Study of cell form and function through microscopy: macrophages in action for undergraduate students

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Microscopy at the undergraduate level can be pursued over a wide range of activities, from a relatively passive experience where students use microscopy to look at prepared slides to record their findings in the form of sketch diagrams, to inquiry-based labs, where students learn to appreciate many dimensions of observation and discovery through microscopy. The laboratory activity we describe here, stemmed from our research on mechanisms of fungal recognition and phagocytosis of a murine macrophage cell line. Phagocytosis of zymosan particles (cell wall derivatives of the yeast *Saccharomyces cerevisiae*) by macrophages can be easily quantified, using a compound microscope, by counting the number of cells ingesting zymosan as well as the number of particles ingested by each particular cell. Phagocytosis can be assessed after different pre-treatments of both particles and macrophages, involving students in qualitative and quantitative data analysis. Furthermore, this research-inspired microscopy laboratory activity exposes undergraduates to many aspects of research, ranging from technical and logistical skills to ethical aspects of scientific inquiry.

Keywords: light microscopy; macrophage form and function; phagocytosis; undergraduate biology

1. Introduction

For most undergraduate biology students, the light microscope is a tool employed to observe tissues and cells using prepared slides, or in the best of cases, using wet mounts of buccal or elodea cells. Inasmuch as these experiences may provide students with insights into cell structure as well as some technical skills for proper use of the microscope, they fail to illustrate the full potential of microscopy for the study of cell function. Based on our research work [1] we embarked on the development of an inquiry-based laboratory that would incorporate various activities that exemplify the potential for research into dynamic changes in cell structure and cell function. This laboratory integrates different fields of biology, including cell biology, immunology, and fungal biology, as well as other disciplines such as chemistry, mathematics and philosophy. By requiring close observation and visual interpretation at the microscopic level, students gain appreciation of new dimensions and organization in cell biology, making this a transformative experience. Finally, skills such as performing Köhler illumination, photomicroscopy and digital capturing of images, cell counting and cell staining, learned during this exercise, prepare students for future investigations.

In this laboratory, students use the murine macrophage cell line RAW 264.7, that can be maintained in culture over several generations; for most students this is their first exposure to *in vitro* cell culture. Students learn the importance of using aseptic techniques, appropriate growth media, as well as suitable incubation conditions when handling these cells. Macrophages are phagocytic cells, and are essential in the development of the innate immune response. Through these laboratory activities students gain appreciation of the importance of macrophage recognition of Pathogen Associated Molecular Patterns (PAMPs) found on the surfaces of microbes [2] by specific macrophage cell surface Pattern Recognition Receptors (PRRs) to promote phagocytosis. Exposure of cells to zymosan particles, *Saccharomyces cerevisiae* cell wall derivatives, rich in the carbohydrate PAMPs beta-glucan and mannan, illustrate this interaction. Further steps involved in phagocytosis, including dynamic changes in the cytoskeleton of cells, are demonstrated by comparing the response of macrophages treated with colchicine, an inhibitor of actin polymerization [3], with non-treated cells. Quantification of phagocytosis can be easily performed by counting the number of cells that have ingested particles.

2. In vitro phagocytosis Laboratory

2.1 Cell cultures and preparation of unopsonized and serum-opsonized zymosan.

RAW 264.7 macrophages, obtained from American Tissue Type Culture Collection, are maintained in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES, and 10 mM L-glutamine -all from Life Technologies, Burlington, ON (D^{10}), and passaged every three days when more than 90 % confluent. In preparation for phagocytosis assays, cells are harvested and centrifuged at room temperature for 7 min at 1100 rpm, and resuspended in RPMI 1640 medium (Life Technologies, Burlington, ON), supplemented in the same way as D^{10} to make R^{10}. Prior to the student lab activity, sterile 4 well chambered Millicell EZ glass slides (Millipore Corporation, Bedford, MA) are seeded with 3 x 10^5 RAW

264.7 cells in each well, in 300 µl of R^{10} medium and macrophages are allowed to adhere in a humidified 5% CO_2 incubator for 30 min at 37° C. Prior to seeding in the well, cells are counted using the trypan blue dye exclusion viability assay and the cells are used if viability is greater than 90%.

A couple of hours before beginning the laboratory, zymosan from *Saccharomyces cerevisiae* (Sigma St. Louis, MO) is resuspended at 1mg/ml, which contains 3 x 10^7 particles/ml, in R^{10}. Aliquots of 120 µl unopsonized zymosan (at a concentration of 3 x 10^7 particles /ml) are prepared for distribution to students. Complement opsonization of zymosan is achieved by incubating 3 x 10^8 zymosan particles suspended in R^{10} with an equal volume of calf serum at 37°C for 30 min with frequent shaking. The opsonized zymosan particles are then washed four times in R^{10} and aliquots of 320 µl (at a concentration of 3 x 10^7 particles /ml) are prepared and distributed to students for use in the phagocytosis assays.

2.2 Processes for the treatment, staining, and microscopic examination of macrophages

Each student will be investigating the effect of opsonization of zymosan with normal fetal bovine serum on phagocytosis, as well as the importance of the cytoskeleton for phagocytosis. Before initiating the experiments, they must clearly identify the different treatments cells will be subjected to, and draw a diagram representing each numbered chamber and the respective treatments (Table 1.). During this time, instructors can discuss the set-up, what each experiment will test, as well as the importance of running controls for future data interpretation.

Table 1 Example of the table with the different treatments and controls students prepare before conducting their experiments.

1	2	3	4
Zymosan	Opsonized zymosan	Opsonized zymosan 10 µM colchicine	Cells in R^{10}

Wells 1 and 2 will be used to examine phagocytosis of zymosan and serum opsonized zymosan, respectively. The role of the cytoskeleton in the phagocytic process is tested by determining the effect of colchicine on zymosan ingestion. Throughout the laboratory, students are asked to prepare working solutions from stock solutions, encouraging them to practice quantitative skills learned in general chemistry laboratories. From a colchicine stock at 100 µM, students prepare a solution of 40 µM, used to treat cells in well 3. They then add 100 µl of R^{10} to wells 1, 2 and 4 and 100 µl of colchicine at 40 µM to well 3. Since the colchicine will be diluted four-fold after addition to the well, the final well concentrations of this agent will be 10 µM. By the end of this step, all wells should have the same total volume of 400 µl; students can visually determine whether all wells are filled to the same degree. Slides are incubated at 37° C for 15 min, as part of the cell pre-treatment process.

Serum-opsonized zymosan as well as unopsonized zymosan particles are then added to the cells. Students are supplied with zymosan suspensions of 3 x 10^7 particles/ ml, and must calculate the volume required in order to add 3 x 10^6 particles to each well to achieve a zymosan to macrophage ratio of 10:1. After vortexing the zymosan suspensions, the appropriate volume (100 µl) of opsonized zymosan is added to wells 2 and 3, and unopsonized zymosan to well 1, and the slides are incubated at 37° C for 50 minutes. Students are asked to also place a falcon tube with 10 ml R^{10} they have been supplied with, in the 37° C incubator; this will ensure the medium is at the appropriate temperature for the following washes and thus prevent the cells from dislodging from the slides.

Following incubation, the supernatant in each well is removed using a plastic Pasteur pipette, and the cells are washed three times to remove any zymosan particles that were not internalized, by gently adding 500 µl of the warm RPMI. After the final wash, the Millicell slide chamber separator is removed, and the slides are allowed to dry for 5-10 minutes. Macrophages are stained with Wright's stain (Sigma) for 5 minutes, transferred into 1:1 Wright's-water solution for 10 minutes, gently rinsed in tap water, then left for 1 minute in a staining jar with water, after which they are left to dry completely. Once dried, students fix a glass microscope coverslip on the slides using clear nail polish.

2.3 Microscopic observation, image capturing and quantification of phagocytosis.

Our laboratory has a common working area with four Nikon Eclipse 50i photomicroscopes (Fig 1), and every student working station is equipped with a Nikon Eclipse E200 microscope. In every exercise involving use of a microscope prior to this particular lab activity, students practice Köhler illumination; encouraging this practice early on effectively allows students to be confident when using a microscope as well as to obtain better results when assessing changes to cell morphology or phagocytic activity.

Each student begins by examining cells in well 4 to observe the appearance of stained macrophages, appreciate variability in cell form and note the presence of pseudopods. Phagocytosis is quantified, at 400X magnification in each of wells 1, 2 and 3. A total of one hundred cells, from three or four different fields of view, must be examined for each treatment, and the number of cells that have ingested zymosan recorded. Ingested zymosan appears as very distinct light oval particles with a pink center. Students must be careful observers, as in some instances, activated macrophages will present multiple vacuoles, which are round and do

not have a pink center, yet may be mistaken for zymosan. This represents an opportunity to discuss the importance of honesty and accuracy when collecting data, regardless of expectations of anticipated results based on knowing treatments applied to particular cells.

The laboratory classes consist of about 14 students and they rotate in order to use the photomicroscopes; while some students quantify phagocytosis, others capture images of cells from each one of the four wells. Before taking the photomicrographs, Köhler illumination is obtained for the 40X objective and white balance is set up; cells are captured at X600, using a Nikon Eclipse 50i photomicroscope (see Fig. 1). Students print pictures and present these together with the rest of their data. Figure 2 represents an example of macrophage photomicrographs from four different wells; differences in morphology, as well as percentage of cells phagocytising zymosan, are noted by comparing the cells in different culture conditions.

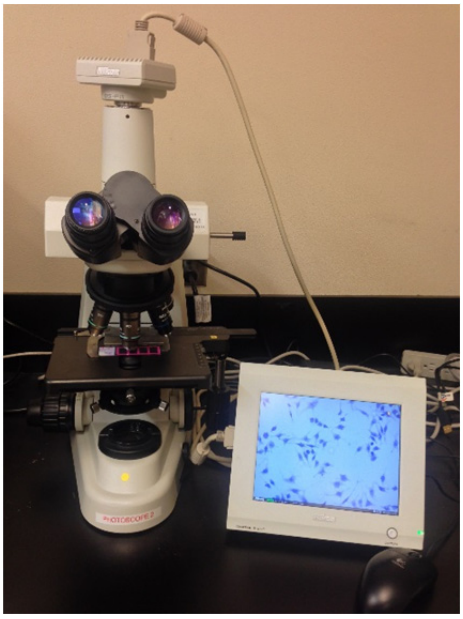

Fig. 1 Three Nikon Eclipse 50i photomicroscopes located in a common working area are available for image photographing cells from different treatments.

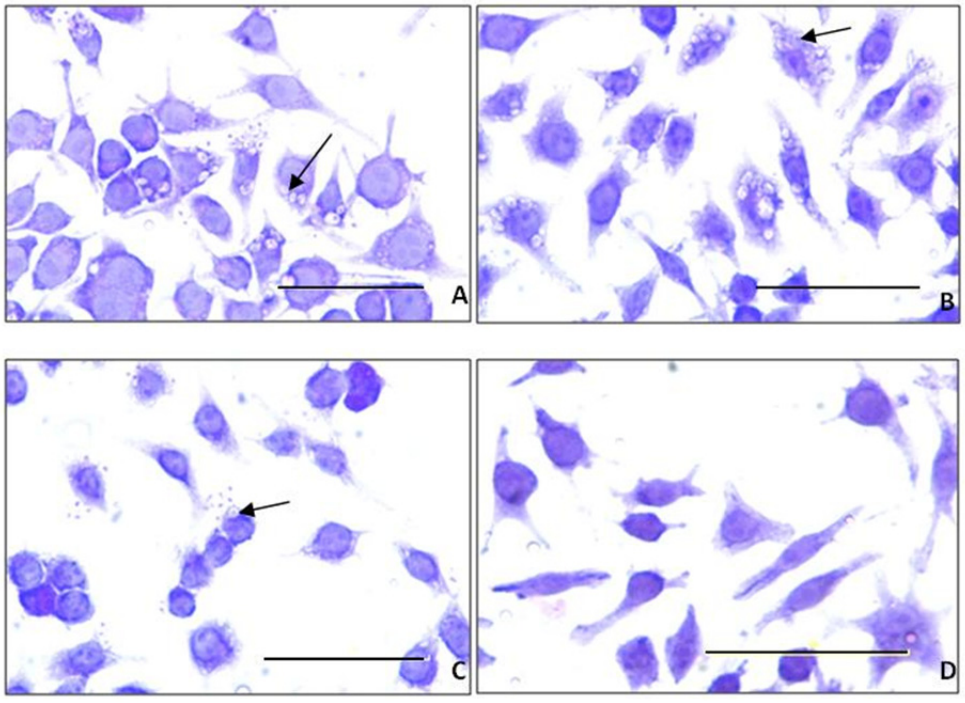

Fig. 2 Macrophages stained and photographed by students after treatments with A) zymosan alone, B) opsonized zymosan, C) opsonized zymosan and colchicine at 10µM and D) medium alone. The size bar in each photomicrograph represents 50µm. The arrows indicate internalized zymosan particles within macrophages.

Once students have quantified the number of phagocytic cells in a sample of one hundred cells for each of wells 1, 2 and 3, they are asked to present the data collected in the form of a histogram in order to demonstrate the effects of zymosan opsonization as well as colchicine treatment on macrophage phagocytosis. Zymosan, opsonized zymosan and colchicine treatment are placed on the x axis, and % phagocytosis is represented on the y axes (data not shown). In other laboratory classes students have examined the dose effects of colchicine on macrophage phagocytosis and found that 38% of cells ingested opsonized zymosan particles, colchicine at 1 μMol suppressed the percentage of phagocytic cells to 12% and this effect was more profound at 10 μMol with only 5% of cells ingesting zymosan. The data recovered for each treatment by all the students can be pooled and graphed and used to demonstrate calculations of standard deviations and statistical significance, illustrating the importance of doing multiple repetitions when trying to establish correlations and drawing conclusions from the data collected.

3. Discussion and conclusion

This laboratory exposes students to multiple features of microscopy, including its use as a tool for the investigation of changes in morphology and function through the examination of phagocytosis by macrophages. Working with RAW 264.7 cells provides a means to investigate many morphological and functional features of cell biology; this is especially advantageous for undergraduate colleges where no animal facilities are available for *in vivo* studies. Furthermore, by performing these *in vitro* studies, students become aware of the possibilities of investigating fundamental aspects of cell biology in innovative ways using a microscope, a basic tool for studies in cell biology.

The importance of early exposure of students to inquiry-based laboratories for development of qualitative and quantitative reasoning has been extensively investigated [4]. The phagocytosis lab we have outlined here requires that students understand the importance of an experimental design that includes the use of proper controls, when trying to address questions relating to the effects of different molecules on cell form and function. Students must observe the differences in cell morphology resulting from each treatment, underscoring the importance of proper staining and optimal illumination set up when using the microscope. The laboratory also emphasizes the significance of counting under different fields of view when quantifying phagocytosis, and stresses the importance of honest and accurate recording of data. The possibility of pooling data gathered by different groups of students allows for statistical analysis and comparison of the significance of small samples versus larger samples, as well as discussion on the importance of repeating experiments to ensure observations are consistent.

One important feature of the phagocytosis laboratory we have presented here is the possibility of introducing other variables which would allow students to further develop their qualitative and quantitative reasoning as well as their microscopy skills. Although students do not determine avidity in this particular exercise, this measure could easily be introduced into the lab, where students would count not only the number of cells phagocytizing zymosan, but also the number of particles phagocytized by each cell, or cell avidity. Further possibilities include kinetic studies to record phagocytosis over time, as well as determination the effect of other immunomodulators such as lipopolysaccharide (LPS) and dexamethasone on macrophage phagocytic activity.

The great majority of biology courses include laboratories requiring the use of the microscope yet, in our experience, few of these activities illustrate the processes involved in preparation of slides for their visualization, the importance of proper illumination for the study of qualitative and quantitative features of cells, or the possibilities microscopy offers for the study of changes in cell form and function. By performing the phagocytosis laboratory we present here, students learn to appreciate these aspects, as well as realize the possibility of using the microscope as a tool for discovery.

Acknowledgements We gratefully acknowledge the consent of students H. Tom, J. Kenny and S. Gill for the use of data generated during Cell Biology labs at Douglas College. Our research work has been supported by the Douglas College Research and Scholarly Activity Fund.

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