



***Using Fijiwings to Understand the Genetic Control of Cell Growth and Proliferation: A Computer-Based Laboratory Exercise***

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**Synopsis:**

We present three lab modules designed to demonstrate how the computer-based analysis tool Fijiwings can assist in measuring cell growth and proliferation phenotypes associated with changes in gene expression. Together, these interrelated modules demonstrate the core concepts of gene expression and regulation, in particular the concepts of how genes control development and how mutations affect genes and the corresponding mRNAs and proteins. In the first module, which can be used in introductory labs, students analyze changes in tissue and cell size following manipulation of insulin signaling in a model organism. This exercise illustrates how a computer-based tool can be used to measure precisely the phenotypic effects of cell signaling on cell and tissue size. Module 2 directs students through several simple exercises designed to illustrate how the underlying JavaScript code in Fijiwings is assembled and can be used to write macros controlling simple computer tasks. The first two modules of this lab require little instructor preparation and no materials beyond a computer with an internet link. In an optional open-ended third module, we provide ideas for the use of Fijiwings to generate and test hypotheses of how genes affect cell/tissue size. From these exercises students develop skills in experimental design and data evaluation and better understand how model organisms can be used to understand key signaling pathways linked to human disease, such as diabetes and cancer.

**Introduction:**

How organisms achieve their proper size through cell growth and proliferation is a central question in biology, but the signaling pathways underlying size control are poorly understood. Body size varies impressively among diverse insect species and the body size of mammals can spread over a 75-million-fold range – from the Etruscan tree shrew to the blue whale. Species-specific adaptations, such as insect antenna and the bat forelimb, can show incredible size scaling from a basic template (Koyama et al., 2013) and within a species development is plastic, as well: organisms adjust the rates of cell growth and proliferation in response to a host of environmental cues, including nutrient availability, temperature and O<sub>2</sub>. The genetic mechanisms regulating body size are subject to intense evolutionary selection pressure with impacts on mate selection, predation and tolerance to environmental changes (Nijhout et al., 2014).

Animal size is the result of an adaptable developmental program that coordinates cell division, growth and differentiation with the availability of nutrients (Nijhout et al., 2014). While cell division is tightly controlled by the cell cycle toolkit (reviewed in Berridge, 2012), cell growth – the macromolecular synthetic events necessary for cells to double in size prior to mitosis – is coordinated in large part by insulin and insulin-like ligands (Hyun, 2013). Insulin/TOR signaling pathways are conserved from yeast to humans, and research in *Drosophila* has provided key insights into how systemic and local signals act via these pathways to regulate cell growth, body size, sexual maturation and longevity (Baker and Thummel, 2007).

The insulin/TOR pathway senses nutrients to regulate growth and homeostasis. In the insulin signaling pathway, insulin or insulin-like peptides bind a receptor on the cell membrane and initiate a signaling cascade. In this cascade, phosphatidylinositol 3-kinase (PI3K) phosphorylates Akt (also known as Protein Kinase B), leading to inhibition of the transcription factor FoxO (Grewal, 2009). In the TOR pathway, TORC1 (Target of Rapamycin Complex) is activated in response to oxygen, amino acid and glucose levels, or by PI3K/Akt signaling via inhibition of negative regulators of TORC1. TORC1 inhibits catabolic cellular processes and activates anabolic cellular processes to control growth (Soulard et al., 2009). The components of the insulin/TOR pathways are structurally and functionally conserved between

Drosophila and mammals (Garofalo, 2002), making the fruit fly an accessible way to study these pathway components.

Epidemiological studies confirm that obese individuals and type 2 diabetics are at higher risk for various types of cancers, but the signaling pathways that link cell growth regulated by insulin with misregulation of cell proliferation associated with cancer are unclear. Testing how signaling pathways control growth is beyond the intellectual and practical abilities of most undergraduate and high school labs and requires extensive expertise and pre-planning on the part of the instructor. This lab makes use of Fijiwings, a computer-based tool that is free for download, to measure the effect of insulin signaling on cell and tissue size in the Drosophila wing (Dobens and Dobens, 2013). FijiWings constitutes a set of computer macros that harness the plugins on ImageJ to accurately measure (1) cell size (seen in wing photomicrographs indirectly by the trichome bristles each cell produces) and (2) overall wing size. Fijiwings then calculates trichome number and density to give insight to how cell number and cell size combine to establish organ size.

In module 1, we use photomicrographs of wings from different genetic backgrounds to furnish students examples with which to apply the features of Fijiwings to detect the effect of gene expression on cell size and overall tissue size. Module 2 presents two simple exercises to shed light on how JavaScript code is written. These two lab modules are computer-based entirely and require no significant prep work. A third optional model illustrates how open-ended labs may be created using Fijiwings, which can form the basis of more complex, hypothesis-driven experiments. Together these three modules shed light on how model organisms are used to understand well-conserved pathways with well-established links to colon, breast and thyroid cancers and obesity and type 2 diabetes (Jackson, 2006; Sansal and Sellers, 2004).

### ***Approach/Method: (Instructor Guidelines)***

#### ***Introduction of Lab:***

The first lab module uses existing data wing photomicrograph examples to test all aspects of Fijiwings, a computer program tool; this module requires no advanced planning and can scale up to any class size. Module 2 focuses on the simple computer code used to drive Fijiwings. For advanced classes, we outline an optional module 3 where students set up crosses to use the fly GAL4 misexpression system to manipulate gene activity in the wing tissue, prepare, photograph and measure cell/tissue size in their experimental wings. This advanced optional module will require some planning by the instructor to order and establish fly stocks; the instructor will be required to understand how fly crosses are set up and have a basic understanding of the GAL4 system. This advanced optional third module can be a jumping off point for undergraduate research projects, as well.

Depending upon the scope of the course and the background of the instructor, module 1 can be used alone or as an entrée to either module 2 or module 3; alternatively, all three modules can be used in sequence. For background to each module, it will be important to cover the molecular basis of cell growth control for module 1, to introduce students to Java script for module 2, and to introduce the GAL4 misexpression system for optional module 3.

**Module 1: Introduction to Fijiwings**

**Prelab.** The instructor can choose to set up and test run Fijiwings on lab computers prior to class; alternatively, students can spend the first 15-20 minutes of downloading and setting up Fijiwings on their own laptops. It is also recommended that the students watch the demonstration of Fijiwings at <https://www.youtube.com/watch?v=kkwbRaN51ww&feature=youtu.be>.

Detailed instructions for downloading Fijiwings and setting it up are available at <http://sourceforge.net/projects/fijiwings/files/?source=navbar>. Fijiwings for the Mac platform is straightforward to download as a zip file, open and begin work directly. Fijiwings for Windows requires downloading and installing Fiji is Just Image J from <http://fiji.sc/Fiji> and also several files from the 'Fijiwings for Windows' folder on the Fijiwings website and placing them in the correct directory.

**Lab overview.** Detailed instructions for using Fijiwings are available at (Dobens and Dobens, 2013). We recommend that students download Fijiwings 2.1, and use Fijiwings 2.1 to work through all its features using the micrograph of normal, wild type wings available for download at <http://sourceforge.net/projects/fijiwings/files/Wing%20example%20files/>. As detailed in the student lab, the student will (1) open the wild type wing file (enGAL\_y.jpg) in Fijiwings, (2) measure trichome density using the "75px sq trichome density" button and clicking on an intervein region uninterrupted by wing vein material, and (3) measure overall trichome density and wing size using the "polygon trichome density" feature of Fijiwings. (4) Students can assess the ability of Fijiwings to detect trichomes by using the magnifying glass feature of ImageJ to evaluate how well Fijiwings detects trichomes. (5) Students can use segmentation analysis to re-draw the wing as a cartoon and evaluate trichome density and size of various vein areas in the wing using the "segmentation (8GB RAM or 4GB RAM)," "intervein trichome density" functions, (6) can create a heat map of intervein areas (7) a heat map of trichome density.

To examine the effect of insulin signaling on wing cell and tissue size, two other wing micrograph files are available (available for download at <http://sourceforge.net/projects/fijiwings/files/Wing%20example%20files/>): (1) a wing expressing high levels of the gene Tribbles, which reduces insulin signaling in the posterior wing compartment and the overall wing size (Mata et al., 2000), and (2) a wing expressing an RNAi to the phosphatase Pten, effectively increasing insulin signaling in the posterior compartment and increasing wing size (Gao et al., 2000). Students use Fijiwings to measure these wings and compare them to normal wild type wings in a graph format. Finally, students can measure and graph the effect on cell size, which are opposite for each gene: Trbl increases cell size and Pten RNAi reduces cell size.

Once students have collected measurements for the wildtype, Trbl and Pten RNAi wings and analyzed their own data, the instructor should compile the data and give the data set to the students to analyze statistically. The students should calculate the mean and standard deviation for the area and trichome density of the marginal cell and 3<sup>rd</sup> posterior cell for all three wing types.

**Answer Key.** Measurements for each wing will vary to a certain degree, but they should be similar to the values below. Instructors can also opt to have students calculate the trichome density themselves, by dividing the trichome count by area, to ensure the program has calculated it correctly.

	Marginal Cell			3 <sup>rd</sup> Posterior Cell		
	Area	Trichome Count	Trichome Density	Area	Trichome Count	Trichome Density
Y (wildtype)	188496	788	0.00418	491494	3135	0.006379
Trbl	143391	960	0.006695	332647	1706	0.005129
PtenRNAi	128605	998	0.00776	588830	4362	0.007408

4a) By looking at the graphs for area, students should be able to come to the conclusion that in the Trbl wing, the 3<sup>rd</sup> posterior cell area decreases compared to wild type while in the Pten wing, the area of the 3<sup>rd</sup> posterior compartment increases compared to wild type. They should also notice that the marginal cell area decreased in both the Trbl and Pten RNAi wings.

4b) Students should see that the 3<sup>rd</sup> posterior cell is denser in the wild type wing, and in the Trbl wing, the marginal cell is denser. In the Pten RNAi wing, the marginal cell is slightly denser than the 3<sup>rd</sup> posterior cell. Their results should agree with their trichome heat maps.

4c) The actual mean and standard deviation values will vary depending on the measurements taken by students. The instructor should calculate these values to ensure the students are calculating them correctly.

5a) Students should predict that a wing overexpressing Pten would look similarly to a Trbl wing. They may also predict that it could look like a wild type wing.

5b) Answers will vary. In general, they should explain that a gene that normally suppresses insulin signaling (such as Pten) would increase the wing area if that gene is knocked down, or it would decrease the wing area if it were overexpressed (like Trbl). Conversely, genes that would boost the effects of insulin signaling would cause the wing area to increase if overexpressed, and cause the wing area to decrease if expression is knocked down.

### **Module 2: Fijiwings JavaScript macros (Module 1 required)**

**Prelab.** Once Fijiwings is working on a computer, no set-up is required for this module.

**Lab overview.** The Fijiwings “Action Bar” collects a set of JavaScript macros that run the features of Fiji/ImageJ and presents them in one place for easy access. A macro is a simple program that automates a series of commands, and in the case of Fijiwings, each macro directs plug-ins that are part of the underlying application, Fiji/ImageJ. Once students are familiar with Fijiwings in module 1, they can take two approaches to understand macros that are used in Fijiwings.

First, students record a macro, which is the easiest way to create a macro, and run it. To do this, students open the “macro recorder” (available by switching to the Fiji tool bar and choosing from the Macro Recorder from the menu drop-down list: Plugins>Macros>Record; note that when Fijiwings is

opened, an “Action Bar” is active and behind this is the Fiji/ImageJ tool bar). Students open a wing image file and then find local maxima in the file; all these actions will be recorded in the “macro recorder” window. Subsequently, students run the macro they have recorded by clicking the “Create” button to open the recorded instructions in an editor. This series of steps, detailed in the lab protocol, serve to shed light on how macros can be written without a detailed knowledge of JavaScript. In this first exercise, students will alter the number of trichomes counted by changing the noise setting.

Finally, students manipulate the macros in Fijiwings directly, by accessing the file “Fijiwings\_2.1.txt” located in the folder Application>Fiji (Right click>Show Package Contents) >Plugins>ActionBar on a Mac, or C:/Program Files/Fiji.app/plugins/ActionBar on a Windows. Opening this file in a dedicated text reader (do not use Microsoft Word), students effectively look ‘under the hood,’ as it were, and make changes directly to the underlying Fijiwings macros. The provided example – searching (Command-F) the file (“Fijiwings\_2.1.txt”) for the phrase “lut=(Cyan Hot)]” and replacing it with “lut=(Red Hot)]” and then saving the file, re-opening Fijiwings, opening the wildtype wing file and running “heat map trichomes” – which will redraw the map with a different color pallet. Again, this striking change in the output of Fijiwings is described step-by-step in the lab protocol. While these are simple tasks, they open the door to student self-directed or instructor-led projects to explore plug-ins in Fiji and to write macros controlling these.

More information about macros as they apply to Fiji is available at [http://fiji.sc/Introduction\\_into\\_Macro\\_Programming](http://fiji.sc/Introduction_into_Macro_Programming).

### ***Module 3 (optional): Using Fijiwings to explore the effect of gene activity on cell/tissue size***

**Prelab.** For this lab, an engrailed GAL4 driver stock is crossed to a stock bearing a UAS-regulated component of the insulin signaling pathway. The Indiana Stock Center maintains a wide variety of these UAS-regulated stocks (collected on a single page at <http://flystocks.bio.indiana.edu/Browse/HD/Metab.php>) to test the effect of a particular component in the insulin pathway on cell and tissue size. As a control, EnGAL4 should be crossed to UAS-lacZ (<http://flybase.org/reports/FBst0003955.html>), which does not affect cell growth or proliferation. The instructor can provide the hypothesis, which we find is appropriate for large classes, or students may read the relevant literature to generate their own, which is a good approach for independent research. The instructor may assign literature listed in the references section for students to read in order to design their own hypotheses. This lab will require planning and preparation by the instructor to obtain stocks from the stock center and rear these flies in preparation for crosses. Slides, coverslips and mounting media are necessary. Dissecting scopes, tools to anesthetize and sort flies, brightfield microscopes with a 4x objective to photograph wings must be available.

**Lab overview.** After obtaining fly stocks and establishing these in the lab, over the course of three lab periods, students (1) virgin flies and set up the cross, (2) at least two weeks later sort progeny and mount wings on slides and (3) at least a day later photograph slides and subject the wings to Fijiwings analysis. For large classes, we have set up the crosses and allowed the students to do steps 2 and 3.

From the first two modules, students will be familiar with the use of Fijiwings to counting bristles and measuring overall wing size. Wing preps can be a challenge and we encourage students to practice on a wild type stock first to become adept at the practice. While this lab is straightforward to carry out,

students should be versed in the signaling pathways regulating growth and in the basic features of the GAL4 system in the fly (Brand and Perrimon, 1993), which is used here to manipulate cell and tissue size.

**Lab Safety:**

Euparal is reported to be inflammable and an irritant so that eye and skin contact should be avoided (<http://www.microbehunter.com/euparal-mounting-medium/>). We find that the odor of Euparal is minimal and stray drops can be cleaned with 95% ethanol.

**Student Evaluation:**

There are several options for a final written submission from the students. You may want to have them write a formal lab report in scientific paper format. Alternatively, students assemble a poster and present their data in undergraduate science fair settings. In addition to this report, students are asked to write their conclusions in proper scientific writing in their lab notebook, which are graded after each lab project. In this way, students communicate their ideas and conclusions to different audiences.

**Justification:**

This lab addresses a recurring question that students pose: how do animals achieve their characteristic sizes? Students come to understand the role of conserved signaling pathways in regulating tissue size and gain insight into the genetic basis of animal diversity, the mechanism of evolution, the effect of environmental conditions on a developmental program, and the underlying causes of developmental aberrations and metabolic disease. From these modules, students learn the effect of genes on cell and organ size, the power of computer-based tools to precisely analyze phenotypes, and gain an increased understanding of how model organisms can be used to understand diseases such as diabetes and cancer, areas of intense interest for all students.

The core competencies addressed by this lab directly relate to all aspects of the scientific process: students evaluate experimental evidence, develop and analyze graphs and apply statistical methods. In the more advanced lab, students formulate hypotheses based on their reading of the literature and confirm their predictions through analysis of misexpression genotypes.

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