Optical Leaf Area (version 1.0.0)

Optical Leaf Area (OLA) is a user-friendly ImageJ macro, which has utilized ImageJ/or Fiji facilities to provide a simple tool for leaf area measurement.

This macro measures the conventional (optical) leaf area. The "Optical leaf area" term has been introduced in contrast to the "Volumetric leaf area" concept, which was introduced in the preprint:

> Haghshenas, A., & Emam, Y. (2021). Accelerating leaf area measurement using a volumetric approach. bioRxiv, 2021.2007.2003.451015.
>
> https://doi.org/10.1101/2021.07.03.451015
>
> Also, the manuscript has been submitted to Plant Methods.

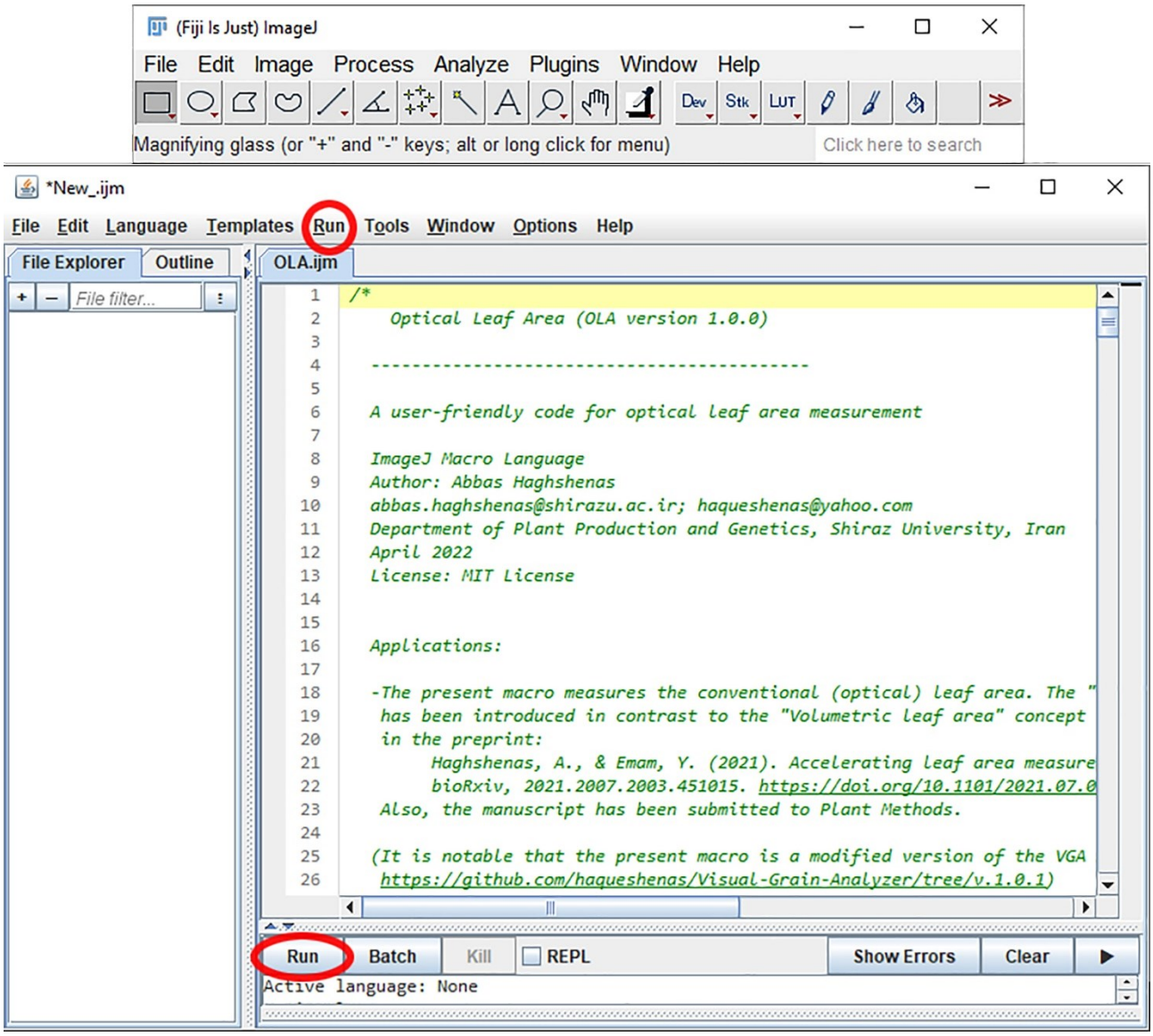
(It is notable that the present macro is a modified version of the VGA macro which is available at:

> https://github.com/haqueshenas/Visual-Grain-Analyzer/tree/v.1.0.1)

How to run?

For running this user-friendly macro, no scriptwriting or image processing skills are required. Just follow the below steps to process your own images, and extract the quantitative information:

1) Download the free and open-source Fiji (or ImageJ) software from: https://imagej.net/software/fiji/downloads
2) Create two input and output folders, and put your images in the input folder.
3) Open the OLA.ijm macro in the Fiji. For this, you can either drag & drop the file into the Fiji head, or follow: File> Open.
4) In the macro editor, click “Run” (if the Run button is hidden, you can follow Run> Run from the top bar).



5) Follow the successive pop-up dialog windows of the macro, to initiate the processing. After clicking Ok in the last window, status of processing will be displayed on the Log window. Please wait for the message: "*Processing completed successfully*".
6) Find the results in the output folder (you have determined the output path previously in the respective pop-up window).

Inputs

- RGB images

Outputs

- Single .csv files: include the quantitative results extracted from the individual images

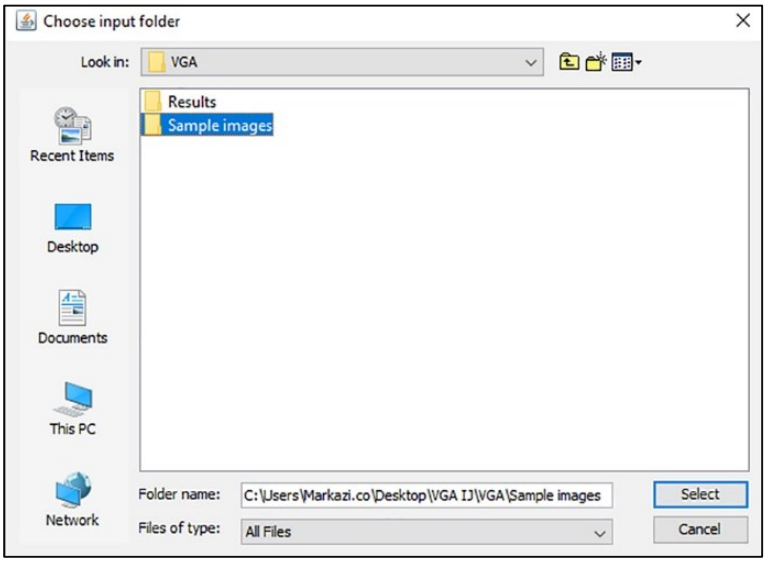
- "*Total values .csv*" file: provides the sum of the leaf area of each image (i.e. the total cumulative value of each individual .csv file).
- Drawings: represent the visual output of image processing.
- Log: general information about processing and settings are saved in this file.

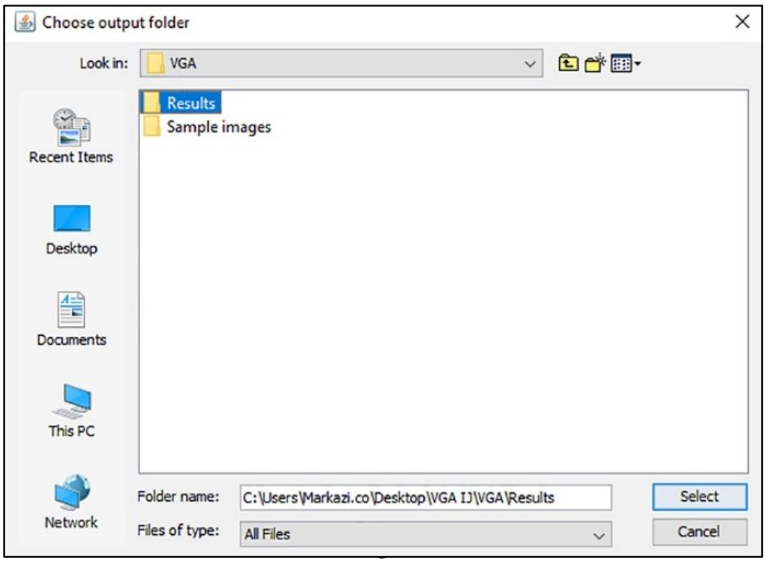
User interface & settings

The order of appearance of pop-up dialog windows is as below:

1. **Choosing the input and output folders**

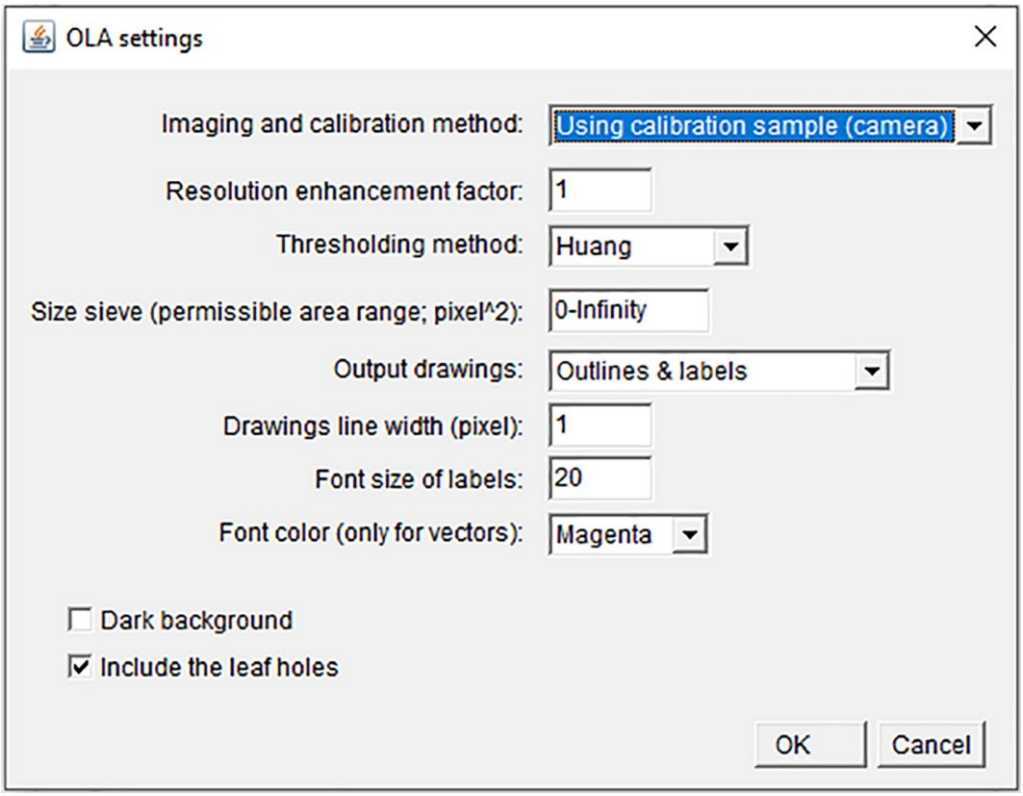
   In these two successive windows, the user is asked to determine the paths of the input and output folders (the "input" and "output" folders might have any other names).





2. OLA Settings

This window includes the main settings of the OLA macro.



Depended on the conditions and purposes of the study, user can set the options, according to the below instruction:

- **Imaging and calibration method:**

    - **Using calibration sample (camera)**
      Use this option, if the size of image pixel (or resolution) is unknown. To calibrate the size, take an image from a circular object with known diameter exactly under the light and imaging condition in which the input images have been acquired (e.g. from similar camera height). Also see Size calibration.

    - **Using known resolution (scanner)**
      In this mode, the image resolution is known, and will either be extracted from the image metadata (for TIFF format), or could be entered manually by the user in the next steps.

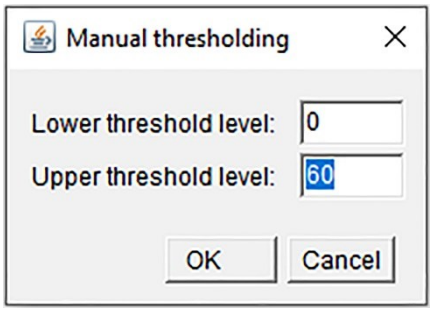
- **Resolution enhancement factor (REFactor)**

  If the input images have relatively low quality, this option may be used to improve the resolution and enlarging the images. Before the main processing, the image dimension will be enhanced using bicubic interpolation. Since both the image height and width are multiplied by the enhancement factor, the final dimension of the enlarged images will be equal to the dimension of the original image × (REFactor^2). For instance, choosing an enhancement factor of 10, would convert a 960×720-pixel image into a 9600×7200 one.

  Note that choosing higher values of resolution enhancement factors could increase the processing time, and also make large output images. Thus, it is recommended that if the input images have high quality, set the enhancement factor equal to one. Besides, this quality enhancement approach cannot be an alternative for acquisition of high-quality images.

- **Thresholding method**

  "Thresholding" is the most important process for distinguishing the leaves (objects) from background, and segmenting the image into these two parts (i.e. to create binary images). In general, this step is carried out by determining the lower and upper light intensity thresholds of the pixels which will be assigned to the objects (vs. background). Since the purpose of OLA is batch processing (i.e. a set of images will be analyzed unsupervised), and the result of image segmentation using fix thresholds may vary from image to image, logically it is recommended to use an appropriate auto-thresholding method for the whole image set. This part on the dialog box, provides a list of ImageJ built-in auto thresholding methods, which could be used depended on the imaging condition. Before running the macro, a pre-test using one to several sample images could determine the best thresholding methods. In the cases where the fix lower and upper thresholds should be used for image segmentation, the last choice of the list (i.e. "Manual") can be selected. Then, in the next steps, a new window will be opened and ask the user to enter the lower and upper thresholds manually (the values should be integers between 0 to 255):



If you need more help to select the best auto thresholding method, this simple instruction may be helpful:

a) Open one of your images with Fiji (File>Open).
b) Follow Image>Type>8-bit.
c) Then follow Image>Adjust>Auto threshold.
d) In the window of Auto threshold, select "*Try all*" from the method list. Depended on your image, select or deselect the "*White objects on black background*". Click Ok.
e) A montage including the outputs of various available auto thresholding methods will be appeared. Evaluate the results, and choose the best thresholding method (title of the methods can be found below each binary image. Use the *Magnifying glass* and/or *Scrolling tool* which are available in the main Fiji toolbar, if necessary).
f) Close the sample image and windows. Then, re-run the OLA macro using the selected thresholding method.

- **Virtual size sieve:**

In order to (a) remove unwanted noises, objects or reflections from background, virtual size sieve has been added into the setting window. By default, permissible ranges of size (area) has been set to "0-Infinity" pixel^2. If the size range, for instance, has been set to 10-Infinity, the particles with an area less than 10 (squared) pixels will be excluded from the analyses.

- **Output drawings**

  Two types of output drawings have been provided in the OLA macro. By default, the drawings option has been set to "*Outlines & labels*", which creates simple and low-size output images. The "*Overlay, outlines, & labels*" output also draw a type of vectors on the grayscale output image. The vectorized drawing of such output is only visible in the Fiji/ImageJ (so open it in Fiji either by drag & drop, or using File > Open). Besides, since this type of output is saved in TIFF format, large-size output images would be produced, particularly if the input images have high quality, or high values of resolution enhancement factors have been used.

  In the next two fields, user can set the line width of drawings, and font size of labels (only applicable in the "*Outlines & labels*" outputs), and also font color of the labels (only in the vectorized output).

- **Dark background**

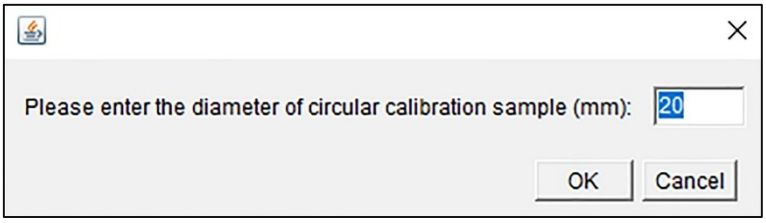
  Checkmark this option if the background of the input images is dark.

- **Include the leaf holes**

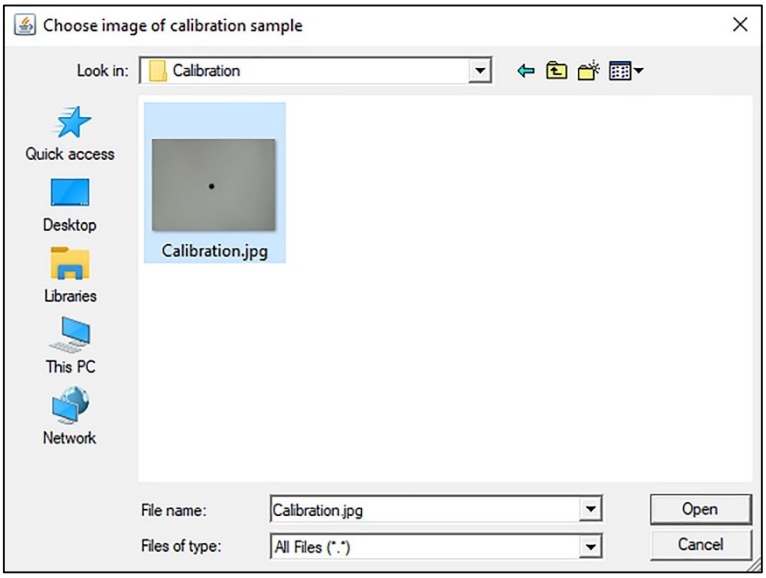
  Choosing this option will include the inner enclosed pixels (holes) of the objects, in the analyses, despite that their grayscale values are more similar to background.

3. Calibration settings

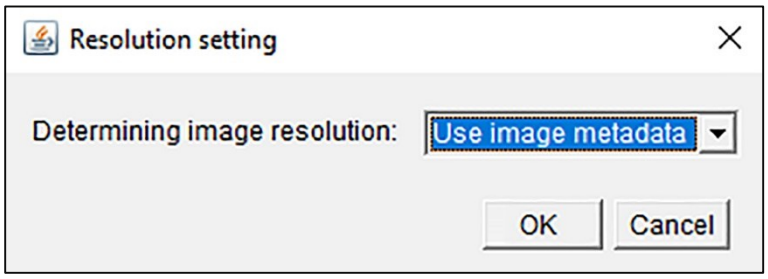
Following the "*OLA settings*" window, user will be asked to set the size calibration, according to the chosen imaging mode. If the "*Using calibration sample (camera)*" mode is selected, the diameter (mm) of the circular calibration sample should be entered manually.

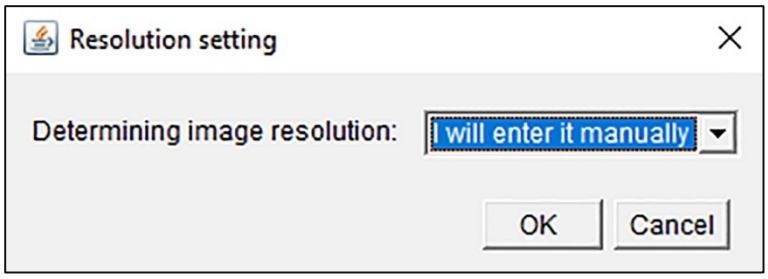


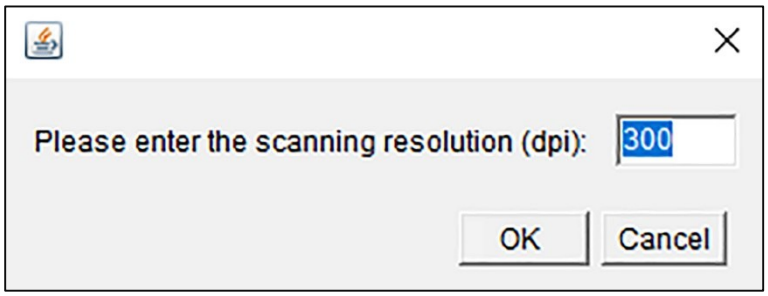
Thereafter, the image of calibration sample should be chosen:



In the case of choosing "*Using known resolution (scanner)*" mode, user will be asked to determine the image resolution (dpi) either using image metadata (if available, e.g. in TIFF formats), or by entering the value manually.







List of measurers in the output .csv files

In the individual result files, various types of data for each leaf is recorded in a row. The dataset includes:

- Regular output of ImageJ particle analysis, including:
  - General information such as labels and number of object,
  - Mean grayscale color value,
  - Size indices (based on pixel), e.g. area, perimeter, Feret and Minimum Feret diameters,
  - Shape indices, e.g. Circularity, AR, Solidity, Roundness, etc.

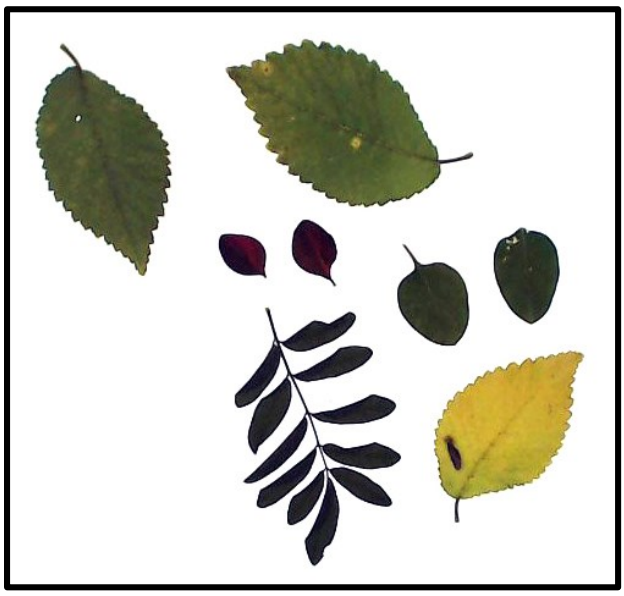
  For the complete list and definitions of the measurers provided by ImageJ particle analysis, see: https://imagej.nih.gov/ij/docs/menus/analyze.html#set

- Leaf size and dimensions based on mm or mm^2, including:
  - Area (mm^2)
  - Perimeter (mm)
  - Feret and MinF diameters (mm)

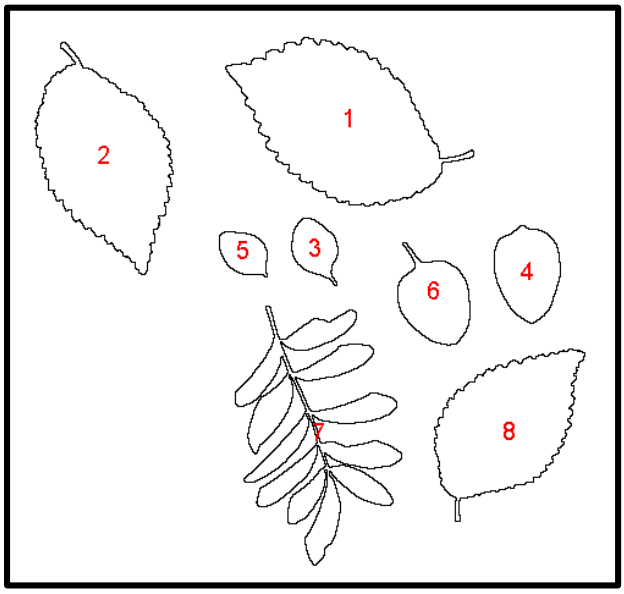
Note:

- Although the grayscale color measurers have been provided here, they may be not reliable for colorimetry and reporting the absolute values, unless standard image acquisition tools, conditions, and procedures have been used.

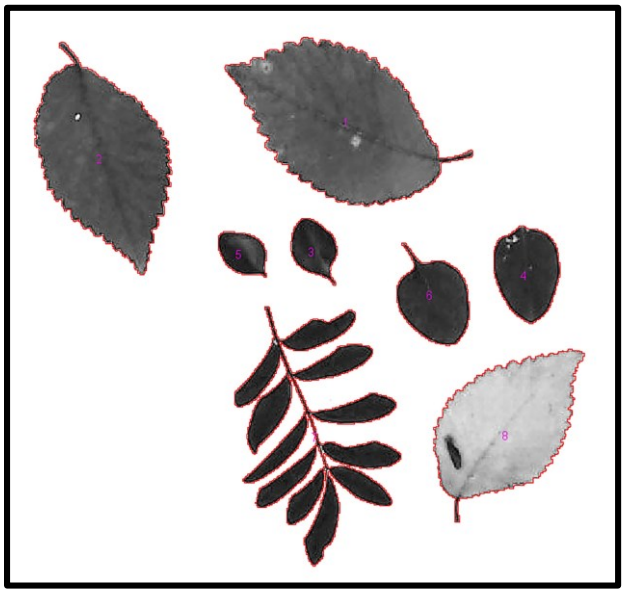
Samples of output drawings



Original image



Outlines & labels



Overlay, outlines, & labels

- As noted before, drawings of the last type (i.e. "*Overlay, outlines, & labels*") would be visible only if opened in the Fiji/ImageJ. Furthermore, as this type is saved in TIFF format, it may create a comparatively large file.

Brief instruction for image acquisition and size calibration

- Image acquisition:

Irrespective that camera or scanner is used for image acquisition, the imaging for scientific approaches should provide high quality and standard images. Please consider below brief points:

  - As a whole, all samples should be imaged under similar conditions e.g. using same models of camera/ or scanner, camera height, device settings, scanner resolution, etc.
  - In order to facilitate image segmentation and leaf (object) recognition, high contrast between foreground and background is required. Therefore, background should be an even surface with a different color with the objects, and free of unwanted reflections. Also, it must hide the shadow of the object, to avoid the interference of shadow in image processing (object recognition). For this purpose, usually two kinds of backgrounds have been used: (i) a flicker-free illuminated surface (non-point light) behind/ or under the object; (ii) a dark matte surface (if such background is used, the "*Dark background*" option should be check marked in the OLA settings window).
  - Although utilizing the uncompressed formats (such as TIFF) may create large files, in scientific image processing approaches they are preferred to the compressed formats (e.g. JPEG); because they provide higher quality and information.

- Size calibration:
  - For leaf area measurement using OLA macro, if the pixel size is unknown (i.e. camera mode), size calibration can be carried out using a circular object with a known diameter. Put the sample on the imaging surface/table, and take an image exactly under the same condition in which the leaf (or desired objects) have been imaged. For instance, the calibration sample provided in the OLA code package is a piece of circular metal with 20 mm diameter, which has been imaged from the 43.5-cm height (the sample images were also taken from this height). The calibration sample might have any other custom diameter, provided that its value has been entered in the respective dialog window (appears following the OLA settings window). As a readily available choice, a lithium coin type battery (i.e. CR series) could be used as the calibration sample (depended on the imaging condition, the potential problem of

light reflection must be solved). For example, based on our measurements (carried out using a ASIMETO 0.01 mm micrometer), the diameter of the 3v CR lithium batteries of several brands were constant, and equal to 20.45 mm in the room temperature (their thickness may vary).

Software update

It is recommendable to keep the Fiji/ImageJ software up to date. For this purpose, type “Update” in the search field, select your update choice from the left panel, and click “Run”: