Using ImageJ to measure leaf area from photos or scanned images

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1 Installation

Install the Fiji software package from this URL: `https://imagej.net/software/fiji/`. There are versions for Windows, macOS, and Linux. Fiji is like a deluxe version of ImageJ, and includes lots of extra features. I recommend Fiji over stock ImageJ, but everything in this manual will probably work in stock ImageJ. Throughout the rest of the manual, I use "Fiji" and "ImageJ" interchangeably.

2 Scanning and photographing images

If storing leaves before scanning/photographing, try to store them flat otherwise they may roll up as they dry.

If using a scanner, set the resolution of the scanner to 300 DPI (dots per inch) for each scan, scan the whole A4 area of the scan, and save as a `.jpeg`.

Store each leaf as a separate image. Include a ruler or scale bar in the image. Make sure the leaf is flat and away from the edges of the image.

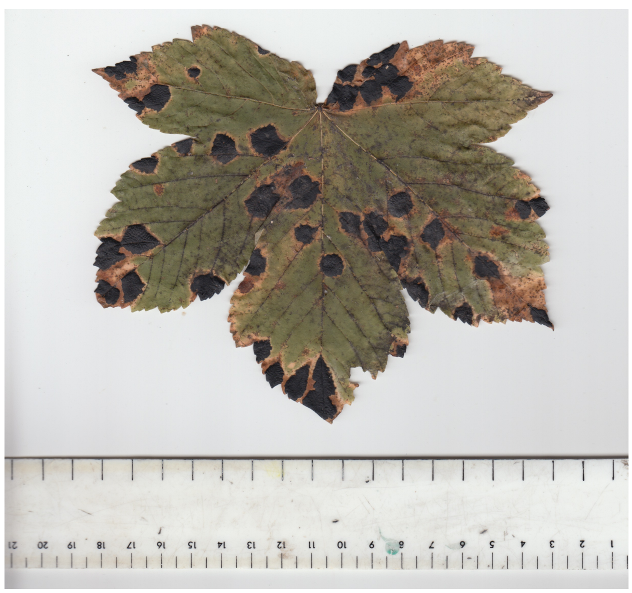

Figure 1: A good example of a scanned image of a leaf.

3 Measuring leaf area

Open Fiji, then click `File → Open...` and choose a leaf image.

First, set the scale of the image. This tells ImageJ how many pixels in the image make up one unit of measurement in the real world. To do this, first select the line tool from the tool bar and draw a straight line of known distance across the scale bar by clicking and dragging the line across the image. Then select `Analyze → Set Scale...`. The `Distance in pixels` will be automatically filled in with the length of the line in pixels. Fill in the `Known distance` with the length on the scale that the line traverses, e.g. 4 cm. Fill in `Unit of length` with the unit of the scale, e.g. cm. `Pixel aspect ratio` can be left as 1.0, as your images will most likely have square pixels.

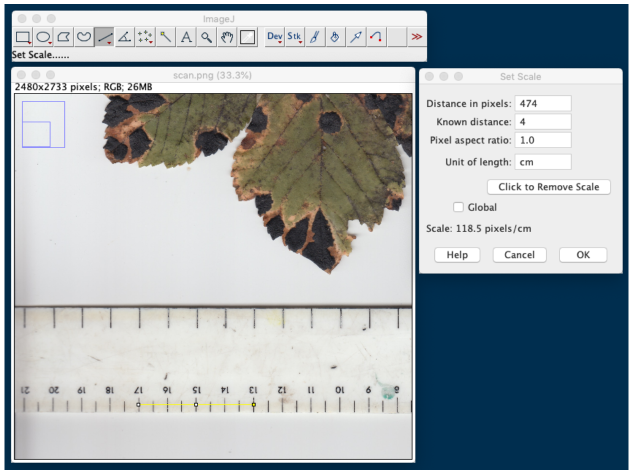

Figure 2: Example of setting the image scale using the line tool to draw a line of known length on the image.

Next, convert the image so that everything which is leaf is black and everything else is white. To do this, first convert the image to "8-bit" (grayscale) by clicking `Image → Type → 8-bit`. Then threshold the image by clicking `Image → Adjust → Threshold...`. Adjust the sliders in the `Threshold` box so the leaf is completely red, but none of the background is red. Ensure that `Dark background` is not checked, then click `Apply` and close the thresholding window. The leaf should now be black, and the background should be white. It doesn't matter if there are few small black objects such as the ruler leftover on the image, but it is important that the leaf is well defined.

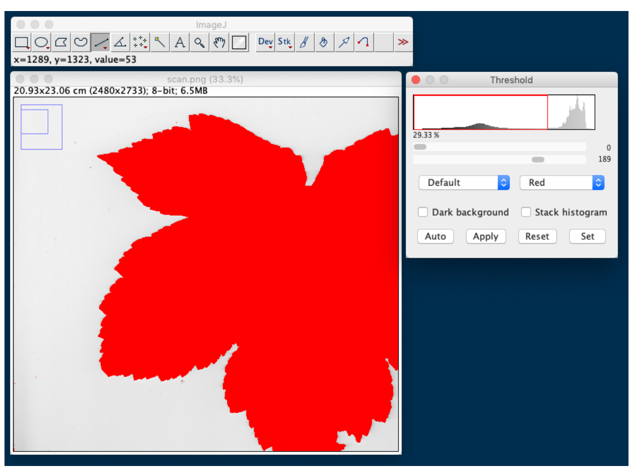

Figure 3: Example of the image binarization procedure.

To find the area of the leaf, go to `Analyze → Analyze Particles...`. `Size (unit^2)` sets the minimum size of contiguous black areas that will be included in the analysis, by default it's set to `0-Infinity` which includes everything, but feel free to change this to something larger to remove little black areas caused by dust. The exact value will depend on the size of the leaves in the image. `Circularity` defines a range of circumference:area ratio that will be included in the analysis. Objects with a value close to 1 are completely circular, values close to 0 are thin lines. I normally set this to `0.20-1.00`, but it will depend on the particular leaves being measured. Select `Show Outlines`. Check `Display Results`, then click `OK`.

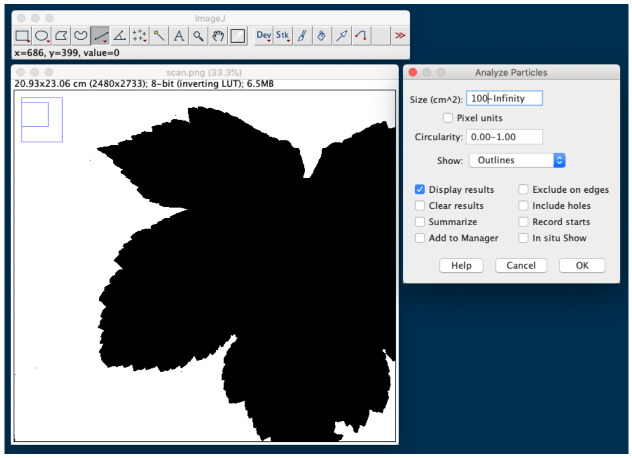

Figure 4: Example of the Analyze Particles procedure on a binarized image.

If you want to exclude certain things from the area analysis, like the petiole or the scale bar, you have two options. The first is to simply open the original image in an image editing program like Microsoft Paint or Adobe Photoshop and draw white over the objects to be excluded and use the edited image in ImageJ, ensuring that the resolution and dimensions are the same as the original image. The second is to use the `Polygon Selections` tool in ImageJ to draw a polygon around the objects of interest. Then proceed to `Analyze → Analyze Particles...`. The area calculations will only be performed inside the polygon selection.

After clicking `OK` a table is displayed which gives the area values of each black object in the image. If the image is thresholded correctly, the leaf should be by far the largest area value. If the image scale is in cm, these area values will be in cm^2. Each area value will be linked to an outlined region on the image. You can click the row in the table corresponding to the leaf and copy it, then paste into a spreadsheet.

4 Using macros

ImageJ has a built-in macro editor which can be used to write scripts to automate the process above if using scanned images with a consistent scale. Note that this method cannot be used for photos, as the scale will be different in each photo. Save the code below to a text file called `leaf_area.ijm`. Use a plain text editor to do this, not a rich text editor like Microsoft Word, and ensure that the file extension is `.ijm`, rather than `.txt`.

```
// Calculate the area of dark objects (leaves) against a white background.

// User inputs
input_path = "/Users/user/input/";
output_path = "/Users/user/output/";
dpi = 300;
min_obj_size = 0;
max_obj_size = "Infinity";
min_circ = 0.0;
max_circ = 1.0;
algorithm = "Default";
// END user inputs

list = getFileList(input_path);

for (i=0; i<(list.length); i++) {
  open(""+input_path+list[i]+"");
  file_name = getInfo("image.filename");
  px_cm = (dpi * 10) / 25.4;
  run("8-bit");
  run("Set Scale...", "distance=px_cm known=1 pixel=1 unit=cm global");
  setAutoThreshold(algorithm);
  setOption("BlackBackground", false);
  run("Convert to Mask");
  saveAs("Tiff", ""+output_path+file_name+"_binary");
  run("Analyze Particles...",
        "size=min_obj_size-max_obj_size circularity=min_circ-max_circ show=[Outlines]
    display clear");
  setOption("Display Label", true);
  saveAs("Results", ""+output_path+file_name+".csv");
  run("Clear Results");
  saveAs("Tiff", ""+output_path+file_name+"_outlines");
  image_id = getImageID();
  selectImage(image_id);
  close();
  selectWindow(""+file_name+"_binary.tif");
  close();
}
```

Code 1: ImageJ macro to calculate leaf area of many images in a directory.

Edit the script to change the `User inputs` to suit your setup. The `input_path` should point to the location of a directory on your computer which contains all the leaf images. Similarly, `output_path` should point to a directory which can be filled with thresholded images and excel files containing the results of the analysis. `min_obj_size` and `max_obj_size` define the range of object sizes to be analysed. `min_circ` and `max_circ` define the range of object circularity to be analysed. `dpi` sets the resolution of the image in DPI. The `algorithm` can also be changed. See other auto-thresholding algorithms here: `https://imagej.net/Auto_Threshold`. The script assumes that the scanned images are A4 size. If this is not the case, you will need to change the `px_cm` value to the actual ratio of image pixels per cm.

To run the macro, open ImageJ and go to `Plugins → Macros → Run...`, then select the `leaf_area.ijm` file, which you should have edited following the instructions above. Wait until the process has finished, then you should be able to extract the leaf area values from the `.csv` files created by the macro. You can use the `*_binary.tif` and `*_outlines.tif` files to check the object detection was successful.