VGA

Visual Grain Analyzer

Visual Grain Analyzer (version 1.0)

Visual Grain Analyzer (VGA) is a user-friendly ImageJ macro, which has utilized ImageJ/or Fiji facilities to provide a simple tool for grain analysis, seed technology, and phenomics studies.

This macro:

A) Simulates the processing and calculations reported in the manuscript:
Haghshenas, A., Emam, Y., & Jafarizadeh, S. (2021). Wheat grain width: A clue for re-exploring visual indicators of grain weight. bioRxiv, 2021.2010.2013.464205. https://doi.org/10.1101/2021.10.13.464205

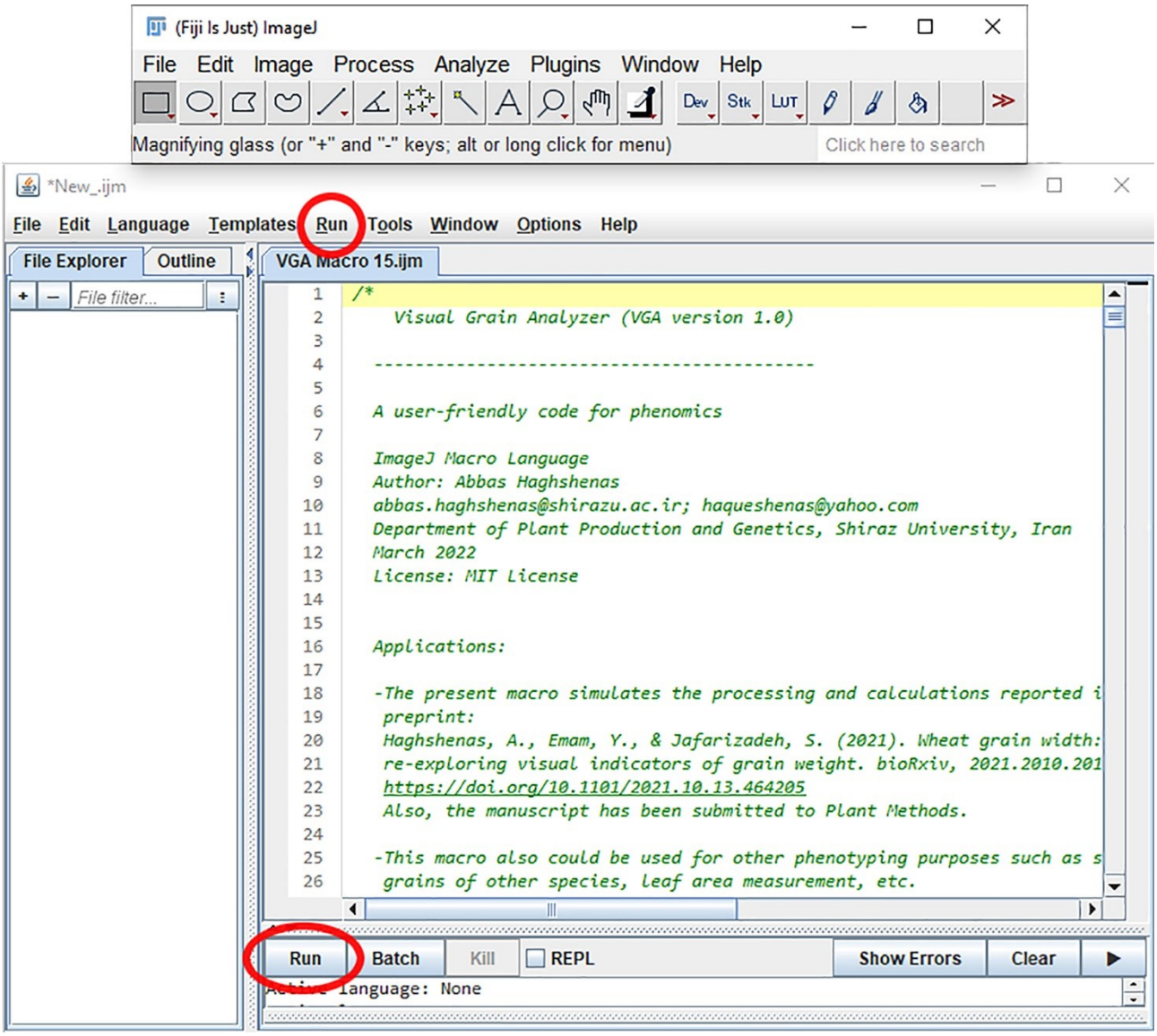
In particular, as reported in the paper, VGA provides various **estimations of wheat grain weight**, based on the image-derived indices.

B) Also, VGA might be used for other phenotyping purposes such as size & shape analyses of grains of other species, leaf area measurement, etc.

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 VGA.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 mean values .csv*" file: provides the mean values of all individual .csv files.

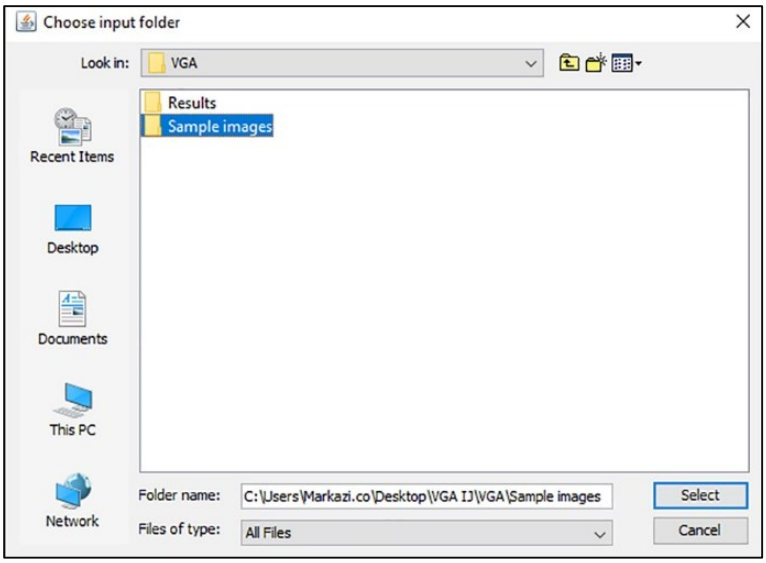
- Drawings: various types of drawings represent the visual output of image processing, including segmentation, ellipse fitting, etc.
- Log: general information about processing and settings are saved in this file.

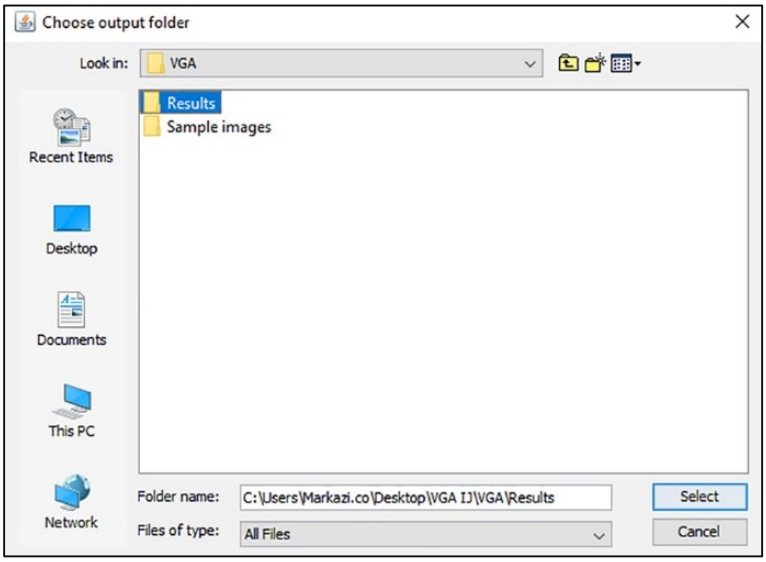
User interface & settings

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

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

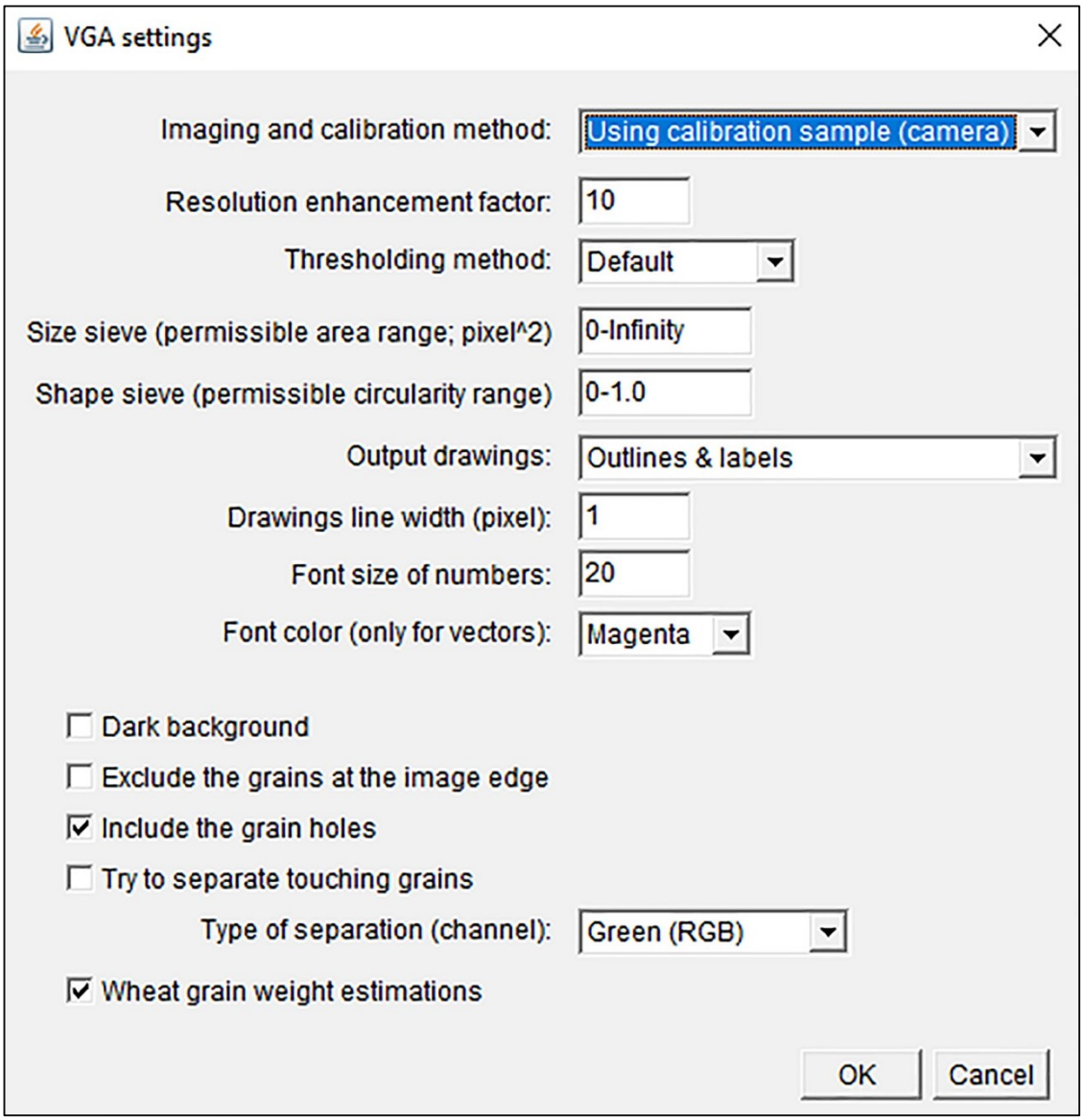
   In these two successive windows, the user is asked to determine the paths of the input and output folders (the “input” and “output” folders might have any other names).





2. VGA Settings

This window includes the main settings of the VGA 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)**

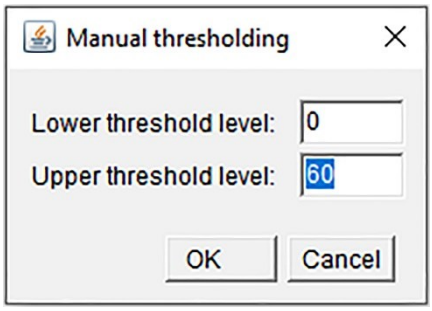
  If the input images have relatively low quality, this option may be used to improve the resolution and enlarging the images. Before the main processing, the image dimension will be enhanced using bicubic interpolation. Since both the image height and width are multiplied by the enhancement factor, the 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 grains (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 VGA 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 VGA macro using the selected thresholding method.

Note: The “Default” thresholding method is an exclusive version of IsoData thresholding, named “IJ-IsoData” (i.e. ImageJ-IsoData).

- **Virtual size and shape sieves:**

  In order to (a) remove unwanted noises or reflections from background, or impurities from the grain mass, and also (b) classify the grain/seed mass, virtual size and shape sieves have been added into the setting window. Here, the degree of grain circularity has been utilized as the shape criteria for sieving. By default, permissible ranges of size (area) and shape have been set to "0-Infinity" pixel^2, and "0-1", respectively. 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. For shape sieve, it is notable that the circularity of the elongated objects tends to zero, while this value approaches 1, in complete circle.
  Note: Although the theoretical range of circularity is between 0 to 1, sometimes in practice, the values might exceed 1 (e.g. for tiny object). Therefore, the user might set the upper circularity limit to values higher than one, to prevent unwanted data losing.

- **Output drawings**

  Various types of output drawings have been provided in the VGA macro. By default, the drawings option has been set to "*Outlines & labels*", which creates simple and low-size output images. Among the list, two choices i.e. "*Overlay, outlines, & labels*", and "*Overlay, outlines, labels, & ellipses*" also draw different types of vectors on the grayscale output image. The vectorized drawings of such outputs are only visible in the Fiji/ImageJ (so open them in Fiji either by drag & drop, or using File > Open). Besides, since these two output types are 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 non-vector outputs), and also font color of the labels (only in the vectorized outputs).

- **Dark background**

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

- **Exclude the grains at the image edge**

  If this option is activated, the incomplete grains at the image edge will be ignored and filtered out from the analyses.

- **Include the grain 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.

- **Try to separate touching grains**

  Although in the ideal imaging and grain sampling, there should be no touch among the adjacent grains (e.g. by using a vacuum seed sampler/ or counter), it could be a time-consuming practice and is not always possible. If this check box is marked, the ImageJ built-in watershed technique will be applied to separate the touching grains.

  - **Type of separation (channel)**

    This sub-choice will be activated only if the "*Try to separate touching grains*" is check marked. Try to choose the color channel which provides a comparatively higher contrast between the grains and background. A pre-test using one to several images could determine the best channel for applying the watershed technique and grain separation. All other steps of image processing will be also limited to this channel.

  Note:

  - If still some exceptions of touching grains have remained after separation, the virtual sieves could be utilized to filter out the unusual large size grains (check the .csv result files).
  - In some cases, separation of the touching grains might change the real shape of grains. Furthermore, since in watershed technique, the input images will be binarized, the grayscale quantities are not valid in the .csv result file (e.g. values of *mean*, *min*, *StdDev*, *max*, etc. will be either recorded as 0 or 255).
  - An appropriate separation may require testing the interactions between resolution enhancement and the utilized channels (i.e.

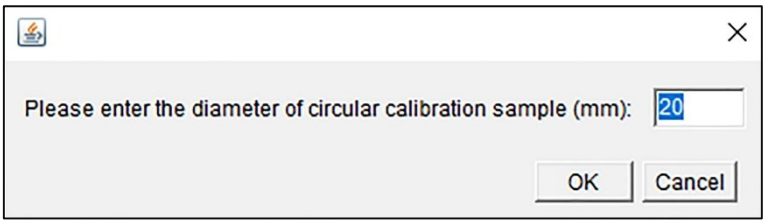
separation type). So, carrying out some pre-tests may be helpful. For instance, test the sample images (of touching grains) provided with the code package using the resolution enhancement factor of 3 and the green channel.

- **Wheat grain weight estimations**

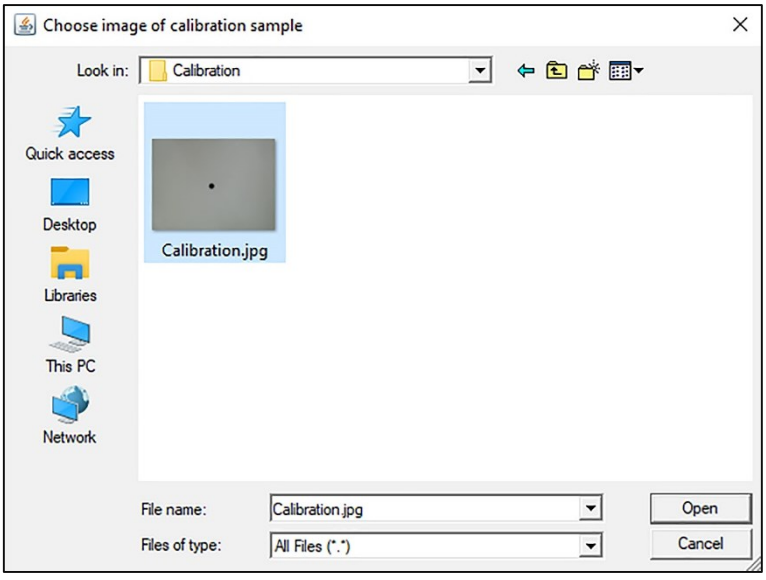
  Wheat grain weights will be predicted using the image-derived indices, if this option is activated (for more information, see the paper).

3. Calibration settings

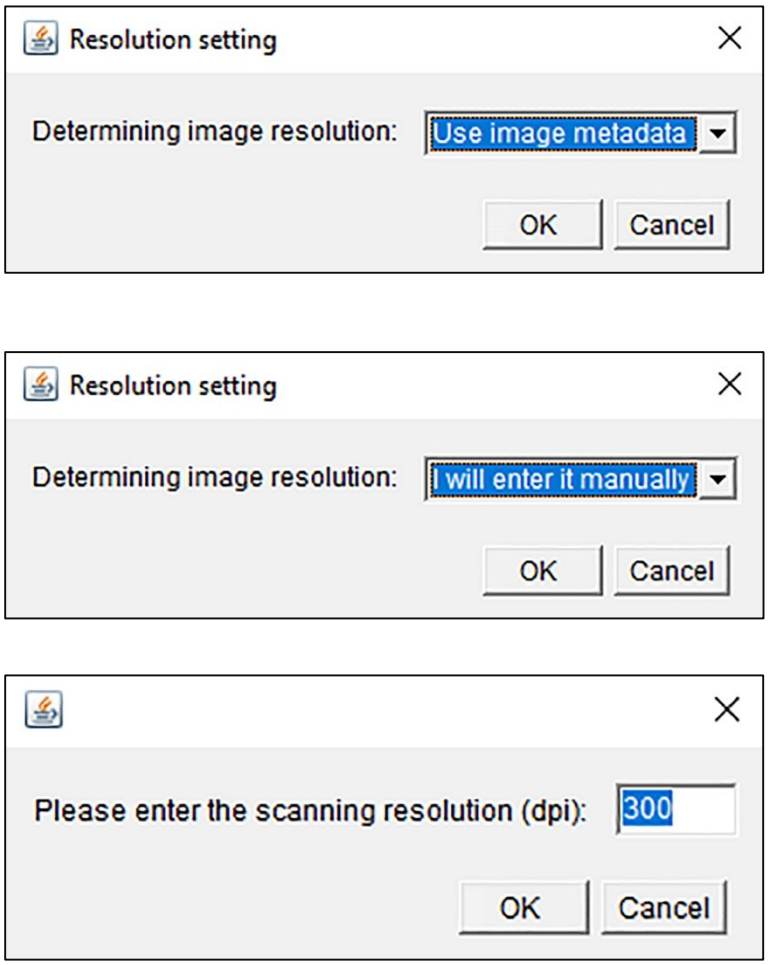
Following the "*VGA settings*" window, user will be asked to set the size calibration, according to the chosen imaging mode. If the "*Using calibration sample (camera)*" mode is selected, the diameter (mm) of the circular calibration sample should be entered manually.



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 grain is recorded in a row. The dataset includes:

- Regular output of ImageJ particle analysis, including:
  - General information such as number of grains, labels, location of grains in the image (X & Y), etc.
  - Grayscale color measurers, e.g. Mean, StdDev, Mode, Min, Max, etc.
  - Size indices (based on pixel), e.g. area, perimeter, major & minor axes of the best fitted ellipse, Feret and Minimum Feret diameters, etc.
  - 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

- Empirical synthetized image-derived indices used in wheat grain weight estimations (based on pixel; for definitions, see the paper).

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

If the option of "*Wheat grain weight estimations*" has been activated in the VGA settings window, the below cases will be added into the result file:

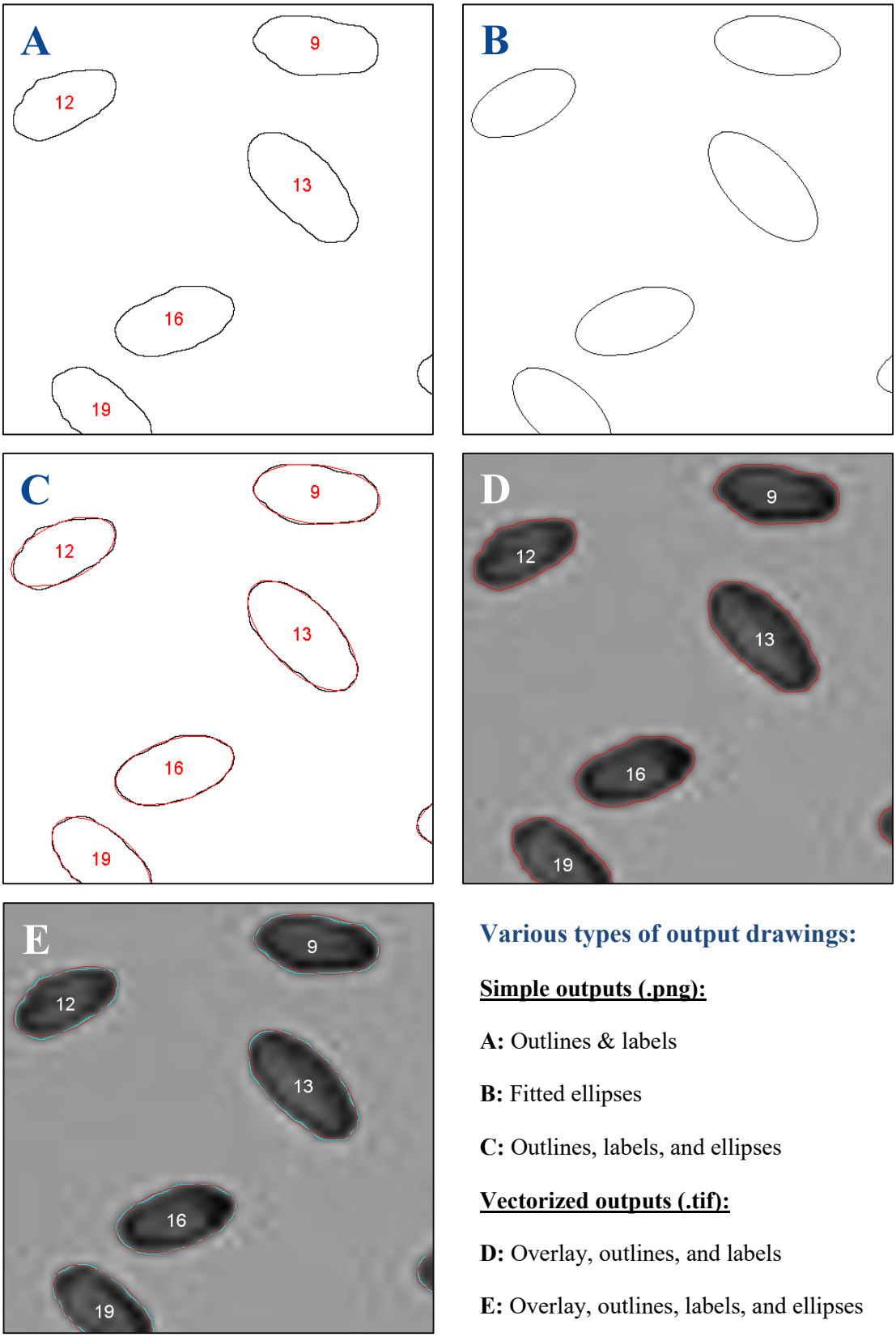
- Empirical synthetized image-derived indices used for wheat grain weight estimations (calculated after size conversion to mm; for more information, see the paper).
- Results of wheat grain weight estimations using 12 linear models (here, weight unit is mg; for more information, see the paper).

Note:

- *Width* and *Height* should not be mistaken with the grain (particle) width and length. Here, these two indices (*Width* & *Height*) are only the width and height of the horizontal peripheral rectangle of each grain; so they may change by rotation of the grain. For measuring the real width & length of the grains, use Feret & MinF diameters, or Major and Minor (axes of the fitted ellipse; see the paper).

- 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



Various types of output drawings:

Simple outputs (.png):

A: Outlines & labels

B: Fitted ellipses

C: Outlines, labels, and ellipses

Vectorized outputs (.tif):

D: Overlay, outlines, and labels

E: Overlay, outlines, labels, and ellipses

- As noted before, drawings of types D and E would be visible only if opened in the Fiji/ImageJ. Furthermore, as these two types are saved in TIFF format, they may create comparatively large files.

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 grain (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 (for instance, see the paper of the current study); (ii) a dark matte surface (if such background is used, the "*Dark background*" option should be check marked in the VGA 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 grain analyzing using VGA 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 grain mass (or desired objects) have been imaged. For instance, the calibration sample provided in the VGA 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 VGA 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”: