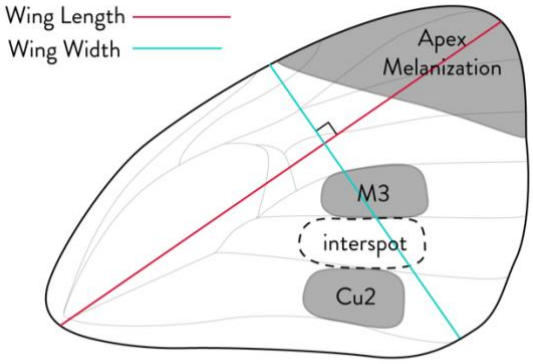
BCEENET
BIOLOGICAL COLLECTIONS IN ECOLOGY AND EVOLUTION NETWORK

Measuring Wing Apex Melanization 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 area the **Apex Melanization** of *Pieris rapae* butterfly wings. These directions assume that you have already measured the wing widths and wing lengths of your butterflies.

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:

- Use the Versatile Wand tool to remove the background from an image
- 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

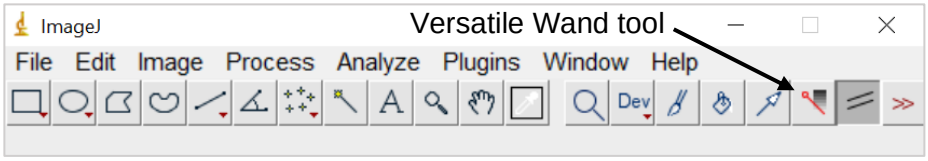
Related documents

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 fourth 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 3 or to complete it after part 4.

INSTRUCTIONS

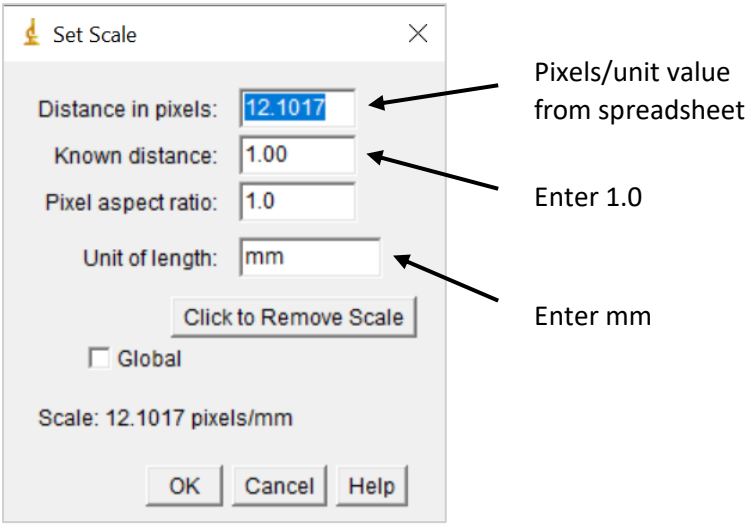
1. **Open ImageJ**
    a. We will primarily be using the **Versatile Wand (tracing) tool** to measure the areas of the wing spots. This ImageJ tool is indicated by the black arrow below.



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).

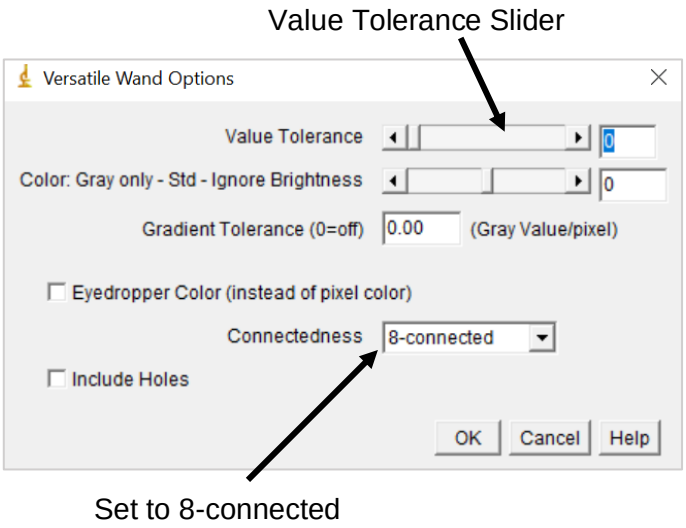
4. **Set the scale**
    a. Find the scale for this image on your spreadsheet. The column will be titled **pixels/unit**.
    b. On ImageJ, navigate to **Analyze >> Set Scale**.
    c. Enter your pixels/unit value from your spreadsheet in the **Distance in pixels** box.
    d. Enter 1.0 in the **Known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



5. Remove the background

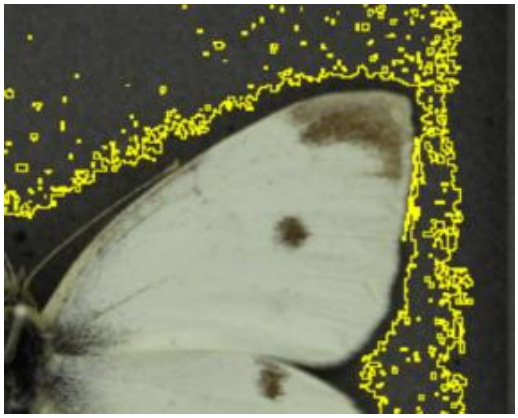
a. Click on the **Versatile Wand Tool,** then click on the background color of your image.

b. Double-click on the **Versatile Wand Tool** to open the **Versatile Wand Options** dialogue box.



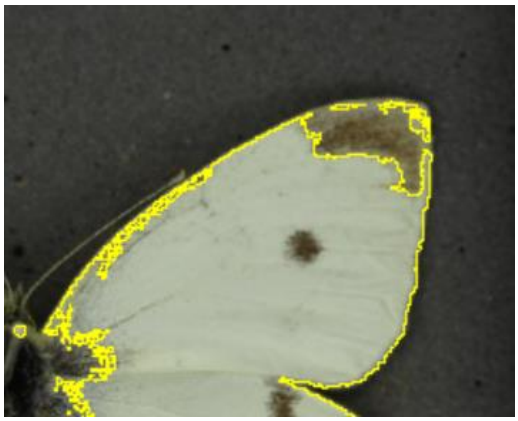
c. Make sure the **Connectedness** is set to **8-connected**.

d. Adjust the **Value Tolerance slider** to select as much of the background as possible *without <u>any</u> of the butterfly near the wing apex selected*. Higher values select more area.



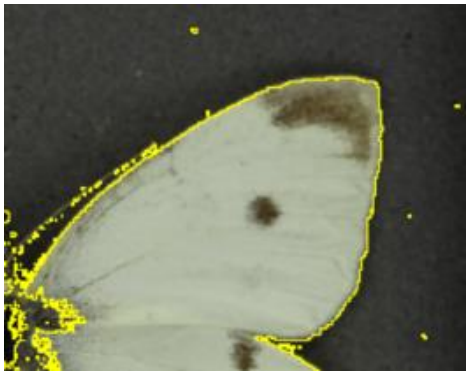
This image has the **Value Tolerance too low**.

Notice that the selected area does not follow the wing apex margins.



Here, the **Value Tolerance is too high**.

The selected area includes part of the melanized area of the wing.

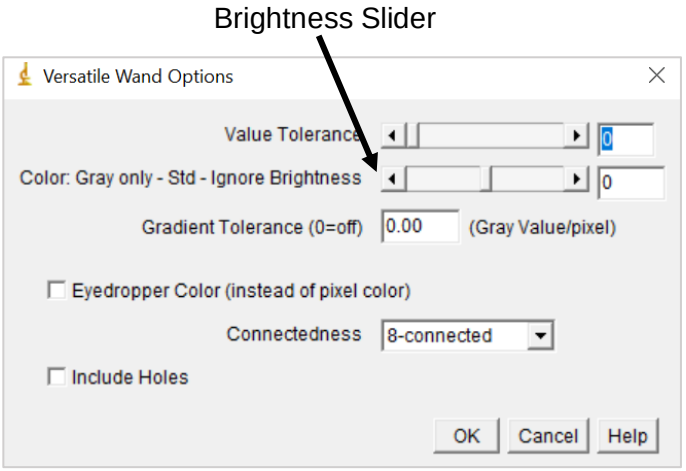


The **Value Tolerance looks good**!

The yellow line follows the margin of the wing apex nicely.

e. If you're having trouble, try closing the **Versatile Wand Options** dialogue box**,** then clicking on different areas of the background. This will reset your reference color and may change the way the selection is made by the tool. You will then need double-click on the **Versatile Wand Tool** again to re-open the **Versatile Wand Options** dialogue box.

f. Once you have set the correct tolerance, adjust the **Brightness slider** to see if you can capture any more of the background without going over the wing apex area.



g. Triple-check that none of the area on the wing near the wing apex is selected (the body and some of the wing near the body will be selected, but that's okay). Once you're satisfied, click **OK**.

h. Now press '**f**' or '**f5**' on your keyboard to flood the selected area with white.

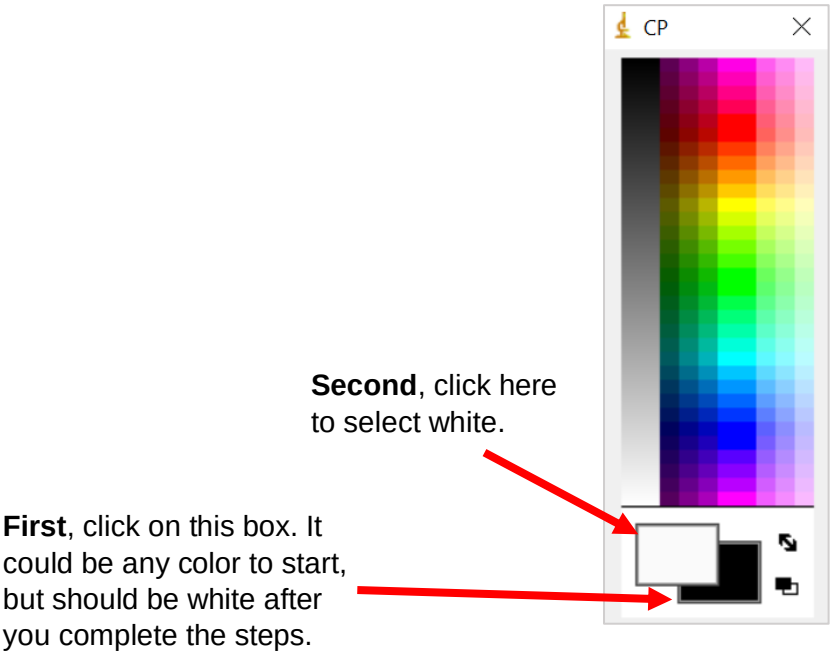
   i. If your program fills with a different color, use the **color picker tool** to select white as your fill color.

      1. The **color picker tool** looks like an eyedropper. It may or may not appear on your toolbar. If the eyedropper is <u>not</u> visible on your toolbar, navigate to **Image >> Color >> Color Picker** to open the **CP dialogue box.**

      2. If you can see the **color picker tool** on the toolbar: The color of the eyedropper matches the drawing color. The eyedropper above would fill the selected area with black. Click on the **color picker tool** to open the **CP dialogue box.**

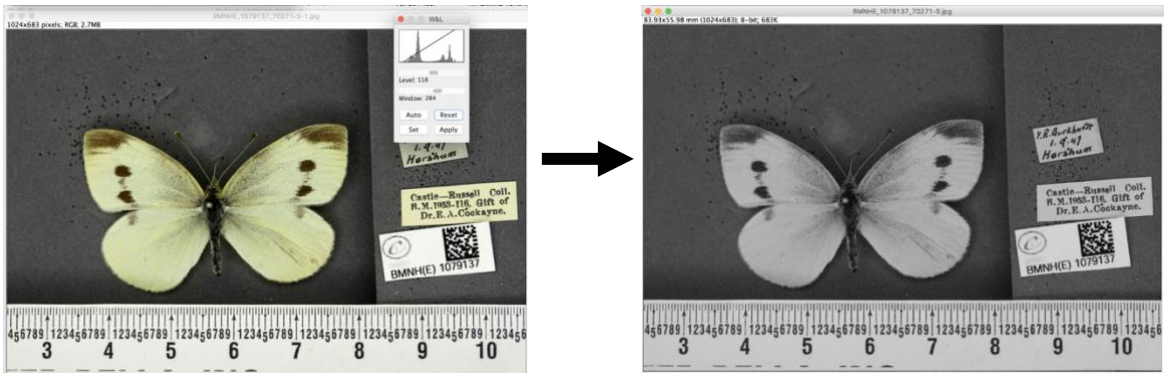
3. In the colorful **CP dialogue box**, click on the big box on the bottom left, then click on the white part of the color bar.
4. The first box should turn white, like in the image to the right.

ii. Try again pressing '**f**' or '**f5**' on your keyboard to flood the background with white fill.



6. Adjust image color

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



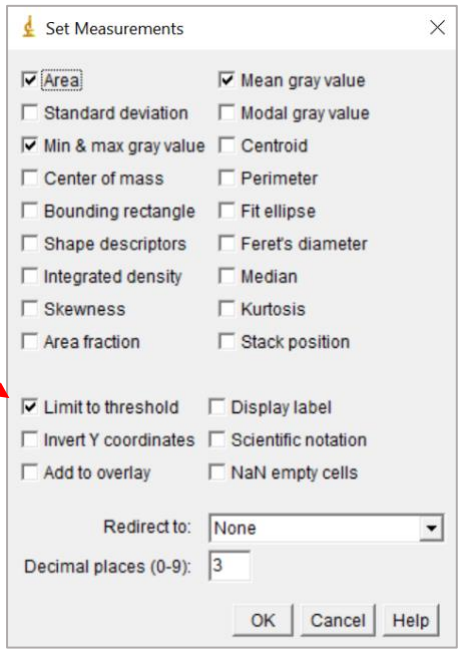
Sorojsrisom, E., Krumm, J.L., & Trillo, P.A. (2022). Measuring size and area of digitized specimens using ImageJ. BCEENET- Biological Collections in Ecology & Evolution Network, QUBES Educational Resources. https://qubeshub.org/publications/3120/1

7. Set the image threshold.

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

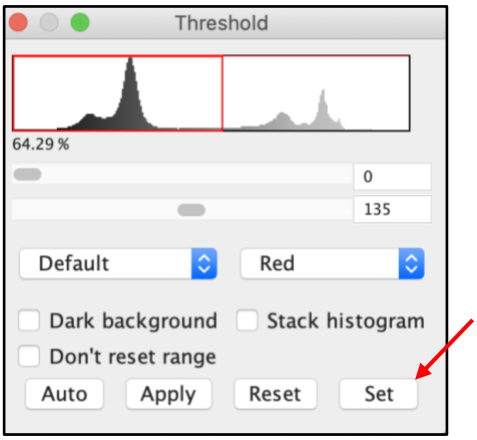
i. Check **Limit to Threshold** Box.

ii. Click **OK.**

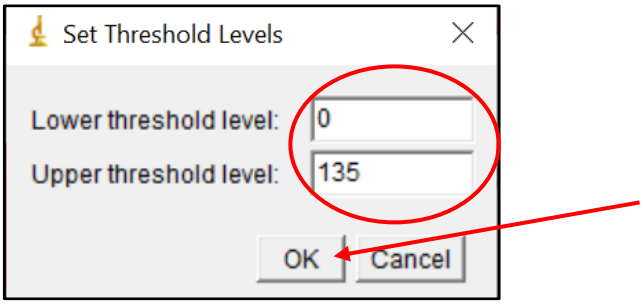


b. Navigate to **Image >> Adjust >> Threshold** to open the threshold dialogue box.

i. Click on **Set**.



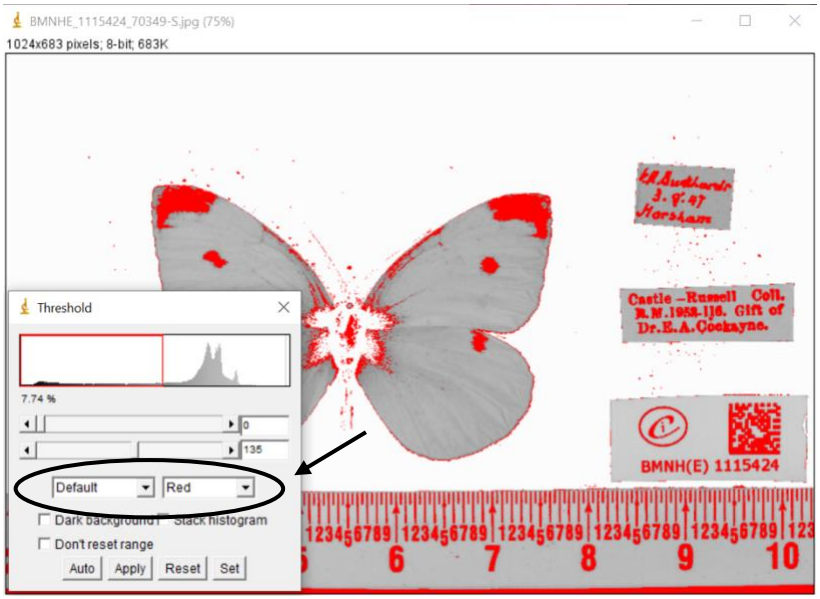
ii. In the **Set Threshold Levels dialogue box**, set the lower level to **0** and the upper lever to **135** (0 = black, 255 = white).
**Note: These values are appropriate for measuring melanized spots on P. rapae specimens. If you are examining a different organism, your instructor may provide different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



iii. Click **OK**.

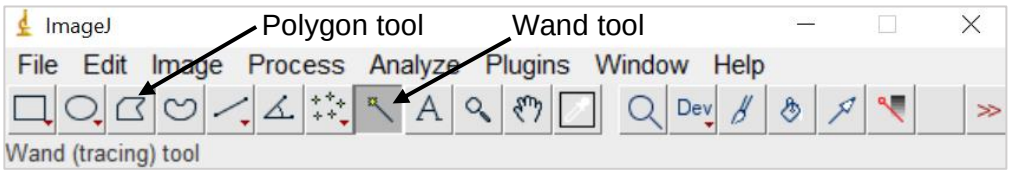
iv. Set the drop-down selections to **Default** and **Red.**

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



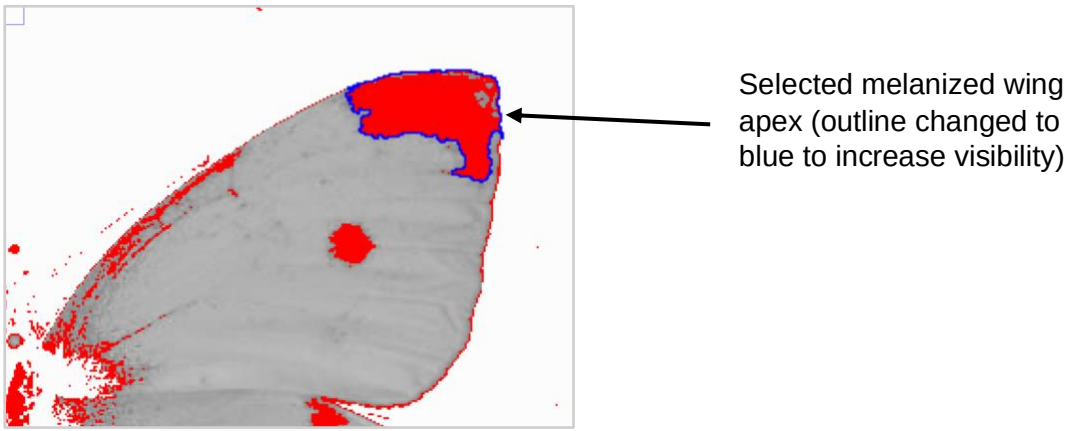
8. Select and measure spots

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.

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



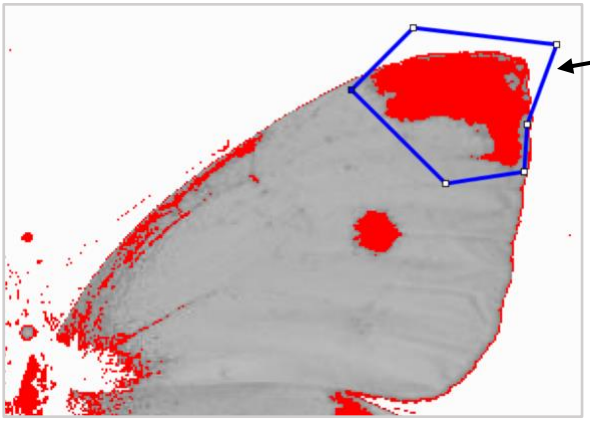
d. If the area selected by the wand tool is larger than the measurement you wish to take, then you can select a smaller area using the **Polygon tool** that excludes the problem area.

   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.



Defining the area measured using the **Polygon tool**

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