Tandem tag assay optimized for semi-automated autophagy activity measurement in *Arabidopsis thaliana* roots

Adrian N Dauphinee[1], Jonas A Ohlsson[1] and Elena A Minina[1, 2 *]

[1]Department of Molecular Sciences, Uppsala BioCenter, Swedish University of Agricultural Sciences and Linnean Center for Plant Biology, P.O. Box 7015, Uppsala, SE-750 07, Sweden

[2]COS, Heidelberg University. Im Neuenheimer Feld 230. 69120 Heidelberg, Germany

*For correspondence: alena.minina@slu.se

Abstract

Autophagy is the main catabolic process in eukaryotes and plays a key role in cell homeostasis. *In vivo* measurement of autophagic activity (flux) is a powerful tool for investigating the role of the pathway in organism development and stress responses. Here we describe a significant optimization of the tandem tag assay for detection of autophagic flux *in planta* in epidermal root cells of *Arabidopsis thaliana* seedlings. The tandem tag consists of TagRFP and mWasabi fluorescent proteins fused to ATG8a, and is expressed in wildtype or autophagy-deficient backgrounds to obtain reporter and control lines, respectively. Upon autophagy activation, the TagRFP-mWasabi-ATG8a fusion protein is incorporated into autophagosomes and delivered to the lytic vacuole. Ratiometric quantification of the lytic pH-tolerant TagRFP and low pH-sensitive mWasabi fluorescence in the vacuoles of control and reporter lines allows for a reliable estimation of autophagic activity. We provide a step by step protocol for plant growth, imaging and semi-automated data analysis. The protocol presents a rapid and robust method that can be applied for any studies requiring *in planta* quantification of autophagic flux.

Keywords: plant autophagy, autophagy activity, Arabidopsis, Tandem-tag assay, autophagy activity in vivo, high-thoughput autophagy activity measurement, autophagic flux

Background

The Tandem Tag (TT) assay is a widespread approach for quantifying autophagic flux in yeast and mammalian cells[1–3]. It has been previously described to be also applicable for plant cells in a study using tobacco BY-2 cell suspension cultures[4]. Here we provide a detailed protocol for *in planta* quantification

of autophagic flux in epidermal root cells of *Arabidopsis thaliana* seedlings. The TT assay employs ratiometric quantification of red and green fluorescence and allows to quantify relative induction or inhibition of autophagy. Another advantage of this method is that it provides valuable morphological data including: subcellular localization of ATG8, visualizing puncta formation, and translocation from the nuclei[5].

For this protocol we used stable transgenic *Arabidopsis* lines expressing the optimized Tandem Tag[3] fused to AtATG8a and driven by a double 35S promoter. The TT-AtATG8a fusion was introduced into wild-type or *atg5atg7* double knockout backgrounds, to produce reporter and control lines, respectively. Under normal growth conditions TT-AtATG8a is localized in the cytoplasm and in the nuclei of the root epidermal cells, but upon induction of autophagy it is gradually translocated to the cytoplasm, then incorporated into autophagosome membranes and delivered together with the cargo to the lytic vacuole. While TagRFP shows relatively high tolerance to the pH of the lytic compartments[3], fluorescence of mWasabi is significantly reduced under the same conditions. Thus, ratiometric measurement of the TagRFP and mWasabi fluorescence allows to estimate the delivery rate of the fusion protein to the lytic compartment and eliminates potential bias coming from differences in fusion protein expression. Furthermore, since the assay relies on confocal microscopy image acquisition and analysis, it is possible to obtain time-resolved and dose-dependent data about the changes in autophagic activity. Although we describe only a protocol for detection of the TT-AtATG8a delivery to the vacuole, it is also possible to use the same imaging data to also visualize and quantify dynamics of autophagosome formation.

We observed that response to known modulators of autophagy activity, such as AZD8055 and concanamycin A[1] in *Arabidopsis* roots significantly varied depending on the area of the root zone scanned. By comparing data obtained on different root zones, we established that most reproducible measurements could be obtained by analyzing images of root epidermal cells located in the beginning of differentiation zone. Nevertheless, we still observed quite significant variation between responses in tricho- and atrichoblast cells. Hence, a relatively large number of images was required for the data analysis to obtain a reliable mean value representative of average autophagy activity in the root zone.

To facilitate high-throughput image analysis, we developed a semi-automated image analysis protocol described below. It relies on the use of macros written in ImageJ Macro Language (IJL) and R scripts. The protocol was tested using images obtained with Zeiss and Leica confocal laser scanning microscopes (CLSMs) and should be applicable for data from other manufacturers of CLSMs. Furthermore, to enable applicability of the protocol for images obtained on different CLSM systems that will naturally vary in efficacy of detection, we added an extra step of threshold values adjustments that will maximize the capacity of the vacuole area selection tool.

Importantly, the TT assay described here allows quantification of autophagy-dependent delivery of ATG8 to the lytic vacuole. Although it can be used as a very good indication for estimating autophagic activity, it is still preferable to combine this assay with other techniques verifying degradation of autophagic cargo e.g. the GFP-ATG8 cleavage assay or long-lived proteins assay.

Materials and Reagents

1. Bleach solution (Klorin, Colgate Palmolive)
2. Tween-20, Polysorbate (VWR, catalog number: 97062)
3. Murashige and Skoog, MS medium (Duchefa Biochemi, catalog number: M0222) liquid medium
4. MES, 2-(N-morpholino)ethanesulfonic acid (Duchefa Biochemi, catalog number: M1503)
5. Sucrose (Duchefa Biochemi, catalog number: S0809)
6. KOH (VWR, 470302)
7. Plant agar (Duchefa Biochemi, catalog number: P1001)
8. DMSO (Sigma-Aldrich, catalog number: 276855) or other vehicle
9. AZD8055 (Selleckchem, catalog number: S1555)
10. Immersion oil (Zeiss, catalog number: 444960)
11. Transgenic Arabidopsis lines used to establish this protocol were published in (Dauphinee et al., 2019, Accepted for publication in Plant Phys journal)

Equipment

1. Petri dishes (Thermo Fisher Scientific, catalog number: 15213338)

2. Forceps (Dumont, catalog number: 11251-10)
3. Pipettes for 100-1000 µl and 1-10 µl
4. 6-well tissue culture plates (Thermo Fisher Scientific, catalog number: 15213338)
5. 4 °C fridge
6. *Arabidopsis* growth cabinet/growth room: 20-22 °C, 50-70 % humidity, 150 µM light
7. Cover slips 25 x 50 mm and 25 x 25 mm (VWR, catalog numbers: 48382-136 and 48366-089)
8. Confocal Laser Scanning Microscope (CLSM; Zeiss, LSM 800)

Software

1. Fiji, the version of ImageJ with included set of plugins (https://fiji.sc/) for this study, we utilized versions 1.51s and 2.0.0-rc-69/1.52i).
2. R (https://www.r-project.org, we used 3.5.2 and 3.5.1)
3. RStudio (https://www.rstudio.com/, we used versions 1.1.453 and 1.2.1186).

Procedure

1. Seed sterilization (40 min)
    - Place ca 15 µl of seeds of reporter and control lines into 1.5 ml Eppendorf tubes
    - Add ca 1.5 ml of the bleach solution
    - Incubate the seeds for ca 30 min, agitating
    - Under sterile conditions pipette out the bleach solution
    - Add sterile water to the seeds. Mix and pipette it out
    - Perform the wash with sterile water at least three times to wash out the leftovers of the bleach solution
    - Seeds can be vernalized either in the sterile water or as described in the step three.

2. Seed plating (40 min)
    - Use 1 ml pipette to transfer single seeds onto a Petri dish with solid 0.5x MS medium. Keep ca 2–5 mm distance between the seeds in the same row to avoid entanglement of roots. Keep ca 5 cm distance between the rows (Fig. 1a)

- Seal the plates with a strip of Parafilm or Saran wrap

3. Vernalization (24–48 h)

- Incubate the plates in the dark at 4 °C for 24–48 h

4. Growth on vertical plates (5 d)
    - Place the plates into growth conditions (150 µM light, 22 °C for 16 h, 0 µM light, 20 °C for 8 h). Make sure the plates are perfectly vertical to ensure root growth on the top of the medium (Fig. 1b)
    - Grow the seedlings on the plates for ca 5 d, the root length should reach ca 2 cm.

5. Drug treatment (2-24 h)
    - Pipette 3 ml of liquid 0.5x MS into wells of a 6-well tissue culture plate. Add the chemical compounds of the required concentration
    - Using soft foreceps, gently pick seedlings from the plates and transfer into liquid 0.5x MS (not more than 20 seedlings/well)
    - Gently pipette the medium from the well onto the seedlings in the well to submerge the roots (white arrow, Fig. 1c)
    - Seal the plate with a strip of Saran wrap or Parafilm
    - Incubate the plate under the same growth conditions for the required amount of time
6. Mounting the samples (1 min)
    - Pipette ca 50 µl of medium from a well on the 25x75mm cover slip
    - Using soft tweezers, gently pick seedlings from the well and place it onto a drop making sure that the root is straight
    - Place up to six seedlings on the cover slip
    - Cover the roots with 25 x 25 mm cover slip (Fig. 1d)
    - Apply immersion if needed to the objective lens and place the sample on the microscope stage

7. Setting up scanning parameters for confocal laser microscopy (30 min, required only once)
    - To estimate optimal range of settings for scanning, it is advisable to perform a pilot experiment using three control treatments with 500 nM AZD 8055 for 4 h, 500 nM ConA for 6 h and corresponding amount of vehicle (0.05% DMSO).
    - Configure settings for sequential scanning of two channels:
        - Channel 1 for detection of mWasabi: excitation at 488 nm, emission detection range 490–564 nm
        - Channel 2 for detection of TagRFP: excitation at 561 nm, emission detection range 564–700 nm
        - It is advisable to use the most sensitive detectors available in the system (i.e. GaAsp or HyD detcors for Zeiss or Leica CLSM, respectively).
        - Using 40x objective is advisable to obtain images most applicable for automated analysis
        - Set switching between channels for each frame to minimize the crosstalk between channels and optimize the pinhole size. If possible, use the pinhole of 1 AU for each of the channels
        - If possible, use 16-bit resolution to increase the resolution of intensities.
        - The sample treated with AZD 8055 will have the weakest fluorescence and should be used to adjust laser intensity and the Detector Gain (Master Gain) to the lowest possible values that do not result in oversaturated pixels.
        - The sample treated with ConA will have the strongest fluorescence at least in the green channel and should be used to re-adjust laser intensity and the Detector Gain (Master Gain) to the lowest possible values that do not result in oversaturated pixels.
        - The sample treated with the DMSO can be used to verify the applicability of the adjusted settings.
        - Additionally, noise can be decreased by ramping down the scanning speed or increasing the averaging number. To our experience, scanning at the speed of ca 10 seconds per frame produced images of the quality appropriate for further analysis and also resulted in acceptable time for experiment.

8. Scanning (ca 30 min per treatment)

- Most of CLSM software will have an option of scanning at selected positions that will significantly decrease the time required for the experiment
- Mark the positions at the beginning of the differentiation zone of the root (Fig. 1e)
- Start fast scanning mode (live scan) and readjust the focal plane for each position to the middle section through the vacuole of the epidermal cells (Fig. 1f with and without cortical cytoplasm)
- When all positions are readjusted, acquire and save the images. Please note, that automated statistical analysis will use information provided in the names of images. Please use the following rules to introduce the required parameters into the name of your images:
    - separate parameters by underscores (_)
    - Name the files as following: ***line_treatment***_seedling***X***_image***Y*** (e.g. *Reporter_50uM.C12_seedling1_image2.czi*)

  Where *line* and *treatment* are text variables or strings identifying the lines (either "Reporter" or "Control") and treatments (i.e. "50uM.C12") used. One treatment must be named "Vehicle". *X* and *Y* are numbers indicating seedling and image replicates thus representing biological and technical replicates, respectively. Note that "seedling" and "image" are fixed strings and only the numbers *X* and *Y* are changed from image to image.

Data analysis

Data needs to be processed prior to the analysis. The processing is done in four steps: (i) confocal images are converted into .TIF files; (ii) adjustment of the threshold values to optimize recognition of the vacuoles must be performed while using the protocol for the first time and, if needed, can be redone for individual experiments; (iii) fluorescence intensities in the vacuoles are quantified and saved; (iv) quantification of the ratios and plotting of the data as control vs reporter lines, or ratios vs time and concentration.

- Place all images for processing into a single folder. If several experiments should be processed, place them as subfolders into a single folder.
- Download and install Fiji, the version of ImageJ with included set of plugins (https://fiji.sc/) for this study, we utilized versions 1.51s and 2.0.0-rc-69/1.52i).
- Download and install R (https://www.r-project.org, we used 3.5.2 and 3.5.1) and RStudio (https://www.rstudio.com/, we used versions 1.1.453 and 1.2.1186).
- Either clone the GitHub repository or download it as a zip file (https://github.com/jonasoh/AuTToFlux). If cloning the repository, RStudio can be used to automate this task: In RStudio, start a new project (File -> New Project -> Version Control -> GIT and input the following URL: https://github.com/jonasoh/AuTToFlux.
- Run the Image Processor: launch ImageJ and go to Plugins -> Macros -> Run -> locate ImageProcessor.ijm.
- The image processor macro opens all compatible images in the target folder and saves them as .TIF files; original acquisition dates of the images are saved in separate files. Multi-image files from experiments scanned using the “Positions“ function are split so that each image is saved as a separate .TIF file.

- Optimize the threshold parameters for selecting the vacuoles:
    - Copy at least three tiff images generated by the ImageProcessor into a separate folder. Aim to select images representing the best the worst and average quality.
    - Launch ImageJ and go to Plugins -> Macros -> Run -> locate CalibrateThreshold.ijm. This runs the macro. In the file picker immediately presented, locate the folder with representative images. Macro will record the area sizes corresponding to the vacuoles and also save masks as tiff files containing threshold values in their names, e.g. file named *.thr0-3.tiff would correspond to the mask created with the threshold values (0;3). The results overview will be generated as threshold-overview.tif (Fig. 1g).
    - Launch RStudio.
    - Open the RStudio project file (AuTToFlux.Rproj).

- To estimate what threshold values provide the largest selected vacuole area for all analyzed images, run the EvaluateCalibration.R script (select the R script, click Code -> Source and then choose the folder containing the .CSV files generated in the previous step – note: on non-Windows systems you need to type in the pathname of the folder).
- The script will plot average sum vacuole areas selected on all analyzed images vs threshold values. Select the threshold value that corresponds to the largest area (Fig. 1h).
- Using the information gathered from the threshold-overview and the threshold graph, select an appropriate upper threshold value for marking. The masks should not contain any background areas.
- Before proceeding with image thresholding and data analysis, ensure that the calibration folder is removed from the directory containing the processed images.

- Run the threshold macro: launch ImageJ -> Plugins -> Macros -> Run -> locate and open the macro file FluorescenceIntensity.ijm. The macro will prompt you to select the threshold value determined earlier in the calibration process. For each image the threshold macro automatically selects areas corresponding to the vacuoles using the GFP channel and saves the masks for the selected areas as .TIF files. The mask is then used to quantify intensities of RFP and GFP fluorescence in the corresponding channels of the image. Ratios of vacuolar RFP/GFP fluorescence intensities are saved to .CSV files with matching filenames.

Data analysis

- Launch RStudio.
- Locate the R project file in the directory created from the GitHub repository and open it.
- The first time you run the script you need to make sure its dependencies are installed. Either use RStudio's interface for installing packages, and install the packages ggplot2, dplyr, and readr, or install them manually by typing the following into the console:

```
install.packages(c("ggplot2", "dplyr", "readr"))
```

- To estimate dose and time-dependent responses the RFP/GFP ratios are plotted vs time using Flux-vs-Time.R (select the R script, click Code -> Source and then choose the folder containing the .CSV files generated in step 9).
    - The initial time of treatment (format YYYY-MM-DD HH:MM, 24-hour time) is user-specified by creating a tab-delimited text file (using any spreadsheet software, e.g. Microsoft Excel) with two columns, which should be saved as info.txt in the folder containing the .CSV files for analysis.

      e.g.

Treatment	StartTime
Vehicle	2018-07-06 09:50
Treatment	2018-07-06 10:00

    - A summary of the experiment data is generated with ratios normalized to the vehicle and grouped by treatment and seedling number (Fig. 1i). To plot the data, we recommend ggplot2 (https://ggplot2.tidyverse.org/), axes (x=Elapsed time, y=Normalized ratios, color=Treatment)
    - Further statistical analysis will depend on the treatments present in the experiment and can be performed using R or other software, e.g. Origin, JMP.
    - The data can be also used to estimate IC50 using R or other software, e.g. Prism.
- To demonstrate autophagy-dependent response, RFP/GFP ratios of control lines are plotted vs ratios of reporter lines. For this, run Control-vs-Reporter.R (select the R script and then click RStudio -> Code -> Source) and then choose the folder containing the .CSV files generated in step 9).
    - This script may take as its argument either a folder containing several treatments as subfolders, or a folder without subfolders that contains a single experiment.
    - The script summarizes RFP/GFP ratios (normalized to vehicle) and groups by line (control or reporter), treatment and seedling number. An unpaired, two-tailed Student's t-test is used to compare log-transformed normalized means of the control and reporter groups. A summary table named pvals.txt is generated. If p-

values derived from permutation (i.e. exact p-values) are desired, uncomment the appropriate sections marked in the R script.

Note: for both Control-vs-Reporter.R and Flux-vs-Time.R data is saved both as raw data (as summary-full.txt in the experiment directory) and as per-seedling summary statistics (summary-perseedling.txt), to facilitate analysis using other statistical software.

Notes

Troubleshooting		
Problem	**Possible cause**	**Solution**
Low number of data points	High noise on the images	1. Adjust scanning settings to decrease the noise 2. Adjust threshold values during data analysis
	Focusing not on the middle section of the vacuoles	1. Make sure that the roots on the samples are not drifting during imaging and are placed in the thinnest possible layer of solution
	Wrong threshold values in the FluorescenceIntensity.ijm macro	Follow the instruction in the procedure for estimating optimal values and adjusting the macro
Incorrectly assigned groups in Flux-v-Time.R	Different number of data points for each timepoint	The script requires the number of data points for each group to be roughly equal. Remove or add data points to balance the experiment.
Time-v-Flux.R exits with error "Error in eval(ei, envir) : file.exists(infofile) is not TRUE"	There is no info.txt in the directory.	Create the info.txt file according to the instructions.
Time-v-Flux.R gives unexpected results	Times in info.txt do not match the acquisition times of the images.	Make sure that times in info.txt correspond to times as recorded by the microscope.
	Treatments in info.txt do not match filenames.	Make sure that treatment names are given exactly the same in filenames and info.txt.

Data points are duplicated	ImageProcessor has been run twice on the same set of images.	Delete all .tif files in the directory and rerun ImageProcessor on the files directly from the microscope.
Script exists with error "*file* does not match naming scheme".	File is incorrectly named.	Double check that the filename matches thje naming scheme described in the protocol.

Recipes:

1. Liquid x0.5 MS medium: 0.5x MS complete with vitamins, 10 mM MES, 1% sucrose dissolved in MQ water. Adjust pH to 5.8 using KOH and autoclave for 20’ at 120°C. Store at 4°C for max. 2 months.
2. Solid x0.5 MS medium: after adjusting pH of th liquid x0.5 MS medium add 0.8 % Plant agar and autoclave for 20’ at 120°C. Store at 4°C for max. 2 months. If needed the medium can be liquified in the microwave,
3. Bleach solution: 5% Klorin (final concentration: 2.7 g/L sodium hypochlorite), 0.01% Tween -20 in MQ water. Prepare in 50 mL Falcon tube and store at RT. After ca 1 month Tween might form precipitates in the solution, it does not impact the efficacy of the sterilization though..
4. AZD 5 mM stock in DMSO, store at -20 °C for max. 2 years
5. ConA 1 mM stock in DMSO, store at -20 °C for max. 1 year

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Competing interests

The authors declare no competing financial interests.

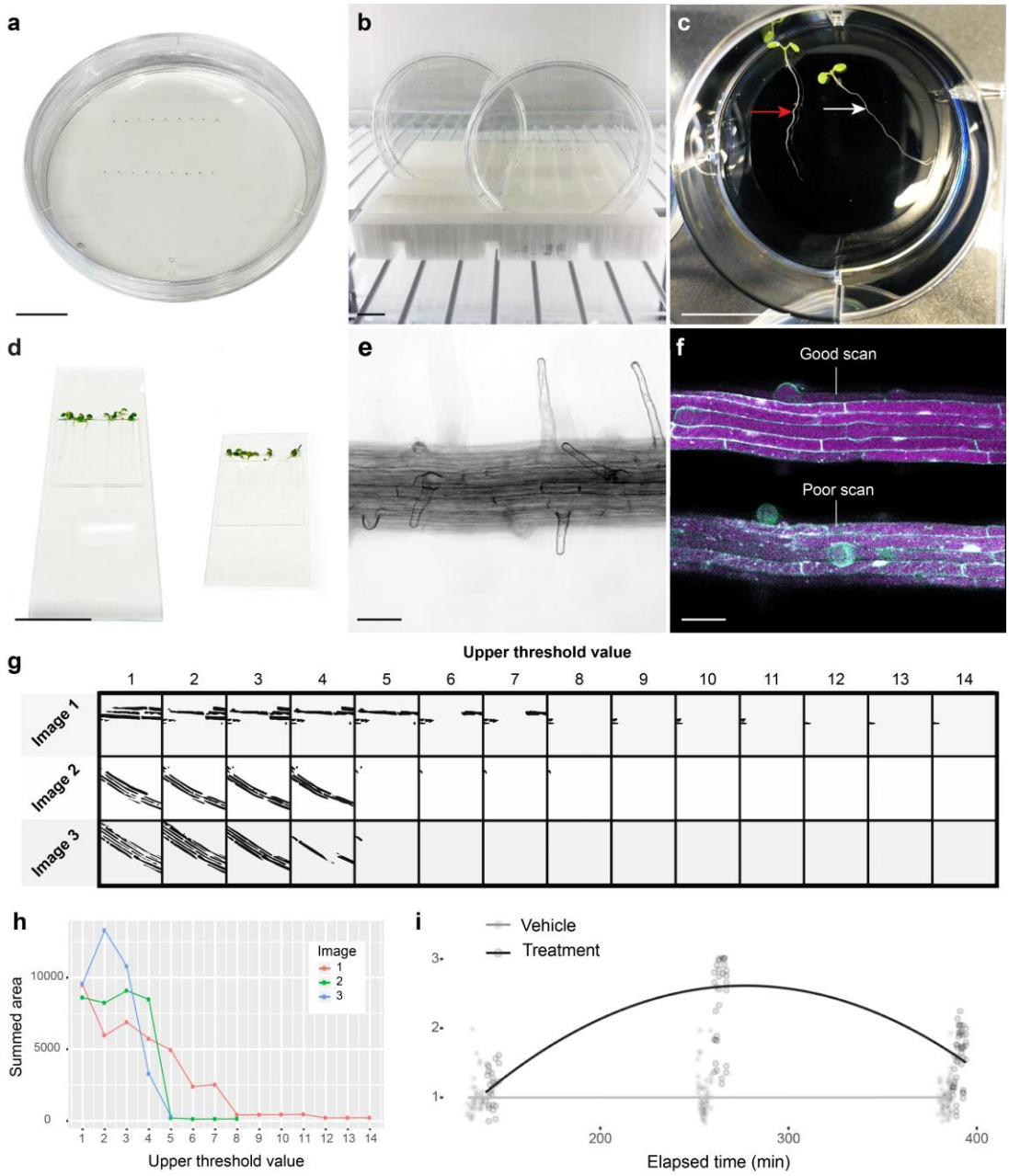

Fig. 1: Arabidopsis tandem tag autophagic flux assay.

a, Tandem tag (TT; TagRFP-mWasabi-ATG8a) reporter (TT in wildtype background) and control (TT in autophagy-deficient background) *Arabidopsis thaliana* seeds plated on ms media and grown vertically (**b**)**. c,** *Arabidopsis* seedlings placed in a 6-well plate for treatment. Note that the roots should be submerged gently (white arrow) and should not be floating on the surface (red arrow). **d,** Seedlings mounted on a glass slide (left) or coverslip (right) for laser scanning confocal microscopy. **e,** Epidermal layer of the root differentiation zone. **f,** Good and poor quality scans of differentiation zone vacuoles in the reporter line. **g,** Vacuole layer masks generated during calibration using the CalibrationThreshold.jim macro. Note that unsuccessful scans (**f**) may lead to complete or partial failure of vacuole selection. **h,** Sum vacuole areas selected on all analyzed images (3 recommended) vs threshold values generated by the EvaluateCalibration.R script. **g,** Flux-vs-Time.R script output for vehicle and a single concentration for one treatment generated in RStudio using the ggplot2 function. Scale bars, 1.5 cm (**a-d**); 50 µm (**e-g**).

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