subsequent Tukey's HSD to determine significant differences for the data they have collected during this lab.

Conclusion

The above protocol offers instructors a dynamic teaching exercise that can be altered to accommodate students at a variety of levels, from an introductory genetics course, to an advanced behavioral or developmental genetics course. This teaching protocol gives students an opportunity to gain experience working with *Drosophila*, a model species widely used in the study of behavior, development, and genetics. The protocol provides students with hands-on experience in the acquisition of scientific data, from the basic level of sample collection and preparation, to the more advanced data analysis and presentation. The protocol also provides the opportunity for direct experience assaying for mutant phenotypes and in more involved versions the possibility of incorporating actual mutagenesis experiments with this behavioral assay.

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Drosophila adult eye model to teach Scanning Electron Microscopy in an undergraduate cell biology laboratory.

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Abstract

We have devised an undergraduate laboratory exercise to study tissue morphology using fruit fly, *Drosophila melanogaster*, as the model organism. *Drosophila* can be reared in a cost effective manner in a short period of time. This experiment was a part of the undergraduate curriculum of the cell biology laboratory course aimed to demonstrate the use of **S**canning **E**lectron **M**icroscopy (**SEM**) technique to study the morphology of adult eye of *Drosophila*. The adult eye of *Drosophila* is a compound eye, which comprises of 800 unit eyes, and serves as an excellent model for SEM studies. We used flies that were mutant for *Lobe* (*L*), *eyeless* (*ey*), and *pannier* (*pnr*) for our studies. The mutant flies exhibit different morphologies of the adult eye. We employed a modified protocol, which reduces sample preparation steps and makes it practically feasible to complete the protocol in

assigned time for the cell biology laboratory. The idea of this laboratory exercise is to: (a) familiarize students with the underlying principles of scanning electron microscopy and its application to diverse areas of research, (b) to enable students to sharpen their observation and quantitative microscopy skills, and (c) minimize the preparation time for the instructor.

Keywords: *Drosophila melanogaster*, eye, tissue morphology, Scanning Electron Microscopy (SEM), cell biology, undergraduate education.

Introduction

Research is an important component of habits of inquiry and learning in the undergraduate curriculum. A large array of laboratory courses has been developed for undergraduate students in order to expose them to techniques used in biomedical research. Interestingly, many new text books and accompanying supplementary materials provide exhaustive and detailed information through images and movies on diverse subject material studied using the **S**canning **E**lectron **M**icroscopy (**SEM**) technique. Although animations and videos can provide an overall idea, it is important that students get a "hands-on" exposure to learn the techniques like how to use SEM to capture high resolution images. We devised a laboratory to introduce students to the SEM technique, its principle and applications, which will allow them to get a hands-on experience on the scanning electron microscope. Furthermore, this exercise can be finished in a single laboratory session with some preparation done prior to the demonstration to the students.

For this laboratory exercise, we chose to study the morphology of the well studied adult eye model of *Drosophila*. This model is highly versatile as in addition to studying the pattern and morphology of the normal flies, the variations in eye development can be easily demonstrated. There are several molecularly characterized mutants that directly or indirectly affect the morphology of the adult eye. Using the normal and mutant flies, we can demonstrate the limitations of conventional light microscopy in terms of resolution and magnification. This may help the students to appreciate (i) SEM has a much greater resolving power than light microscopes, (ii) SEM uses electromagnetic radiation instead of light, and (iii) SEM can obtain much higher magnifications of up to a million times.

SEM produces images by probing the specimen with a focused electron beam that is scanned across a rectangular area of the specimen (raster scanning). The electron beam of SEM is generated from a filament, which may be made up of various types of materials. The most common filament is made up of a loop of tungsten which functions as the cathode. A beam of electrons is produced at the top of the microscope by heating this metallic filament. The electron beam follows a vertical path through the column of the microscope and makes its way through electromagnetic lenses that focus and direct the beam on the sample. Electrons in the beam interact with the atoms constituting the sample material and are scattered back, producing the back scattered electrons or the secondary electrons. A detector collects the secondary or backscattered electrons, and converts them to a signal that is sent to a viewing screen similar to the one in an ordinary television, producing an image (Figure1). These signals contain detailed information about the sample's surface topography, composition and other properties such as electrical conductivity.

Vacuum is an essential requisite for SEM. If the sample is in a gas filled environment, the beam is unstable as gases could react with the electron source, causing it to burn out or result in ionization of beam. Alternatively, other molecules, which come from the sample or the microscope itself, may form compounds and condense on the sample and thereby reduce contrast and obscure details in the image.

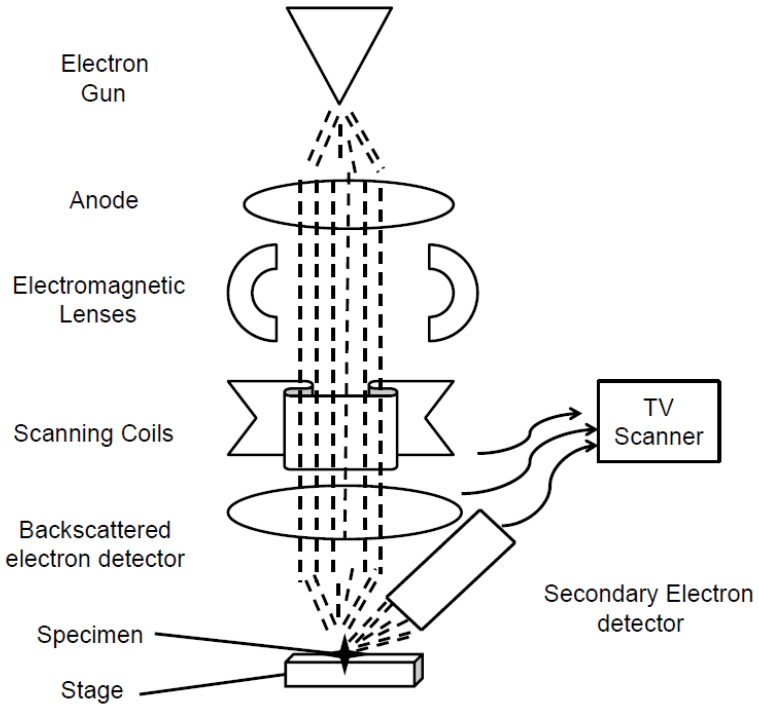

Figure 1. Schematic flow diagram of a Scanning Electron Microscope. Electron microscopes use a particle beam of electrons to illuminate a specimen and create a highly-magnified image. The electrons from electron gun pass through anode, electromagnetic lenses, coils, detectors and strike the gold coated specimen placed on the stage. The electromagnetic lenses focus electron beam to a specific plane relative to the specimen and thereby forming the image. The secondary electrons generated because of electrons striking specimen surface are detected by secondary electron detector and converted into signal that is sent to a TV scanner.

Protocol

We have employed *Drosophila melanogaster* to study the morphology of the adult eye. *Drosophila* eye is a compound eye made up of 750-800 unit eyes referred to as ommatidia. We selected the flies that were mutant for genes involved in eye development (obtained from the Bloomington Stock Center, Indiana; http://flystocks.bio.indiana.edu/). The Bloomington Stock center is a repository of various fly strains and mutations, which are available upon request to the scientific community. We selected fly mutant strains for genes *eyeless* (*ey*) [ey^2 (BL 648), which shows complete loss of the eye field], *Lobe* (*L*) [L_2/CyO (BL 319), a mutant which shows selective loss of the ventral eye], *pannier* (*pnr*) [$pnr^{vx6}/TM6B$, (BL 6334)], which is an embryonic lethal mutation that can generate dorsal eye enlargement in genetic mosaics where *pnr* function is eliminated in patches of cells (Xue and Rubin, 1993; for review see, Blair, 2003). These three different mutants exhibit a range of phenotypes of eye size from enlarged eye to half eye, and to no-eye in comparison to the wild-type eye (Figure 2). However, each of these mutants show a range of phenotypes due to penetrance. Therefore, for our lab exercise, we selected flies that showed distinct eye phenotypes from a large population of each mutant stock.

This exercise helped students to learn two basic experimental operations: (a) sample preparation, and (b) basic operation of the scanning electron microscope. Students also learned some background information on the development and morphology of the normal eye.

The entire methodology of the SEM can be divided into three major steps: (1) sample preparation, (2) sample mounting and sputter coating, and (3) imaging.

1. Sample Preparation:

This step includes preparation of sample and is carried out prior to the research laboratory. For SEM, biological samples need to be dehydrated and dried. Dehydration is carried out to

gradually reduce the water content of the tissue to the point that the tissue is completely into a non-aqueous solvent. Dehydration is done using ascending concentration series of ethanol or acetone. The flies of different eye mutants were passed through a series of ascending concentrations of acetone to dehydrate the sample. The adult fly samples were dehydrated by incubating for 24 hours each in 30%, 50%, 70%, 90%, 95%, and 100% concentrations of acetone. Thus at the end of the seventh day, the sample is completely dehydrated and is present in 100% acetone. To achieve best results, sample was dehydrated in 100% acetone twice.

In earlier protocols, dehydrated samples were subjected to critical point drying. The presence of surface tension during drying is disruptive to tissues and causes visible distortions. Therefore, the critical point drying is carried out in vacuum where fluid and gaseous phases exist together and there is no surface tension. The critical point drying is achieved using liquid carbon dioxide (CO_2). However, drying can also be achieved using commercially available chemicals. Here, we employed **H**exa**m**ethyl **D**i **S**ilazane (**HMDS**), which is a chemical of choice used for drying SEM samples that mainly include insect tissues, large fleshy tissues or soft invertebrates (Braet *et al*., 1997). Furthermore, it does not require vacuum. Drying with HMDS prevents the tissue morphology from getting damaged in freeze drying or liquid CO_2 drying procedures. Following 100% acetone, samples were incubated overnight in 1:1 mixture of 100% acetone: HMDS (Electron Microscopy Sciences Cat# 16700). Samples were then incubated in 100% HMDS solution for 24 hours and they were allowed to air dry at room temperature in a fume hood. The lids of the tubes were left open to allow the HMDS to evaporate.

2. Sample Mounting and Sputter Coating:

Each HMDS treated dehydrated sample was mounted on a metallic stub (a sample holder for electron microscope, available from Electron Microscopy Sciences Cat# 75944). Sample was held onto the stub by a conductive carbon tape (Electron Microscopy Sciences Cat# 77825-12). Maximum contact of the sample with the tape was ensured so that sputter coating is good. Sample was arranged on the stub in such a way that the area of interest (in this exercise the eye tissue) in the sample is perpendicular to the plane of the observation in the microscope. Mounted tissue on the stub was then sputter coated in vacuum with an electrically conductive layer of gold (or some other inert heavy metal). This step is important since it makes the sample conductive, enhances the secondary and backscattered electron emission and increases the mechanical stability of the tissues. Coating is an essential step to prevent accumulation of static electric charge on the specimen during electromagnetic irradiation. Improper coating on the tissue results in charging, which may result in deflection of electron beam, deflection of secondary electrons and periodic burst of secondary electrons. Gold is the preferred metal for coating the samples because of its high atomic number. Further, sputter coating with gold produces high topographic contrast and resolution. Depending on the type of sample, there are several other coating materials like Gold Palladium alloy, Platinum, Iridium, Tungsten, Osmium, Graphite and Carbon. The sample stub was subjected to sputter coating at pressure of 100 psi for a period of 35 seconds and a current of 45 milli amps under vacuum using sputter coater (DV 502) from Denton Vacuum Company.

We have described a protocol for sample preparation for SEM. However, there have been continuous improvements in the processes of sample fixing, drying and coating methods. A variety of new adaptations to SEM have also emerged that enables a large spectrum of samples to be analyzed using SEM technique. There are alternative methods for fixation, dehydration and coating depending on the nature of sample and approach used summarized in Table 1.

Table 1. Alternative materials used in SEM for fixation, dehydration, drying and sputter coating in different model systems.

Sample Type	References	Fixative	Dehydrating agent	Drying process	Mounting/ Sputter coating
Bacteria, virus on surfaces, as parasites	Sangetha et al., 2009; Robinson et al., 1984	Glutaraldehyde, Osmium tetra Oxide (OsO_4)	Ethanol	Critical point drying in amyl acetate	Gold, Gold-Palladium alloy
Plant tissues	Pathan et al., 2008	Glutaraldehyde, Osmium tetra Oxide (OsO_4)	Ethanol/ Acetone	Critical point drying	Mounting using Aq. Silver, Coating with chromium
Insect tissues	Braet et al., 1977, (modified by Naoto Ito)	Fixing is usually not required	Acetone	HMDS (Chemical drying)	Gold
Mammalian tissue	Wierzchos et al., 2008; Lehman et al., 1983	Aldehyde/ Formalin/ Osmium tetra Oxide (OsO_4)	Ethanol	Critical point drying in acetone	Gold, Gold-palladium alloy, Carbon coating

3. Imaging:

The final step in this exercise is to image the samples using SEM. Sputter coated sample stub was then imaged using the Hitachi S-4800 High Resolution Scanning Electron Microscope (HRSEM) available in the Nanoscale Engineering Science and Technology (NEST) facility at University of Dayton. The samples on the stub were placed in vacuum and subjected to electron beam. A voltage of 5kV was applied. The electrons from the gun strike the surface coating of gold, electrons are reflected back off the specimen to a detector, this is transmitted to a TV screen where the image is viewed and photographed. The images were taken at 130×. At magnification of 130×, the entire *Drosophila* head fits in an image plane and is a suitable resolution to study morphology of head and the compund eye. As shown in the Figure 2, each unit eye or the ommatidium is clearly visible, and this would not have been possible using a compound light microscope of 10× magnification. The high resolution SEM images provides detailed information about different kinds of bristles present among the ommatidia. Depending on the model, SEM allows the magnification of a sample up to 500,000- 1000000 times.

Advantages

1. The greatest challenge to teaching a Cell Biology laboratory is the capital investment/ commitment that a university/college must make to laboratory. The use of cost effective exercises can facilitate the execution and implementation of these laboratory programs in an undergraduate academic institution setup.
2. The students get general overview of SEM and hands-on experience of the technique starting from sample preparation to visualizing the sample on the monitor attached to SEM.
3. The sample preparation in conventional method for SEM is a little time consuming procedure. It requires critical point drying in vacuum. In our protocol, we eliminated the critical point drying method which requires vacuum.

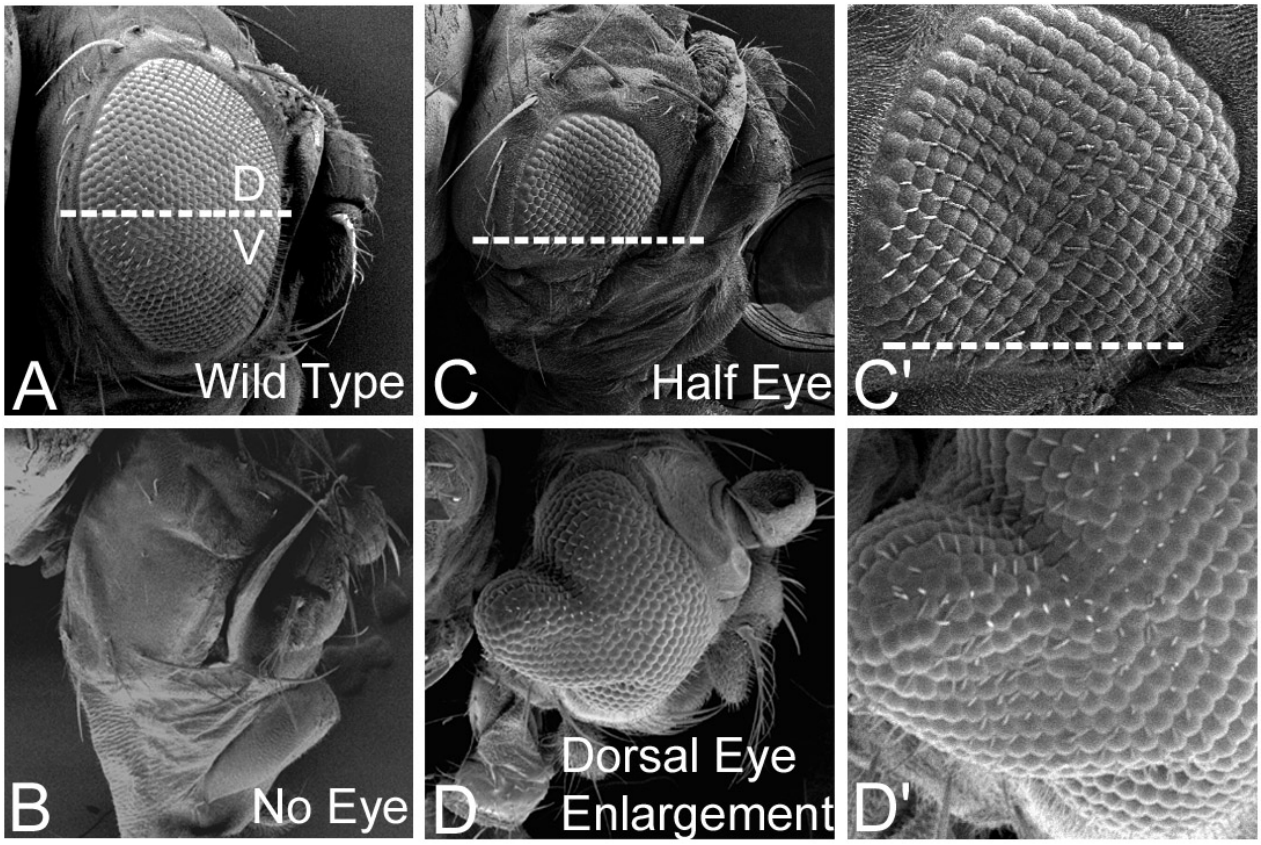

Figure 2. Scanning Electron Micrographs showing morphology of *Drosophila* eye mutants. (A) Wild type eye of *Drosophila* showing 600-800 unit eyes. The dotted line marks the equator where dorsal (D) half of the eye is above the equator and ventral (V) half is below the equator. (B) *eyeless* mutant (ey^2), showing complete loss of eye field. (C) *Lobe* mutant (L_2) shows selective loss of the ventral half of the eye. (C') Magnified view of the L_2 mutant eye. (D) Ectopic dorsal eye generated when *pnr*, a GATA 1 transcription factor, function is eliminated in patches of cells in genetic mosaics (Xu and Rubin, 1993; for review see, Blair, 2003). (D') Magnified view of the dorsal eye enlargement generated due to genetic mosaics of *pnr*.

4. Our protocol does not require post fixation treatment with Osmium tetra Oxide (OsO_4), which is highly carcinogenic and may not be an ideal chemical to use in an undergraduate laboratory. Instead, we use HMDS for final processing after dehydration in acetone series.

5. These exercises does not require educational demonstration kits that minimize the exposure of experimental details and reagents to the students.

6. This experience adds to their skill-set and helps generate a core of trained individuals who can function in academics as well as corporate settings.

High magnification images are powerful sources of communication which are preferred to words. Most laboratory science courses do not actively teach students skills to communicate effectively through images (Riemeier and Gropengießer, 2007). Our laboratory exercise meets this requirement by teaching students to (a) develop basic laboratory skills and learn tissue handling,

sample preparation and scanning electron microscopy, (b) capture digital images using the software, (c) process the image using the Photoshop or imaging software, and (d) develop a series of image portfolios to present their results.

Acknowledgments: Authors are thankful to Dr. Madhuri Kango-Singh and Shilpi Verghese for comments on the manuscript. This laboratory exercise was designed in the Department of Biology, at the University of Dayton. MT, ORP are supported by graduate program of the University of Dayton. SMO is a student in the Premed program of the University of Dayton. AS would like to acknowledge the support of Learning Teaching Center Grant at the University of Dayton, for the development of this laboratory exercise. AS is supported by grants from Ohio Cancer Research Associates and University of Dayton Research Council.

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Heat shock effects upon cell death in *Bar* eye quantified by scanning electron microscopy.

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Bar (*B*) is a well-known sex-linked dominant mutation that arose spontaneously in *Drosophila melanogaster* as a tandem duplication in cytological location 16A1-2 (Tice, 1914; Lindsley and Zimm, 1992). The vertical bar-eye phenotype is due to cell death, especially in the anterior region of the eye disc (Fristrom, 1969), or disruptions in the pattern of mitosis. But the extent of cell death can be influenced genetically (*e.g.*, variegated position effect; Brosseau, 1960) and by environmental conditions like temperature (*e.g.*, developmental temperature and log facet number are inversely proportional; Hersh, 1930) and chemical treatments (*e.g.*, being raised on media supplemented with acetamide, lactamide, cytosine, and other chemicals; Fristrom, 1972; and references in Lindsley and Zimm, 1992). Given its sensitivity to modifying factors, the severity of *Bar* eye cell death can be a model for quantifying experimental influences on development. But for this model system to be sensitive enough to detect comparatively small effects, eye facet (ommatidium) number must be measured very accurately. In spring 2009, the Experimental Genetics and Cell Biology Lab course taught in the Department of Zoology at the University of Oklahoma undertook to test experimental design options and the feasibility of using scanning electron microscopy of *Drosophila Bar* eyes to evaluate the effect on cell death by an experimental treatment, exposure to heat shock that activates chaperone proteins of the stress response. Additional data were