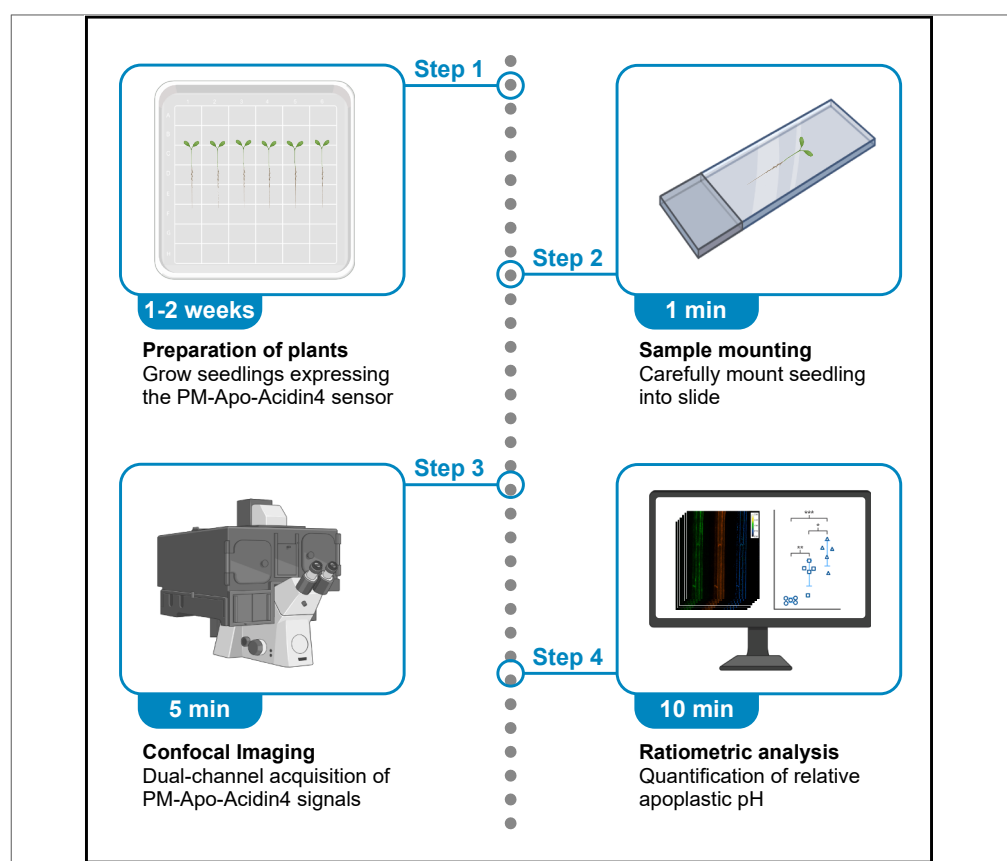


Protocol

Protocol for cell-specific ratiometric quantification of apoplastic pH in *Arabidopsis* seedlings



Apoplastic pH dynamically regulates plant intercellular communication, but its measurement in internal tissues, such as the vasculature, remains technically challenging. Here, we present a protocol for ratiometric quantification of apoplastic pH in *Arabidopsis* seedlings using genetically encoded sensors. We describe seedling preparation, confocal imaging, and ratiometric image processing. Companion cell-specific expression of the pH sensor enables apoplastic pH readouts in the vasculature and supports *in vivo* comparative analyses of apoplastic pH across genotypes, treatments, and growth conditions in young seedlings.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Guidance on *Arabidopsis* seedling growth and sample preparation

Workflow for confocal imaging using genetically encoded pH sensors

Instructions for ratiometric image processing and analyses of relative apoplastic pH

Steps for quantitative analysis of apoplastic pH specifically in companion cells

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Protocol

Protocol for cell-specific ratiometric quantification of apoplastic pH in *Arabidopsis* seedlingsLu Xiong¹ and Paloma Mas^{1,2,3,4,*}¹Centre for Research in Agricultural Genomics (CRAG), CSIC, IRTA-UAB-UB, Campus UAB, 08193 Barcelona, Spain²Consejo Superior de Investigaciones Científicas (CSIC), 08028 Barcelona, Spain³Technical contact⁴Lead contact*Correspondence: paloma.mas@cragenomica.es
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SUMMARY

Apoplastic pH dynamically regulates plant intercellular communication, but its measurement in internal tissues, such as the vasculature, remains technically challenging. Here, we present a protocol for ratiometric quantification of apoplastic pH in *Arabidopsis* seedlings using genetically encoded sensors. We describe seedling preparation, confocal imaging, and ratiometric image processing. Companion cell-specific expression of the pH sensor enables apoplastic pH readouts in the vasculature and supports *in vivo* comparative analyses of apoplastic pH across genotypes, treatments, and growth conditions in young seedlings. For complete details on the use and execution of this protocol, please refer to Xiong et al.¹

BEFORE YOU BEGIN

The apoplast is a highly dynamic extracellular compartment that contributes to the regulation of plant intercellular communication.^{2,3} Changes in apoplastic pH affect multiple physiological processes, including cell expansion, transporter activity, solute movement or the function of pH-sensitive cell-wall remodeling factors, making its quantitative assessment important for understanding plant growth, development and responses to stress.^{4–6} In this protocol, we describe the *in vivo* measurement of apoplastic pH in young *Arabidopsis thaliana* seedlings using the genetically encoded ratiometric sensor PM-Apo-Acidin4.⁷ Fluorescence ratio imaging with this sensor enables the quantitative analysis of apoplastic pH dynamics in intact plant tissues. The protocol described here includes an imaging workflow amenable to epidermal cells in the elongating hypocotyl. The use of the *Commelina yellow mottle virus* (CoYMV) promoter^{8–10} also enables expression of the sensor specifically in companion cells, allowing analyses of pH dynamics in the apoplast of phloem tissues in hypocotyls and roots. This approach provides substantially improved signal-to-noise ratios for reliable pH quantification in phloem-associated cells. The protocol also includes conversion of ratio values into relative pH estimates. In principle, the approach could also be applied to other tissues and developmental stages, provided that sensor expression, tissue accessibility and optical properties support accurate signal detection and ratiometric analysis.

Innovation

Quantitative ratiometric imaging of apoplastic pH in phloem companion cells of *Arabidopsis* seedlings is technically challenging due to tissue thickness and variable sensor signal intensity. Existing protocols primarily focus on epidermal tissues, limiting their applicability to phloem-specific analysis.^{4,7,11} This limitation is particularly relevant because apoplastic pH within the phloem influences processes such as solute transport, long-distance signaling and growth. The protocol presented here extends the usability of existing sensors by expressing the pH sensor under a cell-type specific promoter. The



approach allows for sensitive and reproducible pH measurements in companion cells enabling comparative analyses among distinct genotypes and growing conditions. The *in vivo* imaging also preserves the native tissue context and allows apoplastic pH to be measured without the disruption caused by tissue excision or invasive manipulation. These features support robust and physiologically relevant analysis of apoplastic pH dynamics in the phloem.

Preparation of growth medium

⌚ Timing: 2–4 h

1. Prepare Murashige and Skoog (MS) medium.
 - a. Dissolve 4.4 g/L MS salts and 0.5 g/L MES hydrate (2-(N-morpholino)ethanesulfonic acid monohydrate) in distilled water. Adjust the pH to 5.8 using 1 M KOH.
 - b. Add agar to a final concentration of 1% (w/v), then autoclave.
 - c. After sterilization, allow the medium to cool to approximately 50–60°C. Pour the medium into sterile Petri dishes under aseptic conditions, in a laminar flow hood, to prevent contamination.
 - d. Allow the medium to cool and solidify at room temperature. Seal the plates with parafilm and store at 4 °C for up to 4 weeks. Plates can be also used immediately.

Note: Sugar can be added according to experimental needs. Agar concentration can also be adjusted depending on the desired medium firmness. For liquid MS medium, agar should be omitted.

Preparation and growth of *Arabidopsis* seedlings

⌚ Timing: 1–2 weeks

2. Seed sterilization and plating.
 - a. Place at least 100 *Arabidopsis thaliana* seeds in a sterile microcentrifuge tube and add 70% ethanol to cover the seeds. Invert gently for 1 minute.
 - b. Carefully remove the ethanol with a pipette and add sterilization solution, for example 15% (v/v) commercial bleach containing 0.01% Tween-20. Invert the tube occasionally for 10 min.
 - c. Remove the bleach solution with a pipette and rinse the seeds with 1 mL sterile distilled water. Repeat the rinse step 4 times to remove all traces of bleach and keep the seeds in a small volume of sterile water until sowing.

Note: For this protocol, we use seedlings expressing the pH sensor (PM-Apo-Acidin4)⁷ either under the constitutive Cauliflower Mosaic Virus 35S promoter¹² (35S::PM-Apo-Acidin4). or under the *Commelina yellow mottle virus* (CoYMV) promoter,^{8–10} which drives companion-cell-specific expression (CoYMV::PM-Apo-Acidin4).

Note: Avoid sterilizing very large numbers of seeds in a single tube, as this may reduce sterilization efficiency. For example, >2000 seeds per tube may result in incomplete sterilization.

3. Sowing and stratification.
 - a. Prepare a seed suspension in sterile water using a water-to-seed volume ratio of approximately 1:1 to 1.5:1. Gently mix the seeds with a sterile pipette tip to ensure uniform dispersion.
 - b. Using a pipette fitted with a sterile tip, transfer small droplets of the seed suspension onto the surface of the solid MS medium.
 - c. Cover the plates with their lids and seal the edges with laboratory tape, such as Micropore tape, to prevent accidental opening while allowing gas exchange and minimizing contamination.
 - d. Wrap the plates with aluminum foil to maintain darkness and incubate at 4 °C for 4 days to synchronize germination.

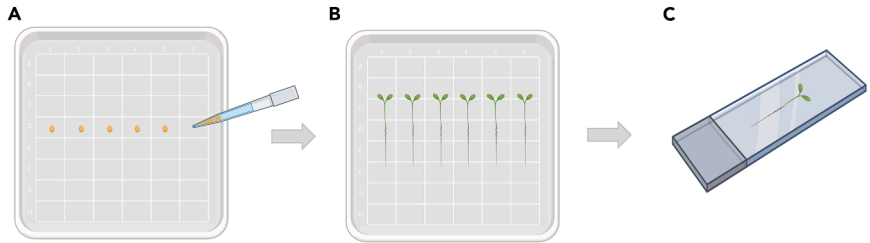


Figure 1. Schematic illustration of the experimental workflow for *Arabidopsis* seed sowing, seedling growth and sample preparation for confocal imaging

(A) *Arabidopsis* seeds are sown on solid MS medium. A pipette is used to dispense small droplets of seed suspension onto the plate, and seeds are distributed individually to avoid crowding.
(B) Seeds are grown under controlled conditions until seedlings reach the desired developmental stage.
(C) Seedlings are transferred onto a microscope slide, mounted in a small drop of liquid MS medium and covered with a coverslip.

Note: All seed handling and sowing steps should be performed in a sterile laminar flow hood to maintain aseptic conditions. Handle plates and pipettes carefully, avoid touching the medium surface, and minimize exposure of open plates to air to reduce contamination.

Note: Deposit seeds individually, leaving enough space between seeds to avoid crowding. For example, on a 12 × 12 cm square plate, seeds can be arranged in a single row along the center of the plate, avoiding the 1 cm border at the edges (Figure 1A).

4. Seedling growth.
 - a. Transfer the plates to a growth chamber and grow seedlings under the desired conditions (Figure 1B).
 - b. Grow seedlings until the desired developmental stage.

Note: Light intensity, photoperiod and plate inclination can be adjusted according to experimental needs. For example, in our diel experiments, plates were placed at an inclination of approximately 70° and seedlings were grown under 12 h light / 12 h dark cycles at 22 °C and 15 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Note: Three-day-old seedlings can be used for imaging hypocotyl companion cells and epidermal cells. Eight-day-old seedlings can be used for imaging the root meristem phloem in 35S::PM-Apo-Acidi4 lines.

△ **CRITICAL:** Ensure uniform seed germination and seedling growth. Use healthy seedlings to obtain reliable and reproducible sensor signal.

△ **CRITICAL:** For analyses of hypocotyl companion cells, low-intensity light, approximately 15 $\mu\text{mol m}^{-2} \text{s}^{-1}$, might be better to prevent hypocotyl thickening and maintain optical clarity for imaging.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Murashige & Skoog basal medium with vitamins	Duchefa Biochemie	M0222
2-(N-morpholino) ethanesulfonic acid hydrate (MES hydrate)	Sigma-Aldrich	M8250

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Plant agar	Duchefa Biochemie	P1001
Potassium hydroxide (KOH)	Merck	1.05033
Ethanol 70% v/v	Scharlab	ET0001005P
TWEEN-20	Sigma-Aldrich	P1379
Commercial bleach	LEJIAS PONS	LEJ3019
Experimental models: organisms/strains		
<i>Arabidopsis thaliana</i> : PM-Apo-Acidin4/Columbia	Moreau et al ²	N/A
<i>Arabidopsis thaliana</i> : CoYMV::PM-Apo-Acidin4/Columbia	Xiong et al ¹	N/A
Software and algorithms		
ZEN	Zeiss	http://www.zeiss.com
Fiji (ImageJ) Life-Line version (2015 December 22)	Fiji (ImageJ)	https://imagej.net/software/fiji/downloads
FIJI-MACRO FOR RATIONOMETRIC IMAGE CONVERSION	Barbez et al ⁴	N/A
Other		
LSM 980 laser scanning confocal microscope (integrated with the Zeiss Elyra 7 platform)	Zeiss	LSM 980
Objective LD LCI Plan-Apochromat 40x/1.2 Imm Corr DIC M27	Zeiss	Item no. 420862-9970-000
Ultrapure water system	Thermo Scientific	Nanopure Diamond D11901
Precision balance	Ohaus	OH30429812-1EA
Borosilicate glass reagent bottles with polypropylene cap and pouring ring	Pyrex	1516
PIPETMAN P1000 pipette	GILSON	F144059M
Pipette tips (1250 µL)	VWR International Eurolab	613-6439
Fine forceps (Dumont Tweezers #55)	Bionic Iberica	11295-51
Square petri dish (120 × 120 mm)	BELLES DIAGNOSTIC	200204
Microscope Slides (26×76 mm)	Epredia SuperFrost	17214894
Coverslips (24×50 mm)	ACEFE, S.A.U.	COVN-050-1K0
Plant growth chamber	Inkoa Sistemas	LRmAIT-RS485
Aluminum foil	Nerium Scientific	LM-0403
Parafilm	FISHER SCIENTIFIC	11762644
Micropore tape	3M	115-8172
Laminar flow hood	Telstar	AH-100
Autoclave	Matachana	S1000

MATERIALS AND EQUIPMENT

Potassium hydroxide (KOH) solution (1 M)		
Reagent	Final concentration	Amount
Potassium hydroxide (KOH, ≥85% basis)	1M	6.6 g
Milli-Q water	N/A	To 100 mL
Total	N/A	100 mL

Note: The mass of KOH was adjusted according to the stated purity (≥85% basis). Dissolve KOH pellets in approximately 70–80 mL of Milli-Q water under stirring. The dissolution process is strongly exothermic. Allow the solution to cool to room temperature before adjusting the final volume to 100 mL with Milli-Q water. The solution was prepared in a chemical-resistant container and stored tightly closed at room temperature.

Murashige and Skoog (MS) media		
Reagent	Final concentration	Amount
Murashige & Skoog basal medium with vitamins	4.4 g/L	4.4 g
MES hydrate	0.5 g/L	0.5 g
Plant agar	1% (m/v)	10 g
Milli-Q water	N/A	To 1 L
Total	N/A	1 L

Note: Adjust the pH to 5.8 using 1 M KOH before adding agar. For liquid MS medium, agar is omitted. Autoclave to sterilize. Allow the medium to cool until it can be handled safely, then pour into Petri dishes. Solidified media can be stored at 4°C for up to 4 weeks.

Sterilization solution		
Reagent	Final concentration	Amount
Commercial bleach	15% (v/v)	15 mL
Tween-20	0.01% (v/v)	0.1 mL
Milli-Q water	N/A	To 100 mL
Total	N/A	100 mL

STEP-BY-STEP METHOD DETAILS

Preparation of *Arabidopsis* seedlings for imaging

⌚ Timing: 1 min

This step prepares seedlings on a microscope slide for confocal imaging.

1. Using fine forceps, gently transfer 3-day-old or 8-day-old seedlings from MS plates to a microscope slide.
2. Add a small drop of liquid MS medium to prevent desiccation. Use the same medium composition as in the growth plates, without agar, unless the experiment requires a specific treatment medium.
3. Carefully place a coverslip over the seedling (Figure 1C) and immediately proceed to imaging.

Note: Maintaining the same medium composition during imaging preserves physiological conditions comparable to those used for seedling growth and minimizes perturbations, including changes in external pH conditions.

⚠ **CRITICAL:** Do not apply excessive mechanical pressure when placing the coverslip as this may damage the seedling or alter tissue morphology. Avoid bubble formation, as bubbles can interfere with imaging.

Confocal imaging of PM-Apo-Acidin4

⌚ Timing: 2–5 min per sample

This step acquires confocal images suitable for ratiometric analysis.

4. Turn on the confocal microscope (we used a LSM 980 laser-scanning confocal microscope integrated with the Zeiss Elyra 7 platform). Allow the laser system to stabilize.

5. Select the 40× water-immersion objective (LD LCI Plan-Apochromat 40x/1.2 Imm Corr DIC M27) for imaging.
6. Locate the region of interest under brightfield or low laser power fluorescence mode.
7. Set up two-channel acquisition for ratiometric imaging:
 - a. Excite SYFP2 at 488 nm and collect emission at 510–554 nm.
 - b. Excite mRFP at 561 nm and collect emission at 580–650 nm.
8. Configure confocal acquisition parameters:
 - a. Set GaAsP-PMT detection or appropriate detector type.
 - b. Set pinhole to 1 Airy Unit (AU) for each channel.
 - c. Set laser power at 2%–5%.
 - d. Set detector gain at 750–850 or adjust according to the microscope and signal intensity.
 - e. Set LSM scan speed at 6 (pixel dwell time: 4.10 μs).
 - f. Set scan direction at bidirectional.
 - g. Enable averaging at 2.
9. Acquire two-channel images simultaneously for the region of interest.
10. Adjust laser power and detector gain to avoid pixel saturation.
11. Export raw two-channel images without additional processing.

Note: The imaging parameters listed above were optimized for the ZEISS LSM 980 confocal microscope used in this study and are provided as a reference. Laser power, detector gain, scan speed, and detector settings may require adjustment depending on the microscope configuration, detector type, objective lens, and sample fluorescence intensity. A critical requirement is to obtain sufficient signal while preventing detector saturation and minimizing photobleaching.

Note: Appropriate controls for ratiometric imaging include wild-type seedlings lacking the fluorescent reporters to assess background autofluorescence and lines expressing a single fluorescent protein to evaluate potential spectral bleed-through between channels.

Note: Use SYFP2 as channel 1 and mRFP as channel 2. This channel order is required for the image-processing macro to calculate the ratiometric signal by dividing channel 1, excited at 488 nm, by channel 2, excited at 561 nm.

⚠ **CRITICAL:** Ensure that pixel intensities are not saturated in either channel, as saturation will lead to inaccurate ratiometric calculations.

⚠ **CRITICAL:** Maintain identical imaging settings (laser power, gain, pinhole, scan speed, etc.) across all samples in the same experiment to ensure reliable comparative ratiometric analyses.

Ratiometric image processing and quantification of apoplastic pH

⌚ **Timing:** 5–10 min per batch (20 images)

This step generates ratiometric images and quantitative outputs from two-channel confocal data using a Fiji-based macro for semi-automated processing and analysis.⁴

12. Organize image files for batch processing.
 - a. Place raw two-channel images in a source folder (Folder 1).
 - b. Create an empty destination folder (Folder 2) for output files.
13. Open Fiji (ImageJ, Life-Line version, 22 December 2015).
14. Load and run the ratiometric analysis macro.
 - a. Navigate to *Plugins* → *New* → *Macro* (Figure 2A).

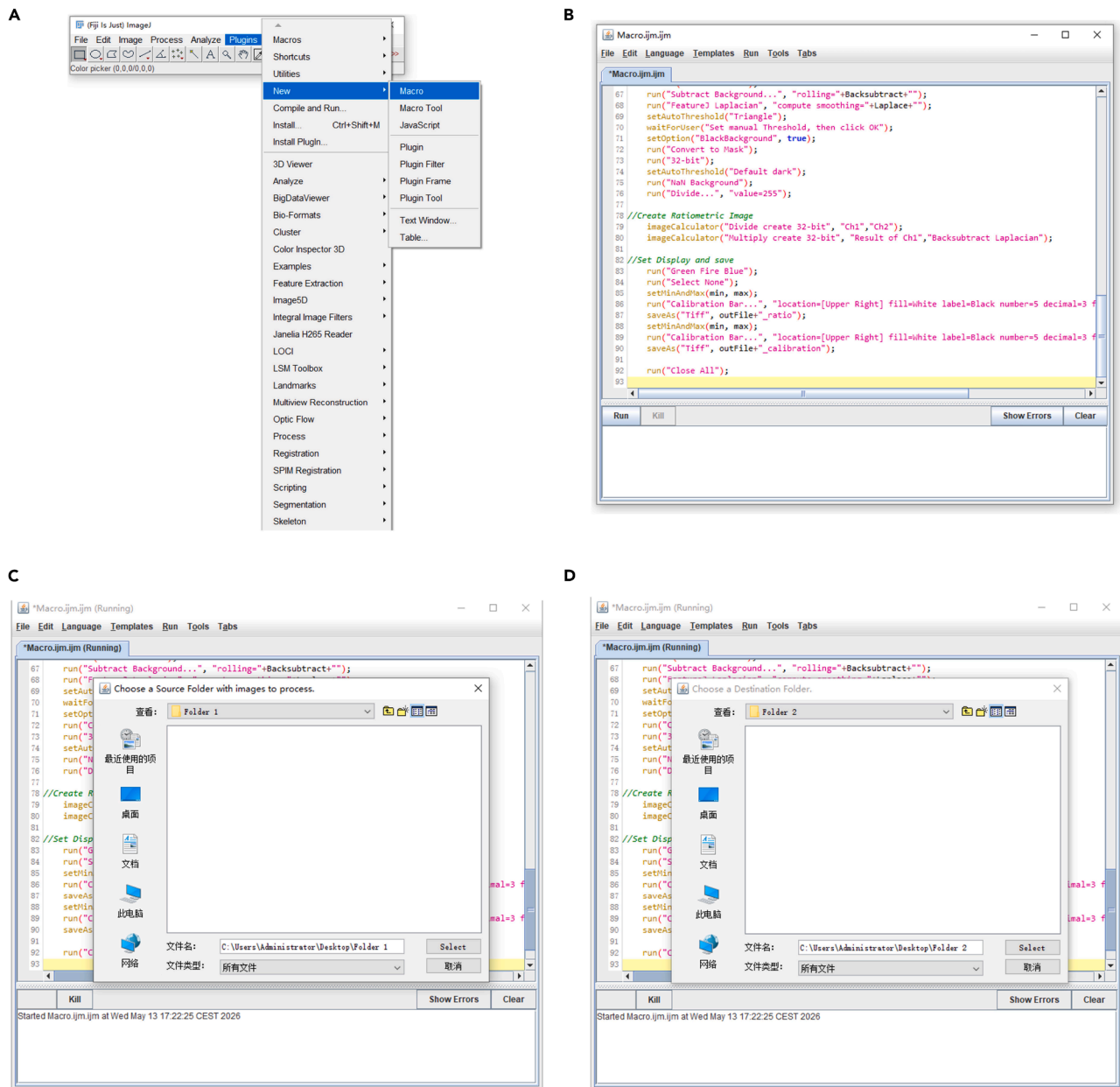


Figure 2. Workflow for ratiometric image analysis using a custom macro in ImageJ/Fiji

- (A) Opening the macro editor in ImageJ/Fiji via **Plugins** → **New** → **Macro**.
 (B) Pasting the ratiometric analysis macro into the editor window and running the script. The full macro code is provided in [Data S1](#).
 (C) Selecting the source folder, Folder 1, containing the images to be processed.
 (D) Selecting the destination folder, Folder 2, for saving the output files.

- b. Paste the macro code into the editor window (see [Data S1](#) for the full script) ([Figure 2B](#)).
 - c. Click **"Run"** ([Figure 2B](#)).
 - d. Select Folder 1 as the source folder containing images to process ([Figure 2C](#)).
 - e. Select Folder 2 as the destination folder for output files ([Figure 2D](#)).
 - f. During processing, manually adjust the threshold for each image when prompted, then click **"OK"** to continue.
15. Locate output files in Folder 2:
- a. *ratio.tif*: raw ratiometric image (32-bit) for quantitative analysis.

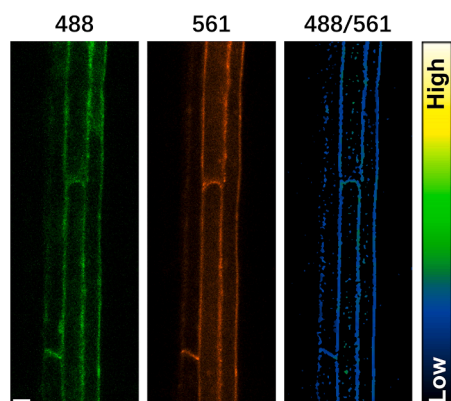


Figure 3. Confocal images and color-coded ratiometric output images

Confocal images of the 488 nm and 561 nm channels and the corresponding color-coded ratio image showing apoplastic pH in the root phloem of seedlings expressing the CoYMV::PM-Apo-Acidin4 pH sensor. Scale bar, 5 μ m.

b. *calibration.tif*: color-coded image with a lookup table (LUT) and calibration bar for visualization.

16. Import *ratio.tif* into Fiji using drag-and-drop or *File* $\rightarrow$ *Open*.

17. Select the region of interest (ROI) and measure the mean gray value to obtain relative apoplastic pH values.

Note: The Fiji macro used here is adapted from a previously published ratiometric imaging workflow⁴ (see [Data S1](#) for full script). In this macro, segmentation and mask generation are performed using channel 2 (mRFP; Segment = 2 in the script). Key processing steps include Gaussian smoothing, Laplacian of Gaussian filtering, background subtraction, and user-defined thresholding prior to mask generation.

Note: The accuracy of apoplastic pH measurements in epidermal cells can be validated using HPTS (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt) staining.⁴

⚠ **CRITICAL:** Always use raw, unprocessed images exported directly from the microscope. Prior image manipulation may distort channel intensities and alter ratiometric values.

⚠ **CRITICAL:** Key parameters (e.g., *Segment*, *Blur*, *Laplace*, *Backsubtract*, *min*, *max*) are pre-defined within the macro and can be adjusted before running, depending on image quality and experimental conditions. The *min* and *max* values should be optimized to match the dynamic range of the dataset for proper visualization.

⚠ **CRITICAL:** Maintain identical imaging settings across samples, since ratiometric analysis assumes comparable acquisition conditions. The same display range (*min/max*) must be applied to all samples within one experiment to ensure comparability.

EXPECTED OUTCOMES

Completion of this protocol generates high-quality ratiometric images of apoplastic pH in *Arabidopsis* seedlings with cell specificity. Two-channel confocal images are processed into ratio files for pixel-wise quantification of the SYFP2/mRFP ratiometric signal, which can be used as a relative readout of apoplastic pH, and into color-coded ratiometric images for visualization (Figure 3). In young seedlings, companion cell-specific expression of PM-Apo-Acidin4 enables reliable signal detection in phloem-associated cells, where the tissue remains sufficiently thin for confocal light penetration and fluorescence detection. In eight-day-old seedlings, constitutive expression of PM-Apo-Acidin4 enables visualization of the root phloem near the root tip, where sieve elements

are sufficiently developed and relative differences in apoplastic pH-associated sensor signal can be detected. The processed images enable quantitative measurement of relative ratiometric values and visual assessment of pH-associated signal distribution across distinct cell types. This supports comparative analyses between genotypes, treatments or developmental stages, and can be applied to both single-time-point and time-course experiments.

LIMITATIONS

This protocol enables cell-specific ratiometric quantification of apoplastic pH in *Arabidopsis* seedlings, but several limitations should be considered. First, signal detection is constrained by tissue thickness and confocal imaging depth. For companion cell-specific *PM-Apo-Acidin4* expression, young seedlings provide optimal conditions because the hypocotyl phloem remains sufficiently thin for reliable fluorescence detection. In older seedlings, increased tissue thickness can reduce light penetration and compromise imaging of deeper vascular layers. Second, constitutive *PM-Apo-Acidin4* expression generates broad fluorescence across multiple cell types, which limits cell-type resolution compared with cell-specific expression. Third, this method provides relative rather than absolute pH measurements. Therefore, consistent imaging settings and analysis parameters are required for accurate comparisons across samples.

TROUBLESHOOTING

Problem 1

Weak or undetectable fluorescence signal (related to step 9).

Potential solution

- Increase laser power or detector gain while avoiding pixel saturation.
- Confirm correct excitation/emission settings for both channels (488 nm and 561 nm).
- Ensure seedlings remain hydrated during mounting (steps 1–3).

Problem 2

Phloem near the root tip in 35S::PM-Apo-Acidin4 seedlings is not clearly resolved (steps 9–11).

Potential solution

- Use older seedlings (e.g., ~6–8-day-old) to ensure proper phloem development.
- Focus on regions close to the root tip where phloem structure is more distinguishable.
- Adjust the focal plane carefully to identify vascular strands.

Problem 3

Reduced amplitude of pH changes detected in hypocotyl epidermal cells (steps 12–17).

Potential solution

- Use younger seedlings, as hypocotyl elongation is more active and pH differences are more pronounced.

Problem 4

Apparent lack of color differences (steps 14–15).

Potential solution

- Apply identical min/max settings across all samples within the same experiment to ensure visual comparability.
- Avoid using overly broad ranges (e.g., 0–3) when actual values fall within a narrower interval.

Problem 5

Variability between samples reduces statistical confidence (steps 17).

Potential solution

- Analyze multiple regions of interest (ROIs) per seedling.
- Analyze multiple seedlings per biological replicate.
- Avoid selecting biased regions during quantification.
- Apply the same ROI selection criteria across all samples.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Paloma Mas (paloma.mas@cragenomica.es).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Paloma Mas (paloma.mas@cragenomica.es).

Materials availability

This study generated PM-Apo-Acidin4 companion cell-specific *Arabidopsis* line. Seeds and the plasmid are readily available upon request to the [lead contact](#), Paloma Mas (paloma.mas@cragenomica.es).

Data and code availability

This study did not generate new data or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.M.; investigation, L.X.; writing – original draft, L.X. and P.M.; writing – review and editing, L.X. and P.M.; funding acquisition, P.M.; supervision, P.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2026.104761>.

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