Teaching Phagocytosis Using Flow Cytometry

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Investigative microbiology on protists in a basic teaching laboratory environment is limited by student skill level, ease of microbial culture and manipulation, instrumentation, and time. The flow cytometer is gaining use as a mainstream instrument in research and clinical laboratories, but has had minimal application in teaching laboratories. Although the cost of a flow cytometer is currently prohibitive for many microbiology teaching environments and the number of trained instructors and teaching materials is limited, in many ways the flow cytometer is an ideal instrument for teaching basic microbiology. We report here on a laboratory module to study phagocytosis in *Tetrahymena* sp. using flow cytometry in a basic microbiology teaching laboratory. Students and instructors found the flow cytometry data analysis program, Paint-A-Gate^{PRO-™}, to be very intuitive and easy to learn within a short period of time. Assessment of student learning about *Tetrahymena* sp., phagocytosis, flow cytometry, and investigative microbiology using an inquiry-based format demonstrated an overall positive response from students.

Using an experimental approach to teaching about microbes is rare in basic microbiology laboratories for lack of easy-to-use laboratory methods appropriate to undergraduate use. Yet students learn best when actively engaged in the process of intellectual discovery (4, 5, 11). The goals of the laboratory series described in this paper were to use an inquiry-based process in teaching a basic microbiology laboratory exercise and give students ownership of the design, implementation, and analysis of their experimental work. Students formulated a research question that involved the evaluation of phagocytosis of prey objects by *Tetrahymena* sp. Experimental data files were acquired using a flow cytometer and analyzed using the academic version of a commercial software program, Paint-A-Gate^{PRO-™}. Through this process undergraduate biology majors learned about flow cytometry and experimental biology during 5 to 6 hours of laboratory activities.

The flow cytometer is an instrument that uses a laser beam to evaluate the properties of individual cells as they flow through an interrogation point. It rapidly generates an "optical fingerprint" of each cell within the population as it passes through the laser beam. When cells are labeled with fluorescent probes, several parameters of each cell can be determined simultaneously. Since the cells are interrogated at a rapid rate, data on a statistically meaningful number of individual cells can be acquired in a few minutes, saved, and computer analyzed in a two-dimensional plot format. The flow cytometer has been used extensively in the study of mammalian cells. More recently, it has been used to study microorganisms (3, 8). A search of the American Society for Microbiology journals under "flow cytometry" yielded 4,273 citations from 1992 through 2003. Half of these citations (2,262) appeared in the last 3 years (<http://www.journals.asm.org>). Flow cytometry is presented in modern basic microbiology

textbooks, along with microscopy, as a method to characterize microbial cells (12, 13). In spite of the emerging eminence of flow cytometry in microbiology research, we are aware of no published reports describing its use in the microbiology teaching laboratory.

Tetrahymena sp. has been studied in research laboratories using flow cytometry and in teaching laboratories using microscopy (7, 9). As Bozzzone reported, this ciliate is ideal for teaching about experimental microbiology using an investigative approach (1). *Tetrahymena* sp. require no special equipment and are easy to grow and manipulate in pure culture on the benchtop. They are not pathogenic and phagocytize a wide range of prey quite rapidly.

We describe here microbiology laboratory activities using flow cytometry where first time microbiology students design and carry out basic experiments on phagocytosis in *Tetrahymena* sp. using fluorescent yeast and beads as prey objects. The dynamic aspects of phagocytosis, coupled with putting the student in charge of all aspects of the experimental process, create an ideal learning opportunity (2, 6).

MATERIALS AND METHODS

Microbial and prey preparations. *Tetrahymena* sp. cultures with known characteristics are available at the American Type Culture Collection (<http://www.atcc.org>). For most experiments *Tetrahymena* sp. were grown in a liquid medium containing 2% proteose peptone, 0.1% yeast extract, 0.5% glucose, and 0.1% NaCl under well-aerated (one revolution per second), ambient temperature conditions for 24 to 48 hours. Concentrations were adjusted to approximately 10⁵ cells per test volume. Fluorescein isothiocyanate (FITC)-labeled *Saccharomyces cerevisiae* (14) and fluorescent beads (red fluorescent ~2 µm and green fluorescent ~6 µm; SpheroTech, Inc., Libertyville, Ill.) were used as prey in feeding studies.

Microscopy. Students observed wet mounts of *Tetrahymena* sp. using either their laboratory microscopes fitted with phase-contrast objectives or a Zeiss Axioscope 2 fluorescence microscope with camera and computer attachments.

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Flow cytometry. Data acquisition was performed using a FACSCalibur or a FACScan flow cytometer (BD Biosciences, San Jose, Calif.) set to acquire forward scatter (FSC), side scatter (SSC), and fluorescence (FL1 and FL2) with log amplification using an FL1 threshold set just below the minimum *Tetrahymena* sp. autofluorescence. Depending on the experiment, either 10,000 total prey and *Tetrahymena* sp. events were acquired, or the flow cytometer was set to acquire between 500 and 2,000 *Tetrahymena* sp. and the number of prey varied.

Analysis program. Paint-A-Gate^{PRO} (BD Biosciences, Immunocytometry Systems, San Jose, Calif.) was used by students for computer analysis of data files acquired on the flow cytometer. This program is intuitive and simple to learn. Two-dimensional dot plots are created in which each cell becomes a dot defining two values obtained from the flow cytometer (eg., relative size and relative fluorescence). The student analyzing the data file chooses a color and encircles the cell population of interest with the cursor. All of the cells in this population appear in the plot in the chosen color (painting) and can be selected (gated) for further analysis.

Student preparation. The material presented here was used in an upper division microbiology course for biology majors with concentrations in microbiology, molecular biology, physiology, and organismal biology (botany, zoology, ecology, and evolution), and biochemistry majors at San Jose State University (SJSU). Students were familiar with distinguishing cellular characteristics of *Tetrahymena* sp. and *Saccharomyces* sp., and light microscopy, fluorescence microscopy, and flow cytometry theory from lecture. Skills in basic culture manipulation were developed in earlier laboratory exercises.

Student activities. During 5 to 6 hours, students completed three exercises (Worksheets 1-3: <http://www2.sjsu.edu/depts/Biology/specialprogs/flocyto/html/fc-p05.html>) designed to progressively develop a conceptual framework requiring increasing knowledge about experimental microbiology and flow cytometry (http://www2.sjsu.edu/depts/Biology/specialprogs/flow_cytometry.html). Worksheet 1 addressed basic concepts in microbiology, microscopic observation, and cell characterization and required a *Tetrahymena* sp. culture, fluorescent yeast, and microscopes. Worksheet 2 focused on basic concepts in flow cytometry: fluidics, optics, SSC, FSC, fluorescence, and dot plots. A computer exercise included in Worksheet 2 introduced the students to Paint-A-Gate^{PRO} through the use of archived data files. Worksheet 3 (Fig. 1) addressed scientific investigation. Students proposed a question, established an information base

about phagocytosis and prey, designed and conducted an experiment, and acquired and analyzed data files. This experiment required using laboratory facilities and a flow cytometer. The complexity of the experiment dictated the size of the student groups. Students working in groups of two to four cultured *Tetrahymena* sp. in the presence of prey (fluorescent-labeled yeast or beads) under specific conditions, acquired their data files using a flow cytometer, and performed analysis using Paint-A-Gate^{PRO} software.

Student assessment. The laboratory curriculum described here was developed as part of an ongoing grant from the National Science Foundation to two of the authors of this paper (J.T.B. and R.K.), and the assessment of this curriculum has been conducted in accordance with the procedures in the grant proposal. Although an assessment comparing the previous way of teaching about protists to our present methodology would be beneficial in assessing the efficacy of using the flow cytometry experiment, this option was not available because the present methodology was immediately integrated into the laboratory curriculum upon funding of the grant. The outcomes that we report here were derived using flow cytometry laboratory experience assessments among the students enrolled in laboratory sections of General Microbiology (Microbiology 101) at SJSU over a 3-year period. Students were asked about their academic level,

Experimental question:

- Question on phagocytosis in *Tetrahymena* sp. determined by lab group:
Does the size of prey affect ingestion by *Tetrahymena* sp.?

Experimental design:

Purpose: determine if prey size affects ingestion of beads by *Tetrahymena* sp.

- *Tetrahymena* sp. concentration: $\sim 10^5/200\mu\text{l}$.
- Beads used as prey objects for *Tetrahymena* sp.:
 - Large beads: 6 μm green fluorescence emission detected in FL1.
 - Small Beads: 2 μm red fluorescence emission detected in FL2.
- Bead concentration: 10^6 beads/ $20\mu\text{l}$ in *tetrahymena* medium (~ 10 beads per *tetrahymena*).
- Time of phagocytosis held constant at 5 minutes.
- Mix $200\mu\text{l}$ *Tetrahymena* sp with $20\mu\text{l}$ beads and incubate on the bench top for 5 minutes.
- Stop phagocytosis by adding $50\mu\text{l}$ formalin and mixing.
- Evaluate experiment using fluorescence microscopy. Prepare a wet mount to determine the number and size of beads inside 50 formalin fixed *tetrahymena*.
- Evaluate experiment using flow cytometry – Determine the percentage of *tetrahymena* ingesting beads of two different sizes using the following plots:
 - Forward scatter vs. side scatter (gate on *Tetrahymena* sp)
 - FL 1-Tet & 6 μm Green vs. FL 2-Tet & 2 μm Red

Experimental protocol:

- Each group of three students obtain the following:
 - Test tube rack
 - Falcon (flow cytometer) tubes
 - Microfuge tube containing Green beads, Red beads, or a mixture of the two
 - Microfuge tube containing $100\mu\text{l}$ formalin (**Note: toxic/carcinogenic**)
 - pairs Gloves (**wear gloves when handling cultures and tubes**)
 - Micropipettors: Eppendorf P20 & P200
 - Box of pipette tips (small tips)
 - Tube *Tetrahymena* sp. $\sim 1\text{ ml}$ of a 24-48 hour culture
- Label Falcon tubes with your group letter and (1) 'Flow cytometry' or (2) 'Microscopy'.
- Set your P200 micropipettor to $200\mu\text{l}$ and the P20 micropipettor to $20\mu\text{l}$.
- When directed by your instructor, mix the *Tetrahymena* sp. Culture and place $200\mu\text{l}$ *Tetrahymena* sp. Culture in each of the two tubes (**Mix before you add to each tube**).
- Mix the microfuge tube containing the beads (**Mix well!!**). When indicated by the instructor, add $20\mu\text{l}$ of the beads to each tube and mix the tubes quickly.
- Set the P200 micropipettor to $50\mu\text{l}$.
- After 5 minutes, add $50\mu\text{l}$ formalin to **both of your tubes** and mix.
- Make your experimental observations:
 - Prepare a wet mount from tube marked 'Microscopy' and observe your slide under fluorescence microscope. Count the number and size of beads inside 50 *Tetrahymena* sp. And record your results.
 - Collect 10,000 events on the flow cytometer in a file identified by your group letter and date. These files will be transferred to the computer lab for your analysis.

FIG. 1. Example of Worksheet 3 experimental question, design, and protocol.

where they had taken their prerequisite courses (cellular biology and organic chemistry), previous experience with flow cytometry, instructor competence, facilities and materials, and learning experiences. In all, 230 students responded to the questionnaire.

RESULTS

Student activities from Worksheet 1 on basic concepts in cell characterization. Essential terms and concepts were presented in about 45 minutes. These included relative size, relative complexity, fluorescence, autofluorescence, fluorescence microscopy, flow cytometry, FSC, SSC, data files, and dot plots. A theoretical experiment was conducted addressing the question "Does *Tetrahymena* sp. ingest yeast?" Students observed a live wet mount preparation of *Tetrahymena* sp. phagocytizing fluorescent-labeled yeast (Fig. 2A and B) and were asked to predict characteristics of the resulting cell populations and draw the positions of the hypothetical cell populations on the graphs in dot plot format (Fig. 2C).

Student activities from Worksheet 2 on basic concepts in flow cytometry. A theoretical experiment was conducted and analysis was performed on archived data files from a previously run experiment addressing the question of how long it takes *Tetrahymena* sp. to reach its maximum yeast

uptake. Students were asked how the question could be addressed experimentally using a flow cytometer and then to predict how long it takes *Tetrahymena* sp. to ingest yeast. Students were to imagine mixing *Tetrahymena* sp. with fluorescein-labeled yeast for specified times. With help from the instructor, students chose a time point of interest (5 seconds to 20 minutes) and then analyzed the data files to determine the percent of *Tetrahymena* sp. that had ingested yeast for their time point.

This analysis was accomplished by gating the high FSC, high SSC events (red) on the left plot (FSC versus SSC) (Fig. 3A) and then painting the high FL1-FITC (green) events on the right plot (FL1-FITC yeast versus SSC) of Fig. 3B. The green number across from the "%=" (in this case 64.64) represents the percentage of *Tetrahymena* sp. that had phagocytized fluorescent yeast at 5 minutes. The results for all the time points were compiled and summarized in graphical form during a class discussion (Fig. 3C).

Student activities from Worksheet 3 on scientific investigation. Student groups worked to develop possible questions about phagocytosis in *Tetrahymena* sp. that could be addressed using flow cytometry, and each laboratory section chose one question to address in their section's experiment. Below are examples of questions investigated by several student laboratories:

- What is the optimum temperature range for ingestion of yeast by *Tetrahymena* sp.?
- Does the amount of glucose in the medium affect the ingestion of yeast by *Tetrahymena* sp.?
- Does the size of prey affect ingestion by *Tetrahymena* sp.?

An example of a student laboratory investigation of one of these questions is described here.

Example of Worksheet 3 experiment, protocol, acquisition, analysis and summary of experimental results exploring the question "Does the size of prey affect ingestion by *Tetrahymena* sp.?" With instructor guidance, students developed an appropriate protocol for this question (Fig. 1) and performed the experiment using beads of two different sizes each with a different fluorescence emission. Students observed a wet mount of their *Tetrahymena* sp.-bead mixture to assess visually the number and size of beads inside *Tetrahymena* sp. (Fig. 4A). They acquired their data files using the flow cytometer (under instructor supervision). The data files were transferred to a computer laboratory equipped with the Paint-A-GateTM software, and students analyzed their data individually by opening their data files in dot plot format and gating *Tetrahymena* sp. as in Worksheet 2 (Fig. 3A and B).

FIG. 2. Students observed a mixture of fluorescent-labeled *Saccharomyces cerevisiae* and *Tetrahymena* sp. under phase-contrast (A) and fluorescence (B) microscopy displayed on a 15-inch monitor via a video camera. Students identified the cells present and characterized them in terms of relative size (FSC), complexity (SSC), and fluorescence. Students were asked to predict the placement of each cell type (event) on dot plots of FSC versus SSC and fluorescence versus SSC. Example of a student plot where T = *Tetrahymena* sp. and Y = yeast, fluorescent-labeled *Saccharomyces cerevisiae* (C).

FIG. 3. Students determined the percentage of *Tetrahymena* sp. with ingested yeast over time. Students chose a sample time for analysis, opened an archived datafile for their time point in Paint-A-Gate^{PRO}, made two dot plots (FSC versus SSC and FL1-FITC yeast versus SSC), and painted and gated the *Tetrahymena* sp. population (red events) (A). Students then painted the high FL1 events, representing the *Tetrahymena* sp. cells with ingested yeast, on the right plot (SSC versus FL1-FITC yeast) green (B). Students compiled their results to determine the time it takes for *Tetrahymena* sp. to ingest fluorescent-labeled yeast (C).

FIG. 4. An example of student experimental results from investigating prey size preference (2- μ m red fluorescent beads and 6- μ m green fluorescent beads). *Tetrahymena* sp. were fed fluorescent beads (inset) (A). Dot plots (side scatter versus forward scatter and green versus red fluorescence) show the distribution of *Tetrahymena* sp. populations (B). A graphical summary of student data is shown using the means of five to six replicates of each condition (C).

Students then opened a dot plot (Fig. 4B) for two fluorescence parameters (FL1-Tetra and 6- μ m green versus FL2-Tetra and 2- μ m red) and identified the four possible populations of *Tetrahymena* sp. cells present.

The microscopic experimental results show that *Tetrahymena* sp. can ingest 2- μ m red fluorescent beads and 6- μ m green fluorescent beads (Fig. 4A). The flow cytometry experimental results identified four *Tetrahymena* sp. populations based on their ingestion of prey (Fig. 4B). The four *Tetrahymena* sp. populations are (i) those that have not ingested any beads (gray events, 50.15%), (ii) those that have ingested the 6- μ m green beads only (green events, 2.33%), (iii) those that have ingested the 2- μ m red beads only (red events, 43.06%), and (iv) those that have ingested both the 6- μ m green beads and the 2- μ m red beads (blue events, 4.46%). In this laboratory section the students elected to perform six experimental replicas. Figure 4C gives a graphical representation of the results submitted by a student indicating that almost 50% of the *Tetrahymena* sp. ingested beads and that they preferred the smaller beads. The results were discussed in class, and students were asked to submit a summary of the class results in graphical form with their conclusions from the experiment.

Student learning. Student perceptions of their learning experiences were assessed using questionnaires after the flow cytometry exercises. Students felt that these flow cytometry experiences had enhanced their ability to understand protozoology, analyze data, and appreciate computer-aided data analysis (Table 1). Responses indicated that students had learned about flow cytometry and microbiology, that instructional and instrument support for the activities enhanced their experience, and that they would like to see flow cytometry incorporated into other classes (Table 2).

DISCUSSION

Learning can be optimized when students with sufficient background determine the questions to be addressed, design and conduct an experiment, and analyze and communicate their findings (10, 11). Experiences are best when the results are meaningful and feedback on results is immediate. In many basic microbiology laboratories the laboratory skills and content knowledge of the students, time, and expense limit such opportunities. This is especially challenging in teaching protist microbiology where most published laboratory protocols are limited to microscopic observation. Our experiences in teaching basic microbiology laboratories using phagocytosis in *Tetrahymena* sp. as a model system and flow cytometry to acquire data, provide a teaching strategy

that transcends most of these limitations.

By following Worksheet 1, students observed a living culture of *Tetrahymena* sp. ingesting fluorescent yeast and used critical observation skills to characterize the three types of cells present (large, complex *Tetrahymena* sp. cells containing smaller green fluorescent yeast cells, empty *Tetrahymena* sp. cells, and extracellular green fluorescent yeast cells). Based on these observations, students learned to translate the cell characteristics into relative flow cytometry parameters (SSC, FSC, and fluorescence) and predict the placement of these cells (events) in dot plot format. Both the microscope and the flow cytometer provide essential and unique information about cells.

Using Worksheet 2, students determined the time course of *Tetrahymena* sp. ingesting fluorescent yeast using archived data files. In doing so, they learned about experimental design and the importance of controls, data manipulation through painting and gating cell populations, and data analysis (percent *Tetrahymena* sp. with ingested fluorescent yeast) using computer software (Paint-A-Gate^{PRO}).

In doing Worksheet 3, students designed and executed an experimental protocol, operated a flow cytometer to acquire their data, analyzed their data, and interpreted their results. The students provided the initiative in all aspects of the experiment with the instructor acting as a resource. This experience built on content about protists and yeasts learned from lecture and previous laboratory experiences with culture manipulation and microscopic observation.

Questions designed to measure the students' perception of these laboratory activities were included in the post-laboratory assessment (Table 2). Student perceptions of the flow cytometry laboratory experience clearly indicated that they felt it was a valuable educational experience and that the experience enhanced their ability to understand the subject area being studied, analyze data, and appreciate data analysis. Students agreed strongly that they had learned about flow cytometry and that they wanted to see flow cytometry used in other classes. For each of the 3 years included in the table, the over-all median for the ratings is 5.0 (data not shown). Thus, the evidence presented here demonstrates that students viewed their exposure to the flow cytometry curricula as a highly positive experience.

Flow cytometry laboratory exercises proved to be ideal for teaching about microbes using inquiry-based formats. The flow cytometer provided rapid acquisition of experimental data and was easy for students to operate. The Paint-A-Gate^{PRO} analysis software was intuitive. Extracting meaningful data from 10,000 cells for up to four parameters as

TABLE 1. Student assessment of the impact of their experiences with flow cytometry over a 3-year period

Question	Student assessment ^a							
	Year 1		Year 2		Year 3		All years	
Has your experience with flow cytometry enhanced your...	Yes	No ^b	Yes	No ^b	Yes	No ^b	Yes	No ^b
ability to understand biology?	43	6	50	2	118	11	211	19
ability to analyze data?	49	0	51	1	120	9	220	10
appreciation for computer-aided data analysis?	48	1	52	0	115	14	215	15

^a Frequency of given answer.

^b Answer either "No" or "Not applicable."

TABLE 2. Responses to questions included on a post-lab assessment to measure students' perceptions of their flow cytometry laboratory experiences

Questions	Responses ^a			
	Year 1 ^b	Year 2 ^b	Year 3 ^b	All years ^c
This class enhanced my general knowledge of flow cytometry.	4.59 ± 0.76	4.33 ± 0.81	4.33 ± 0.88	4.42
This class enhanced my ability to understand microbiology.	4.27 ± 0.78	4.35 ± 0.40	3.64 ± 1.20	4.09
The written material for this class was useful.	4.49 ± 0.68	4.46 ± 0.80	4.20 ± 0.87	4.38
The instructors were well prepared and knowledgeable.	4.69 ± 0.55	4.56 ± 0.83	4.57 ± 0.73	4.61
The facilities were adequate to support the class.	4.59 ± 0.73	4.44 ± 0.80	4.25 ± 0.89	4.43
I would like to see flow cytometry used in other classes that study cells.	4.73 ± 0.72	4.61 ± 0.60	4.29 ± 0.90	4.54
Overall rating	4.56 ± 0.70	4.46 ± 0.80	4.22 ± 0.96	4.41

^a Responses on a 5-point scale, strongly disagree (1) to strongly agree (5).

^b Mean and standard deviation of results.

^c Mean.

presented here was straightforward. Although a flow cytometer represents a substantial capital outlay, the cost per sample is relatively low, and its applicability to any laboratory that studies cells makes its cost justifiable in many cases. Furthermore, we have established a shared-use facility at SJSU. Student laboratories conducted at schools near SJSU can submit their fixed, experimental samples to us for data acquisition, and then the data files are returned to the off-site campus for analysis using their computer facilities. The one drawback is that students from nearby schools are not able to operate the cytometer unless the class comes to SJSU. With low-cost flow cytometers becoming increasingly available, it is likely that they will be a common feature of student laboratories in the near future.

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