

METHODOLOGY

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A workflow for absolute apoplastic pH assessment during live cell imaging in plant roots

Ann-Kathrin Rößling^{1,2} , Niklas Mayle¹ and Elke Barbez^{1,2*}

Abstract

Background Apoplastic pH is a central regulator of plant growth, development, and environmental adaptation, influencing cell expansion, nutrient uptake, and extracellular signaling. Many studies have successfully used HPTS to monitor relative changes in apoplastic pH in plants. At the same time, research increasingly targets pH-dependent biochemical and biophysical processes. Many enzymatic activities, ion binding events, and receptor–ligand interactions depend on defined proton concentrations. Accordingly, the development of reliable approaches to measure absolute pH in living tissues is gaining importance.

Methods A calibration-based workflow was developed to enable quantitative assessment of absolute apoplastic pH using ratiometric HPTS imaging. The approach integrates a simplified two-point normalization strategy with an in-vitro derived sigmoidal calibration model, thereby minimizing the need for extensive in-vivo calibration curves. Confocal imaging was performed using HPTS excited at two wavelengths followed by ratiometric image processing. Data analysis is supported by a custom Fiji plugin, Ratio2pH, which converts ratiometric images into pixel-resolved maps of absolute pH.

Results In vitro characterization revealed a robust, non-linear relationship between normalized HPTS ratios and pH, enabling accurate pH estimation within the physiologically relevant range of pH 5.0–7.0. When applied in-vivo to *Arabidopsis thaliana* roots, the workflow yielded extracellular pH estimates consistent with the pH of the incubation medium and detected reproducible pH shifts in response to pharmacological treatments.

Conclusions This workflow enables reproducible, spatially resolved measurement of absolute apoplastic pH in living plant tissues. By combining a simplified calibration strategy with accessible image analysis tools, it facilitates quantitative extracellular pH measurements and their integration into biochemical and biophysical analyses.

Keywords Apoplastic pH sensor, HPTS, Ratiometric imaging, Confocal microscopy, Fiji plugin, Live-cell imaging

*Correspondence:

Elke Barbez

elke.barbez@biologie.uni-freiburg.de

¹Institute of Biology II, Molecular Plant Physiology (MoPP), University of Freiburg, 79104 Freiburg, Germany

²Center for Integrative Biological Signalling Studies (CIBSS), University of Freiburg, 79104 Freiburg, Germany



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Introduction

The pH of the plant apoplast plays a central role in regulating growth, development, and environmental adaptation [1–3]. Classical models, such as the acid growth theory, highlight how apoplastic acidification in maize coleoptiles promotes cell elongation by activating wall-loosening enzymes like expansins [4, 5]. In addition, auxin-triggered apoplast alkalization steers root bending during gravitropism in *Arabidopsis* [6]. Abiotic stresses, including salinity and drought, also induce changes in apoplastic pH that help initiate systemic defense responses [3].

Despite its physiological importance, accurately quantifying apoplastic pH in living tissues remains technically challenging [7]. In previous work, we demonstrated that 8-hydroxy-pyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) is a reliable and effective dye for the relative assessment of apoplastic pH in *Arabidopsis thaliana* roots [6]. HPTS is a cost-effective, non-toxic fluorescent indicator that passively diffuses into the root apoplast and performs well in live-cell imaging applications [8]. Like other pH-indicators, HPTS exists in a dynamic balance between its protonated and deprotonated states. Interestingly, the protonated and deprotonated HPTS form distinct wavelength (λ) excitation maxima of 405 nm and 465 nm respectively. These spectral characteristics enable imaging of the protonated and the deprotonated HPTS version in 2 different imaging channels. The ratio of these two signals provides a reliable readout that correlates with the surrounding pH. So far, HPTS enabled many researchers to assess relative pH in the apoplast of *Arabidopsis* roots [8–14].

The assessment of relative pH dynamics has dissected substantial plant physiological processes in the past and will continue to do so in the future. However, the study of specialized biophysical and biochemical processes may require the measurement of absolute apoplastic pH values. The activity of enzymes, the behavior of pH-sensitive binding sites, and the charge state of biomolecules all depend on defined pH thresholds. Subtle differences in proton concentration can influence molecular conformation, reaction rates, and interaction affinities. Spatially resolved measurements of absolute pH are therefore essential for linking extracellular pH dynamics to their functional consequences in plant physiology. Using HPTS, absolute pH approximations are currently possible but require the generation of an extensive in vivo calibration curve in each experiment to account for variability [6]. Generating an in vivo calibration curve is therefore helpful, but laborious and time-consuming.

Here, we introduce a novel calibration strategy that enables the quantification of absolute apoplastic pH values in vivo. This approach relies on a normalization step

that reduces experiment-to-experiment variability. The simplified workflow removes the need for generating multiple in vivo pH standards while maintaining measurement accuracy. To support image analysis, we provide a user-friendly Fiji plugin that transforms HPTS ratio images into absolute pH maps, where each pixel reflects the local extracellular pH.

Results

Absolute pH assessment in vitro

To characterize the pH-dependent behavior of HPTS under controlled conditions, we prepared liquid $\frac{1}{2}$ MS medium supplemented with 1 mM HPTS and adjusted the pH across a range from 4.0 to 8.0 in steps of 0.5 pH units. Fluorescence emission at 514 nm (± 10 nm) was recorded upon excitation at 405 nm (protonated form) and 465 nm (deprotonated form) using a microplate spectrophotometer. Signal intensities were measured across different detector gain settings to assess the robustness of the ratiometric signal across variable imaging conditions. For each sample, we calculated the 465/405 fluorescence intensity ratio and implemented a min-max normalization strategy to account for variation in signal strength due to detection gain, laser power, or sample handling.

Normalization was performed according to the formula:

$$x_{norm.} = \frac{x - \bar{x}_{pH4}}{\bar{x}_{pH8} - \bar{x}_{pH4}}$$

x = measured 465 nm/405 nm fluorescence intensity ratio for any sample

$\bar{x}_{pH4}$ = mean ratio at pH 4.0 (lower calibration point)

$\bar{x}_{pH8}$ = mean ratio at pH 8.0 (upper calibration point)

$x_{norm.}$ = normalized fluorescence ratio

This normalization allows for comparability across independent measurements. Plotting the normalized HPTS ratios against the known pH of each solution revealed a strong, non-linear correlation (Fig. 1a). Regression analysis yielded a logistic sigmoidal equation that accounted for 99.77% of the variance, suggesting an excellent fit to the data (Fig. 1). The inverted calibration equation allows the calculation of pH values from normalized HPTS ratios. However, because the calibration curve follows a sigmoidal shape, the reliability of pH extraction varies along the curve. In the steep central region, small changes in normalized ratios correspond to well-defined pH differences, providing high predictive accuracy. In contrast, near the upper and lower plateaus, similar ratio changes can result in disproportionately large shifts in calculated pH, reducing precision. Consequently, identical levels of ratio variability yield different

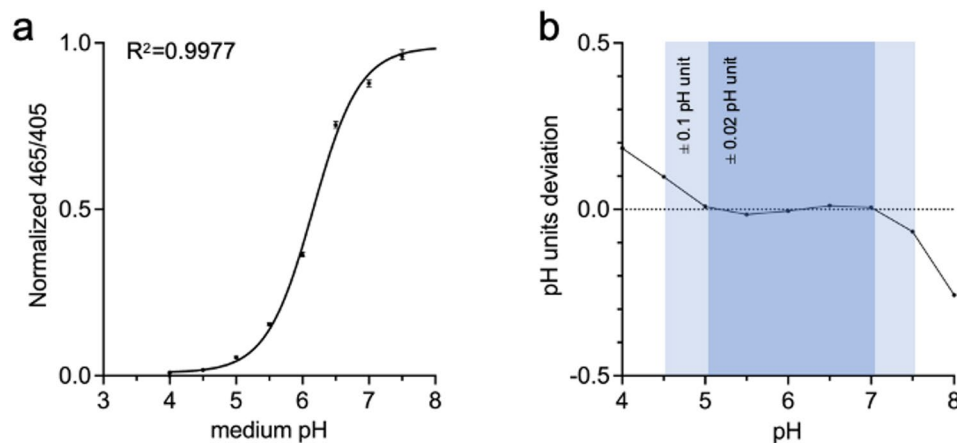


Fig. 1 In vitro HPTS characterization. (a) Graph depicts the normalized 465/405 fluorescence ratio values of HPTS-supplemented liquid medium with pH values ranging from 4.0 to 8.0 as measured using a microplate spectrophotometer. The sigmoidal regression curve with $R^2 = 0.9977$ is shown. (b) Graph depicts how much the pH, determined by the regression curve in (a), can deviate (y-axis) from the actual pH (x-axis), assuming an average SD in normalized HPTS ratios of 0.01

accuracies depending on the pH range. To determine the usable range of our model, we simulated how the average standard deviation of normalized HPTS ratios (0.01) would affect the resulting pH calculation (Fig. 1b). The model indicates that pH values between 4.5 and 7.5 can be determined with a precision of approximately ± 0.1 pH units. Within the narrower range of pH 5.0 to 7.0, the accuracy is even higher (± 0.015 pH units), allowing highly reliable extraction of absolute apoplastic pH values. Based on these findings, we focused further modeling and analysis on the pH 5–7 range.

We next prepared HPTS-supplemented medium in a pH range from pH 5.0 to pH 7.0 in steps of 0.1 pH units. We recorded fluorescence emission at 514 nm upon excitation at 405 nm (protonated form) and 465 nm (deprotonated form) using a microplate spectrophotometer and normalized the 465/405 fluorescence intensity ratio according to:

$$x_{norm.} = \frac{x - \bar{x}_{pH5}}{\bar{x}_{pH7} - \bar{x}_{pH5}}$$

x = measured 465 nm/405 nm fluorescence intensity ratio for any sample.

$\bar{x}_{pH5}$ = mean ratio at pH 5.0 (lower calibration point).

$\bar{x}_{pH7}$ = mean ratio at pH 7.0 (upper calibration point).

$x_{norm.}$ = normalized fluorescence ratio.

We performed a logistic sigmoidal regression in which $x = \log(\text{concentration})$ and did not fix the asymptotes for better data fitting (more detailed description of the regression analysis can be found in the material and methods section). The resulting model (Fig. 2a, b can be described by the following parameters:

Top:	upper asymptote	1.117
Bottom:	lower asymptote	-0.029
LogIC50:	inflection point	6.211
HillSlope:	slope factor	1.213

$$x_{norm} = Bottom + \frac{Top - Bottom}{1 + 10^{(LogIC50 - pH) * HillSlope}}$$

$$x_{norm} = -0.029 + \frac{1.147}{1 + 10^{(6.211 - pH) * 1.213}}$$

Since the normalized HPTS ratios robustly correlate with pH ($R^2 = 0.998$), this data set enabled us to formulate an equation that converts normalized HPTS ratios to absolute pH values.

$$pH = LogIC50 - \frac{1}{HillSlope} \log_{10} \left(\frac{Top - Bottom}{x_{norm} - Bottom} - 1 \right)$$

$$pH = 6.211 - \frac{1}{1.213} \log_{10} \left(\frac{1.147}{x_{norm} + 0.029} - 1 \right)$$

Using this equation, we calculated pH values from normalized ratios and confirmed that the predicted values closely matched the actual pH of the samples (Fig. 2c).

We next calculated how much the calculated pH values deviate from the original medium pH. This analysis helps us understand how well HPTS can be used to differentiate between small changes in pH levels (Fig. 2d). The absolute deviation from the original medium pH was on average 0.034 ± 0.001 pH units. We do observe slightly higher deviations above pH 6.5, presumably due to the reduced buffer capacity of MES at higher pH. Overall, this data indicates that HPTS is able to reliably

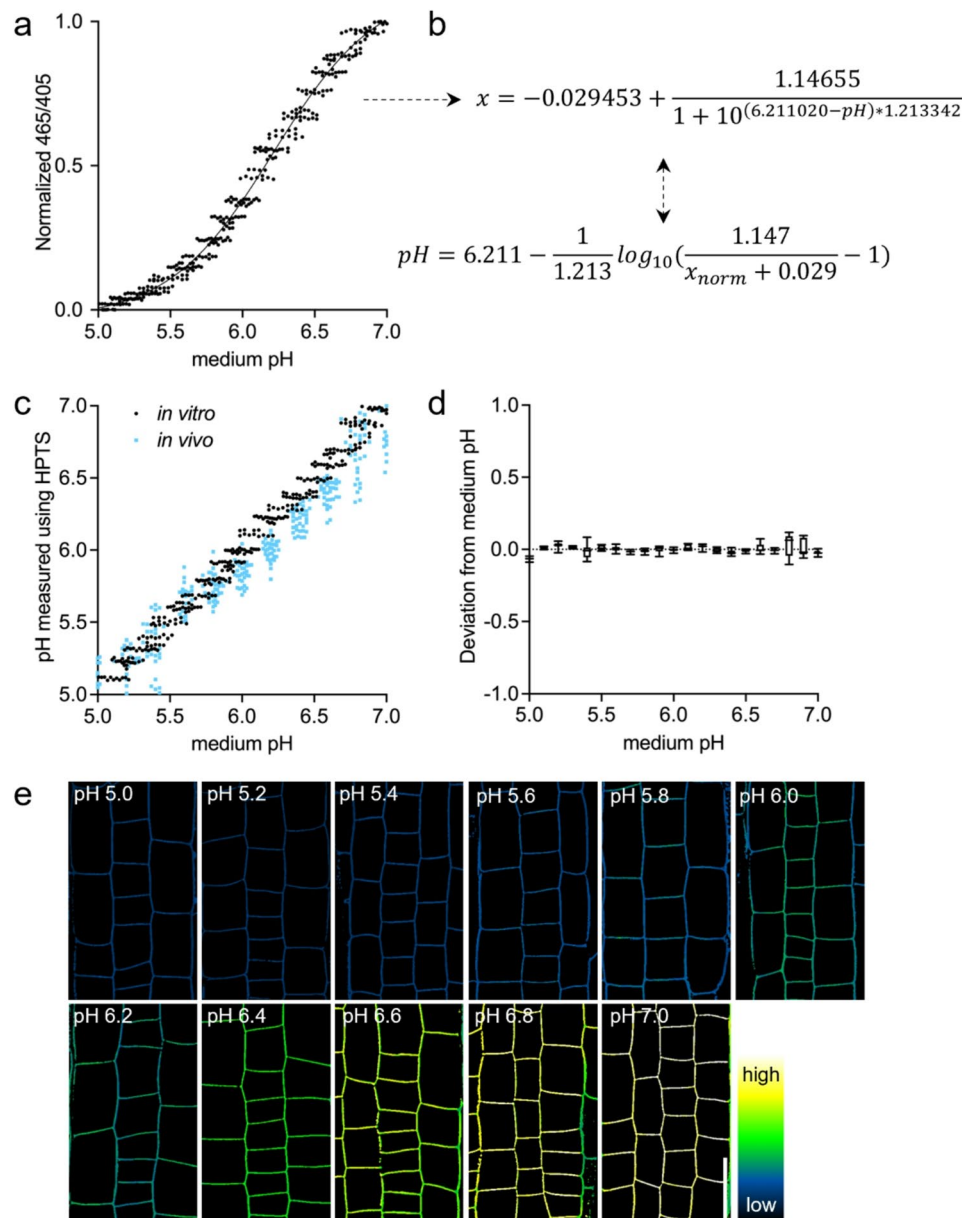


Fig. 2 HPTS calibration in vitro and in vivo. **(a and b)** In vitro HPTS characterization. **(a)** Graph depicts the min-max normalized 465/405 fluorescence ratio values of HPTS-supplemented liquid medium with a pH across a range from 5.0 to 7.0 as measured using a microplate spectrophotometer. **(b)** The sigmoidal regression curve with $R^2 = 0.9980$ is shown, as well as the inverted function that converts normalized HPTS ratios into absolute pH values. **(c)** The pH measured in vitro and in vivo via HPTS normalization and conversion is plotted against the medium pH. **(d)** Box-plots depict the deviation of HPTS-measured pH in vitro from the original medium pH. **(e)** In vivo HPTS characterization. Representative confocal images of seedlings incubated for 10 min and subsequently imaged in HPTS-supplemented medium with varying pH between pH5 and pH7. Scale bar = 25 μ m, $n = 9-12$

distinguish pH differences smaller than 0.1 pH units (Fig. 2d).

Absolute pH assessment in vivo

To test our calibration strategy in a biological context, we incubated *Arabidopsis thaliana* Col-0 seedlings for 10 min in HPTS-supplemented liquid medium adjusted to pH values between 5.0 and 7.0. Via confocal

microscopy, we imaged the protonated ($\lambda_{ex} = 405$ nm) and deprotonated ($\lambda_{ex} = 465$ nm) HPTS versions in 2 separate channels.

The resulting fluorescence images were processed using our “ratiometric image conversion” Fiji plugin [6, 8], which provides an output image where each pixel depicts the ratio of the 465 nm/405nm signal intensities (Fig. 2e). In order to normalize the HPTS intensity ratios in

analogy to the in vitro approach above, we measured the average HPTS intensity ratios of the roots incubated in pH5 ($\bar{x}_{pH5}$) and pH7 ($\bar{x}_{pH7}$). We subsequently measured the average HPTS intensity ratios of the roots incubated in all pH solutions and normalized those values using the same min-max approach as described above. The resulting normalized ratios were then used to calculate absolute pH values via the calibration equation derived from the in vitro dataset (Fig. 2b).

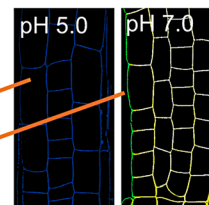
Plotting the calculated apoplastic pH values against the pH of the incubation medium revealed a strong correspondence between predicted and actual values (Fig. 2c). This confirms that the normalization and calibration approach developed in vitro is applicable to in vivo imaging and can reliably estimate extracellular pH in living plant tissues.

The Ratio2pH Fiji plugin

To facilitate data analysis, we developed Ratio2pH: a custom Fiji plugin that converts ratiometric HPTS images into absolute apoplastic pH maps (Fig. 3). The plugin requires a pre-processed ratio image as input (Fig. 3a). The latter are the output images from the “ratiometric image conversion” plugin [6, 8]. Ratio2pH will perform a min-max normalization step to correct for daily variation in laser stability and experimental conditions. Users can choose to normalize the image using either manually entered reference values or by loading representative calibration images from samples imaged at pH 5.0 and pH 7.0 (Fig. 3b). The plugin performs min-max normalization on every pixel of the ratio image using the following equation:

a. Input/output files and folders

b. Calibration



c. Conversion to absolute pH using our sigmoidal model:

$$pH = \log IC50 - \frac{1}{Hillslope} \log_{10} \left(\frac{Top - Bottom}{x_{norm} - Bottom} - 1 \right)$$

d. Output style and pH range

Fig. 3 Converting HPTS ratios into absolute pH values: The Fiji Plugin Ratio2pH. Our custom-made Fiji macro enables users to convert HPTS ratio images into images depicting absolute pH values. **(a)** Define the location of the input files (HPTS ratio files) and the location where the output images should be stored. Define the file extension of the input files. **(b)** Decide to enter calibration values manually (manual) or to upload representative calibration images (from calibration images). The plugin can consider only one option. **(c)** Enter the sigmoidal equation parameter from our default analysis (Top=1.1171; Bottom = -0.0295; LogIC50=6.2110; Hillslope = 1.2133) OR from an adjusted in vitro HPTS calibration and regression analysis. **(d)** Choose a color code (LUT) that will depict absolute pH values, and define the range of pH values that will be shown

$$x_{(x,y)norm.} = \frac{x - \bar{x}_{pH5}}{\bar{x}_{pH7} - \bar{x}_{pH5}}$$

(x, y) = pixel in position x, y

$x_{(x,y)}$ = 465 nm/405 nm intensity ratio per pixel

$\bar{x}_{pH5}$ = mean 465 nm/405 nm intensity ratio of the loaded calibration image OR manual value

$\bar{x}_{pH7}$ = mean 465 nm/405 nm intensity ratio of the loaded calibration image OR manual value

$x_{(x,y)norm.}$ = normalized fluorescence ratio per pixel

Subsequently, it converts each pixel's normalized value into an absolute pH using the calibration function derived from in vitro experiments (Fig. 2a, b):

$$pH = 6.211 - \frac{1}{1.213} \log_{10} \left(\frac{1.147}{x_{norm} + 0.029} - 1 \right)$$

For users working with different buffer systems or experimental conditions, the Ratio2pH offers the option to input a custom calibration function. By performing their own in vitro calibration using HPTS in the relevant buffer system, users can determine a sigmoidal regression curve that best fits their data. The Ratio2pH interface allows manual entry of the corresponding parameters (Top, Bottom, logIC50, and HillSlope), enabling flexible adaptation to alternative calibration curves and ensuring accurate pH conversion across diverse experimental setups (Fig. 3c).

$$pH = \text{LogIC50} - \frac{1}{\text{HillSlope}} \log_{10} \left(\frac{\text{Top} - \text{Bottom}}{x_{norm} - \text{Bottom}} - 1 \right)$$

The Ratio2pH plugin outputs a 32-bit image in which absolute pH values are color-coded using a user-defined lookup table (LUT) applied across a specified pH range (Fig. 3d). These images support precise quantification of apoplastic pH and can be further analyzed in Fiji, allowing users to extract pH values from defined regions of interest.

Workflow for absolute apoplastic pH assessment

In order to assess approximate absolute apoplastic pH values, users have to implement 2 calibration samples in each of their experiments. The samples are mock-treated WT seedlings incubated in liquid HPTS-supplemented medium at pH 5.0 ($\bar{x}_{pH5}$) or pH 7.0 ($\bar{x}_{pH7}$), each imaged using identical confocal settings. These reference roots — typically 3 to 5 wild-type seedlings per calibration pH — serve to define the lower ($\bar{x}_{pH5}$) and upper ($\bar{x}_{pH7}$) bounds of the 465/405 ratio range for normalization. Since HPTS signal intensities are sensitive to imaging depth, laser power, and detector gain, it is important to define calibration values for each experiment and

each tissue type of interest. This approach allows the normalization step to correct for experimental variation, enabling reliable conversion of ratiometric HPTS data into absolute apoplastic pH values (see overview in Fig. 4).

Dynamic regulation of absolute apoplastic pH in roots

To verify the functionality of our calibration-based workflow, we tested the effect of several pharmacological treatments on the absolute apoplastic pH in the root epidermis of six-day-old Arabidopsis seedlings. To calibrate our experiment, we incubated Col-0 wild-type seedlings for 10 min in HPTS-supplemented liquid ½ MS medium of pH 5.0 and pH 7.0. The obtained images were ratiometrically converted using our previously described ratiometric image conversion Fiji plugin [6, 8]; <https://doi.org/10.5281/zenodo.15658668>), as well as the Ratio2pH Fiji plugin for absolute pH annotation.

In general, fusicoccin activates, while DCCD inhibits proton pump activity at the plasma membrane, resulting in a lower and higher apoplastic pH, respectively [15]. Fusicoccin resulted in a 0.6 pH unit decrease on average, while DCCD increased the apoplastic pH by 0.3 pH units on average compared to mock-treated seedlings. Previous work showed that the phytohormone auxin triggers a fast apoplast alkalization in Arabidopsis roots [6]. Here, we are able to show that IAA treatment results in a 0.35 pH unit increase on average in apoplastic pH (Fig. 5).

Conclusion and outlook

Apoplastic pH is a highly dynamic parameter that steers a plethora of plant developmental aspects. The assessment of relative pH dynamics in the apoplast has been very valuable for plant biological research so far. However, biochemical and biophysical applications often require information on absolute apoplastic pH values at a cellular resolution.

Our workflow addresses this gap by combining a simple yet robust normalization strategy with a flexible Fiji plugin, making absolute apoplastic pH imaging more accessible and reproducible. The modular design of the Fiji plugin allows users to tailor the calibration function to their own imaging conditions or buffer systems, extending the method's flexibility. The ability to derive absolute pH maps at cellular resolution provides a bridge between visual observations and biochemical interpretation. Since HPTS is not toxic and bleach-resistant, our workflow can be used to measure apoplastic pH assessment during root growth over several hours or days [8]. As plant biologists increasingly recognize the apoplast as an active regulatory compartment, accessible tools like this will be key to decoding its complex contributions to adaptive plant development.

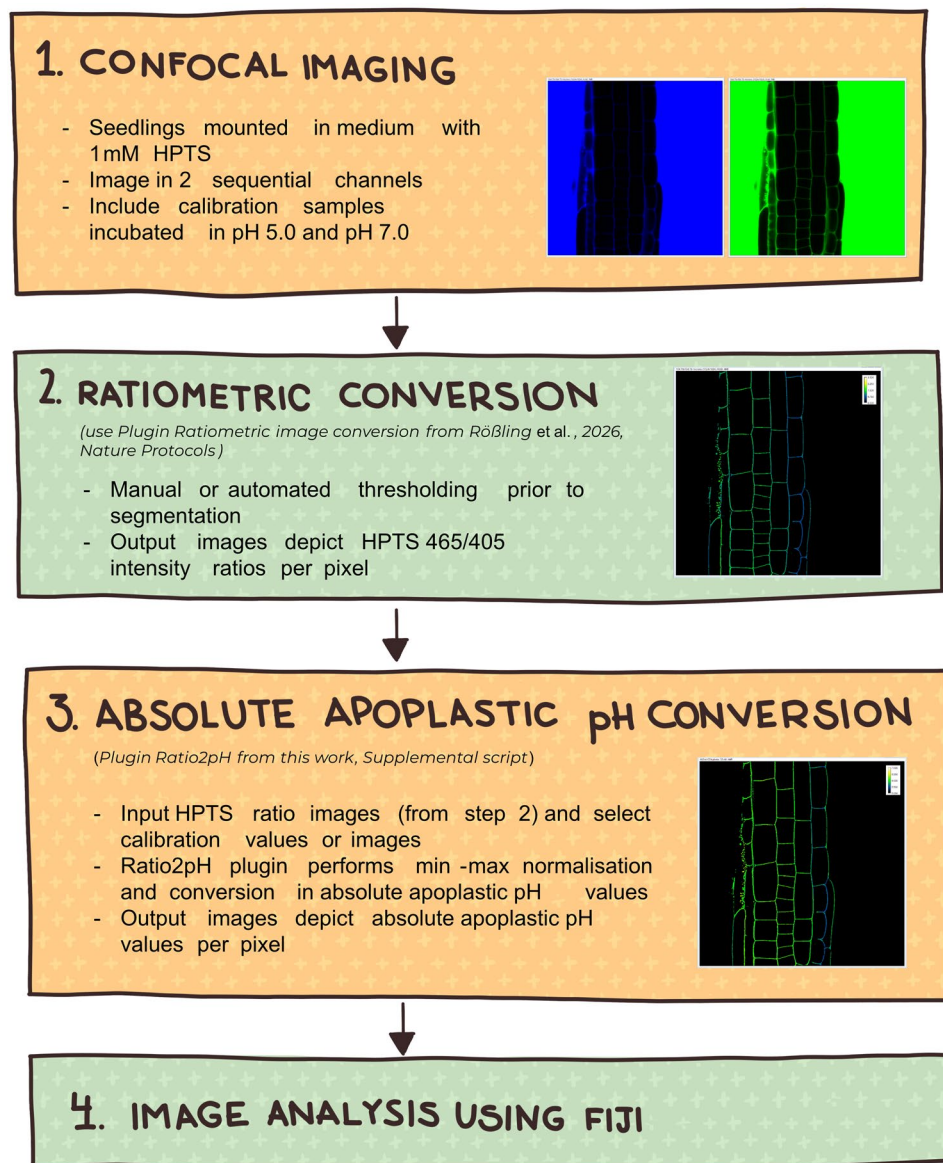


Fig. 4 Workflow of absolute apoplastic pH assessment using HPTS. The first step **(1)** is confocal imaging using 1 mM HPTS for mounting and staining. The imaging setup should allow for imaging sequentially in two channels to get the raw images. **(2)** Using the Ratiometric Image Conversion Fiji Plugin [8], the raw images are converted into HPTS ratio images. These images can be used for determining relative pH differences. **(3)** The Ratio2pH plugin generates 32-bit pH maps from the ratio images by applying the in vitro calibration equation and the experimental reference points (pH 5.0 and 7.0). Each pixel in the output image corresponds to an absolute pH value, displayed using a color Lookup table (LUT). **(4)** The Ratio2pH output images can be further analyzed in Fiji

Materials and methods

Plants

All experiments were carried out in *Arabidopsis thaliana*, ecotype Col-0, obtained from the Nottingham Arabidopsis Stock Centre (NASC, UK). After surface sterilization using 70% and 100% ethanol, the seeds were sown on Murashige and Skoog (MS) plates with 1% sucrose and a pH of 5.7, stratified for 2 days at 4 °C in darkness. Afterwards, they were grown vertically in a long-day regime (16 h light, 8 h darkness) at 21 °C/18°C.

Chemicals

Chemicals were dissolved in dimethyl sulfoxide (DMSO), which then also served as a solvent control. Fusicoccin (FC) (Sigma, MO, USA), from *Fusicoccum amygdali*, was used as a proton pump activator, whereas we used N, N'-Dicyclohexylcarbodiimide (DCCD) (Sigma (MO, USA)) to chemically inhibit proton pumps. For auxin treatment, 3-Indoleacetic acid (IAA) was utilized (OlChemIm, Czech Republic).

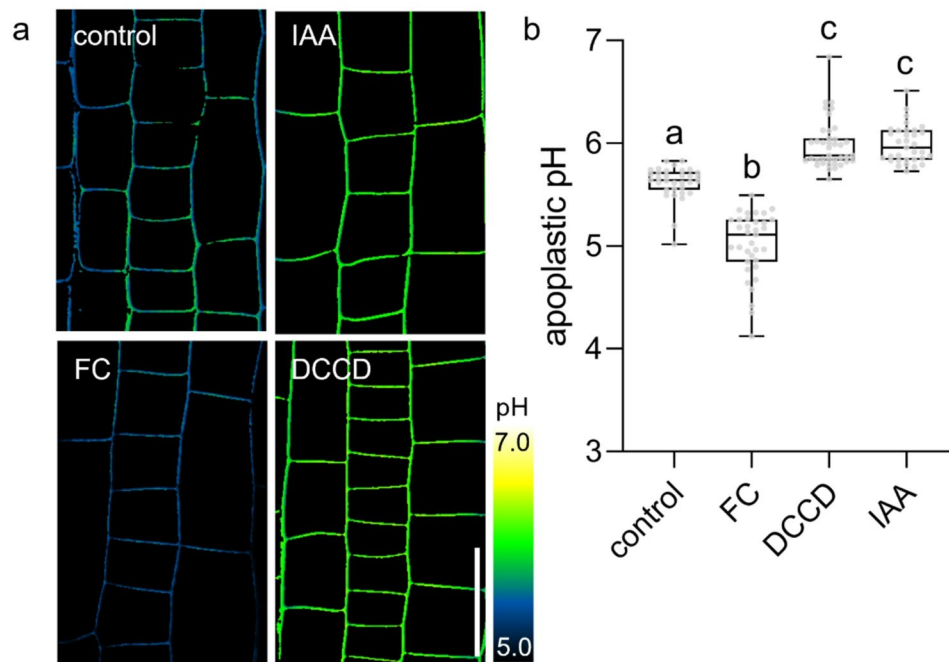


Fig. 5 Dynamic regulation of absolute apoplastic pH in Arabidopsis roots. **(a)** HPTS-based assessment of apoplastic pH in roots of 6-day-old Col-0 wild-type seedlings, treated with 5 μ M FC for 3 h, 5 μ M DCCD for 3 h or 250 nM IAA for 1 h or solvent control. $n=8-12$ roots per line/condition. **(b)** Boxplots depict absolute apoplastic pH from treatments shown in **(a)**. Scale bar = 25 μ m. Boxplots: Box limits represent the 25th and 75th percentile, and the horizontal line represents the median. Whiskers display min. to max. values. Representative experiments are shown. Statistical significance was determined using a one-way ANOVA with a Tukey Post Hoc multiple comparisons test ($p < 0.05$, letters indicate significance categories)

In vitro characterization of HPTS fluorescence

To assess the spectral behavior of HPTS in response to pH, we performed an in vitro calibration using HPTS in liquid plant growth medium. Liquid $\frac{1}{2}$ Murashige and Skoog (MS) medium was prepared and adjusted to pH values ranging from pH 4.0 to 8.0 in 0.5 pH unit increments (Fig. 1) or from 5.0 to 7.0 in 0.1 pH unit increments (Fig. 2). All solutions were autoclaved and cooled to room temperature prior to use. HPTS (8-hydroxy-pyrene-1,3,6-trisulfonic acid trisodium salt; Sigma-Aldrich) was added to each solution to a final concentration of 1 mM. For each pH condition, 200 μ L of HPTS-supplemented medium was transferred in quadruplicates into a black, flat-bottom 96-well microplate. Fluorescence intensity was measured using a microplate reader with excitation wavelengths at 405 nm and 465 nm, and emission recorded at 514 $\pm$ 8 nm for both channels. The ratio of fluorescence intensities (465/405) was calculated for each replicate. Ratio values were min-max normalized using the mean ratios from the lowest and highest assessed pH values respectively.

$$x_{norm.} = \frac{x - \bar{x}_{pH_{lowest}}}{\bar{x}_{pH_{highest}} - \bar{x}_{pH_{lowest}}}$$

x = measured 465 nm/405 nm fluorescence intensity ratio for any sample

$\bar{x}_{pH_{lowest}}$ = mean ratio of the samples with the lowest pH (lower calibration point)

$\bar{x}_{pH_{highest}}$ = mean ratio of the samples with the highest pH (upper calibration point)

$x_{norm.}$ = normalized fluorescence ratio

To generate the calibration model, normalized HPTS fluorescence ratios were plotted against the corresponding medium pH values ranging from 4.0 to 8.0 (Fig. 1) and 5.0 to 7.0 (Final model, Figs. 2, 3, 4 and 5). Data were fitted to a four-parameter sigmoidal (4PL) regression model of the form:

$$x_{norm} = Bottom + \frac{Top - Bottom}{1 + 10^{(LogIC50 - pH) * HillSlope}}$$

where Top and Bottom represent the upper and lower asymptotes, LogIC50 defines the inflection point, and HillSlope describes the curve steepness. Regression fitting and parameter extraction were performed using a custom-made python script. The model accounted for >99% of the data variance ($R^2 > 0.99$), confirming an excellent fit. We subsequently inverted the model, enabling pH calculations from normalized HPTS ratios.

To assess model reliability (Fig. 1), the standard deviation of normalized HPTS ratios obtained from independent technical replicates (SD=0.01) was propagated through the inverse function of the sigmoidal equation

to estimate the corresponding pH uncertainty across the entire range. This simulation yielded a reliability curve (Fig. 1b), indicating that pH values between 4.5 and 7.5 can be determined with an accuracy of ± 0.1 pH units, and between 5.0 and 7.0 with a precision better than ± 0.02 pH units. All curve fitting and error propagation analyses were conducted using GraphPad Prism 10.2.3 (GraphPad Software Inc., Boston, MA, USA) and Python 3.10 using PyCharm version 2024.3 Community Edition (JetBrains; Prague, Czech Republic) along with the packages NumPy 2.2.6, Matplotlib 3.10.6, pandas 2.2.3, and SciPy 1.15.3.

Python script for the generation of a curve fit of a sigmoidal model

For users who want to adjust for their experimental conditions or use a different buffer, they can generate their own HPTS in vitro calibration. Our Python script (Supplemental Script 1) offers a quick way of generating the parameters for an equation for user-defined calibration data. This equation can then be put into the plugin Ratio2pH. Users can use a csv file as input (see example file for formatting), where the file path can be entered in the console. In brief, the script reads in the data from the csv input file and then does a sigmoidal fit with initial guess parameters. Then it calculates the quality of the fit using R^2 , RMSE, and MAE. The relevant parameters, such as top, bottom, hillslope and logIC50 are then displayed in the console, along with the equation metrics, and the sigmoidal model is shown in a graph in a separate window. The parameters can then be entered into the plugin for the use of a user-defined calibration curve.

Confocal microscopy

For imaging, an upright Leica TCS SP8 FALCON FLIM confocal laser scanning microscope equipped with a Leica HC PL APO Corr 63 $\times$ 1.20 water immersion CS2 objective was used. Apoplastic pH assessment using 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS; Sigma-Aldrich) was carried out as introduced by Barbez et al., [6, 8]. The protonated form of HPTS was excited at 405 nm using a diode UV laser at 0.5% intensity, whereas the deprotonated form was excited at 458 nm utilizing an Argon laser, running at 30% intensity. Images were obtained in sequential scanning mode. As detectors, PMTs were used. The PMT gain stayed constant during imaging. For in vivo imaging of apoplastic pH in *Arabidopsis thaliana* roots for the calibration curve, six-day-old seedlings were incubated in HPTS-supplemented $\frac{1}{2}$ Murashige and Skoog (MS) liquid medium with different pH values between 5.0 and 7.0 in steps of 0.2 prior to confocal imaging. The treatments

were carried out as described in the figure legend. Confocal images were acquired sequentially using excitation at 405 nm and 458 nm and a common emission window at 514 nm. In this manuscript, we quantified late meristematic cells right before the onset of elongation (as described in Dünser et al., [16] and Müller et al., [17]) for analysis. However, the cells and tissue of interest can be freely chosen by the user.

The Fiji plugin

The here developed Ratio2pH plugin is available at Zenodo: <https://doi.org/10.5281/zenodo.15599805>. Ratio2pH converts 32-bit ratiometric HPTS images into absolute apoplastic pH value images and is implemented in Jython (Python for ImageJ). The macro prompts the user to input lower and upper calibration values (i.e., mean fluorescence intensity ratios at pH 5.0 and pH 7.0, respectively) or select representative pictures of seedlings incubated in liquid medium of pH 5.0 and pH 7.0.

The calibration values (manual mode) or the mean intensities of the calibration images (automated mode) will be used for normalization. For the automated mode, the plugin retrieves the mean grey value of the uploaded calibration images of the upper and lower calibration points. The mean gray value is obtained by calculating the sum of all pixels and dividing it by the number of pixels using Fiji's image statistics. For a very specific regions of interest, we recommend the manual mode for calibration. During normalization, the Ratio2pH plugin applies a *clamping step* to prevent extreme ratio values from producing infinite or undefined pH values and extrapolation errors when the inverse logistic equation is applied. Values at the upper or lower asymptote are slightly adjusted (by ± 0.0001) to remain within the valid range of the model. The normalized values are then converted to absolute pH using a logistic sigmoidal regression equation derived from in vitro HPTS characterization (Fig. 2). If a custom calibration equation, for instance, for a different buffering system, is desired, the parameters of the sigmoidal equation can be changed. Users select a Lookup table (LUT) and the image processing range, with the default being pH 5.0 and 7.0.

The resulting images display absolute pH values per pixel and are saved in 32-bit format for further analysis. For displaying the images, they need to be converted to RGB images first.

Other software

Fiji plugin: Ratiometric image conversion ([8]; <https://doi.org/10.5281/zenodo.15658668>).

Fiji plugin: Ratio2pH (This work, Supplemental script 2; <https://doi.org/10.5281/zenodo.15599805>) were used.

Fiji (image J): Schindelin et al., [18]

Illustrations

The workflow summary figure (Fig. 4) was created using the Concepts App for iOS (Version 6.9.2, TopHatch, Inc (Turku, Finland)) and edited using AffinityDesigner (version 1.10, Serif (West Bridgford, UK)) by Ann-Kathrin Rößling.

Abbreviations

HPTS	8-hydroxy-pyrene-1,3,6-trisulfonic acid trisodium salt
MS	Murashige and Skoog
FC	Fusicoccin
DCCD	N,N'-Dicyclohexylcarbodiimide
IAA	Indole-3-acetic acid
LUT	Lookup table

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Author contributions

A.-K.R. and E.B. conceived the study. A.-K.R. performed the experiments and data analysis. N.M. assisted in Fiji plugin and Python script development and image analysis. E.B. and AKR wrote the paper. All authors approved the final manuscript.

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Data availability

The Python Script for generating a user-defined sigmoidal calibration curve is available at Zenodo: <https://doi.org/10.5281/zenodo.17303477> The Ratio2pH Fiji plugin for absolute pH conversion is available at Zenodo: <https://doi.org/10.5281/zenodo.15599805> All data supporting the findings of this study are included in the article and supplementary files. Source data files and raw images are uploaded at Freidata, the data server of the University of Freiburg, available under <https://doi.org/10.60493/t29wb-7my86>.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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