RRQuant: High-throughput quantification of seedling's epidermal integrity

Protocol

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Overview

We provide a workflow to quantify and compare epidermal defects in hypocotyls of *Arabidopsis thaliana* seedlings. This protocol is based on ruthenium red (RR) staining dye penetration assay and optimized to be performed on dark-grown Arabidopsis seedlings imaged with an automated stereomicroscope. The workflow then relies on ImageJ macros, the use of the deep learning segmentation software RootPainter (Smith *et al.*, 2022), and R scripts and shiny apps, to process, segment and quantify images, yielding publication-ready quantitative comparison with statistical tests.

Quick steps overview:

1) Seedling growth in the dark
2) RR staining and imaging plate preparation
3) Large RGB tile scan acquisition with a stereomicroscope
4) Image splitting based on genotype and/or condition
5) Hypocotyl segmentation using RootPainter
6) Mask conversion and correction using Fiji
7) Quantification by running RRquant in Fiji
8) Data analysis and visualization using R shiny app

Materials and reagents

Biological material

1. *Arabidopsis thaliana* L. seeds

Reagents

1. EtOH (Ethanol)
2. Sodium hypochlorite (NaClO) in water solution (VWR Chemicals, catalogue number: 27900.365)
3. Sterile dH_2O (distilled water)
4. Murashige & Skoog (MS) medium including vitamins (Duchefa Biochemie BV, catalogue number: M0222)
5. Plant agar (Duchefa Biochemie BV, catalogue number: P1001)
6. MES monohydrate ($C_6H_{13}NO_4S.H_2O$) (Duchefa Biochemie BV, catalogue number: M1503)
7. KOH (potassium hydroxyde) (VWR Chemicals, catalogue number: 1.05029.1000)
8. Agarose (Fisher BioReagents™, catalogue number: 10366603)
9. Ruthenium red (Sigma, catalogue number: 00541)

Solutions

1. ½ MS plates
2. 1% agarose plates
3. Ruthenium red staining solution

Recipes

Half-strength MS medium, 1% plant agar:

Store at 4°C for < 1 month. Adjust the pH to 5,7 using 1M KOH. Add 10g of plant agar prior to autoclaving.

Reagent	Final concentration	Amount
MS medium including vitamins	½-strength	2,2g
MES	0,05% (w/v)	0,05g

dH2O	n/a	Top up to 1L
Total	n/a	1L

Note that other growth media can be used alternatively.

Agarose plates:

Store at 4°C for < 1 month.

Reagent	Final concentration	Amount
Agarose	1% (w/v)	1g
dH2O	n/a	Top up to 1L
Total	n/a	1L

Ruthenium red staining solution:

Store at 4°C in the dark for < 2 months. Do not use if the solution changes color (can become black).

Reagent	Final concentration	Amount
Ruthenium red	0,05% (w/v)	5mg
dH2O	n/a	Top up to 10mL
Total	n/a	10mL

Laboratory supplies

1. Square plates
2. 6/12-well plates
3. 50mL conical tubes
4. 1L glass bottle that can be autoclaved
5. Graduated cylinder
6. Various sizes of pipette tips.

Equipment

1. Autoclave
2. Forceps
3. Stir plate
4. pH meter
5. Stereomicroscope

Software and datasets

- Fiji https://fiji.sc/

1. **MorphoLibJ plugin** ("IJBP-Plugins" update site, (Legland, Arganda-Carreras and Andrey, 2016))
    - To install the plugin MorphoLibJ, turn on the IJBP-Plugins update site:
    - Go in the "Help" menu of Fiji and click on "Update...". This will open the "ImageJ updater".
    - In "ImageJ updater", click on "Manage update sites".
    - In the list, find e.g. "IJPB-Plugins" and click in the square next to it to add it.
    - Then close the window and apply changes on "ImageJ updater".
    - Restart Fiji.
    - If more explanation needed, see https://imagej.net/update-sites/following

2. **RRQuant** macros: available to download: https://github.com/VergerLab/RRQuant/
    - Copy the RRQuant_Workflow_toolset.ijm file to your Fiji "macros/Toolset folder".
    - Access the tools from the right end side of Fiji window ">>" (More tools).
    - SplitLargeImage.ijm, MaskConvert.ijm and RRQuant.ijm

- Rootpainter https://github.com/Abe404/root_painter/

Follow instructions for installation and use as provided with Rootpainter (Smith *et al.*, 2022).
Brief overview:

- Create a virtual environment (cuda, mamba) and install the server, called "trainer" (pre-requisite: suitable GPU and CUDA installed) locally or on a remote machine.
- Run the trainer. The first time, it will create a sync directory.
- Install the client, called "painter"
- Directly segment the target image directory, or create a project based on a specific model, from "RRQuant_DarkHypo_RPWeight_V2.pkl"

- RStudio https://posit.co/download/rstudio-desktop/

Install the following libraries:

- data.table
- readr
- stringr
- tidyverse
- shiny
- plotly
- bslib
- sortable

It can also be done automatically by running the Install_R_packages.R script in the console of RStudio:

- copy/paste the code into the console and press crtl-Enter
- the script checks if the packages are already installed and install only the ones missing

All R scripts needed are available to download https://github.com/VergerLab/RRQuant/ :

- RRQuant_data-table.R is used to combine and organize the data issued from the Fiji macro.
- RRQuant_app.R contains the shiny app to visualize and analyse the data.

Double click on the file name to open it automatically with RStudio.

Procedure

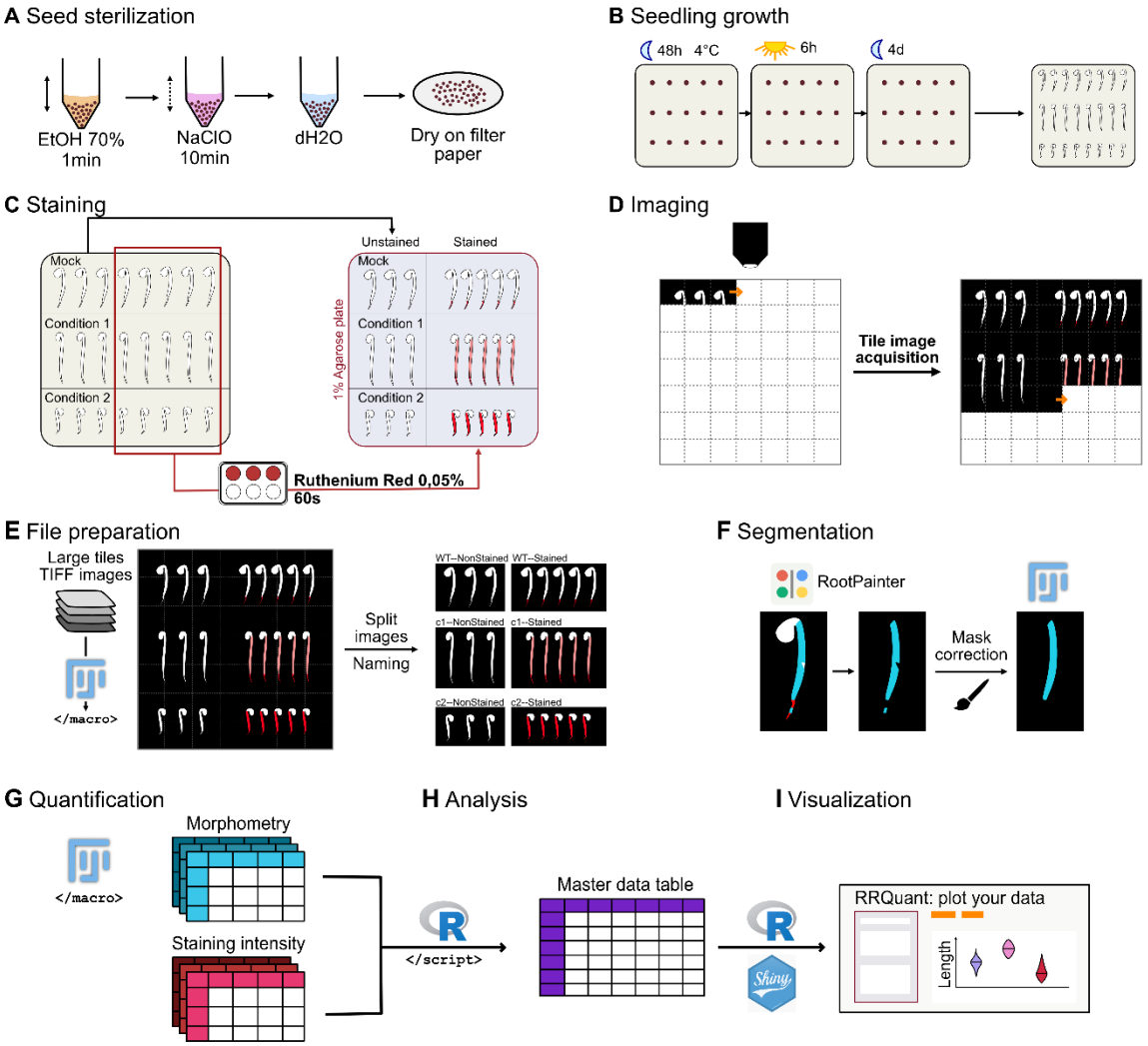

Figure 1: RRQuant workflow

A Seed sterilization: seeds are incubated for 1min in EtOH 70% with constant tube agitation. EtOH is removed and replaced by NaClO for 10min with occasional tube agitation. Seeds are then washed five times with sterile dH2O and let to dry on a filter paper.

B Seeds sowing and seedling growth: after sowing the seeds on growth medium agar plates, the plates are put 48h at 4°C for vernalization. The plates are flashed for 6h to start the growth. Plates are placed in the dark in growth chamber for four days.

C Staining: seedlings are dipped for one minute into Ruthenium Red 0,05% solution in a 6 or 12 well-plate and gently placed on an agarose plate. Some of the seedling should not be stained and directly put on the agarose plate (NonStained).

D Imaging: the agarose plate is entirely imaged using a stereomicroscope with darkfield. All tiles are stitched to form a large image.

E File preparation: images are converted to TIFF. The first step of the Fiji macro is to split large images to have each condition on one image. At this step, the files are also renamed using specific rules (detailed in the macro and the user-guide).

F Segmentation: a trained model of RootPainter segments hypocotyls. The obtained masks can be corrected in Fiji.

G Quantification: an ImageJ macro measures various parameters using the original image and the mask (e.g.: hypocotyl length, staining intensity…).

H Analysis: a first R script compiles and organizes data of all samples.

I Visualization: a Shiny app is used to plot the results.

A. Seedling growth, staining, imaging

I- Seed sterilization

Sterilization takes place under a laminar flow hood. Other seed sterilization methods can be used. (Figure 1A)

1. Put a certain aliquot of seeds in a 1,5 mL Eppendorf tubes.
2. Add 500 µL of 70% EtOH in each tube and invert for 1 minute.
3. Remove EtOH, add 500 µL of 14-15% NaClO and invert the tubes occasionally for 10 minutes.
4. Remove NaClO and wash the seeds 4-5 times with 500 µL of sterile dH_2O.
5. Put the seeds on a sterile filter paper to dry out before sowing them on plates.

The exact seed preparation and growth conditions described here are an example of typical growth conditions that can be used for this protocol, but any other sterilization methods, growth medium and growth conditions can be used as long as it yields etiolated seedlings for the subsequent staining and imaging steps.

II- Growth conditions

The workflow is meant to be used on dark-grown seedlings (Figure 1B).

1. After sterilization, plate at least 30 seeds per genotype/condition/replicate, in rows on a square Petri-dish containing half-strength MS medium (see Recipes). The seeds should be plated with a distance to allow manipulation of single seedlings.
2. Seal the plates with micropore tape and then cover the plates with two layers of aluminium foil. Put the plates horizontally at 4°C for 48 hours of stratification and vernalization.
3. To enable the growth, light induction is necessary. Uncover the plates and transfer to the growth room (22°C with light) for 6 hours.
4. After induction, the seedlings can be grown in the dark. Plates are covered again with 2-3 layers of aluminium foil and grown vertically for another 4 days in the same growth room. After 4 days, plates are then opened under green light for staining and imaging as described below.

III- Ruthenium Red staining

I – Sample preparation

1. Plates used for imaging the seedlings are prepared in the following way: 50 mL of 1% agarose solution is added to each square Petri plate that will be used for imaging.
2. About 20 seedlings are carefully transferred to a well (from 6/12 well plates) to which 1 mL of 0,05% of Ruthenium red dye is added. Then they are stained for 60 seconds (Figure 1C). In this step make sure to grab the seedlings by the roots to avoid damaging the hypocotyl.
3. After staining, the seedlings are washed twice with dH_2O and transferred to the agarose plate.
4. A few of the non-stained seedlings are also directly transferred to the agarose plates as a control (4-5 per genotype/condition; Figure 1C).
5. Samples or genotypes are named accordingly with a marker pen.

II- Imaging

1. Depending on microscope available: Acquire RGB darkfield images of the samples with a stereomicroscope (Figure 1D, Note 2). Adjust illumination, exposure and gain to reach the best image quality possible. Aim for good light and color contrast and avoid background saturation.

Ideally use an automated tiling function with at least 10% to take multiple tile images (approximately 90-110) which are merged into a bigger image for high-throughput imaging.

2. Images are finally exported as TIFF for further processing in ImageJ (Note 3).

B. Data analysis

We developed a series of ImageJ macros bundled into a toolset that can be used directly through the ImageJ graphical interface, for different steps of the workflow. In addition, Rootpainter is used separately for image segmentation.

I – Image splitting: SplitLargeImage.ijm

Starting from large tile stereo microscope RGB images in TIFF format, of ruthenium red stained samples: (Note 3)

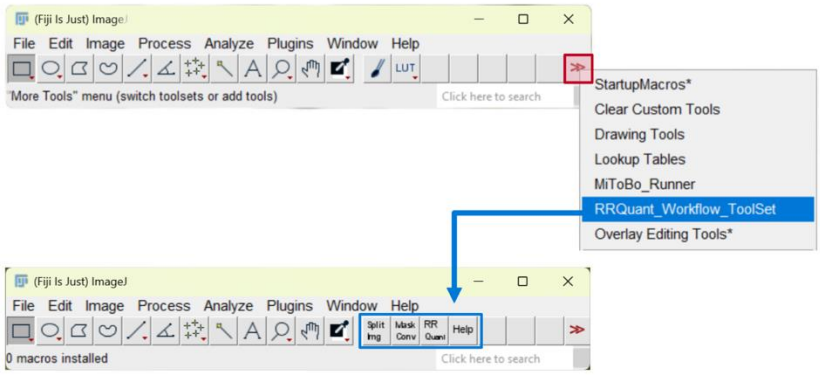

Figure 2: Start RRQuant Tool set
Click on the double arrow to find in the list RRQuant toolset. The different tools are added to the main Fiji interface.

1- Start Fiji and open the large image by drag and drop the file on Fiji user-interface.
2- Check that images are RGB with all channels merged.
3- Split large images per genotype/treatment, stained/non-stained, replicates (Figures 1E,3 SplitLargeImage.ijm in the Fiji macro).
    a. Click on the first button of the RRQuant toolset: Split Img (red in Figure 3A)
    b. A pop-up window opens. Select the folder where to save the split images (Figure 3A).
    c. Follow instructions given to select an area to crop (Figure 3B): select a region containing all seedlings for a condition with the rectangular selection tool.
    d. Name the image according to genotype/treatment, stained/non-stained, replicates (Figure 3C). The name should follow the rules described:
        - Do not leave spaces in the names, use _ instead.
        - Do not use special characters
        - Do not use -- (in other places than expected)
    e. Repeat steps a and b until all the samples have been treated by choosing the right message in the "More?" window (Figure 3D-E).
    f. Close Fiji

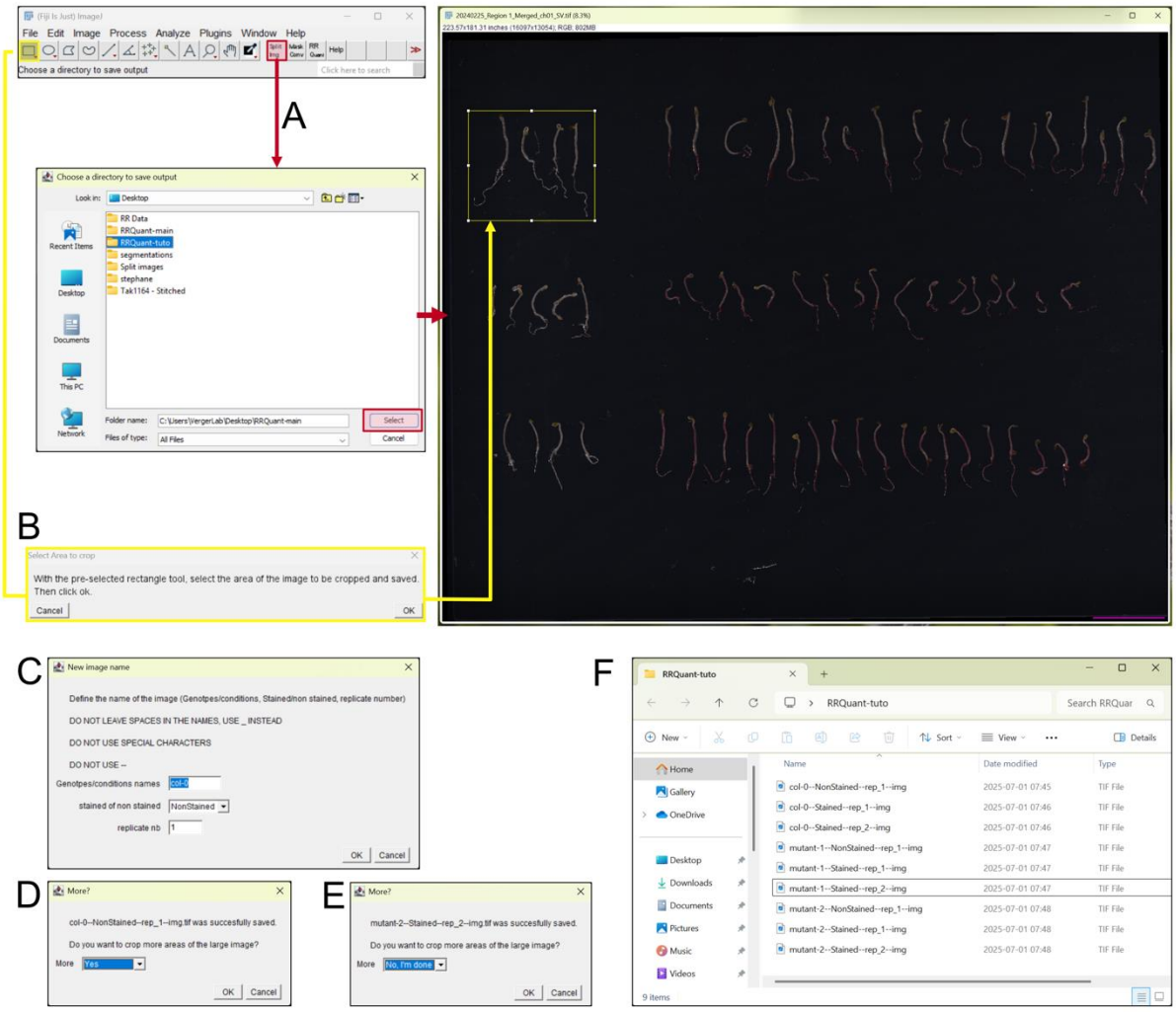

Figure 3: Split images with ImageJ macro
A Starting the macro. Choose an output directory.
B Instructions to split the large image.
C Naming of the cropped image with rules to follow.
D-E Choosing the next step: continue to select areas to crop or all has been cropped.
F Output directory containing the split images.

II – Hypocotyl segmentation: RootPainter

Run segmentation with RootPainter using either our pre-rained model (RRQuant_DarkHypo_RPWeight_V2.pkl), or another custom model of choice (Figure 1F,4):

1- Start RootPainter software (Figure 4A)
2- In the Ubuntu terminal, activate the virtual environment for RootPainter (here called RP, orange box in Figure 4) and start the trainer. A window opens.
3- Click on Network > Segment folder and select the input folder that contains the images to segment, the output folder to save the masks and the trained model (Figure 4C).
4- This will generate segmented images as PNG files (in blue in Figure 4D)

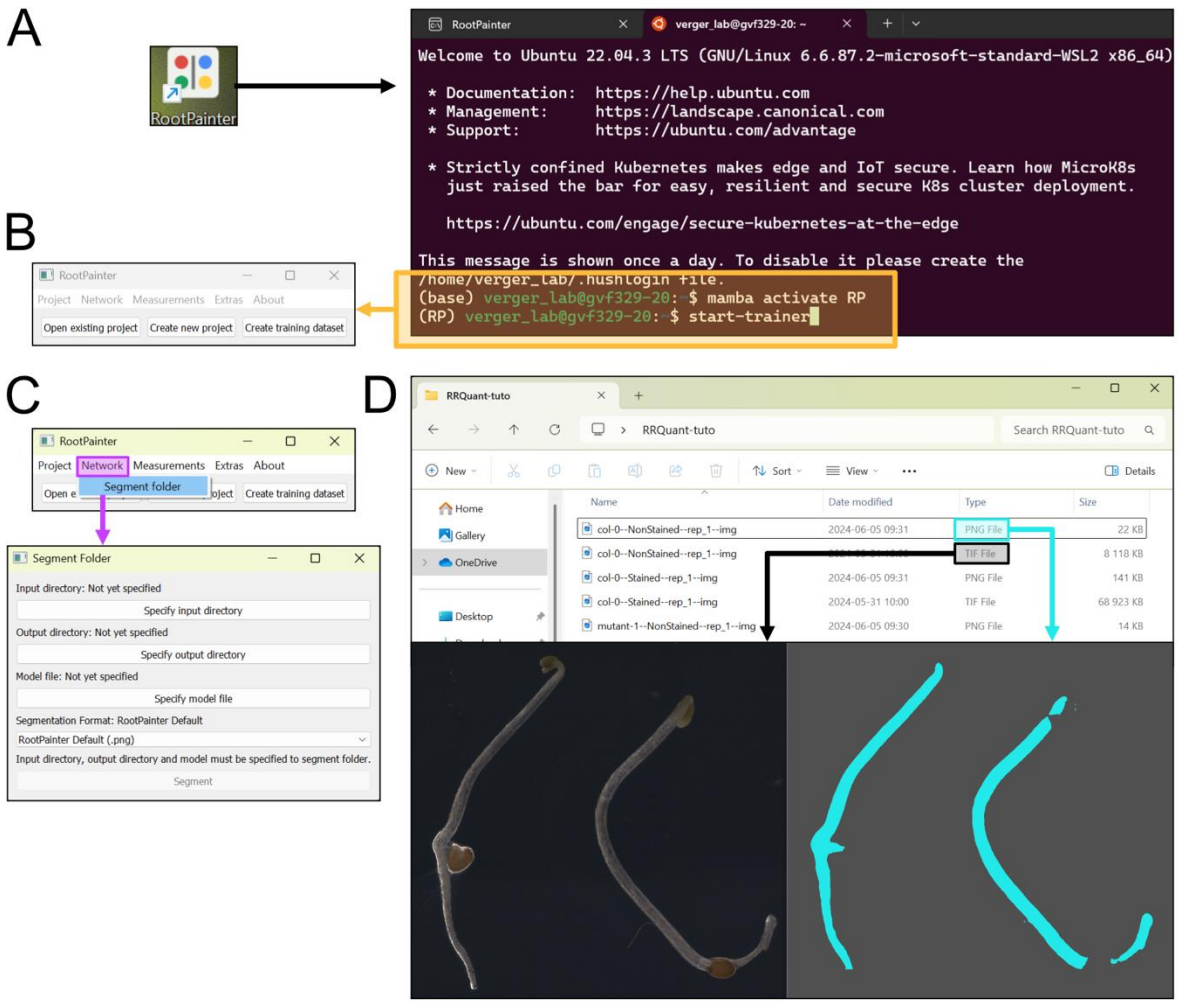

Figure 4: Hypocotyl segmentation with RootPainter
A Start RootPainter and activate the trainer in the terminal.
B RootPainter interface
C Select output and int-put directories and the trained model for segmentation.
D Example of a segmentation result.

III – Mask conversion and correction

To be processed by our workflow, the segmented images need to be converted in binary masks. In this process masks can be manually corrected if needed (Figure 1F, MaskConvert.ijm):

1- Open Fiji and click on Mask Conv button (in blue in Figure 5A).
2- Select the folder containing all microscopy images and segmented result from RootPainter.
3- Correct manually the mask if necessary. Follow the instruction given by the pipeline (Figure 5C):
    a. Select the color picker tool (green in the figure) and select the background, it will make the brush black to erase wrong segmented pattern (e.g.: in red in the figure). Select the segmented object to have the brush white and add surface to the segmented object.
    b. Draw on the image to remove/add on the segmentation image.
    c. Click OK once the mask is corrected.

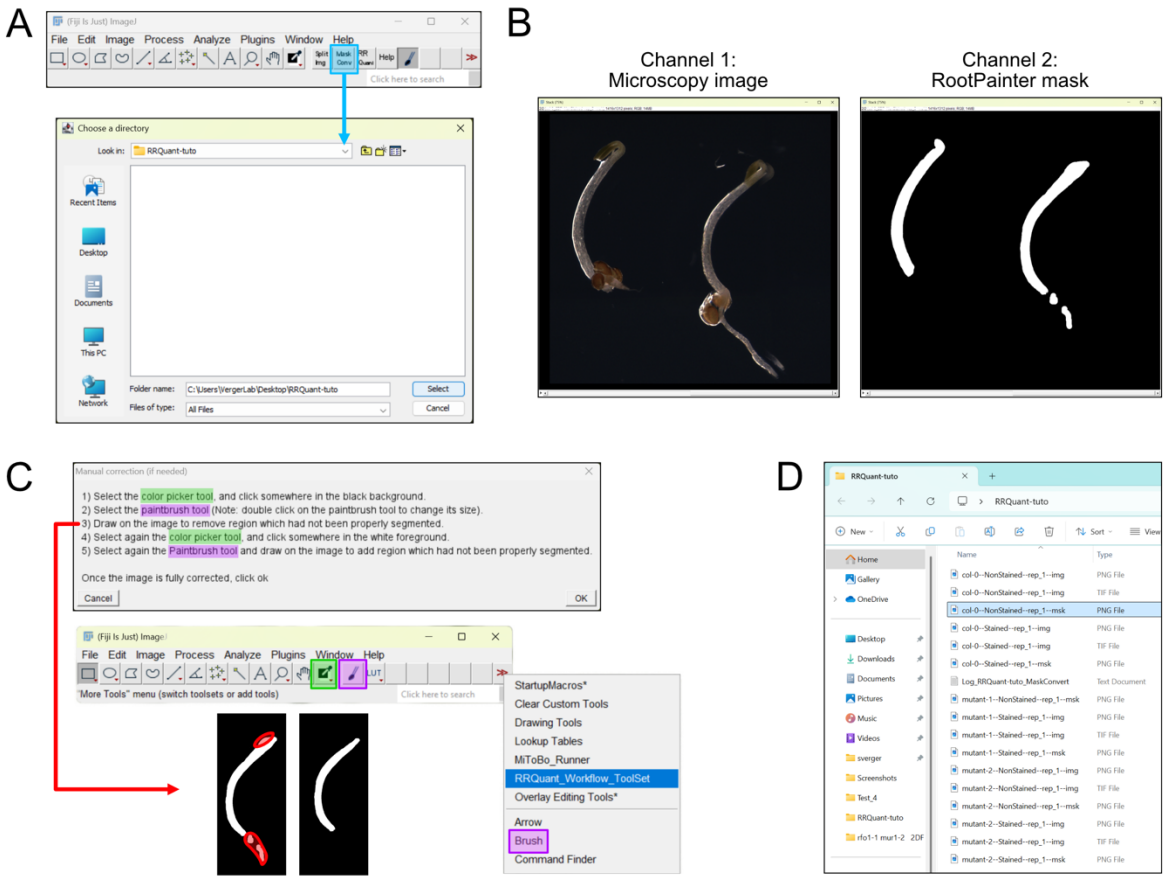

Figure 5: Mask correction in Fiji
A Starting the 2nd step of the RRQuant procedure in Fiji. Choose the directory containing the split microscopy images and the masks from RootPainter.
B Example of an image and its segmentation result.
C Correct the mask by painting with the brush tool.
D Folder ready to use for RRQuant quantification step.

IV – Quantification: RRquant

Finally, the core RRquant Fiji macro (Figure 1G,6, RRQuant.ijm) runs the quantification of staining intensity and hypocotyl morphology.

- Click on the RRQuant button in Fiji to run it (orange in Figure 6). Data will be automatically generated in batch.

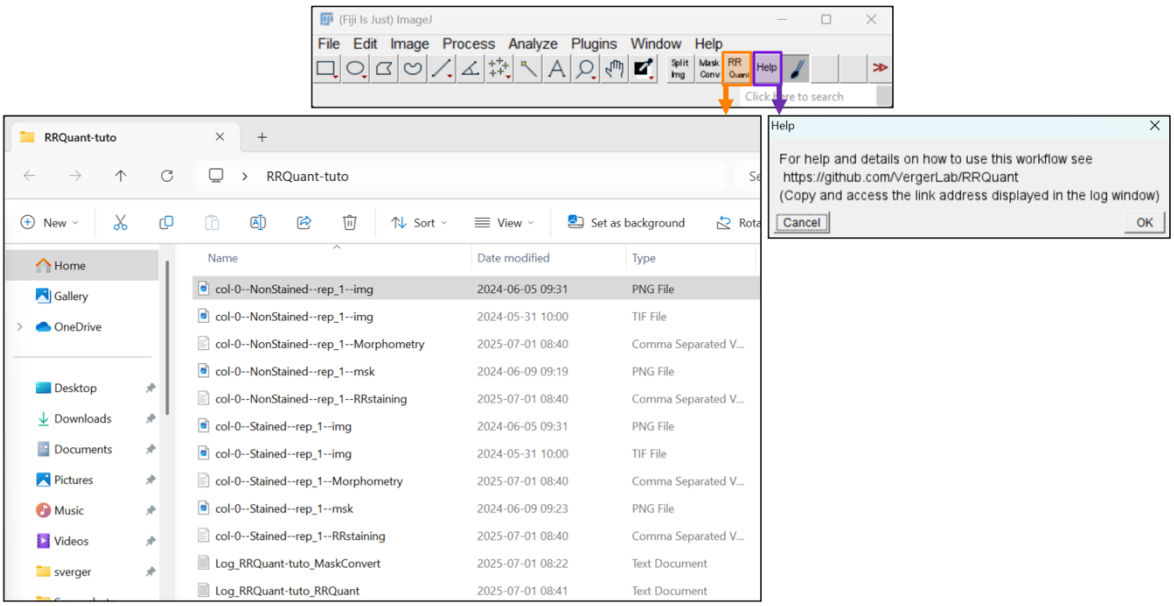

Figure 6: Quantification
RRQuant button activates the quantification macro, the results are saved in the selected output directory. If necessary, more help on how to use the workflow can be found on GitHub.

V – Data processing: R

We then developed a script for data processing, plot generation and statistical analysis in R (Figure 1H, https://www.R-project.org). A tutorial video is available.

1- Run the script RRQuant_data-table.R in RStudio by selecting all the code (crtl+a) and crtl+Enter or click on Run in the user interface (Figure 7A).
2- Select the working directory containing all the CSV files computed by the RRQuant imageJ macro (Figure 7A).

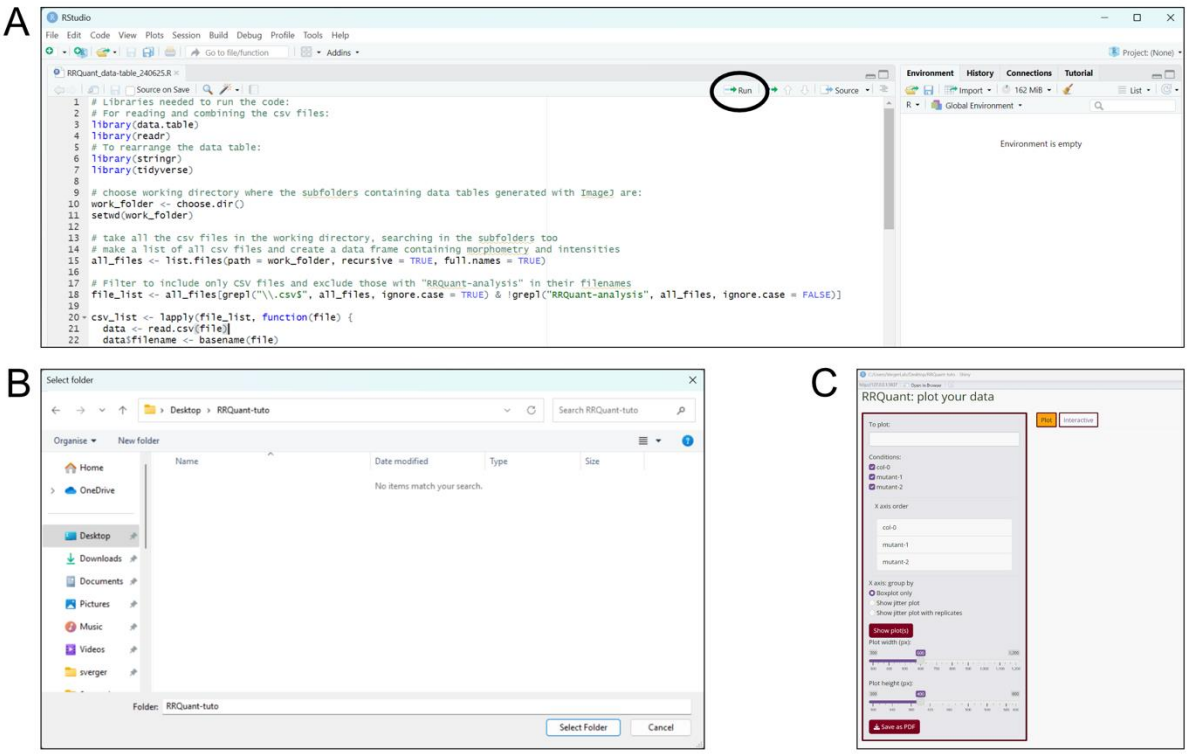

Figure 7: Data analysis and representation using R
A Rstudio window with RRQuant_data-table.R code.
B Select the folder containing all CSV files. It is also the output directory where the analysis results will be saved.
C Shiny app for data representation.

3- A pop-up window opens. Choose the working folder containing all the csv files generated by from the macro (Figure 7B, see Notes 3 to 5). This will combine all the data into one large data table and calculate relative staining intensity (stained vs non stained samples). The script reorganizes and adds labels to the data to enable plotting them in different ways (per genotype, per replicate…). More information about the R script can be found directly in the code as comments. 2 data tables are saved as CSV files directly in the working folder:
   - RRQuant-analysis_*Date*_*Time*: contains all data, with stained and non-stained samples. This table contains all information from the different quantification files generated by the macro. Data are organized but no filtering is applied and the relative intensity between the stained and unstained samples is not yet computed. This raw file can be used to plot the unstained samples against the stained ones.
   - RRQuant-analysis-Stained_*Date*_*Time*: contains only data from stained samples. Filtering is applied based on the pixel count to make sure that no small dots remain from the segmentation: every object below 200 pixels is removed. This file is used to make plots in the next step. See Note 6 for explanation about the columns' content.
4- Run the script RRQuant_app.R
5- A pop-up opens to select the csv file of interest. Choose a RRQuant-analysis-**Stained** file.
6- Once the statistical analysis is done, the RRQuant shiny app opens (Figure 8).
7- Plots can be saved in the location of your choice. All displayed plots in the app are saved in the same document. For more detailed information about how to use the app, see Figure 8 legend.

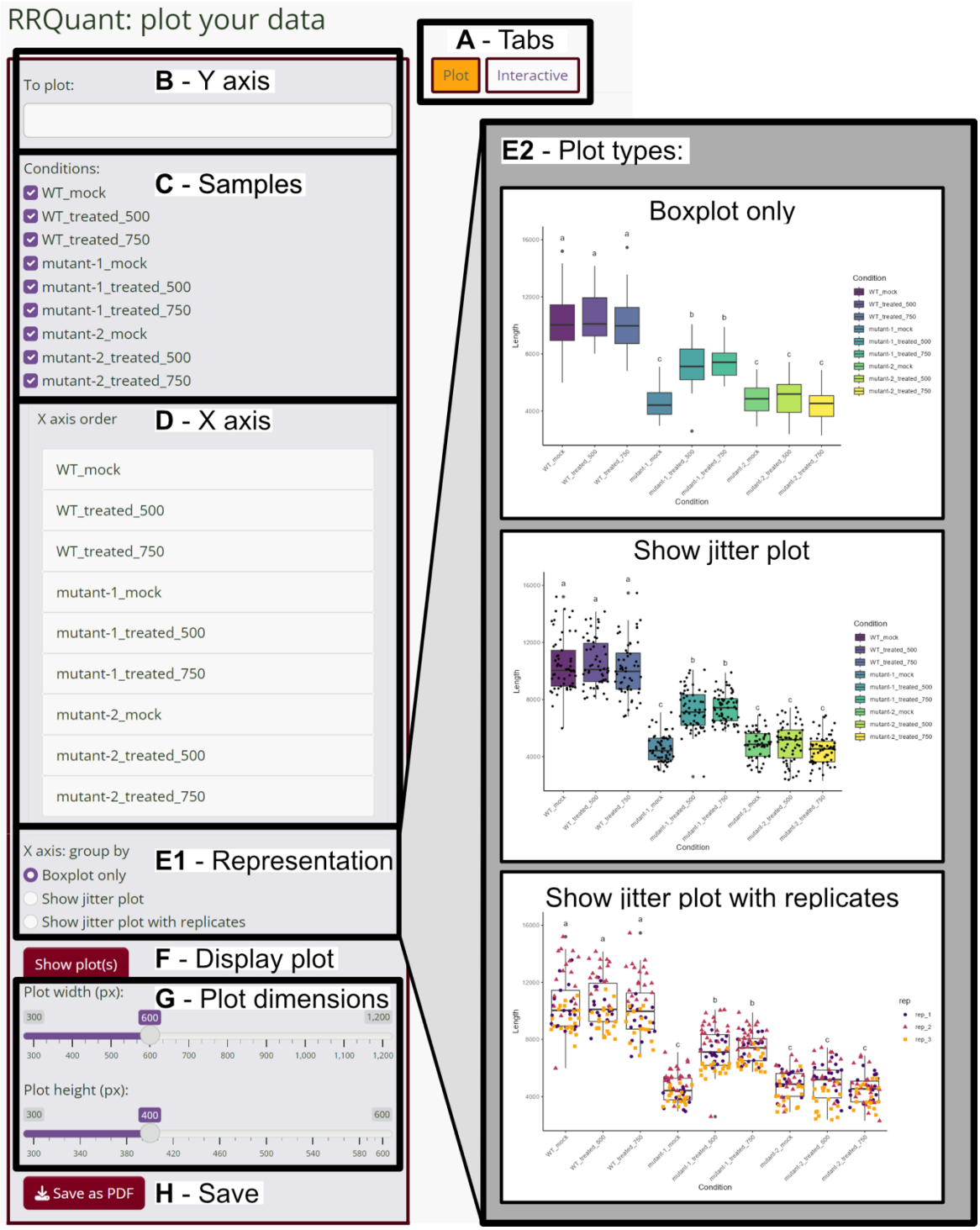

Figure 8: RRQuant app description

A Tabs to select which type of plots to observe. The "Plot" tab displays the plots that will be saved. The "Interactive" tab allows to hover the mouse on the data points and display the sample identity (see following figure).

B Rolling menu to choose the data to plot. It corresponds to the columns of the data table.

C Samples to display. All conditions present in the data table are listed. The box can be unticked to remove unwanted data from the plot.

D X axis organization. List of all the chosen conditions (in C). Each element of the list can be moved to adjust the order of the conditions on the x axis: the top element is displayed at the left most part of the plot.

E Type of plots. E1 – only one type of plot can be chosen at a time. E2 – plot representations corresponding to their chosen plot type.

F Button to display the plots on the right pannel. After choosing all previous parameters, click on Show Plot(s) to display the plots. To visualize plots with new parameters, it is necessary to click again on the button.

G Plot dimensions. Width and height can be adjusted for better representation.

H Save as PDF. A pop-up window opens to choose the directory where to save the plots. All displayed plots in the “Plot” tab will be saved in one PDF file.

Validation of the protocol

Baba et al., 2024. 'Rhamnogalacturonan-II dimerization deficiency impairs the coordination between growth and adhesion maintenance in plants'. *bioRxiv*.

Available at: **https://doi.org/10.1101/2024.11.26.625362**

General notes and troubleshooting

General notes:

1- In our case, we used a stereomicroscope Leica M205CFA with 1X/0.02NA objective, to acquire large tile RGB darkfield images of the full plates (or at least the area containing samples). Large, stitched tile images were generally made of 90 to 110 individual 1920x1080px images with a pixel size of 6.29 µm and 8bit depth that were acquired with 10% overlap and merged with the Leica Application Suite (LAS X) software. No zoom was used.

2- It is possible to bypass the first step of the image processing workflow (Image splitting) if the microscopy images do not require to be split. However, it is crucial to follow the naming rules described:
   - It should be similar to: Sample**--Stained--rep**_01**--img** or Sample**--NonStained--rep**_01**--img** with instead of "Sample" the genotype, condition, etc. and the replicate number (blue parts are variable). Use Stained or NonStained depending on the condition.
   - Do not leave spaces in the names, use _ instead.
   - Do not use special characters
   - Do not use -- (in other places than expected)

3- Images can be first saved in the microscope proprietary format (e.g.: lif for Leica). The images must be converted into TIFF to be used in the later stages.

4- The working folder can also contain images or other file types, just make sure to not have csv files that should not be processed. The script will use ALL csv files to make the table, and errors can occur if the csv does not correspond to what is expected (csv generated by the macro).

5- When choosing the directory, several problems may arise:
   a. The popup window opens in the background: check your taskbar.
   b. The popup window does not open at all. To check the issue, write choose.dir() in the console, it should return NA. In this case, write choose.file() in the console, when the window opens for choose.file(), do not select anything (click cancel). The function choose.dir() should now work as expected.

6- Explanation about the columns in the data table:
   a. **RRmean_absolute** is the RR mean measured by the macro.
   b. **RRmean_NS_genotype** is the mean of all non-stained samples RR mean per genotype (e.g.: mean of RR for all non-stained Col-0)
   c. **RRmean_NS_rep** is the mean of all non-stained samples RR mean per genotype and replicate (e.g.: mean of RR for all non-stained Col-0 for replicate 1)
   d. **RRmean_relative_NS_genotype** is the value of RRmean for each sample relative to the mean of the RR intensity for all the corresponding genotype non-stained samples.
   e. **RRmean_relative_NS_rep** is the value of RRmean for each sample relative to the mean of the RR intensity for all the corresponding genotype and replicate non-stained samples.

Example:
For the sample col-0-Stained-rep_2-2:

RRmean_NS_genotype	**RRmean_NS_rep**	**RRmean_relative_NS_genotype**	**RRmean_relative_NS_rep**
Mean of all non-stained col-0	Mean of all non-stained col-0 rep2	RRmean_absolute col-0-Stained-rep_2-2 **/** RRmean_NS_genotype col-0	RRmean_absolute col-0-Stained-rep_2-2 **/** RRmean_NS_rep col-0-rep_2

f. **Length** corresponds to Geodesic Diameter:

For particles with complex shapes, the geodesic diameter may be of interest. It corresponds of the largest geodesic distance between two points within a region, the geodesic distance being the length of the shortest path joining the two points while staying inside the region (Lantuéjoul & Beucher, 1981). (See MorpholibJ documentation)

7- The script can be re-run several times in the same folder, it will not take in the "RRQuant-analysis files".

Troubleshooting:

1- R scripts:
   a. If there are issues with the pop-up windows, see Note 4.
   b. If errors happen when running one of the scripts:
      i. Check if the chosen file is correct and has the expected structure (see example on GitHub).

References

Legland, D., Arganda-Carreras, I. and Andrey, P. (2016) "MorphoLibJ: integrated library and plugins for mathematical morphology with ImageJ," *Bioinformatics*, 32(22), pp. 3532–3534. Available at: https://doi.org/10.1093
/bioinformatics/btw413.

Smith, A.G. *et al.* (2022) "RootPainter: deep learning segmentation of biological images with corrective annotation," *New Phytologist*, 236(2), pp. 774–791. Available at: https://doi.org/10.1111/nph.18387.