HHMI Fall Semester 2012 module design - Nicola Carraro and Wendy Peer

HORT 590 Confocal Laser Scanning Microscopy for Plant Biology and Agriculture

Objective: design a transferable module that combines quantitative data collection and analysis together with microscopy. The module requires use of a microscopy facility (or a confocal microscope) and biological samples for data collection – it combines reading assignments, writing assignments and laboratory work.

1. Abstract

This is for faculty, not students.

a. Biological question

1. Do the proteins of choice co-localize in the same cellular domains when expressed *in planta*, before and after treatments?

b. Statistical content

- Collecting quantitative data (measurements) from images
- Descriptive statistics (median, mean $\pm$ SD vs SEM)
- Inferential statistics (frequencies of co-localization, significance tests, ANOVA)
- Graphical representations of results

c. Students activities

- Learn how to use a confocal microscope and software to collect images from plant biological samples
- Prepare biological samples for microscopy
- Design an appropriate experiment (sample number, sample life span, etc.)
- Design hypothesis
- Collect measurements from images
- Analyze and interpret the data to verify the initial hypothesis
- Present the results (graphs and tables) and compare them with fellow students
- Analyze and critique a figure/result from a scientific article

d. Skills (those developed and practiced in the exercise, including things like writing, computer skills, etc.)

- Experimental design (formulate hypothesis, make predictions, identify controls, relate findings back to predictions and hypothesis)
- Practice compiling and organizing data sets
- Computer skills
- Microscopy skills
- Learn to use software for statistical analysis (Excel, etc.)
- Determine the best way to analyze the raw data (significance test, descriptive statistics)
- Interpret data (graphical representations and the results of inferential statistics)

e. Student-active approaches (used in the exercise)

- Students design hypotheses and make predictions
- Students collect data
- Students organize, represent, analyze, and interpret their data
- Students compare graphical representations with each other and perform a peer review (constructive feedback)
- Students brainstorm about the appropriate components of good graph (these will be used to compile a rubric for assessment of future graphs)
- Students present their data analysis to the class for guided discussion

f. Assessable outcomes

Depending on the level and experience of the students, after completing this module students will be able to:

1. Design an experiment (formulate a testable hypothesis, make predictions, incorporate experimental controls, design experimental groups)
2. Organize a data set
3. Perform descriptive statistics (mean, median, SD, SEM, each as appropriate)
4. Create appropriate graphical representations of their data
5. Explain the advantages/disadvantages of different graphical representations for this data set
6. Verify the significance of the obtained results (significance test)
7. Draw conclusions from their data and develop ability to distinguish between a correct analysis and an incorrect one
8. Be able to read and understand a figure and relative legend based on a similar experiment

2. Background

This section is written for faculty who can modify the background material as appropriate for their students.

Co-localization of fluorescent tagged proteins (adapted from Zinchuk et al. 2008).

Quantitative co-localization analysis (QCA) is a method to estimate the degree of co-localization of antigens in multicolor confocal immunofluorescence microscopy images. Co-localization is observed when staining of two or more antigens in the same section, labeled by corresponding antibodies with different excitation spectra and therefore visualized in different colors, overlaps. Although co-localization is a mere coexistence of molecules in a very close physical location, it can provide valuable clues clarifying their common characteristics. Scientific applicability of co-localization observed *per se* is, however, limited because it is perceived differently by the eye and thus can frequently be misleading. Thus, the method described here evaluates co-localization objectively by estimating its quantitative characteristics.

Co-localization is determined by calculating a number of specialized values representing the proportion of co-localized pixels. These values are estimated according to co-localization coefficients. The following coefficients are used.

Mander's correlation coefficient,

$$R = \frac{\sum_{i} S_{1,i} S_{2,i}}{\sqrt{\sum_{i} S_{1,i}^2 \sum_{i} S_{2,i}^2}}.$$

where *S*1 represents signal intensity of pixels in channel 1 and *S*2 represents signal intensity of pixels in channel 2. This coefficient was developed specifically for estimating co-localization (Manders et al., 1993). Its advantage is that it is insensitive to the limitations of typical fluorescence imaging, such as efficiency of hybridization, sample photobleaching, and camera quantum efficiency. The values of this coefficient are in the range from 0 to 1.0. If the image has an overlap coefficient 0.5, it implies that 50% of both its objects, i.e., pixels, overlap. A value of zero means that there are no overlapping pixels.

Case study: co-localization of the ABCB19 protein with PIN1 and/or PIN2 proteins in the root of Arabidopsis thaliana.

3. Student Instructions

These are detailed step-by-step instructions for the students to do the exercise.

Pre-laboratory activities ***(assumes the students have read the background information before coming to class and that they have learned about hypothesis-driven inquiry)***

You will be using the transgenic *Arabidopsis thaliana* lines carrying fluorescent tagged ABCB19, PIN1 and PIN2 proteins. Preliminary reading of papers provided by the instructor, regarding fluorescent protein constructs, col-localization experiments, PAT (Polar Auxin Transport) in plants and confocal microscopy is required. For this activity, you will work in pairs. In your laboratory notebook write out the following questions/topics and your answers or responses:

a. ***What do you know about the localization of ABCB19, PIN1 and PIN2 in the wild type Arabidopsis primary root? What do you know about polar auxin movement in the primary root?***

b. ***Predict changes in proteins localization after treatment of the root with auxin and/or auxin transport inhibitors.***

Experimental procedure:

1. Undergo the confocal training procedure and read the provided instructions.
2. Grow *Arabidopsis thaliana* seedlings in vitro (sterile seeds, petri dishes and media will be provided) for 5 days. Make sure you grow also wild type plants to use as negative controls in your experiments.
3. Analyze your wild type samples at the confocal microscope: verify that you can correctly visualize the different fluorescent protein constructs and compare the images to negative controls (non-transgenic plants).
4. Proceed with the analysis of the transgenic lines carrying two fluorescent constructs (for example *proPIN1:PIN1-RFP* and *proABCB19:ABCB19-GFP).* Determine in which plasma membranes and in which cells you can detect the fluorescent signal.
5. Use the co-localization module on the Zeiss confocal microscope to determine where your two proteins co-localize or not (make sure to save the file with the data for subsequent analysis)
6. Proceed with treatment of the seedling using IAA and NPA according to the provided instructions.
7. Image the seedlings (make sure to image also untreated controls) and analyze the images using the co-localization module.
8. Determine if the treatment has changed the co-localization coefficient for the proteins of interest.
9. Determine the number of samples and treatments you want to perform (repetitions) in order to have a robust analysis.

Post-laboratory analysis and results

You have now gathered the results from your experiments. Gather also the results from your fellow students. How do you go about organizing the data and making claims about it and your hypotheses?

1. Summarize the similarities and/or differences between your results and the results obtained from the other groups. (Few sentences)

2. Graphically represent the results from your pair by hand in your laboratory notebook or excel file, in at least two different formats.
3. Choose the type of graph and table you want to use to represent your results.
4. Perform an ANOVA on your data set to verify the significance of your results.
5. Compare the trends in your experimental data with what you and your group predicted would happen to the proteins localization after the treatments.
6. Identify the possible steps or procedures that might have generated errors in the data collection, analysis and in the subsequent results.
7. Discuss the results in class and compare them to published results from the literature.

4. Materials and reagents

- *Arabidopsis thaliana* seed from at least one stably transformed line carrying two fluorescent-tagged proteins (alternatively,one protein with a fluorescent tag and a specific antibody can be used for whole mount immunolocalization, although this technique is more cumbersome and less preferbale).
- Confocal microscope with co-localization analysis module.
- Slides, cover slips, sterile water, forceps.
- Gloves, laminar flow bench for sterile work, micropore tape.
- Growth chamber for plants, petri dishes and MS media.

5. Assessment

The students will be evaluated based on the following aspects:

1. Ability to understand the concept of co-localization and its importance in the study of proteins in plants (this is tied to the ability of reading and understanding the background literature that is provided at the beginning of the module).
2. Ability to formulate a testable hypothesis-driven experiment.
3. Correct choice of sample size, graphic representation of the results and correct data collection.
4. Ability to understand and criticize the results obtained from the experiment and also the results obtained by other students and those published in literature.
5. Participation in class discussion and ability to complete home assignments in a timely and complete manner.