What can the fruit fly tell us about the human eye?

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Overview
This laboratory exercise provides a hands-on way for students to explore the categories of experiments developed by Adams (2003) - “Show it, block it, and move it.” Students observe fruit fly pupae that are ectopically over-expressing the eyeless gene in the imaginal disc tissues using the GAL4/UAS system. The pupae contain ectopic eyes in the legs, wings, and antennae regions of the body. This observation demonstrates that the eyeless gene is sufficient to produce eye tissue. Students then read a primary research article (Halder 1995) in which this effect was reported and explore the idea that eyeless is both necessary and sufficient to induce eye formation. Emphasis is also placed on the proper development of an experimental hypothesis and prediction as well as how to communicate the importance of basic research using model organisms to a broad audience.

Genetics Concepts Addressed
Nature of Genetic Material
Genetics of Model Organisms
Methods and Tools in Genetics

Core Competencies Addressed
Students should be able to locate, read, and comprehend primary literature research papers on genetics topics. Students should be able to generate testable hypotheses. Students should be able to gather and evaluate experimental evidence, including qualitative and quantitative data. Students should be able to effectively explain genetics concepts to different audiences.

Audience: Undergraduate (upper level biology majors, but it can be adapted for use with non-majors)
Activity Type: Laboratory exercise
Activity Length: ~2.5-3 hours, but could be shortened
Resource Justification and Introduction:

One of the important goals in science education is to teach the scientific method, a topic that many students struggle to understand and appropriately apply. A hypothesis is a statement that provides an explanation for an observation; a hypothesis must be testable, falsifiable, and based on prior knowledge. In contrast, a prediction is an “if, then” statement that provides an expected outcome from an experiment (Strode 2015; Hutto 2012; McPherson 2001). In the example from the introduction to the lab packet, the hypothesis is that the caffeine in coffee helps you to feel awake in the morning. A number of predictions can be developed based on that hypothesis that can be explored through experimentation.

One useful resource for teaching students how to develop strong experiments is an article by Dany Adams, then at Smith College, which introduces the idea of utilizing “bins” to teach critical thinking and experimental design in developmental biology (Adams 2003). In this article, Adams outlines the approach that she uses to teach her students how to understand and evaluate experiments in the context of the scientific method. She utilizes a reading from Gilbert’s Developmental Biology textbook (“Evidence and Antibodies”), which walks through the use of this approach. To summarize, Adams assigns experiments to one of three categories: Show It, Block It, or Move It. Show it experiments provide correlative data, and include experimental approaches such as immunofluorescence, in situ hybridization, or the use of reporter assays. Block It experiments are used to demonstrate that a given molecule or protein is necessary. Experimental approaches include loss-of-function mutations, RNAi-based depletion, chemical inhibition, etc. Finally, Move It experiments use gain-of-function to establish that a given molecule is sufficient. These experiments could involve over-expression/ectopic expression (as is used in this activity), tissue transplantation, or the addition of beads soaked in a chemical or molecule.

This approach to teaching experimental design has significantly changed the way that I have taught multiple courses, from a writing-intensive senior capstone course to 400-level lab and lecture based courses (Advanced Cell Biology and Animal Development), and even a cell biology and genetics course for non-majors. Utilizing this framework to discuss the types of data that can be collected from an experiment helps students to categorize the data that they collect and the results that they read about in the literature, as well as to evaluate the strength of that data and of the conclusions that can be drawn.

Adams (2003) found that the students achieved a number of learning goals: “(1) They understand the experimental foundation of information, (2) They have an intellectual technique to use when asked to think, (3) They understand how to
interpret the results of experiments performed with either classical or modern techniques, (4) They can read the primary literature and comprehend it much more, more quickly, (5) They can judge the validity of conclusions, (6) Every student seems to understand, even those who are not at the top of the class, (7) As their confidence grows, they become more active participants in class, (8) They are aware that they are thinking well, and most find that very exciting. That awareness also enhances their self-esteem and builds their confidence. “The first time I utilized this framework was in a speaking and writing-intensive senior capstone course. Students spent the semester reading and presenting primary literature articles and writing a review paper on a topic in developmental biology. Introducing the “Show it, Block it, Move it” terminology facilitated our discussion of the primary literature. Even if students did not understand the details of an experimental technique, they could at least understand the purpose of the experiment and categorize the type of data that was collected. In this course, which did not have a laboratory component, I observed many of the same learning outcomes that Adams (2003) reported. I saw significant growth in the students over the course of the semester, often identifying the types of experiments in their presentations or in our discussions without prompting. When asked about the use of this terminology, the response was overwhelmingly positive. Some of the comments I received from students in this course (Fall 2014) were:

“I liked the show it, block it, and move it. I feel like it really helped to see how and why researchers used the methods in their experiments.”

“I really liked the show it/block it/move it because it helped break down the different experiments into simpler terms. It helped me to see what experiments were more strongly supporting what the authors were experimenting with.”

“I think the show it, move it, block it was extremely helpful. Actually, I wish that it had been implicated in the intro courses. It is a very helpful way to categorize and better understand different types of experiments.”

I found this framework to be so powerful that I wanted to adopt it in other courses, as one student suggested. I wanted to develop a hands-on laboratory activity that could be used to illustrate and train students in this classification method. The Darling Marine Center at the University of Maine hosts a Developmental Biology Teaching Workshop, which I attended in the summer of 2014. There, I was introduced to a laboratory activity in which students dissected fly pupae that were expressing the eyeless gene in the 15 imaginal disc tissues. As a Drosophila researcher, I thought that this could provide an excellent laboratory activity to teach students the concept of “necessary and sufficient” in the context of an Animal Development course. Although
the activity was piloted in an Animal Development course, I have also used it in a non-major’s course as a demonstration, and I plan to use it in an Advanced Cell Biology course as well.

This experiment relies on the use of the GAL4/UAS system in the fruit fly. The GAL4/UAS system is a binary system that was adopted from the budding yeast, Saccharomyces cerevisiae. In yeast, the transcription factor, GAL4, binds to a series of upstream activating sequences (UAS) to regulate the transcription of genes required for galactose metabolism. Because the machinery that regulates gene transcription is evolutionarily conserved, the GAL4/UAS system can and has been utilized in other organisms such as the fruit fly. The GAL4/UAS system allows researchers to express any DNA sequence (coding or non-coding) in a temporally controlled or tissue specific manner. The system involves crossing or mating two fly lines to each other, each containing one half of the system. One line is the “driver” line in which the GAL4 transcription factor is expressed in a specific tissue or pattern based on the endogenous gene regulatory sequences that are near the inserted GAL4 transgene (Elliot 2008). The responder line contains a UAS-transgene that can be used to express either protein coding or non-coding (ex. dsRNA for RNA interference-based knockdown) RNA. For further information on the use of and modifications to the GAL4/UAS system, see (Elliot 2008).

In this activity, students dissect pupae that are expressing UAS-eyeless (UAS-ey) cDNA under the control of the decapentaplegic-GAL4 (dpp-GAL4) “driver.” This is accomplished by crossing UAS-eyeless flies to dpp-GAL4 flies. The offspring from the cross will therefore contain both elements of the GAL4/UAS system and should be expressing the eyeless cDNA in all tissues where Dpp protein is normally expressed. Dpp is a conserved member of the TGF-β/BMP family of signaling molecules, which act as morphogens during development. Dpp is normally expressed in the imaginal disc tissue, and mutations in dpp lead to defects in one or more of the 15 structures (legs, eyes, antennae, mouth parts, wings, genitalia, and the haltere balancing organs) that arise from the imaginal discs (Gelbart 1989).

**Eyeless is a conserved member of the Pax family of genes required for eye development**

Eyeless is a transcriptional regulator that is both necessary and sufficient to induce eye formation. Mutation of the eyeless gene leads to a complete loss of the eye; further, over-expression of eyeless cDNA can lead to the formation of ectopic eyes in tissues such as the leg, antennae, and wing (Ghering 1996). This result, which is the basis for the activity, demonstrates that eyeless is sufficient to induce the formation of eye tissue. Eyeless is a transcription factor whose structure has been conserved through evolution. Eyeless is part of the Pax family of genes. In fact, over-expression of the mouse Pax-6 gene can also induce...
ectopic eye formation in the fly (Halder 1995). Mutations in the mouse and human homolog lead to reduced or absent eyes (Gehring 1996), which demonstrates that despite the significant differences in the structure of the fly and vertebrate eyes, similar genes control their development. Therefore, basic research in the fruit fly can have a significant impact on our understanding of protein function in higher organisms.
Learning Objectives

- Explain what is meant by the phrase “necessary and sufficient” and what types of experiments could be used to demonstrate these roles
- Explain the benefits of using model organisms in research
- Explain the GAL4/UAS system and transgenes
- Design an experiment to test a hypothesis
- Identify the hypothesis and prediction from a primary research article
- Use a database to identify a relevant primary article
- Dissect fly pupae

Instructor Guidelines

1. Laboratory Preparation:

1.1 Materials Needed for Complete Exercise

- Stereomicroscopes (1 per group if students are performing the dissections or at least 2 if using as a demonstration)
- Fly vials and flugs (Carolina Biological Supply Co. Drosophila Culture Vial Set 3, 144 Vials, Plugs, Caps, Item # 173080, $150)
- Fly media (instant food can be purchased from Carolina Biological Supply Co. Formula 4-24® Instant Drosophila Medium, Plain, 1 L, Item # 173200, $8.50)
- Active dry yeast (a small package of yeast comes with the instant fly food from Carolina)
  - Yeast can be ground with a coffee grinder and kept in a salt shaker
- Forceps or Tweezers - 2 pairs per student group
  - I have used Knotting Tweezers #5 from JewelrySupply.com, $3.41/pair
  - Forceps can also be purchased from Fine Science Tools. I would choose the Dumont #5, but others could be substituted (http://www.finescience.com/Special-Pages/Products.aspx?ProductId=335)
- CO₂ anesthesia apparatus or FlyNap (Carolina Biological Supply Co. Item #173010, $13.75)
CO₂ anesthesia apparatus (FlyStuff Flypad) can be purchased from Genesee Scientific (Cat #59-114, $154.05), but this would also require a CO₂ tank, regulator, and tubing

- Paintbrush to move flies around when setting up crosses
- Fly Stocks:
  - dpp-GAL4 flies (Bloomington Drosophila Stock Center #1553)
    - Genotype: w[*]; wg[Sp-1]/CyO; P[w[mW.hs]=GAL4-dpp.blk1\{40C.6/TM6B, Tb[1]
  - UAS-eyeless flies (Bloomington Drosophila Stock Center #6294)
  - For “Control” flies, students could dissect the tubby pupae, which do not contain the dpp-GAL4 transgene, but which do have the UAS-eyeless transgene, or “wild type” flies could be ordered from the Bloomington Drosophila Stock Center (Ex. Canton-S #64349 or Oregon-R-C #5. Just make sure that the control flies have red eyes)
  - Note: Be sure to order these lines well in advance of your lab, as it can take a few weeks to receive the lines from the stock center and multiple weeks to expand the lines to the point that virgins can be collected
  - Additional information about fly maintenance and culturing can be found at http://flystocks.bio.indiana.edu/Fly_Work/culturing.htm
  - Another excellent resource for more information about the fruit fly as a model system is Roote and Prokop (2013)

1.2 Materials for each lab station
- 2 Pairs of forceps
- Stereomicroscope
- Vial of flies that contain the progeny from a cross set up between the dpp-GAL4 flies and the UAS-eyeless flies at the late pupal stage.
- Optional: Vial of control flies

1.3 Setting up crosses between dpp-GAL4 and UAS-eyeless flies
• 3-4 weeks prior to the lab, begin to expand your stocks. For active virgin collection, I like to keep ~5-6 vials of each stock going at a time, staggered by 1-3 days.

• **Note:** The *dpp-GAL4* flies are not the healthiest line, so be sure that you maintain multiple vials of these flies and keep a close eye on them to make sure that they do not die before you are ready to use them. Because the *UAS-eyeless* line is healthier, I usually collect virgin female flies from this line and cross them to males from the *dpp-GAL4* line.

• Crosses are typically maintained at 25°C, but could be done at room temperature if it is stable between ~18°C-28°C. At 25°C, the flies should reach the late pupal stage ~9-12 days after you set up the cross. However, if you are raising your flies at a cooler or warmer temperature, then the flies will develop slower or faster respectively.

• Perform test crosses to determine the best timing for your crosses. Even after performing the test crosses, start to set up crosses ~2 ½ weeks before your lab. Continue to collect virgins and set up crosses until ~1 week before the lab period.

• After setting up a cross, transfer the adults to new vials with fresh ground yeast every 2 days. This will stagger the crosses so that you should have them at the ideal stage for your lab period.

• Set up as many crosses as possible to insure that you have enough vials at the right developmental stage for the activity.

• Crosses must be set up between VIRGIN females of one line and males of the other line.

• Crosses should be set up with ~8-10 virgin females (or as many as you can collect within a 5-day window) and ~3-5 males in a fresh vial of fly food with some dry ground active yeast.

• **Note:** Put a pinch of yeast at the bottom of the vial with food before you put in any flies. Avoid covering the food with the yeast, as this will affect egg laying. Try to put the yeast in a little pile on one side of the vial.

• **Collecting Virgins – Traditional Method**
  
  o Maintain multiple vials of each of the lines
  
  o Stagger the vials by transferring adults to new vials every 2-3 days so that at a given time, virgins can be collected from multiple vials.

  o Look for virgin females multiple times a day (morning, mid-day, and afternoon)
    
    ▪ Anesthetize flies using either CO₂ or FlyNap and dump flies out onto either a Flypad or notecard.
• **Note:** No matter which method is used, always anesthetize flies while holding the vial upside down. Once larvae are present in the vial, the food will become sticky, and if flies are anesthetized with the vial upright, they will get stuck in the media.

• Use a paintbrush to sort flies based on sex. Use the Roote and Prokop (2013) resource for additional information about distinguishing between males and females as well as basic fly maintenance.

  o Virgins can be identified by the pale body color, dark spot (meconium) in their abdomen, and wings that are still folded. Images of virgins can be found: [https://valeriepeeters.wordpress.com/2014/02/18/a-male-fruit-fly-and-his-virgins/](https://valeriepeeters.wordpress.com/2014/02/18/a-male-fruit-fly-and-his-virgins/)

  o Collect virgin female flies in a separate vial with food (no yeast), labeling the vial with the date that the first virgins were added to the vial.

  o Within ~4-5 days of the initial virgin collection, set up the cross. You will need to anesthetize the flies before transferring them.

    ▪ In a new vial of food with a pinch of yeast in the bottom, add the collected virgins from one line (either UAS-ey or dpp-GAL4) and 3-5 males of the other line.

    ▪ Label the vial with the date that the cross was set up.

  o Incubate flies at 25°C (or room temperature if an incubator is not available).

  o Every 2-3 days, transfer the parental generation to a new vial of food with fresh yeast and label with the date of transfer.

• **Collecting Virgins using the P{hs-hid}Y System**

  o To facilitate large scale virgin collection, the UAS-eyeless stock has been combined with P{hs-hid}Y (Starz-Gaiano 2001; Venema 2006). Upon heat shock of larvae, the dominant lethal hs-hid transgene is expressed, killing all males. Therefore, only female flies will eclose and will remain virgins until they can be isolated.

  o To utilize the P{hs-hid}Y system, maintain multiple sets of the X/P{hs-hid}Y; UAS-ey/Cyo cultures, transferring adults to new vials every 1-2 days. After transferring adults from one vial to a new vial, culture the original vial for an additional 2 days. When young larvae are present in the culture, perform a heat shock at 37°C for 2 hours to induce expression of the hs-hid transgene. Allow the flies to recover at 25°C for 22 hours and repeat the heat shock for another 2 hours. Allow flies to recover at 25°C after the second heat shock.
- **Note:** It is important to remove all adults prior to the heat shock so that newly eclosed females do not mate with any remaining adult males in the culture.
  - Once flies begin to eclose, collect virgin female flies in a separate vial with food (no yeast), labeling the vial with the date that the first virgins were added to the vial.
  - Set up crosses between virgin female *UAS-ey* flies and *dpp-GAL4* male flies.
    - **Note:** A second chromosome balancer was introduced when *P{hs-hid}Y* was combined with *UAS-ey*. Therefore, if curly-winged virgins are used to set up the crosses, then half of the progeny will inherit the balancer, and half will inherit the *UAS-ey* transgene, decreasing the number of progeny with the over-expression phenotype.

2. **Lab Period Agenda:**

   2.1 Pre-lecture topics (~30 min)
   - Discuss the scientific method, and the differences between a hypothesis and prediction
   - Reinforce the idea of the different types of experiments (Show it, Block it, Move it) that they read about in the introduction
   - Introduce the fruit fly as a model organism
   - Discuss the basics of the GAL4/UAS system and their application to this experiment

   2.2 Explain the mechanics of the dissection and then allow students to perform the pupal dissections and make their observations (~30-60 minutes)

   2.3 **Note:** The *dpp-GAL4* transgene is not homozygous in the stock. It is carried over a balancer chromosome that is labeled with a mutation that makes the larvae and pupae smaller than normal (tubby). Therefore, only half of the progeny from the cross will have inherited the *dpp-GAL4* transgene. Instruct students to dissect the longer pupae to see the effect of Eyeless over-expression. The shorter, tubby pupae could be dissected as a control. Alternatively, wild type (red eyed) flies could be used as the control dissection

   2.4 Once they have completed the dissections and observations, allow students to answer the lab questions related to their observations and to the Halder (1995) paper. These questions could also be assigned as homework if class time is
limited (~1-1.5 hour). Reinforce the conserved nature of the eyeless gene and how this work can impact our understanding of eye development in other organisms.

3. Answer Key

Lab Questions

1. What type of experiment have you performed today (Show It/Block It/Move It)? Explain your answer.

   Move it. We have over-expressed the Eyeless protein in tissues where it is not normally found.

2. In flies, genes are often named based on the loss-of-function phenotypes. What would you predict would happen if you blocked the function of the eyeless gene?

   If you block the function of eyeless, eye development will be disrupted.

3. From this experiment and your prediction in the question above, what could you hypothesize about the role of the eyeless gene in the developing fly?

   Eyeless is necessary and sufficient for eye development in the fruit fly.

4. Develop a hypothesis about the role for the human homolog of eyeless, PAX6.

   PAX6 is also necessary (and possibly sufficient) for eye formation in humans.

5. Now, read the accompanying Halder (1995) paper. What is the hypothesis and prediction for this paper?
Hypothesis: Eyeless is the master control gene for eye morphogenesis.

Prediction: “If ey is the master control gene for eye morphogenesis, [then] the ectopic expression of ey should induce the formation of ectopic eye structures in other parts of the body...” (p. 1789)

6. In the paper, the authors state, “Our experiments ...identify the gene that is necessary and sufficient to induce ectopic eyes at least in imaginal discs.”
   a. In general terms, what does it mean if something is necessary and sufficient?

   It means that the gene is required for formation of a structure (like the eye), and also that expression of that gene alone is able to induce formation of eye tissue in a part of the organism where it is not normally found.

   b. Which specific pieces of data support each part of this statement? (Hint: they might not all be shown in the paper)

   Previous experiments discussed in the introduction report that eyeless is necessary for eye formation. For example, “The eyeless (ey) mutation of Drosophila was first described in 1915 (1) on the basis of its characteristic phenotype, the partial or complete absence of the compound eye.”

   Figure 2 demonstrates that ectopic expression of Eyeless leads to the formation of ectopic eye structures, which are further characterized in Fig. 3 and 4. In Fig. 5, the authors show that the ectopic expression of the mouse homolog, Pax-6, is sufficient to cause the formation of ectopic eyes as well, demonstrating the conserved role for eyeless and its homologs as master control genes for eye morphogenesis.

7. What is one additional question you have after reading this paper? Use this question to develop an additional experiment that you would like to do if this were your thesis project. You can do this in very general terms. For example, can eyeless induce eye formation in other tissues, not just imaginal discs?
4. Potential Problems

4.1 If the timing of the crosses is not ideal, students can share the vials of flies, or dissections could be performed ahead of time and pupae maintained in 70% ethanol for students to observe. Note: the pigmentation in the body will fade over time, making the ectopic eyes, which are normally bright orange, more difficult to see.

4.2 Students can have difficulty performing the pupal dissections. The pupal cases are very fragile, and if using the inexpensive jewelry tweezers, this can be challenging. If a couple of pupae can be dissected by the instructor ahead of time, this could allow students to make the observations even if they are unable to do their own dissections. There are also images of UAS-ey; dpp-GAL4 flies on the following website: http://hawaiireedlab.com/wpress/?p=477
References


