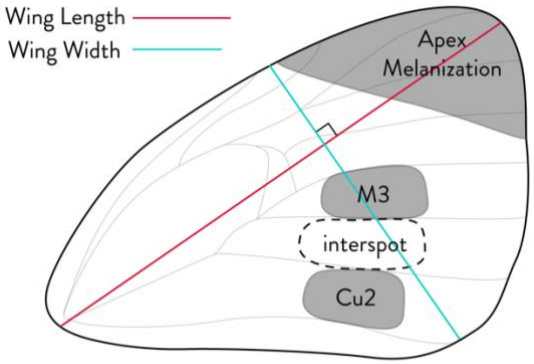
BCEENET
BIOLOGICAL COLLECTIONS IN ECOLOGY AND EVOLUTION NETWORK

Measuring Wing Spot Area using ImageJ

Created by: Elissa Sorojsrisom, Columbia University; Janice L. Krumm, Widener University; Paula A. Trillo, Gettysburg College

INTRODUCTION

These directions will walk you through how to use ImageJ to measure the melanized areas of *Pieris rapae* butterfly wings. These directions assume that you have already measured the wing widths and wing lengths of your butterflies. Closely examine the diagram below to see how the **M3** and **Cu2** spots are delimited by the wing veins- these are the spots you will measure in this activity.



This activity may be used as a standalone activity to gain practice using ImageJ, or as part of the BCEENET Sexual Dimorphism CURE: Exploring Melanized Wing Patterns of Pieridae Butterflies (Trillo, Sorojsrisom, Jordan, & Krumm, 2022). After completing this activity, you will be able to:

- Adjust levels and limit the threshold of the image to select for specific darkness levels
- Use the wand tool or polygon tool to measure the area of a spot

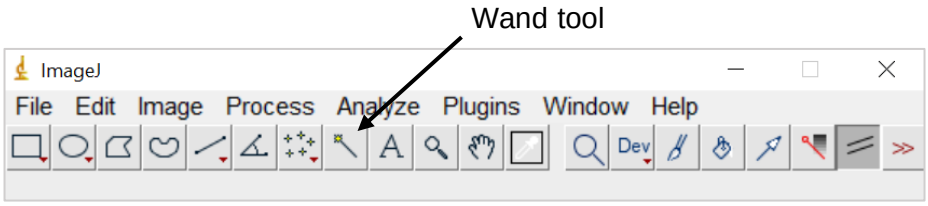
Related documents

Part 1- Installing ImageJ and Tools
Part 2- Measuring Wing Size using ImageJ
Part 3- Measuring Wing Spot Area using ImageJ
Part 4- Measuring Apex Melanization using ImageJ

This is the third part in this series. **Be sure to complete Part 1, Installing ImageJ and Tools, and Part 2, Measuring Wing Size using ImageJ, before you begin.** Your instructor may ask you to skip part 4 or to complete it before part 3.

INSTRUCTIONS

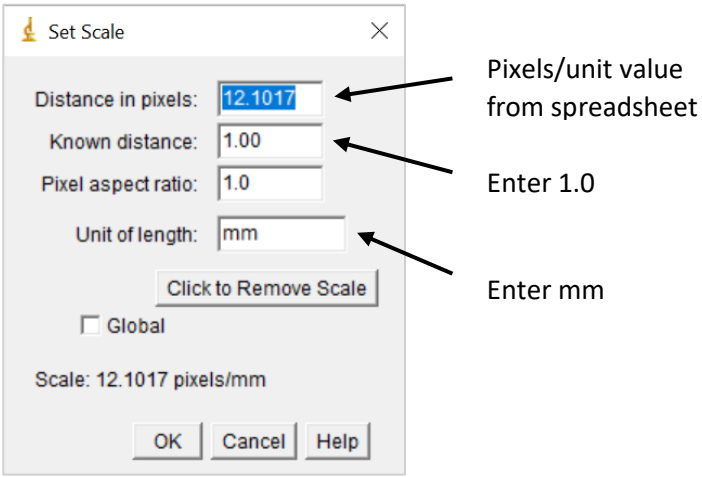
1. **Open ImageJ**
    a. We will primarily be using the **Wand (tracing) tool** to measure the areas of the wing spots. This ImageJ tool is indicated by the black arrow below.



2. **Open your first *Pieris rapae* image**
    a. Navigate to **File >> Open…** and select your image file. Click **Open**.

3. **Open your Spreadsheet for data collection**
    a. Open the data spreadsheet where you previously recorded the wing width and wing length data.
    b. Find the associated specimen record (represented by the row on your spreadsheet with the same **coreID** as your image).

4. **Set the scale**
    a. Find the scale for this image on your spreadsheet. The column will be titled **pixels/unit**.
    b. On ImageJ, navigate to **Analyze >> Set Scale**.
    c. Enter your pixels/unit value from your spreadsheet in the **Distance in pixels** box.
    d. Enter 1.0 in the **Known distance** box.
    e. Enter mm in the **Unit of length** box.
    f. Click **OK**.

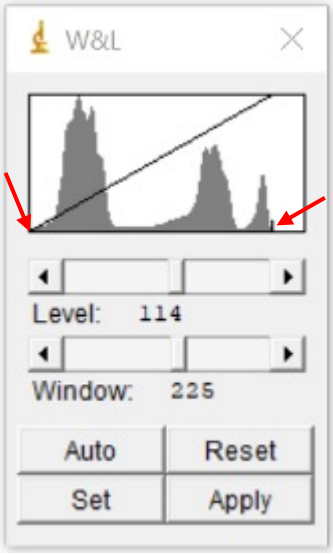


5. Adjust image color & levels

a. Navigate to **Image >> Adjust >> Window/Level**.

b. Click **Auto**.

c. Look at the histogram. Make sure the diagonal line begins before the gray peaks and ends after them (there is a little tick at the bottom to indicate where it ends - see red arrows). If it looks good, click **Apply**.



d. If the diagonal line cuts off any part of the gray area, click **Reset** and try using **Auto** again. Make sure you only click **Auto** once.

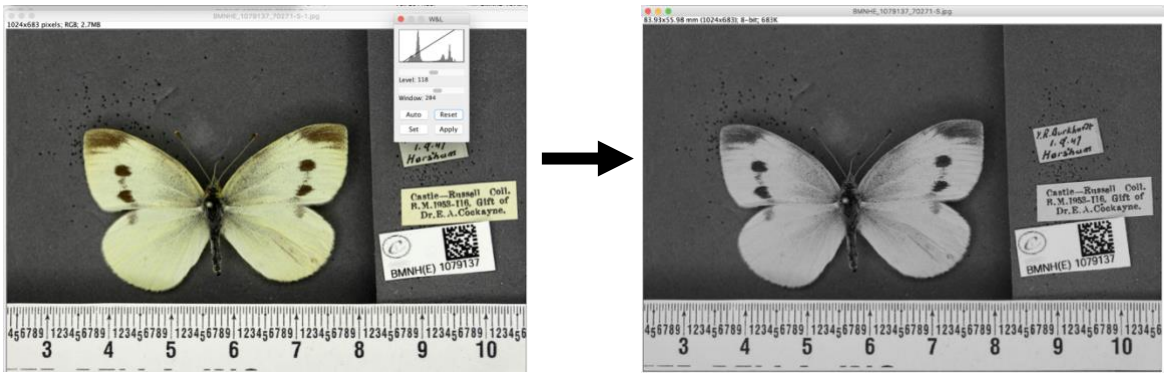
e. If the histogram is still cut off, set the window and level manually:

   i. Using the slider, adjust the window value higher to make it smaller.

   ii. Adjust the level to move the window back and forth.

   iii. You may have to tweak both values until you get the window right outside of where the grey area starts and ends.

f. Navigate to **Image >> Type >> 8-bit** to convert the image to grayscale.

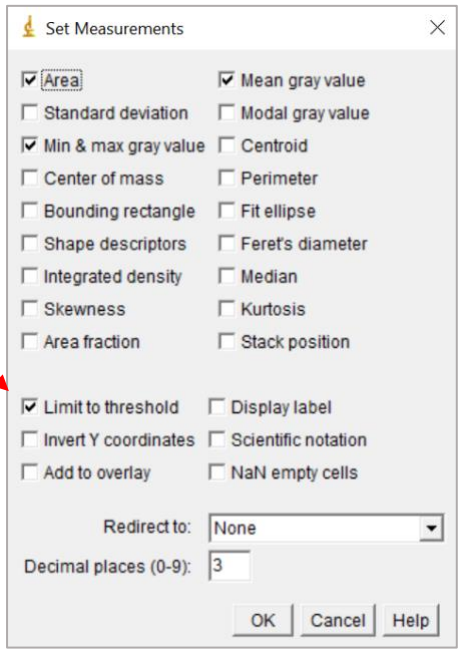


6. Set the image threshold.

a. Navigate to **Analyze >> Set Measurements**.

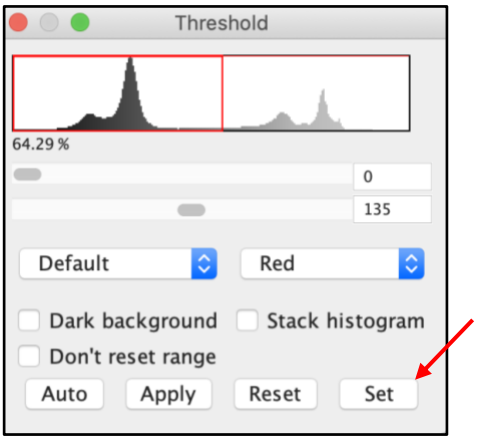
i. Check **Limit to Threshold** Box.

ii. Click **OK.**

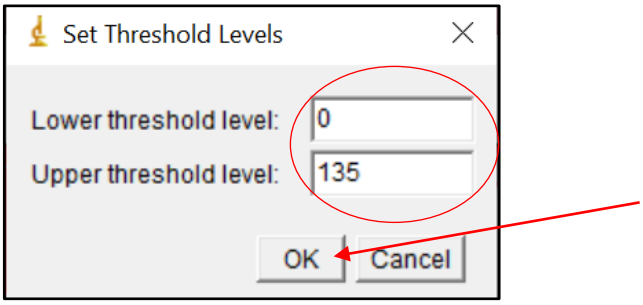


b. Navigate to **Image >> Adjust >> Threshold** to open the threshold dialogue box.

i. Click on **Set**.



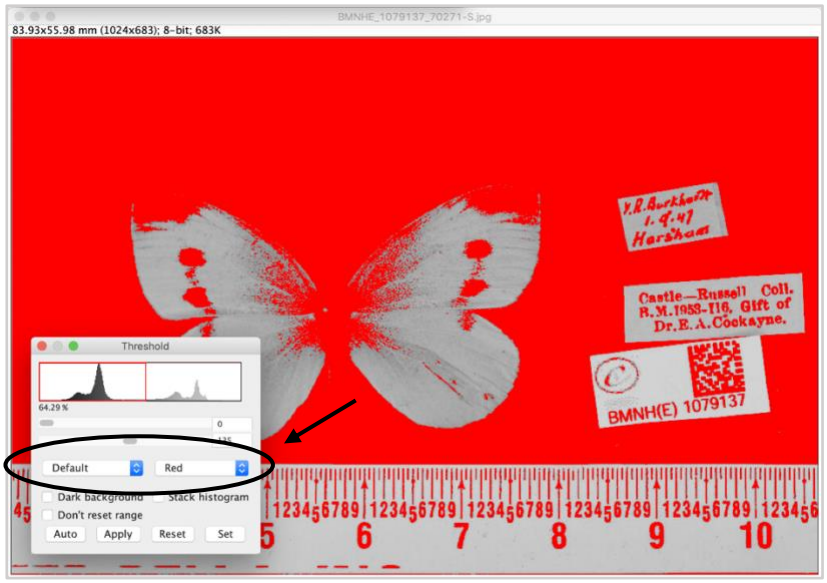
ii. In the **Set Threshold Levels dialogue box**, set the lower level to **0** and the upper lever to **135** (0 = black, 255 = white).
**Note: These values are appropriate for measuring melanized spots on P. rapae specimens. If you are examining a different organism, your instructor may provide different threshold values*.



iii. Click **OK**.

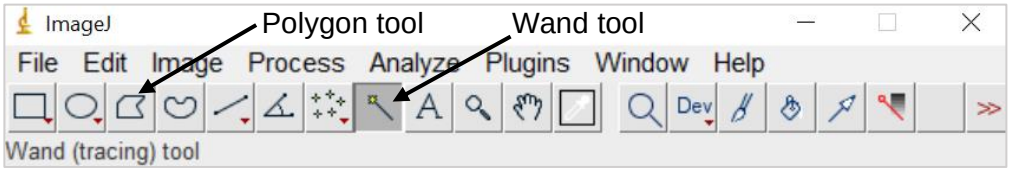
iv. Set the drop-down selections to **Default** and **Red.**

v. **Do *not* hit apply** or your image will switch to black & white, and it will be harder to work with.

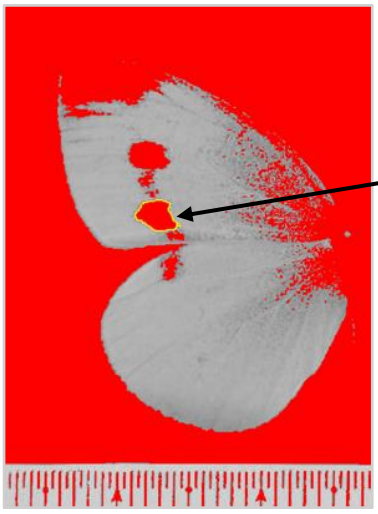


7. Select and measure spots

a. Find the **Wand Tool** and the **Polygon Tool** on the ImageJ toolbar.



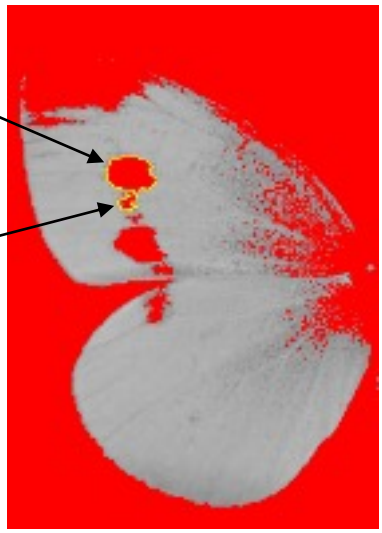
b. Click on the **Wand Tool**. Select the wing spot you want to measure by clicking on it. ImageJ will outline the area that is selected and will be measured.



Selected melanized wing spot Cu2 (outlined in yellow)

c. Navigate to **Analyze >> Measure** to take measurements of the area and record in your spreadsheet.

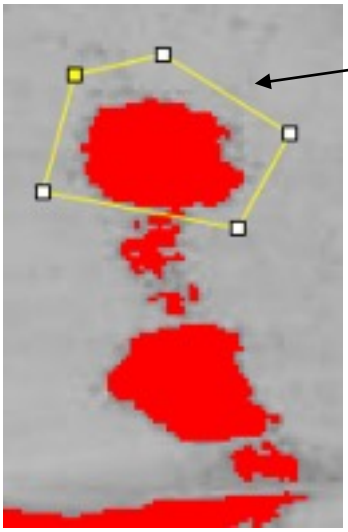
d. If the area selected by the wand tool is larger than the measurement you wish to take, then you can select a smaller area using the **Polygon tool** that excludes the problem area.



   i. Click on the **Polygon** tool.

   ii. Click on the image, just outside of the spot you are trying to select.

   iii. Continue clicking around the spot to form a shape that includes the spot but excludes any undesired areas. To complete the polygon, click again on the first point you made, shaded in yellow.



   iv. Then select **Analyze >> Measure**. Only the thresholded (red) area inside the polygon will be measured.

ADDITIONAL RESOURCES

ImageJ User Guide

REFERENCES

ImageJ. (1997). National Institutes of Health. https://doi.org/10.1038/nmeth.2089

Trillo, P.A., Sorojsrisom, E., Jordan, C.N., & Krumm, J.L. (2022). Sexual dimorphism CURE: Exploring melanized wing patterns of Pieridae butterflies. BCEENET- Biological Collections in Ecology & Evolution Network, QUBES Educational Resources. https://qubeshub.org/publications/3250/1