

Multiparametric confocal fluorescence imaging for quantitative nanocarrier mapping in plant leaves

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Method Article

Keywords: Confocal microscopy, weed plants, nanoparticles, foliar autofluorescence

Posted Date: October 14th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11066653/v1>

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Additional Declarations: No competing interests reported.

Abstract

Background.

Tracking fluorescently labeled polymeric nanocarriers in leaf tissue using confocal laser scanning microscopy is limited by strong autofluorescence from chlorophyll and cell wall compounds, and by the absence of a common spatial reference against which the position of the detected signals can be compared across samples. To address these challenges, we describe an integrated workflow that reduces autofluorescence interference and provides a consistent spatial reference for signal localization. This procedure involves weed cultivation, foliar application of rhodamine-labeled nanocarriers by droplet deposition, and leaf processing along three complementary routes, namely, fixed tissue for localization, cryosectioned tissue for morphoanatomical characterization, and fresh tissue for the oxidative-response assay with 2',7'-dichlorodihydrofluorescein diacetate.

Results.

Tissue autofluorescence is deliberately recorded and employed twice: cell wall and cuticle emission reconstruct leaf architecture, while the intensity-weighted centroid of chlorophyll emission offers an internal axial reference. Axial intensity profiles of the same optical planes were used to derive two parameters: accumulated fluorescence, which integrates the profile after subtraction of paired untreated controls, and axial displacement ($|\Delta z|$), which is the distance between centroids of the label and chlorophyllous tissue. Because both centroids are derived from the same series, $|\Delta z|$ is independent of the acquisition starting point relative to the leaf surface. The applicability of the approach is validated in a monocot (*Eleusine indica*) and a dicot (*Amaranthus viridis*), and two nanocarrier systems. The two species exhibited distinct surface distribution patterns consistent with their epidermal organization, displayed stomatal labeling, and were quantitatively differentiated using both parameters.

Conclusions.

Leaf autofluorescence, typically considered an obstacle to fluorescence-based nanocarrier tracking, can instead serve as a reference for localizing nanocarriers within leaf tissue. Accumulated fluorescence and axial displacement provided complementary information, quantifying signal intensity and its position relative to the photosynthetic tissue; notably, these two parameters were dissociated in one of the species tested. As the reference is biological, requires no additional labeling, and is acquired with the label, the workflow can be readily applied to other nanocarrier compositions and leaf species, thereby facilitating nanoherbicide design guided by the barriers and entry routes of target plants.

Background

Weed control in major agricultural production systems primarily depends on herbicide application [1]. Provided that herbicides are accurately applied, this technology is beneficial due to its low operational cost and effective weed control [2–4]. However, the indiscriminate use of these products generates ecological risks that negatively impact the environment and non-target organisms [5, 6], while also contributing to the selection of resistant weed species [7, 8]. This scenario is further exacerbated by the challenges that climate change poses to conventional agriculture [9, 10].

Accordingly, application of nanotechnology in agriculture has emerged as a promising solution, enabling the development of nanoscale inputs and their targeted delivery at reduced doses to address agricultural constraints and support more sustainable practices [11–13]. Consistent with this, researchers have developed numerous approaches based on herbicide-carrier nanoparticles, while exploring diverse materials and architectures [14–16]. Studies on *Amaranthus hybridus* have revealed that zein nanoparticles loaded with glyphosate (720 g acid equivalent ha^{-1}) can achieve up to 96% control, which is vastly superior to the approximately 40% control achieved by commercial glyphosate. Furthermore, plants treated with nanoparticles display control symptoms quicker than those treated with the commercial product [17]. Furthermore, the use of polycaprolactone (PCL) nanoparticles to encapsulate the herbicide metribuzin has also yielded promising results [18]. Applying the nanosystem at pre-emergence of *Amaranthus viridis* achieved an 84.3% control rate in the tested plants while using 10-fold less than the recommended field dose (480 g a.i. ha^{-1}). Notably, this result was equivalent to that observed in the group treated with the full dose of commercial herbicide.

As new formulations are developed, elucidating the transport pathway of nanoparticles within plants and their interactions with plant tissues has become imperative [19, 20]. Accordingly, complex probe-labeling strategies are used to track these systems in plants. These probes enable nanoparticle migration monitoring through tissues and pinpoint accumulation sites, thereby serving as powerful tools to quantify and visualize nanoparticle accumulation in plant cells and tissues [21, 22]. Currently, confocal laser scanning microscopy (CLSM) is the reference technique utilized for this purpose; it enables thorough microscopic investigation of tissues [23]. In leaves, such tracking has already elucidated nanoparticle entry pathways; following foliar application, nanoparticles enter mesophyll cells via hydrophilic (stomata, necrotic areas, hydathodes, and trichomes) or lipophilic (diffusion via cuticle pores and fissures) pathways [24–26].

Despite its utility, CLSM tracking in leaf tissues has two recurring limitations. First, the strong autofluorescence of plant tissues introduces difficulty in distinguishing between target fluorescence and background autofluorescence signals stemming from endogenous fluorophores, particularly chlorophyll and lignin [27–29]. Second, spectral overlap among markers, probes, and pigments is a particularly critical issue in plant cells, which exhibit intense autofluorescence originating from cell walls and chloroplasts. Spectral unmixing alone does not satisfactorily resolve these issues [30–33].

Considering the rapid diversification of herbicide nanocarriers, standardized and reproducible confocal microscopy protocols are necessary to improve interpretation of nanocarrier-associated fluorescence in

plant tissues. Therefore, the technique proposed here integrates fluorescent labeling of polymeric nanocarriers and the deliberate use of tissue autofluorescence as a structural reference. Collectively, these approaches facilitate the distinction of nanocarrier-associated fluorescence from tissue autofluorescence, along with the separation of fluorophores with overlapping emission spectra.

Methods

Experimental design

Figure 1. Schematic overview of the experimental protocol. Monocot (*Eleusine indica*) and dicot (*Amaranthus viridis*) weeds receive 1 μL droplets of a fluorescently labeled herbicide nanoformulation. Thereafter, leaves are processed along three routes: fresh tissue for the DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) assay, PFA-fixed tissue for nanoparticle localization, and cryosectioned tissue for anatomical analysis. Images are obtained using confocal microscopy with a pulsed white light laser and HyD photon-counting detectors, subsequently supporting morphoanatomical, nanoparticle-tracking, and oxidative stress analyses.

Plant material and growth conditions

Eleusine indica and *A. viridis* were selected as the model weed species for this experiment. Both weeds are agronomically relevant C4 species and share *Kranz* anatomy but differ in leaf traits that may influence penetration of a foliar-applied nanocarrier. These distinct properties include cuticle thickness and epicuticular wax deposition, venation pattern, stomatal distribution, and leaf blade geometry. Additionally, we obtained seeds from Agrococosmos (Cosmos Agrícola Produção e Serviços Rurais Ltda., SP, Brazil).

Seeds were sown in plastic pots containing sterilized commercial substrate, and plants were grown in a greenhouse at $25 \pm 2^\circ\text{C}$ under a 12 h photoperiod, with a photosynthetic photon flux density of approximately $200 \mu\text{mol m}^{-2} \text{s}^{-1}$. Furthermore, we supplemented plants with nutrient solution to maintain non-limiting growth conditions. Nanoparticle suspensions were applied to the plants upon reaching the fully expanded four-leaf stage.

Nanoparticle preparation and fluorescent labeling

Rhodamine-labeled poly(ϵ -caprolactone) (PCL) nanoparticles were prepared using the nanoprecipitation method as described by Takeshita et al. (2024). The organic phase comprised 200 mg of Myritol®, 40 mg of Span® 60, and 100 μL of 18:1 Liss Rhod PE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)) in 30 mL of acetone. Subsequently, this solution was mixed with an aqueous phase containing 60 mg of Tween® 80 in 30 mL of ultrapure water and stirred magnetically for 20 min. Thereafter, the suspension was concentrated by rotary evaporation to a final volume of 10 mL.

We prepared the zein nanosystem using a modified antisolvent precipitation method [34]. Briefly, the aqueous phase included a 2% (w/v) Pluronic (Poloxamer 188) solution adjusted to pH 4.0. The organic phase was prepared by dissolving zein (2% w/v) in aqueous ethanol (85% v/v, 10 mL), followed by the addition of 18:1 Liss Rhod PE (0.1% v/v) and 636 μ L of nonanoic acid. Subsequently, the mixture was stirred for 10 min at room temperature. Following this, the organic phase was added to the aqueous phase (30 mL) under magnetic stirring at 500 rpm, with consistent stirring for further 20 min. Finally, ethanol was adjusted by rotary evaporation to a final volume of 20 mL.

The hydrodynamic diameter, polydispersity index (PDI), and zeta potential were measured by the dynamic light scattering (DLS) technique using a Malvern Zetasizer ZS instrument (Malvern Panalytical, Westborough, MA, USA). Prior to analysis, the nanoparticle suspensions were diluted in ultrapure water (1:100 v/v). Measurements were performed in triplicate at a fixed scattering angle of 90° and samples were maintained at 25 °C.

Nanoparticle concentration and size distribution were determined by nanoparticle tracking analysis (NTA) using a NanoSight LM10 instrument (Malvern Panalytical) equipped with a 532 nm laser and a CMOS camera, which was controlled by NanoSight software (version 3.2). Prior to measurement, samples were diluted in ultrapure water (1:100,000, v/v). For each sample, we recorded five videos (60 s each) at 25°C.

Foliar application and leaf sampling

The labeled nanoformulations were applied to the adaxial leaf surface using droplet deposition with a micropipette. Ten droplets of 1 μ L each were randomly distributed across the leaf blade. Leaves were collected 12, 24, and 48 h following application. Each sampling time comprised three plants per species, with one leaf sampled per plant and one imaging field acquired per leaf. Untreated plants of both species were grown and processed in parallel with the treated material. Thereafter, these were sampled at the same time points and imaged under identical optical settings as the treated plants.

Using leaf autofluorescence to identify structures

This study leveraged tissue autofluorescence, often considered background noise, as a structural reference. Chlorophyll (orange/red fluorescence) and lignin (blue/green fluorescence) are the plant structures most widely investigated for fluorescence acquisition [28, 33]. In leaf cross-sections, the red autofluorescence of chlorophyll maps mesophyll, weaker green fluorescence corresponds to lignin, and intense green fluorescence of the epidermis stems from cell wall-bound flavonoids [35, 36]. Because lignin is characteristic of xylem tissue, its autofluorescence helps identify the vascular system. Thus, simultaneous acquisition of the chlorophyll and blue-green cell-wall channels enables reconstruction of the leaf architecture, including the epidermis, mesophyll, and vascular bundles, along with localization of the labeled nanoparticles within this structural framework.

Tissue processing

Leaf material was processed through three complementary routes, with each starting from 0.5–1 cm segment excised from the median region of the leaf blade adjacent to the midrib. For nanoparticle localization, segments were fixed in 4% paraformaldehyde for 4 h at 4°C, protected from light, and washed twice in 1× phosphate buffered saline (PBS) (10–15 min each). Concurrently, for morphoanatomical characterization, fixed segments were cryoprotected in 10% (w/v) sucrose in 1× PBS until the tissue sank, embedded in OCT medium in molds oriented for transverse sectioning, and frozen. Subsequently, transverse sections (15 µm thick) were obtained in a cryostat (chamber temperature – 16 to – 20°C), collected onto glass slides, and protected from light until mounting.

Further, for oxidative response, fresh, unfixed sections were incubated in DCFH-DA (10 µM, 30 min), protected from light, and washed in 1× PBS to remove excess probe [37]. Intracellular esterases cleave the acetate groups, and subsequent oxidation of DCFH generates fluorescent DCF. Because probe uptake and conversion rely on membrane permeability, esterase activity, cell viability, retention, and local oxidative conditions, DCF fluorescence was interpreted as a relative indicator of intracellular oxidative activity, rather than a measure of any reactive oxygen species (ROS) [38].

Finally, leaf sections from all three analyses were mounted on glass slides with an aqueous mounting medium (Fluoromount, Southern Biotech) followed by covering with a coverslip, and stored at 4°C protected from light until image acquisition.

Confocal microscopy image acquisition

Images were acquired on a Leica STELLARIS 5 laser scanning confocal microscope equipped with a pulsed white light laser (WLL, tunable from 485 to 790 nm), a 405 nm diode laser, and Power HyD detectors, which was operated through LAS X software (version 3.5.7.23225, Leica Microsystems – Danaher Corporation, Washington, DC, USA). Additionally, a 20× objective was used. The 405 nm diode excited the blue/green autofluorescence of the cell wall and cuticle, whereas the WLL lines excited the fluorescent labels and chlorophyll autofluorescence. Table 1 summarizes the channel settings.

Images were acquired at 1024 × 1024 pixels, with an 8-bit depth, scan speed of 400 Hz, and a 1 Airy unit pinhole, thereby yielding a field of view of 581.25 × 581.25 µm and a lateral pixel size of 0.57 µm. Axial image series were acquired with a z-step of 0.68 µm. further, channels were recorded sequentially to minimize excitation crosstalk.

Laser power and detector gain were set on untreated control tissue of each species and maintained identical for all samples included in the same quantitative comparison. Acquisition settings were verified to avoid detector saturation in all channels. The following were recorded for each acquisition: microscope model, software version, detector type, objective, numerical aperture, immersion medium,

excitation wavelength, laser power at the sample, emission window, detector gain, pinhole diameter, pixel dimensions, bit depth, scan speed, averaging, z-stack, and acquisition mode.

Table 1

Channel settings for multiplexed detection of nanocarrier-associated fluorescence, probe fluorescence, and leaf tissue autofluorescence

Signal	Excitation (nm)	Detection window (nm)	Purpose in the workflow
Rhodamine (18:1 Liss Rhod PE)	560 (WLL)	549–640	Nanocarrier-associated fluorescence. Quantitative analysis
DCF	488 (WLL)	515–530	Relative indicator of intracellular oxidative activity
Cell wall/cuticle autofluorescence	405 (diode)	420–549	Structural reference for the epidermis and vascular tissue
Chlorophyll autofluorescence	613 (WLL)	640–753	Internal reference for chlorophyllous tissue

Image quantification and derived parameters

Axial intensity profiles were extracted in the LAS X Quantify module using the Stack Profile tool, with the z dimension selected and region of interest defined to be the full image frame. All channels of a given series were profiled in a single operation, to ensure that the same optical planes and region of interest were applied to each channel. For each channel, the software provided the series axial extent (volume depth), mean and maximum intensities, position of maximum intensity, and intensity-weighted center of mass (CoM) along z.

Consequently, two parameters were derived from these outputs. The accumulated fluorescence (AUC) was calculated as the product of the mean intensity and volume depth, expressed in arbitrary units × μm, which is proportional to the integral of the axial intensity profile, and hence, represents the total nanocarrier-associated fluorescence in the imaged volume. Prior to AUC calculation, the mean intensity measured in the rhodamine detection of the untreated control tissue from the same species and sampling time was subtracted from that of the corresponding treated samples, thereby ensuring that the parameter reflected only nanocarrier-associated fluorescence.

Furthermore, the Axial Displacement ($|\Delta z|$) was calculated as the absolute difference between the CoM of the rhodamine and chlorophyll channels within the same series. Because both centroids were computed from the same optical planes, this parameter was independent of the axial position at which acquisition was initiated relative to the leaf surface. Therefore, this is comparable across samples and species without the need for an external reference. The absolute value was adopted because the comparison of interest is the proximity to the chlorophyllous tissue, regardless of direction.

Chlorophyll autofluorescence was an internal reference for the position of photosynthetic tissue within the imaged volume. Importantly, this marks the centroid of the chlorophyllous tissue sampled by the series and does not delimit the anatomical boundaries of the mesophyll.

Statistical analysis

AUC and $|\Delta z|$ were analyzed using two-way analysis of variance (ANOVA), with species and time after application as fixed factors and their interaction included in the model. Normality of residuals and homogeneity of variances were assessed using Shapiro–Wilk and Levene’s tests, respectively. Both assumptions were met with the two response variables. Means were compared using Tukey’s test at $p < 0.05$. All analyses were performed in Google Colab (Python 3.12.12) using pandas for data manipulation and SciPy and Statsmodels for assumption testing, ANOVA, and post hoc comparisons. Finally, adjusted means, standard errors, and Tukey letter groupings were extracted from the fitted models to construct figures and interpret the results.

Results

Figure 2 illustrates the intensity-based size distribution (%) as a function of the hydrodynamic diameter of the nanoparticles used in this experiment. Both formulations displayed a monomodal distribution, with no secondary peaks indicating aggregation. Rhodamine-labeled poly(ϵ -caprolactone) nanoparticles demonstrated a mean hydrodynamic diameter of 268.4 ± 3.8 nm, polydispersity index (PDI) of 0.082 ± 0.025 , concentration of 1.79×10^{13} particles/mL, and zeta potential of -33.5 ± 1.5 mV (Fig. 2a). Concurrently, rhodamine-labeled zein–nonanoic acid nanoparticles displayed a mean hydrodynamic diameter of 233.0 ± 5.0 nm, PDI of 0.262 ± 0.020 , concentration of 3.14×10^{13} particles/mL, and zeta potential of $+27.2 \pm 1.4$ mV (Fig. 2b).

Anatomical differences between dicotyledonous and monocotyledonous species (Fig. 3) directly influence nanoparticle (NP) interactions with the leaf surface and their subsequent fate within plant tissues [20]. Dicotyledonous species display a dorsiventral mesophyll, which comprises a compact palisade parenchyma and a spongy parenchyma with abundant intercellular air spaces, along with a relatively thin cuticle, predominantly hypostomatous distribution, and reticulate venation [39, 40]. Conversely, monocotyledonous species are characterized by high vein density and short interveinal distance, with the mesophyll radially arranged around the vascular bundle sheath cells [41, 42]. In C4 grasses, this pattern is further linked to a thick cuticle covered by epicuticular waxes and a frequently amphistomatous condition [43]. Within the context of foliar application, these attributes determine surface wettability and droplet residence time, the availability of the stomatal and cuticular entry pathways, and, once internalized, the length of the apoplastic route to the conducting elements. Thus, the morphoanatomical characterization of the target species is a necessary prerequisite for interpreting the uptake and translocation assays described below.

As described in the preceding sections, confocal microscopy enables non-destructive assessment of these features in intact sections. Figure 3 illustrates transverse sections of *E. indica* (3a–3c) and *A. viridis* (3d–3f), which are C4 monocotyledonous and dicotyledonous weed species, respectively. Excitation at 405 nm revealed autofluorescence from cell wall compounds, primarily lignin and phenolic derivatives (3a, 3d), thereby allowing delineation of the conducting vessels and arrangement of vascular bundle sheath cells, which are characteristic of Kranz anatomy (3a). Furthermore, chlorophyll fluorescence, collected in the red range (640–753 nm) revealed the distribution of chloroplasts within the mesophyll (3b, 3e), highlighting the contrast between the dorsiventral organization of *A. viridis*, with a well-defined palisade parenchyma, and the radial, more homogeneous arrangement of *E. indica*. Overlaying the two channels (3c, 3f) integrates cell wall and photosynthetic tissue information, thus, enabling measurement of leaf layer thickness. Importantly, this parameter is directly associated with the barriers that NPs must cross before reaching the vascular system.

Once the morphoanatomical characteristics of the target species have been established, fluorophore-associated signals can be interpreted within the corresponding structural framework. Accordingly, Fig. 4 illustrates the leaf surface of *A. viridis* samples at the three sampling times. In the isolated rhodamine channel (4a, 4f, 4k), nanocarrier-associated fluorescence is heterogeneously distributed across the surface and follows a pattern that outlines the contours of the pavement cells. Superimposed on this pattern are rounded, higher-intensity structures corresponding to the trichome bases. At 48 h (4k), the signal is accompanied by labeling aligned along the vascular bundles.

Figure 4. Localization of rhodamine-labeled PCL nanoparticles in the adaxial surface of *Amaranthus viridis* leaves at 12 h (a–e), 24 h (f–j), and 48 h (k–o) following foliar application. For each sampling time, the upper panel presents the rhodamine channel alone (a, f, k). The three intermediate panels display, from left to right, chlorophyll autofluorescence (b, g, l), cell wall and cuticle autofluorescence excited at 405 nm (c, h, m), and the merging of all channels (d, i, n). The lower panel illustrates the merging between the rhodamine (red) and 405 nm (blue) channels (e, j, o), wherein nanocarrier-associated fluorescence is resolved against epidermal structures. Scale bars = 100 μm (a, f, k), 100 μm (b–d, g–i, l–n), and 50 μm (e, j, o).

Simultaneous acquisition of the autofluorescence channels allowed us to position these labels within the overall structural context of the epidermis. Chlorophyll autofluorescence (4b, 4g, 4l) revealed the underlying mesophyll, whereas excitation at 405 nm (4c, 4h, 4m) outlined the cell walls and the cuticle, thereby illustrating the contours of the pavement cells, stomatal complexes, and trichomes. Overlay of the channels (4d, 4i, 4n) indicates that rhodamine fluorescence does not coincide with chlorophyll distribution and remains restricted to the epidermal plane throughout the entire assessment period.

Detailed views of the overlay between the rhodamine and 405 nm channels (4e, 4j, 4o) enabled identification of surface structures associated with the labeling. In 4e, the signal is concentrated at the trichome bases and along the outline of the pavement cells, whereas individual stomata appears delineated by rhodamine fluorescence. Figure 4j presents the same association, with labeling also

included in the trichome septa. In 4o, the fluorescence clearly outlines the ostiole of a stomatal complex and follows the course of the vascular bundles. Importantly, the presence of this fluorescent signal in the stomata and ostiole is consistent with the stomatal uptake pathway described for foliar-applied nanoparticles [24, 26] in which entry occurs both through the ostiole opening and by diffusion across the cuticle, which has reduced thickness in the region overlying the guard cells.

Figure 5. Localization of rhodamine-labeled PCL nanoparticles in the adaxial surface of *Eleusine indica* leaves at 12 h (a–e), 24 h (f–j), and 48 h (k–o) following foliar application. For each sampling time, the upper panel presents the rhodamine channel alone (a, f, k). The three intermediate panels display, from left to right, chlorophyll autofluorescence (b, g, l), cell wall and cuticle autofluorescence excited at 405 nm (c, h, m), and the merge of all channels (d, i, n). The lower panel exhibits the merging between the rhodamine (red) and 405 nm (blue) channels (e, j, o), wherein nanocarrier-associated fluorescence is resolved against epidermal structures. Scale bars = 100 μ m (a, f, k), 100 μ m (b–d, g–i, l–n), and 50 μ m (e, j, o).

Excitation at 405 nm (5c, 5h, 5m) outlined the cell walls and, particularly intensely, the vascular bundles, which were visible as longitudinal bands spanning the field and reflecting the high vein density and short interveinal distance of the species. Chlorophyll autofluorescence (5b, 5g, 5l) revealed the mesophyll arranged around the bundles. The overlay of the channels (5d, 5i, 5n) places the rhodamine labeling in the intercostal zones, between the vascular bundles.

In the detailed images of the overlay between the rhodamine and 405 nm channels (5e, 5j, 5o), individual stomata appear labeled by rhodamine fluorescence at all three sampling times. This aligns with the parallel stomatal files typical of grasses. Pavement and mesophyll cells are identified in the same fields, thereby allowing us to position the labeling relative to the epidermal and subepidermal planes. In 5o, the trichome identified in the field illustrates only cell wall autofluorescence, with no associated rhodamine signal, in contrast to *A. viridis*, where the trichome bases present the most intense labeling.

Comparing the two figures reveal distinct surface distribution patterns. In *A. viridis*, the signal follows the outline of the pavement cells and concentrates at the trichome bases, whereas it is organized along the longitudinal cell files and intercostal zones in *E. indica*, with no accumulation associated with the trichomes. In contrast, labeling of stomatal complexes was observed in both species and at all sampling times, thereby indicating that the association of the nanocarrier with the stomata is independent of the pattern of epidermal organization.

Figure 6. Quantification of nanocarrier-associated fluorescence in leaves of *Eleusine indica* and *Amaranthus viridis* at 12, 24, and 48 h following foliar application of rhodamine-labeled PCL nanoparticles. (a) Accumulated fluorescence (AUC), calculated as the product of the mean intensity and volume depth in the rhodamine channel, after subtraction of the corresponding untreated control signal. (b) Axial displacement ($|\Delta z|$), calculated as the absolute difference between the intensity-weighted centers of mass of the rhodamine and chlorophyll channels within the same z-series. Bars represent means of three plants per species per sampling time. Error bars represent the standard error of the

mean. Uppercase letters compare species within each sampling time, and lowercase letters compare sampling times within each species. Means followed by the same letter do not differ significantly according to Tukey's test at $p < 0.05$. All images used for quantification were acquired under identical optical settings.

Figure 6 consolidates the two parameters analyzed at the three sampling times. Accumulated fluorescence (Fig. 6a) differed between species at 12 and 48 h, with higher values in *A. viridis* than in *E. indica*, whereas the two species did not differ from each other at 24 h. The temporal patterns also diverged: values remained stable in *E. indica* across the three sampling times, whereas the value at 48 h in *A. viridis* exceeded those recorded at 12 and 24 h.

Axial displacement (Fig. 6b) separated the two species at all evaluated sampling times. $|\Delta z|$ ranged from 3.3 to 4.0 μm in *E. indica* and from 7.9 to 9.3 μm in *A. viridis*, with no significant variation over time in either species. Therefore, the signal associated with the nanocarrier remained approximately twice as far from the chlorophyll centroid in *A. viridis* as in *E. indica*; this separation was already established at the earliest sampling time.

In *A. viridis*, the cumulative signal increased over time while its axial position relative to the chlorophyll-containing tissue remained unchanged, thereby indicating additional fluorescence accumulation without a corresponding shift in the depth distribution. In *E. indica*, both parameters remained stable. Notably, $|\Delta z|$ is measured relative to an internal reference whose own position is governed by mesophyll organization. Hence, the contrast between species should be interpreted in conjunction with the anatomical characterization in Fig. 3, in which the dorsiventral organization of *A. viridis* and radial arrangement of *E. indica* position the chlorophyll centroid at different depths.

As the final step of the proposed workflow, the same confocal microscopy approach can be applied to assess the biochemical response of the tissue herbicide-carrying nanoparticle exposure. Figure 7 presents the ROS detection assay using DCFH-DA in the two weed species previously characterized.

In 7a and 7d, the red channel identifies the location of the Rhodamine-labeled zein–nonanoic acid nanoparticles in the tissues of *E. indica* and *A. viridis*, respectively, thereby reproducing the distribution patterns described in the previous section. In 7b, the DCF signal in *E. indica* is extensively distributed along the cell files, with greater intensity in the regions linked to the vascular bundles. In 7e, the green fluorescence in *A. viridis* is more locally concentrated, thereby delineating the outline of the anticlinal walls of the epidermal cells and forming clusters restricted to certain regions of the leaf blade. Overlaying the channels (7c, 7f) enables assessment of the spatial correspondence between nanoparticle presence and ROS generation. Furthermore, the overlap between the signals indicates that the regions of nanoparticle accumulation correspond to the areas of highest ROS production. This result demonstrates that integrating morphoanatomical characterization, nanoparticle tracking, and ROS detection within a single analytical workflow enabled direct association between the deposition and uptake pattern observed in each species to the tissue's oxidative response, thus, providing a functional overview that none of these approaches could offer in isolation.

Discussion

The protocol described here successfully consolidates, within a single analytical workflow, three levels of information that are typically obtained in isolation: the morphoanatomical characterization of the target species, spatial tracking of nanoparticles within leaf tissues, and assessment of the oxidative response associated with exposure to herbicide nanoformulations. The core strategy focuses on overcoming intense autofluorescence, which is a classic obstacle of confocal microscopy in plant tissues. The autofluorescence of chlorophyll and of cell walls is deliberately employed as a structural reference to reconstruct leaf architecture. Therefore, this protocol supports the rational design of nanoherbicides tailored to the target plant, thereby shifting the development beyond material-centered approach to one that considers plant tissue barriers and entry pathways from the design stage onward.

Quantitative comparison of fluorescence intensity across leaf samples is complicated by two factors that are rarely clarified. First, the axial position at which acquisition begins relative to the leaf surface is operator-defined and varies between samples. Resultingly, raw intensities at a given optical plane are not equivalent across series. Second, the axial extent of the series varies with tissue thickness and mounting, hence, summed intensities are inflated deeper stacks. Importantly, the two parameters adopted here address these limitations separately. Accumulated fluorescence integrates the axial profile and, after the subtraction of the paired untreated control, is unaffected by optical planes lacking nanocarrier-associated signal. Axial displacement is calculated from two centroids derived from the same optical planes within a given series, and therefore, is independent of the acquisition starting position.

Furthermore, employing chlorophyll autofluorescence as an internal reference makes the second parameter transferable. Notably, this requires no additional labeling, is present in any photosynthetically active leaf, is acquired simultaneously with the label in the same optical planes, and occupies a spectral region distant from most fluorophores used to label polymeric nanocarriers. Additionally, the reference is biological rather than instrumental; therefore, $|\Delta z|$ remains interpretable across microscopes and acquisition sessions and provided channel settings are maintained constant within each comparison.

The two parameters proved complementary. In *A. viridis*, accumulated fluorescence increased between 24 and 48 h while $|\Delta z|$ remained unchanged, thereby indicating that additional signal accumulated without a corresponding change in the axial distribution. Notably, a single intensity-based descriptor would fail to distinguish this scenario from one in which the nanocarrier had progressed further into the tissue.

The two species had distinct nanocarrier surface distribution which was consistent with their epidermal organization. This comprised a reticulate pattern, following the pavement cells and concentrating at the trichome bases in *A. viridis*, and a striated pattern along the longitudinal cell files and intercostal zones in *E. indica*. In contrast, stomatal complex labeling was recorded in both species and at all sampling times, thereby indicating that association of the nanocarrier with stomata is independent of the pattern of

epidermal organization. This consistent with the stomatal entry route described for foliar-applied nanoparticles [24, 26].

The $|\Delta z|$ distinction between species must be considered against this anatomical background. As the reference is the centroid of the chlorophyllous tissue, and because the dorsiventral mesophyll of *A. viridis* and the radial arrangement of *E. indica* place that centroid at different depths within the imaged volume, the between-species difference partially reflects leaf architecture rather than nanocarrier behavior alone. Therefore, this is a property of the parameter rather than a technical limitation. This makes $|\Delta z|$ directly comparable across sampling times and treatments within a species, and interpretable between species only when anatomical characterization is available. Our workflow supplies this characterization in the same experiment.

The approach described here depends on sequential acquisition, narrow emission windows, and paired untreated controls to attribute the detected signal to the nanocarrier. This is sufficient when the label emits in a spectral region where tissue autofluorescence is weak, as with rhodamine in the present work. However, this becomes limiting in two scenarios: (1) when the label emits in the green range, i.e., where cell wall and flavonoid autofluorescence are strong, and (2) when two fluorophores with overlapping emission must be resolved in the same tissue, for instance, when the nanocarrier and the oxidative probe are imaged simultaneously.

Nonetheless, photon arrival-time information offers a framework to accommodate both limitations, as it discriminates emitters by their temporal rather than spectral signature [44]. Previous studies have applied label-free chlorophyll fluorescence lifetime in plants to map the effect of a photosystem II-inhibiting herbicide in vivo [45]. Figure 8 illustrates the use of the TauSeparation and TauContrast tools for tracking Rhodamine B-labeled nanoparticles in the leaf mesophyll. Two temporal components, centered at average photon arrival times (AAT) of 0.2 and 1 ns and automatically proposed by the software upon activating TauSeparation, were initially tested to assess any possible contribution of autofluorescence within the Rhodamine B emission range. Figures 8ia and 8ib display near-complete overlap between the signals obtained at the two temporal components, which differ essentially in intensity. Additionally, the same cellular structures are revealed in both channels, with no additional population resolved. Figure 8ic corroborates this finding: TauContrast displays no color transitions across the field, thereby indicating a single emitter population and the consequent absence of significant autofluorescence in this spectral range. Considering this scenario, the component centered at 0.2 ns was selected, due to the higher signal intensity recorded. Notably, throughout this protocol, values such as 0.2 ns denote operational positions on the AAT axis used to define detection windows and not fitted fluorescence lifetimes. Finally, Figure (8id) presents the overlay between the fluorescence of Rhodamine B-labeled nanoparticles (magenta) and the chlorophyll autofluorescence of chloroplasts (red). No overlap between the two signals is observed in the 0.2 ns window, which indicates that the entire magenta signal corresponds to nanoparticles taken up by the leaves.

In the second example, the same lifetime-discrimination approach was applied to separate Rhodamine B from chlorophyll. Prior to analysis, fresh TauSeparation was performed on this field, and it was verified that only the chloroplasts remained emitting in the component centred at 0.7 ns. Therefore, this position on the AAT axis was adopted as the chlorophyll-specific temporal window. After determining the temporal windows that best isolated fluorescence from the labeled nanoparticles and chloroplasts-specific emission, TauGating was applied with detection windows of 0.2 ns for Rhodamine B (Fig. 8iia) and 0.7 ns for chlorophyll (Fig. 8iib) [46].

Notably, these are preliminary observations that were obtained from a separate set of acquisitions. Establishing temporal windows as a routine component of the workflow would require reference distributions derived from untreated leaf, unlabeled nanoparticle, free-dye, and single-fluorophore controls, for each species and preparation condition.

Conclusion

The workflow described in this study demonstrates that leaf autofluorescence, typically considered an obstacle to fluorescence-based nanocarrier tracking, can be utilized as a reference system to facilitate tracking. Cell wall and cuticle emission reconstruct the epidermal and vascular architecture used to localize label, while the chlorophyll emission centroid provides an internal axial reference that enables comparison of nanocarrier signal position across samples, species, and acquisition sessions without the need for an external fiducial marker.

The two parameters derived from this reference—accumulated fluorescence and axial displacement—were complementary descriptors of signal intensity and spatial position relative to the photosynthetic tissue. Furthermore, they dissociated in one of the species tested, which could not have been resolved by a single intensity-based descriptor. Moreover, because the reference is biological rather than instrumental, it requires no additional labeling, and is acquired simultaneously with the label. This approach readily translates to other nanocarrier compositions, as demonstrated here for two systems of opposite surface charge, and to other leaf species for which a morphoanatomical characterization is available. When incorporated at the design stage, this quantitative, structurally anchored approach can guide nanoherbicide development based on the target plant's barriers and entry routes rather than solely on the properties of the carrier material.

Declarations

Ethics approval and consent to participate.

Not applicable.

Consent for publication.

Not applicable.

Competing interests.

The authors declare no competing interests.

Author Contribution

The research was designed by B.C.C, A.C.P, and L.F.F Samples were collected and analyzed by B.C and A.C. Statistical analyses were performed by B.C.C. Manuscript writing was carried out by B.C.C, A.C.P, and L.F.F.

Acknowledgement

The authors gratefully acknowledge the financial support received from the São Paulo Research Foundation (FAPESP) through grants CEPID CBioClima (#2021/10639-5), #2025/02524-4, #2026/03614-0, #2024/07260-2, and #2026/14054-5. Additionally, this study was supported in part by the INCT Nanotechnology for Sustainable Agriculture (CNPq #405924/2022-4), CAPES-MEC #88887.953443/2024-00), and FINEP.

Data Availability

The data supporting the findings of this study are currently stored in the Unesp Institutional Repository. However, access is restricted because the data are part of a master's thesis that has not yet been defended and are therefore not publicly available. The data may be made available upon reasonable request, subject to institutional authorization.

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Figures

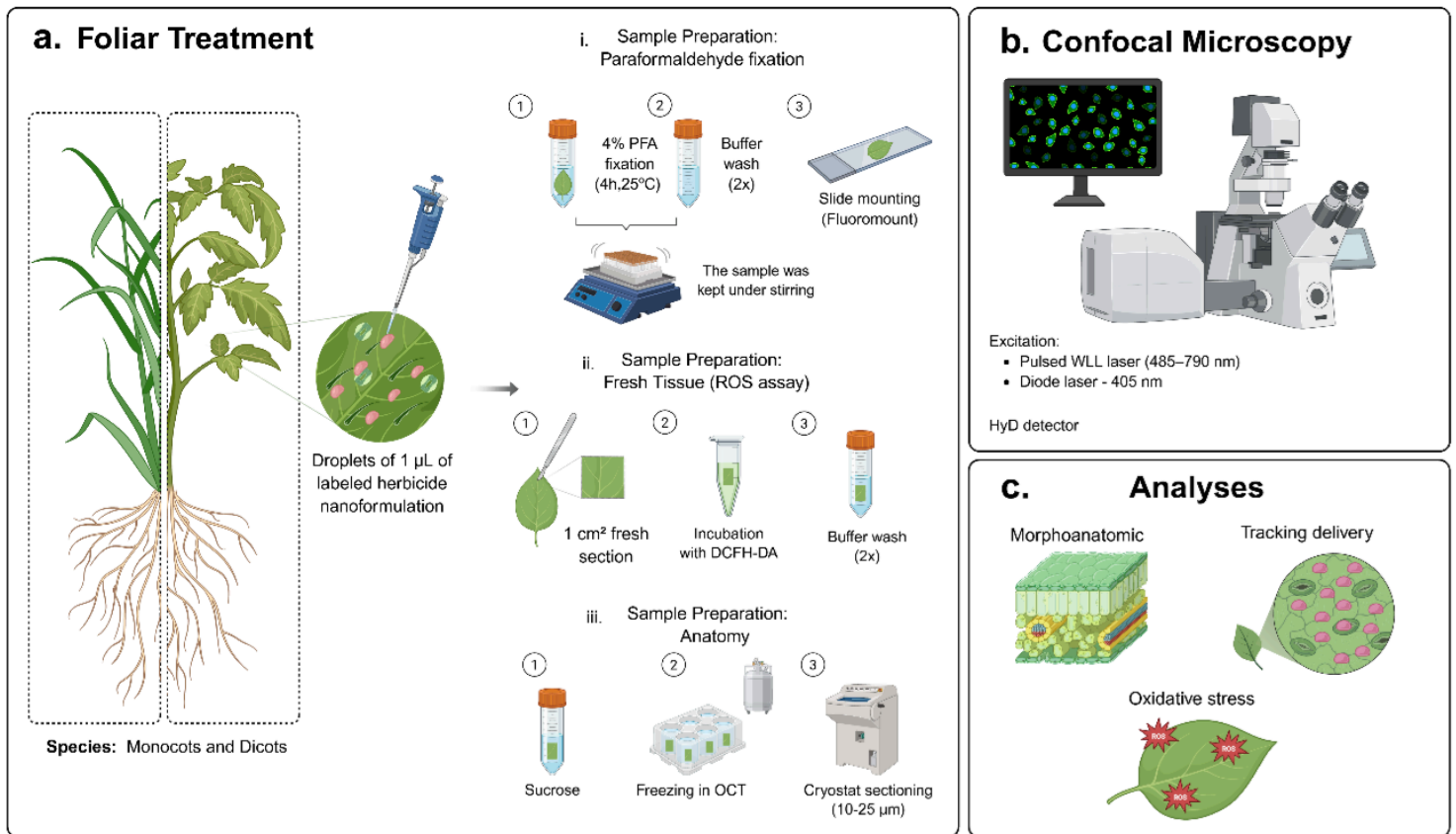


Figure 1

Schematic overview of the experimental protocol. Monocot (*Eleusine indica*) and dicot (*Amaranthus viridis*) weeds receive 1 μL droplets of a fluorescently labeled herbicide nanoformulation. Thereafter, leaves are processed along three routes: fresh tissue for the DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) assay, PFA-fixed tissue for nanoparticle localization, and cryosectioned tissue for anatomical analysis. Images are obtained using confocal microscopy with a pulsed white light laser and HyD photon-counting detectors, subsequently supporting morphoanatomical, nanoparticle-tracking, and oxidative stress analyses.

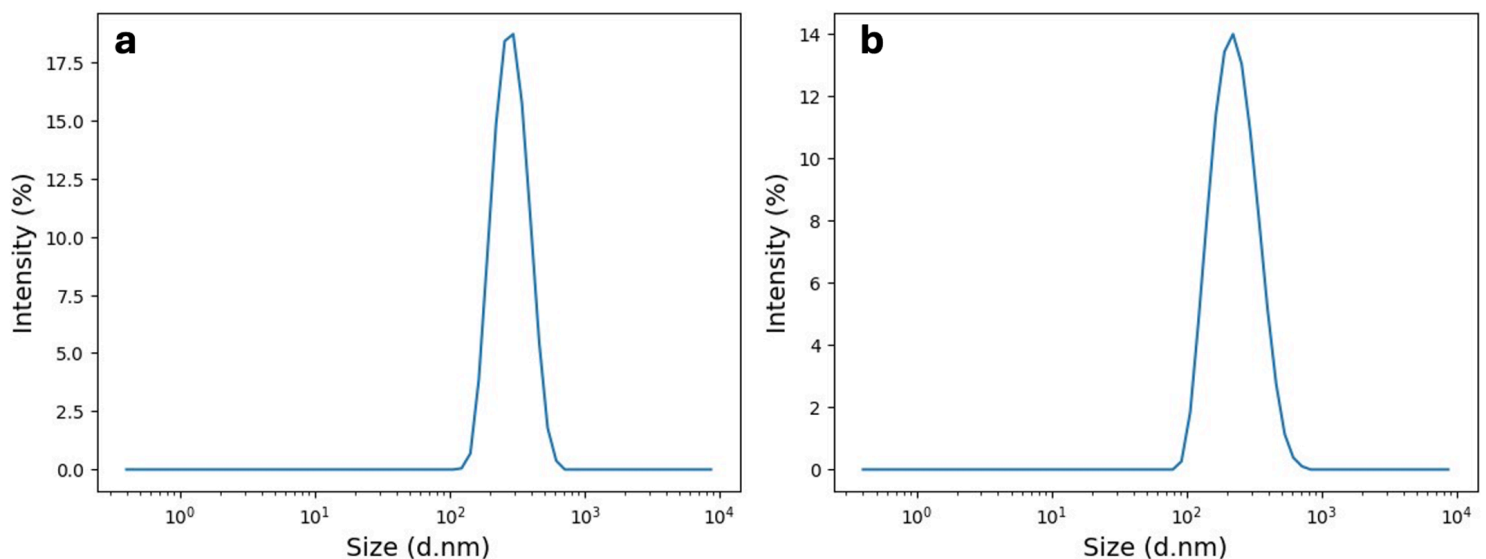


Figure 2

Intensity-based size distribution of the nanoparticles determined by dynamic light scattering (DLS). (a) Rhodamine-labeled poly(ϵ -caprolactone) nanoparticles. (b) Rhodamine-labeled zein–nonanoic acid nanoparticles. x-axis is logarithmic.

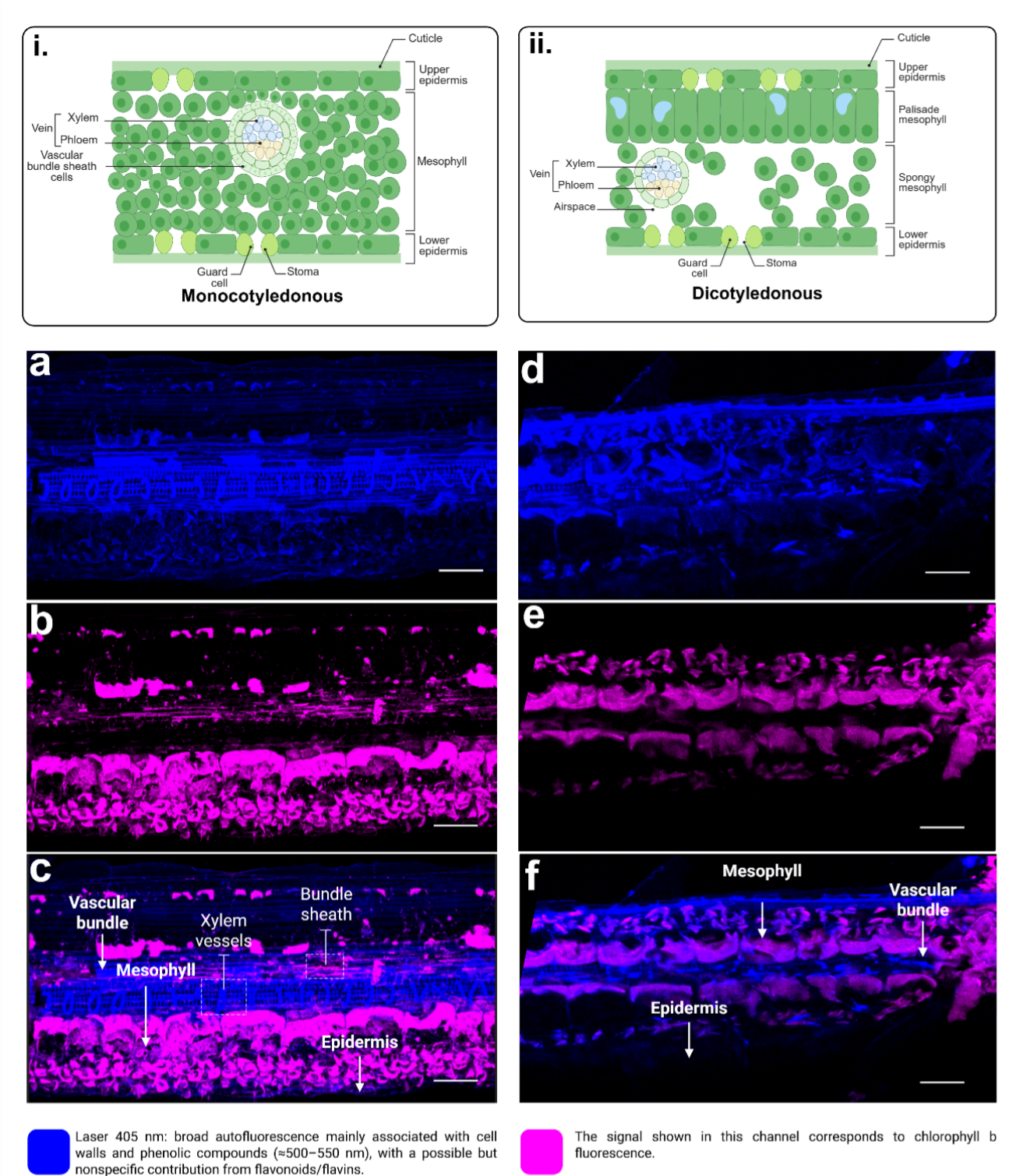


Figure 3

Comparative leaf anatomy of monocotyledonous (i) and dicotyledonous (ii) species in cross-section; epidermis, cuticle, mesophyll organization, and vein structure are highlighted. Transverse leaf cross-sections of *Eleusine indica* (a–c) and *Amaranthus viridis* (d–f) imaged by confocal microscopy. Cell wall autofluorescence excited at 405 nm (blue a, d), chlorophyll autofluorescence collected at 640–753 nm (magenta b, e), and the overlay of both channels (c, f), used to reconstruct leaf architecture as a structural reference for nanoparticle localization. Scale bars = 25 μ m.

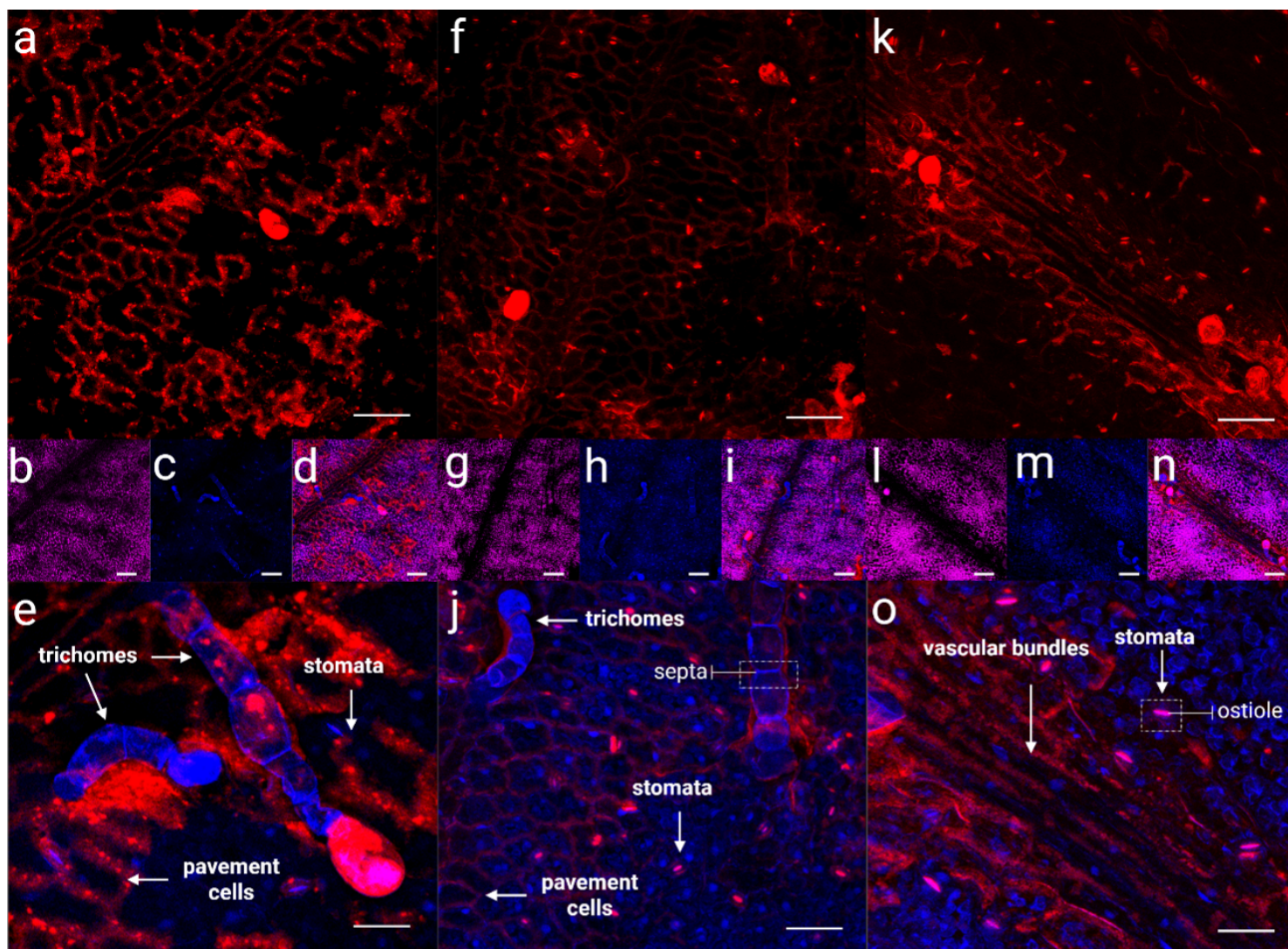


Figure 4

Localization of rhodamine-labeled PCL nanoparticles in the adaxial surface of *Amaranthus viridis* leaves at 12 h (a–e), 24 h (f–j), and 48 h (k–o) following foliar application. For each sampling time, the upper panel presents the rhodamine channel alone (a, f, k). The three intermediate panels display, from left to right, chlorophyll autofluorescence (b, g, l), cell wall and cuticle autofluorescence excited at 405 nm (c, h, m), and the merging of all channels (d, i, n). The lower panel illustrates the merging between the rhodamine (red) and 405 nm (blue) channels (e, j, o), wherein nanocarrier-associated fluorescence is resolved against epidermal structures. Scale bars = 100 μ m (a, f, k), 100 μ m (b–d, g–i, l–n), and 50 μ m (e, j, o).

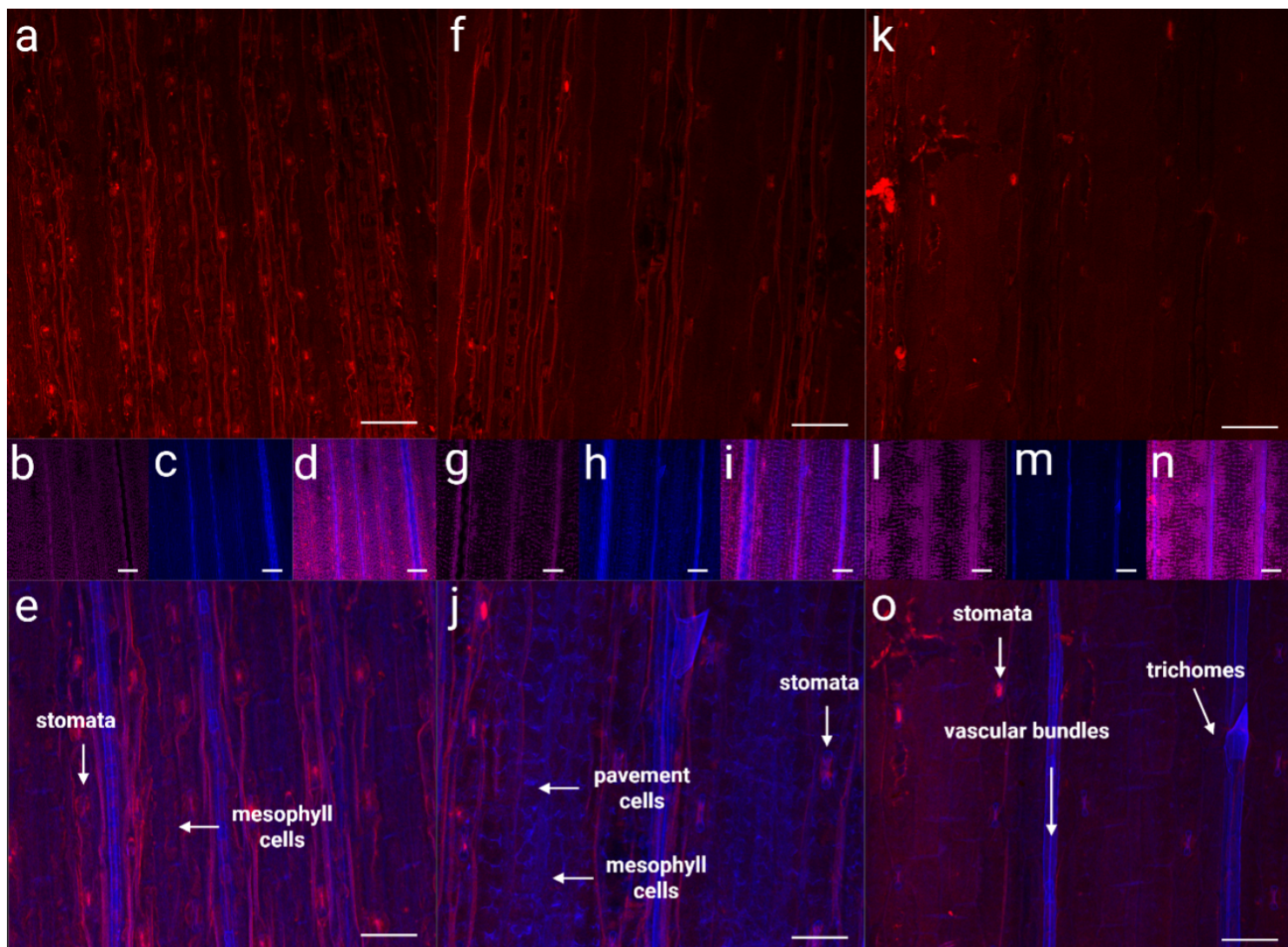


Figure 5

Localization of rhodamine-labeled PCL nanoparticles in the adaxial surface of *Eleusine indica* leaves at 12 h (a–e), 24 h (f–j), and 48 h (k–o) following foliar application. For each sampling time, the upper panel presents the rhodamine channel alone (a, f, k). The three intermediate panels display, from left to right, chlorophyll autofluorescence (b, g, l), cell wall and cuticle autofluorescence excited at 405 nm (c, h, m), and the merge of all channels (d, i, n). The lower panel exhibits the merging between the rhodamine (red) and 405 nm (blue) channels (e, j, o), wherein nanocarrier-associated fluorescence is resolved against epidermal structures. Scale bars = 100 μ m (a, f, k), 100 μ m (b–d, g–i, l–n), and 50 μ m (e, j, o).

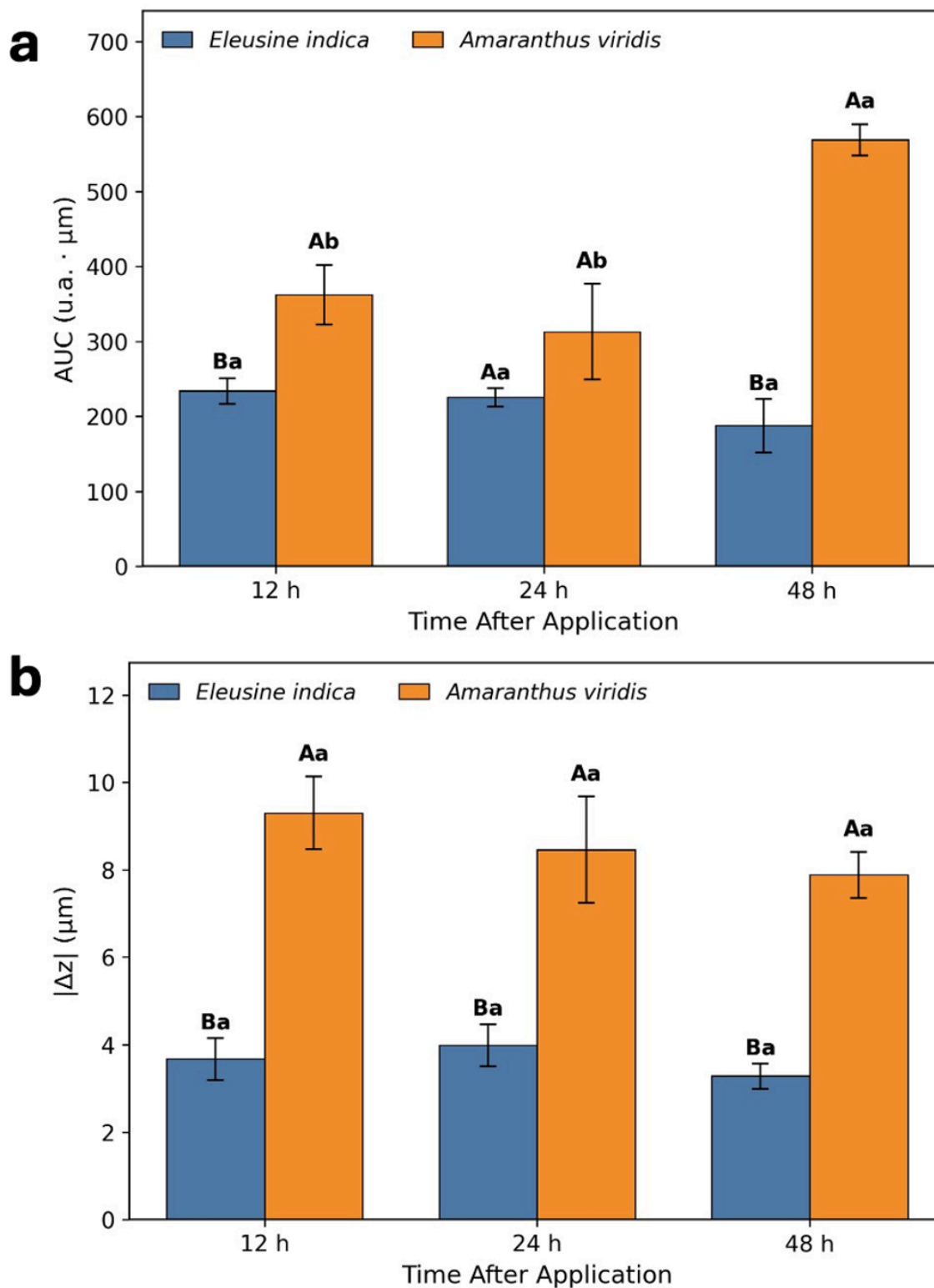


Figure 6

Quantification of nanocarrier-associated fluorescence in leaves of *Eleusine indica* and *Amaranthus viridis* at 12, 24, and 48 h following foliar application of rhodamine-labeled PCL nanoparticles. (a) Accumulated fluorescence (AUC), calculated as the product of the mean intensity and volume depth in the rhodamine channel, after subtraction of the corresponding untreated control signal. (b) Axial displacement ($|\Delta z|$), calculated as the absolute difference between the intensity-weighted centers of mass of the rhodamine

and chlorophyll channels within the same z-series. Bars represent means of three plants per species per sampling time. Error bars represent the standard error of the mean. Uppercase letters compare species within each sampling time, and lowercase letters compare sampling times within each species. Means followed by the same letter do not differ significantly according to Tukey's test at $p < 0.05$. All images used for quantification were acquired under identical optical settings.

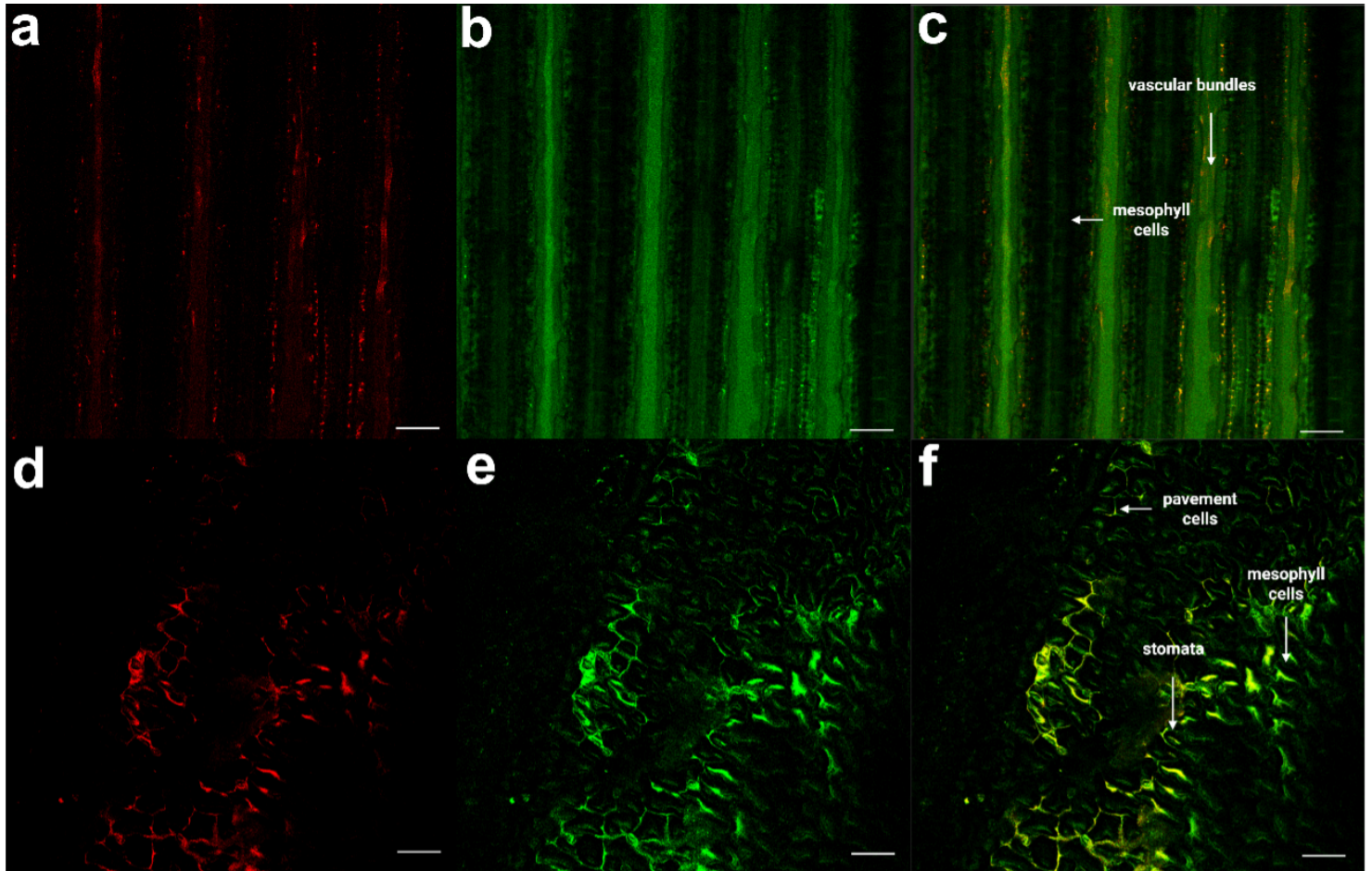
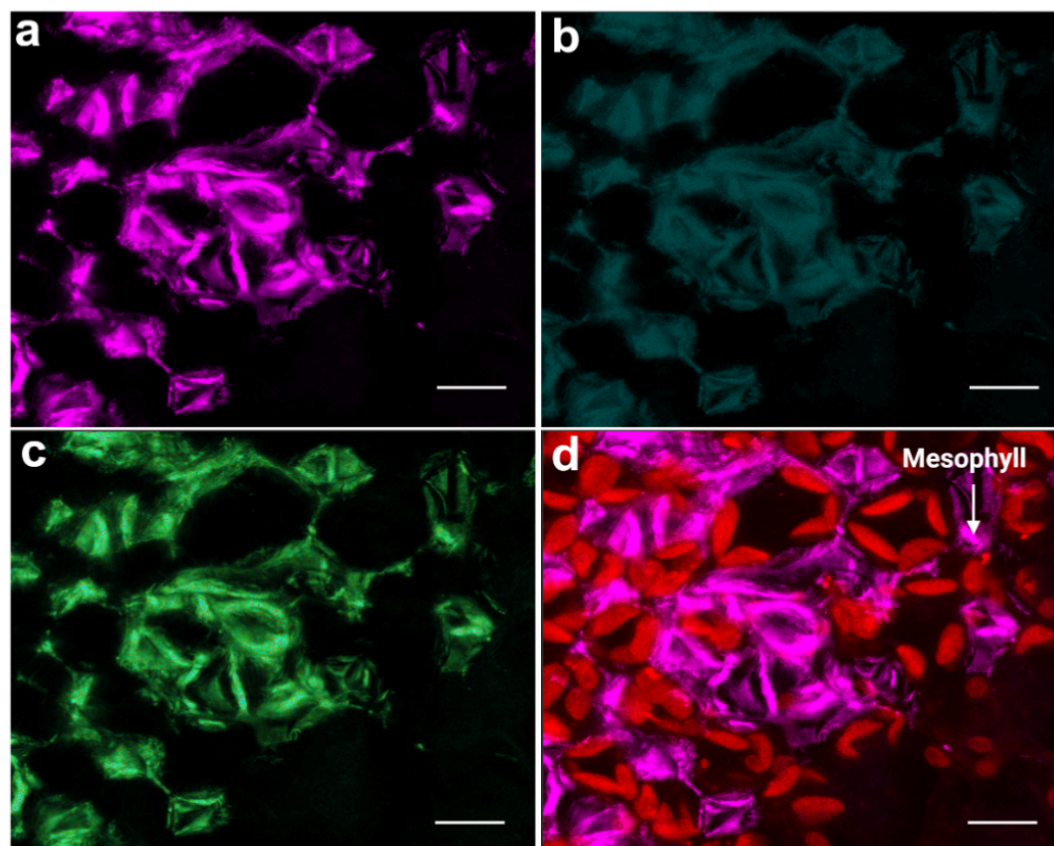


Figure 7

ROS detection with DCFH-DA in *Eleusine indica* (a–c) and *Amaranthus viridis* (d–f) following exposure to Rhodamine-labeled zein–nonanoic acid nanoparticles. Nanoparticle signal (a, d), DCF fluorescence indicating ROS production (b, e), and overlay of both channels (c, f), wherein yellow denotes co-localization.

(i) Lifetime-based separation of the fluorescence of Rhodamine B-labeled nanoparticles in leaf mesophyll



(ii) τ -gating of Rhodamine B-labeled nanoparticles in the stomatal region

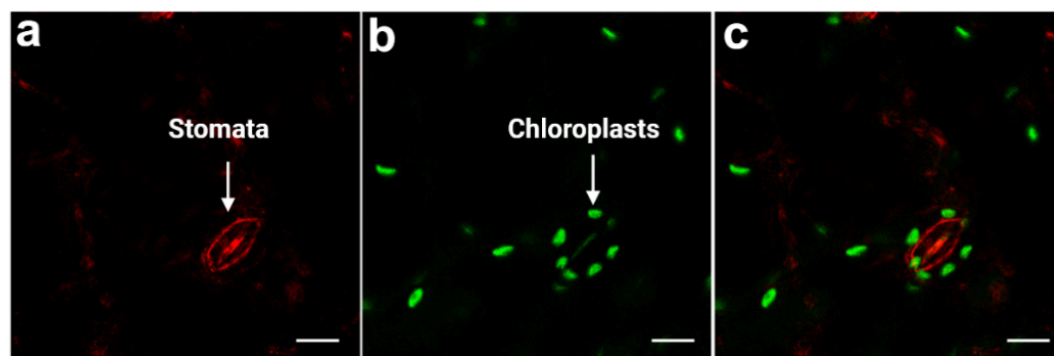


Figure 8

Lifetime-based discrimination of rhodamine and chlorophyll fluorescence in leaf mesophyll, presented as a perspective on the workflow. (i) TauSeparation and TauContrast applied to rhodamine-labeled nanoparticles: (a) channel corresponding to the temporal component centered at 0.2 ns and (b) to the component centered at 1 ns. (c) TauContrast map of the same field, in which the color coding represents the average photon arrival time (AAT) per pixel. (d) Overlay of the 0.2 ns component (magenta,

nanocarrier-associated fluorescence) with chlorophyll autofluorescence (red). (ii) TauGating applied to the same principle: (a) detection restricted to the 0.2 ns window, corresponding to the nanocarrier, (b) detection restricted to the 0.7 ns window, corresponding to chlorophyll autofluorescence, and (c) overlay of (a) and (b). Scale bars = 25 μm .