Tetrahymena are free-living ciliate protozoa that are commonly found in freshwater ponds. Among protozoa, *Tetrahymena pyriformis* and *Tetrahymena thermophila* are the most commonly ciliated model used for laboratory research. They are also popular for student lab exercises as they are fairly easy to cultivate and maintain. Originally cultured in bacterized hay or vegetable matter infusions, *Tetrahymena* was the first animal-like eukaryotic cell to be grown axenically (single species, free of all other contaminating organisms). Axenic proteose peptone-based media are currently the most common choice for laboratory culture but, *Tetrahymena* can be successfuly grown in a wide variety of media. For routine daily use, *Tetrahymena* cultures are generally maintained out of direct light, between 18°C and 24°C (room temperature) in slow growing stock tube cultures.

In nature *Tetrahymena* is a suspension feeder. Particulate matter is moved via cilia to a specialized oral structure, the cytostome, found at the base of the oral apparatus. *Tetrahymena* ingests the particles and form a food vacuole or phagosome. These then travel to the posterior of the cell in a directed manner and are digested. Phagocytosis appears to be rather nonspecific in that many types of particles can be ingested, including bacteria, India ink and latex beads.

Student Outline

Objectives

- Become familiar with the use of a light microscope
- Use the ocular scale bar to obtain measurements using several magnifications
- Determine the minimum dilution at which glycerol can stop the movement of *Tetrahymena pyriformis*
- Determine if there is a difference in the rate at which "old" and "new" *Tetrahymena pyriformis* cultures phagocytose a solution of 5% India Ink
- Determine if there are any visible physical differences in "old" and "new" *Tetrahymena pyriformis* cultures
- Gain experience designing experiments (including controls), data analysis & interpretation
- Practice effective communication with lab mates and general collaboration skills
- Improve scientific writing skills

Microscopy

The lens objectives, at the bottom of the barrel, provide both image magnification and image resolution. The scopes you will be using come with a scanning objective, (4X objective magnification), an intermediate dry lens objective (10X), a high-powered dry lens objective (40X) and a high-powered oil immersion lens objective (100X). Your oculars (eyepieces) have a 10X magnification. The total magnification of the scope is found by multiplying the magnifications of the ocular and the objective. You will not be using the oil immersion lens for today's work.

In addition to magnification, the resolving power of a microscope is very important. This is defined as the ability to resolve fine detail, or to discriminate between adjacent details. Resolving power is the least distance that can be discerned between two objects. For the compound light microscope, objects closer than 0.2 μm cannot be resolved as separate structures. (Interestingly, van Leeuwenhoek, using a simple microscope, but with an excellent lens, obtained a resolving power of 2 μm.)

Compound scopes objectives are both parfocal and parcentral. Parfocal means that the focal point of the objectives is in the same plane, i.e. the focus barely changes when objectives are switched-only minor adjustment with the fine focus knob is necessary. Parcentral indicates that the center of the viewing field remains constant when objectives are changed, so be sure to center the object of interest carefully before changing magnification.

The viewing slide is placed on the fixed stage. Below the stage is a movable sub-stage, consisting of the condenser and the iris diaphragm. The condenser concentrates light on the tissue section and must be centered accurately. The iris diaphragm regulates the amount of illumination and needs to be adjusted for each objective so that peripheral light rays are blocked, and the light passing through the tissue just fills the front lens of the objective. As objectives are changed, the iris diaphragm must also be adjusted.

Care of the Microscope

1. Carry by holding firmly onto base and back support.
2. Use only Kimwipe to clean lens.
3. Take care when changing objectives not to hit the stage or glass slide.
4. Turn off microscope when the scope is not in active use.
5. Be sure that the stage area is clean, and any spill is cleaned using a Kimwipe.
6. Always put away scope with lowest objective, 4X, in viewing position.
7. Have the TA check your microscope before putting away. Get a ✓ not an ✗

Methods

Adjusting the Light Intensity

1. Place the 4X objective lens in viewing position, using the small knob on the right-hand side of back support, turn lamp intensity to its highest position, then turn down a little (~3/4 to full intensity). If you need to change the brightness at any point, DO NOT USE THIS KNOB, use the iris diaphragm found below the stage.

2. At low magnifications (4X objective), low light is generally required. To check it, move the iris diaphragm lever back and forth until the desired lighting is reached. As you move to a higher magnification, you will have to open the iris diaphragm further to allow more light to reach your specimen.

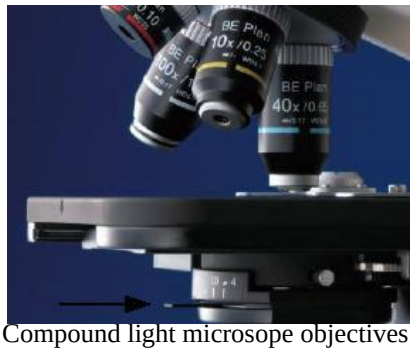

Figure 1. Compound light microsope objectives and stage.

Tetrahymena - Background

Tetrahymena are free-living ciliate protozoa that are commonly found in freshwater ponds. Among protozoa, *Tetrahymena pyriformis* and *Tetrahymena thermophila* are the most commonly ciliated model used for laboratory research. They are also popular for student lab exercises as they are fairly easy to cultivate and maintain. Originally cultured in bacterized hay or vegetable matter infusions, *Tetrahymena* was the first animal-like eukaryotic cell to be grown axenically (single species, free of all other contaminating organisms). Axenic proteose peptone-based media are currently the most common choice for laboratory culture, but, *Tetrahymena* can be successfully grown in a wide variety of media, including bacterized peptone, bacterized infusions of lettuce or rye leaves, skim milk based media, and chemically defined media. For routine daily use, *Tetrahymena* cultures are generally maintained out of direct light, between 18°C and 24°C (room temperature) in slow growing stock tube cultures.

In nature *Tetrahymena* is a suspension feeder. Particulate matter is moved via cilia to a specialized oral structure, the cytostome, found at the base of the oral apparatus. *Tetrahymena* ingests the particles and forma food vacuole or phagosome. These then travel to the posterior of the cell in a directed manner and are digested. Phagocytosis appears to be rather nonspecific in that many types of particles can be ingested, including bacteria, India ink and latex beads.

The life cycle of *Tetrahymena* consists of an alternation between asexual and sexual stages. In nutrient rich media during vegetative growth cells reproduce asexually by binary fission. This type of cell division occurs results in the development of duplicate sets of cell structures, one for each daughter cell. Only during starvation conditions will cells commit to sexual conjugation, pairing and fusing with a cell of opposite mating type.

Materials

5mL *Tetrahymena pyriformis* grown in 2% proteose peptone (Old maintained for 1 year, New ordered 2 weeks ago)
Light microscope & glass slides
5% solution of India Ink
Glycerol & water
Plastic bulb pipettes
Microcentrifuge tubes
Timer
Hemocytometer (google it)*:*

Methods

A. To View Tetrahymena in their Natural State

1. Using a plastic Pasteur pipette, add one drop of *Tetrahymena* to a glass slide and view. Take a few notes of your observations. Just to have an idea of their "natural" state. *Tetrahymena pyriformis* has been grown in 2% proteose peptone for 4 days, in a bacteria free environment.
2. Determine the density per unit volume of both old and young cell cultures using the hemocytometer. Look up use of hemocytometer online – there are many protocols and videos to illustrate

> ***You will be required to write up a detailed Materials & Methods for your lab report. So, take good notes! When creating your flowchart, use simple drawings, arrows, and words to convey methods to ensure it is visually easy to understand and follow.***

B. To Determine the Minimum Dilution of Glycerol to Use for Your Experiment

Q1: What is the minimum dilution of glycerol required to stop the movement of *Tetrahymena pyriformis*?
*Work as a bench, organize yourselves to ensure reproducibility and consistency.

. To avoid contamination, label 2 plastic pipettes; one for glycerol and the other for water.
. Using microcentrifuge tubes to make up solutions, determine the ratio or dilution of glycerol required to stop the movement of cilia in *Tetrahymena*. Make up various dilutions of glycerol to test. For example, 1 drop of glycerol + 9 drops of water, would be a 1/10 dilution, or a 10% glycerol solution.
. Once your glycerol + water dilution is properly mixed, obtain a new microcentrifuge tube and mix one drop of the glycerol dilution with one drop of *Tetrahymena*. Gently mix.
. Add one drop to a glass slide and immediately view. Record observations.
  - Once you (as a bench) have determined the minimum dilution of glycerol to stop the movement of cilia in *Tetrahymena*, you will use this solution strength to "sample" *Tetrahymena* at several time points. To "sample" is referring to "stop phagocytosis" and "count/observe" *Tetrahymena.*
. As a bench, prepare a volume of diluted glycerol to use for the entire experiment. Label and do not contaminate.

C. To Determine the Difference in Rate of Phagocytosis in Old and New Cultures

Q2: Is there a difference in the rate of phagocytosis (vacuole formation) of India Ink in Old and New cultures of *Tetrahymena*?

- Phagocytosis will be determined using 5% India Ink. *Tetrahymena* will "ingest" the India Ink and form vacuoles within their cell? Decide as a group how to set up your bench to determine the rate of vacuole formation in the Old culture as well as the New culture.
- Design an experiment to test if there is a difference in the rate of vacuole formation in old cultures of *Tetrahymena* versus new cultures of *Tetrahymena*. You will work on this as a bench.
- Make sure you have controls, and that observations will be as consistent as possible.
- Record observations. Your experiment may be very simple.
- Take detailed notes for your methods section, and make sure everyone is on the same page. Think about appropriate tables and/or figures to display your results.

You may want to test your control first, and also discuss as a bench. You may deviate from these methods, but use general methods described below

Ideas: Pair #1 uses the Old culture and Pair #2 use the Young culture (in duplicate?)
OR
BOTH pairs use both old and young cultures and compare results (for consistency

. You will be counting the number of vacuoles per cell. Observe 5 *Tetrahymena* cells at the following time points: 5 minutes, 10 minutes, 15 minutes, 20 minutes
. Pipette equal volumes (drops) of *Tetrahymena* and 5% India Ink into a clean microcentrifuge tube. Gently mix. Start timing!
. Sample tubes at 5, 10, 20 and 30 minutes to count # of vacuoles at each time point.
. To stop the movement of cilia in *Tetrahymena*, remove a known volume of culture and pipette into a new microcentrifuge tube. Add pre-determined glycerol dilution to the *Tetrahymena* culture.
. Immediately place a drop on a slide and start counting vacuoles.
. Continue with each time point. To avoid cross contamination, you may want to rinse the transfer pipette with d'H_2O between time points.
. Record %glycerol used and data for both cultures and each time point on spreadsheet found at front bench

D. To Determine Visible Physical Differences in Tetrahymena pyriformis Cultures

Q3: Are there visible physical differences between the "Old" and "Young" cultures.

Ideas: Motility speed, type of motility (directed vs tumbling), size, etc.

Make up your own variables, you should have at least 2.

- Design an experiment to test these variables/differences in both cultures of *Tetrahymena*. You will work on this as a bench. Again, make sure you have controls, and that observations will be as consistent as possible.
- Record observations. Your experiment may be very simple.
- Take detailed notes for your methods section, and make sure everyone is on the same page.
- Think about appropriate tables and/or figures to display your results.

Results

- Summarize lab section results into one table or a maximum of 3 tables. Don't forget text to mention trends and info you want reader to pay particular attention to.
- Table(s) - Reorganize class data in a way that will simplify and clearly show all variables used. Tables are meant to be "stand alone" therefore captions (above table) are also important.
- Extra Table – Create an additional table that includes info/data that support ideas/conclusions to be included in the discussion.

Discussion

Upon making sense of all data, with regards to old versus younger culture, are there differences? What are the major findings? Also touch on the following:

- Are they any changes to your methods that would have improved reliability of your results? How would you modify the current experiment?
- Find a non-hazardous alternative to glycerol to slow down or stop our critters …as some of you may have noticed that *Tetrahymena* are negatively affected by glycerol after a while (any idea how?), include citation.
- State main conclusions

Cited References

Bozzone, D. M. 2005. Using microbial eukaryotes for laboratory instruction and student inquiry. Pages 81-109, in Tested Studies for Laboratory Teaching, Volume 26 (M.A.O'Donnell, Editor). Proceedings of the 26th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 452 pages.

Bozzone, M. D. and D. A. Martin. 2000. An experimental system to study phagocytosis. Pages 405-415, in Tested studies for laboratory teaching, Volume 21 (S. J. Karcher, Editor). Proceedings of the 21st Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 509 pages.

Materials

Each student pair:
5mL *Tetrahymena pyriformis* grown in 2% proteose peptone ("Old" maintained for 1 year, "New" ordered 2 weeks prior to the lab)
Light microscope
Glass slides
Microcentrifuge tubes
Plastic transfer pipettes
Timer
Hemocytometer
Manual counter
5% solution of India Ink (Speedball)
glycerol or potassium iodide solution
Distilled water

Notes for the Instructor

We use the simple model of *Tetrahymena* (Carolina Biological) in Cell Biology to allow students to design experiments (including controls), analyze and interpret data. We also felt that this experience allowed students to practice communicating with lab mates and work on general teamwork.

Each section of this course has 24 students per lab per day, for a total of 4 lab sections. There are 3 benches of 8 students in each lab. One treatment is replicated 3 times for "old" and "new" cultures in each lab section. For reports, students use the data from their own lab section and compare their design and data to other sections.

The first time this lab was done, students knew the identity of the culture they were given. This year, the cultures were labeled X and Y, so that students could make a hypothesis on whether they thought the "old" or the "new" culture would produce more ink vacuoles. Once the experiments were completed, the culture identity was revealed to the students.

Following the mini workshop presentation in the 41st ABLE Conference, the lab was modified for a WISE (Women In Science & Engineering) summer camp for girls in Grades 7 and 8. The methods to determine the minimum glycerol concentration portion was dropped, and potassium iodide solution (Wards) was used instead. One drop was added to the glass slide containing *Tetrahymena* and India Ink at each time point. Potassium iodide worked very well to instantly stop the movement of *Tetrahymena* and students were still able to count the ingested vacuoles of India Ink. Data from each group was compiled and shared with the entire class to create summary plots with means and standard deviations as part of the science camp experience.

For the upcoming Winter 2020 Cell Biology lab, potassium iodide will be used instead of glycerol. Students will spend more time with experimental design to ensure reproducibility of results, as this was noted as an issue the first time.

For the scientific writing portion of the exercise, students were given an outline as a guide to write up their report. The outline contained a list of elements required for each section of the report. A detailed rubric was created for the teaching assistants to keep the marking consistent across sections. The emphasis was placed on experimental reproducibility, data summary and presentation (tables, graphs), formatting etc. and not so much on cell biology content of the lab. Although class data varied across lab sections, the learning outcome of teaching students about experimental design and how to present results and discuss findings was achieved.

This laboratory can easily be adapted to students old enough to understand the main concepts, from Grade 5 students to university sophomore students learning experimental design.

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Bozzone, D. M. 2005. Using microbial eukaryotes for laboratory instruction and student inquiry. Pages 81-109, in Tested Studies for Laboratory Teaching, Volume 26 (M.A.O'Donnell, Editor). Proceedings of the 26th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 452 pages.

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About the Authors

Hélène d'Entremont holds a BSc in Biology from Mount Allison University and an MSc in Microbiology

from Acadia University. Hélène began as a technician at Acadia in 1994 and has been an Instructor at Acadia University since 2001, where she teaches a large number of students in second year microbiology and cell biology labs.

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