Morphogenetic and functional analysis of plant organs with sub-cellular resolutions.

Running title: Quantitative plant cell biology *in situ*.

Abstract

A shortfall of precise plant phenotyping methods at subcellular resolution limits our ability to dissect the genotype-environment interactions that are critical for understanding plant adaptation to different types of stresses and for linking genes with their function, expression, and localization. Here, we describe an effective, high-resolution phenotyping platform that allows the detection of tissues, cell files, and cell shapes, as well as protein localization, chromatin organization, cell cycle kinetics, cell polarity, and protein-protein interactions at subcellular resolution. The platform does not require endogenous markers and is plant-species independent. The platform has been successfully used for the analysis of diverse organs in several plant species, including grasses. Application of the platform requires minimal time and experience from researchers and can be completed in 3 to 4 working days (including complete data extraction for each cell). The method requires a high-resolution microscope and state-of-the-art computer. We present the results of platform applications in diverse developmental contexts. The platform is free and open source, with a user-friendly graphical interface.

- Key words: Phenotyping, 3D imaging; segmentation; cell cycle, gene expression, epigenetics.

- Abberviations: iRoCS- internal root coordinate system.

Introduction.

A large-scale quantitative study of cell shape and cell functionality in a multicellular organ entails an accurate estimation of the quantitative three-dimensional geometry and functions of each cell in that organ (Bassel, G. W., 2019). State-of-the-art confocal and other advanced microscopes with high-resolution volumetric image acquisition allow researchers to scan 3-D images of the cell boundary, chromatin, cell cycle events, and protein localizations for almost all plant organs (Rowland & Nickless, 2000). The volume, spatial position, orientation, and polarity of cells comprising an organ are important phenotyping traits, as they can characterize mutant phenotypes or gene-editing effects under different environmental conditions.

Recently developed methods enable precise segmentation based on cell border labeling (Liu et al., 2013, 2014). This approach, combined with the detection of nuclei and cell cycle events (Schulz et al., 2006; Pasternak et al., 2017), allows a simultaneous analysis of cell geometry, cell cycle duration, and geometry of organelles. Cell positions within organs are defined using an internal organ coordinate system based on the position of the organ tip (e.g., the quiescent center [QC] in the root). Cell boundary labeling facilitates the integration of multiple cell types into organ atlases with a precise description of

cellular neighborhoods. One recently developed technique now directs quantitative protein level analysis (Omelyanchuk et al., 2016) and localization and quantification of the protein complex through Proximity Ligation Assay (PLA) (Pasternak et al., 2018; Teale et al., 2021). All these approaches, including the necessary protocols for whole-mount labeling, image scanning, processing, layer identification, and analysis, have been integrated into the Deep-Resolution Plant Phenotyping Platform (DRPPP), a deep pipeline for volumetric segmentation and analysis of the geometry of plant tissue and the detection and quantitative analysis of cell volume, cell cycle events, chromatin status, gene expression and protein complex at cell-level resolution. The main advantage of the DRPPP is that it provides simultaneous analysis and tracking of all cells in an organ in the context of their positions within that organ. The platform requires no endogenous markers; hence, it can be applied to diverse plant species.

Overview on the pipeline.

The pipeline includes growing plants *in vitro* or in soil, incubation of plant tissues with 5-ethynyl-2′-deoxyuridine (EdU) for cell cycle kinetics or other chemicals according to the task, tissue fixation, labeling, scanning, and image processing.

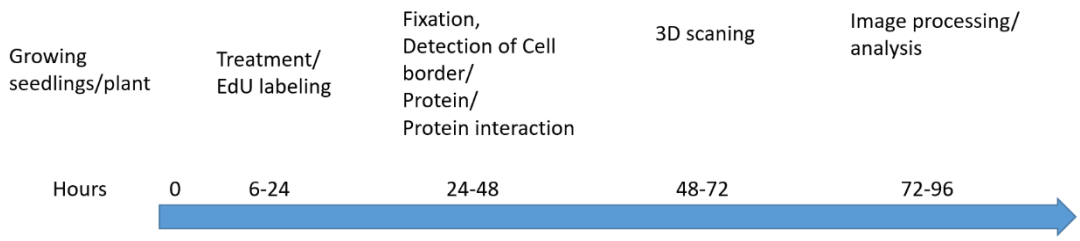


Figure 1. Pipeline step-by-step protocol with timing (in hours). The whole procedure, from treatment to full extraction of the data, requires less than 96 hours.

Labeling methods and parameters extracted.

The platform includes four different tissue labeling options or their combinations: cell border labeling, nucleus labeling (including EdU labeling for cell cycle kinetics), protein localization, and protein complex detection. All four options can be combined to study cell geometry/cell cycle/gene expression simultaneously. Since the quality of labeling is crucial for further analysis, we will describe the procedure in detail in the next parts. Acquisition of the required very high-quality images is quite challenging; therefore, we describe the most important aspects in detail and give protocols in the upcoming sections.

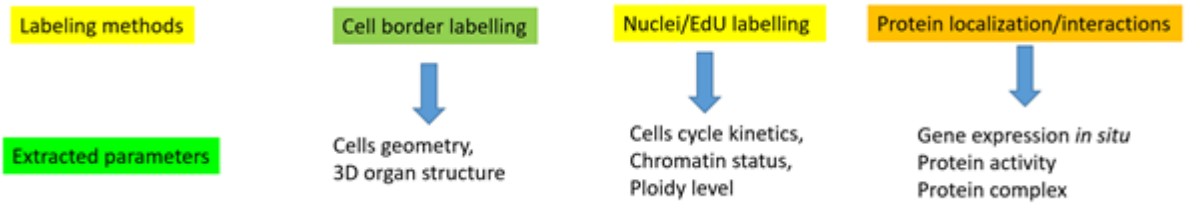


Figure 2. Labeling options.

Live and fixed tissue samples.

The analysis of whole organs at sub-cellular resolution requires the recording of multiple tiles (typically 3–5), with a depth-dependent dynamic adaption of the recording parameters. Thus, living objects (especially roots) are difficult to label and high-quality images are difficult to produce because of the rapid growth and movement of the objects, although the pipeline does not preclude live imaging. High-quality analyses can only be performed on fixed tissues.

Cell boundary detection.

Cell boundaries in plants can be detected based on labeling of the cell wall or plasma membrane. One condition necessary for successful boundary detection by the software is an equal intensity of the boundary of each cell. For this reason, simple cellulose labeling is not suitable for boundary detection because of the variations in the cell wall thickness and various other cell wall properties within an organ. We optimized cell wall labeling by partial digestion of the cell wall to create an almost equal signal intensity for all cell layers after calcofluor white labeling. This labeling can be combined with nucleus detection and gene expression analysis by immunolocalization or PLA assays. This option is especially suitable for the leaves because of their equal cell wall thicknesses compared with root cells.

A second option is to label polysaccharides after normalization of ketone-bound digestion (so-called pseudo-Schiff reagent labeling) (Pearse, 1968). However, this procedure is conducted at pH 1.4 and cannot be combined with any other labeling.

An alternative to cell wall labeling is plasma membrane labelling, which can greatly improve the evenness of the cell boundaries. The use of FM™ 4-64FX, a fixable analog of the FM™ 4-64 membrane stain, gives a satisfactory segmentation quality. However, it does not allow us to combine this labeling successfully with DAPI or immunostaining used for protein expression and localization. A more vigorous fixation procedure using a high Triton-X100 concentration punctures the membrane lipid bilayers that preclude a segmentation step.

We have been able to successfully combine triple labeling using calcofluor white for cell boundaries, antibodies for protein localizationon, and propidium iodide for nucleus labeling. Another possibility is to use DAPI for nuclear labeling, as this also allows the detection of cell position in the organs, although it does not allow cell geometry detection.

Mounting.

A 3D scan requires full organ integrity, so a mounting spacer equal to or larger than the organ thickness must be used. For Arabidopsis roots, a 120 µm spacer is sufficient. However, bigger roots, such as tobacco, require a 250 µm spacer.

Scanning procedure.

Proper image analysis requires equal signal intensity through all organs. However, it is very difficult to achieve in thick objects because of the samples' optical density. Equal signal intensity can be improved in two ways: 1. By dynamically regulated scanning through gradual changes in detector gain, laser power, averaging signal to keep signal-to-noise ratio at a similar level along the sample; 2. By the use of double-sided scanning. The second way is more challenging and can be applied to the samples with a thickness greater than 250 µm.

Software for image analysis.

The intrinsic root coordinate system (iRoCS) Toolbox was used for image analysis (Schmidt et al., 2014). This software allows extraction of cell geometry and analysis of cell cycle kinetics, gene expression, and protein complexes.

Limitations of the platform.

The platform can be applied to most plant organs. However, thick organs (more than 500 µm thickness) may require longitudinal sectioning before processing/scanning.

Materials and methods.

Detailed protocols for the image processing and analysis is in Pasternak T. et al., 2021a.

https://protocols.io/view/deep-resolution-plant-phenotyping-platform-descrip-brsdm6a6.pdf

Reagents.

All reagents were described in detail previously (Pasternak et al., 2015; 2017; 2018).

Microscope.

The confocal or other microscope must provide 3D image scanning at high resolution. For thick objects, we recommend using a 2-photon microscope.

Computer.

The software requires a computer with a minimal RAM size 8 (16) GB (64 GB is recommended for large images with high-resolution segmentation).

Plant material preparation and labelling.

Plants of *Arabidopsis thaliana*, *Nicotiana tabacum*, *Setaria italica*, and *Lycopersicum esculentum* have been used. All procedures for plant cultivation, fixation, treatments, and imaging were described previously (Pasternak et al., 2015; 2017; 2020). The specific differences from the previously published protocols are presented below.

Cell boundary labeling.

Our cell boundary labeling protocol is based on the binding of propidium iodide to de-ketonized cell wall polysaccharides at low pH (1.4) in the presence of sulfur. Although the basic protocol has been described previously (Truernit et al., 2008), significant modifications are required to adapt the protocol for 3D scanning and analysis. One limitation is that the original fixation in acetic acid leads to significant tissue maceration and often damages the softer mature plant parts. For this reason, we recommend fixation with formaldehyde in MTSB buffer at pH 7 before labeling. The de-ketonization level (time of periodic acid treatment) is another crucial parameter. For Arabidopsis roots, a 30 min de-ketonization in 1% periodic acid partially punctured the cell walls, especially in the mature parts. We recommend reducing the de-ketonization time to 15–20 minutes. The mounting procedure is another crucial step. As already mentioned, the spacer should have a similar thickness to the object. A thick object can be scanned using double-sided scanning by mounting the samples between 2 coverslips: a 24 × 60 mm size as a base and a 24 × 32 mm size as a cover. This adjustment allows the object to be scanned from both sides to avoid a low signal-to-noise ratio in the deeper parts.

Scanning procedure.

Note: Always adjust the immersion medium and embedding medium to the average refractive index of the sample for optimum image quality, especially when conducting deep optical sectioning.

A confocal laser scanning microscope with at least four lasers (2P, 488, 543/561, and 633) is required. We recommend the following objectives: ×25 for cell geometry with glycerol immersion and ×40 or ×63 for gene expression, cell cycle, and chromatin structure analysis.

Scanning should be performed with at least 1024 × 1024 pixels per frame at 16 bits and with the amplifier offset close to zero to avoid underexposed image regions. Z-stack corrections are highly recommended to obtain a reasonable signal-to-noise ratio throughout the stack, while avoiding over-exposure. To increase the image quality in the deeper parts, increase the average numbers or reduce the scanning speed in this region. The choice of objective depends on the type of analysis: for cell volumetric analysis ×25 or ×40 objective with immersion correction should be used. For gene expression analysis, cell cycle analysis, and chromatin structure, the minimum is ×40, but ×63 objective is better.

Data extraction/presentation.

After data extraction, each cell/nucleus has three basic coordinates: the distance from the QC, the radial distance, and the radians (unrolled organ). We suggest using a graphical presentation of an unrolled root,

e.g., radians on the x-axis and distance from the QC as the y-axis. The volume can be embedded as a third set of coordinates for the cells as well as for nuclei.

Statistical analysis.

The platform allows extraction of all parameters from at least 5000 cells from Arabidopsis and 30,000 cells for large roots (e.g., tobacco, tomato, millet). The most commonly used method involves analysis of a maximum of 100 cells per root. The platform allows the collection of more detailed information, thereby providing a deeper insight into developmental processes as well as a better understanding of plant responses to environmental stimuli.

CASE RESULTS

Here, we have demonstrated the application of the platform for diverse plant organs and species.
We applied the platform for two tasks: geometrical analysis (geometry of each cell) and functional analysis, when cell function (chromatin organization, cell cycle, protein location and protein proximity) were analyzed.

I. Volumetric plant organ analysis.

The most straightforward platform application is quantitative analysis of Arabidopsis root apical meristem geometry with 3D resolution. This analysis includes the evaluation of cell elongation and cell volume in each cell continuity. The whole pipeline is presented in Figure 3. The simple cylindrical root structure is amenable to application of the root coordinate system, which in turn, allows the extraction of the distance from the QC, the radial distance, and the angle.

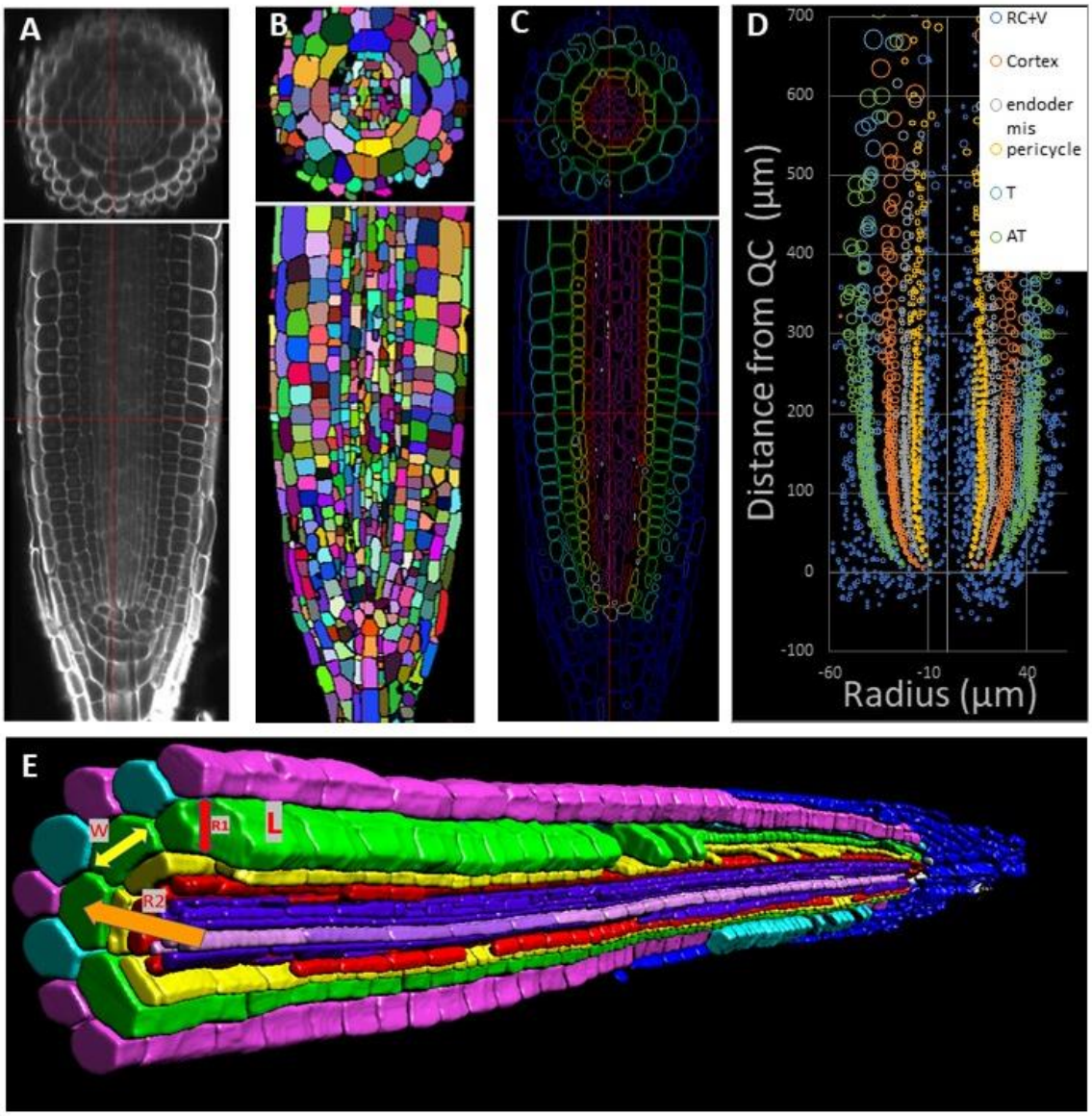


Figure 3. Graphical presentation of the pipeline for the example of the Arabidopsis root apical meristem (RAM). (a) - input image; (b) - segmentation; (c) - layers; (d) - volumetric analysis (circle size proportional to the volume); (e) - parameters extracted for the root cells all cell layers, the detailed structure of xylem/phloem, trichoblast/atrichoblast, root asymmetry in the cortex xylem and phloem poles. L - cell length; W - cell width; R1 - cell radial size; R2 - distance from the axis; R3 - radians; V - cell volume. All parameters are from all 3000 cells in the RAM and the elongation zone until full maturity, including root hairs.

We have also looked at the structure of the outer cell layers because the characterization of these layers provides insight into specific physiological responses. Specifically, (i) cortex length has been used for estimation of root proximal apical meristem length (Hacham et al., 2011); (ii) cell cycle kinetics in the epidermis has been used for estimation of cell cycle kinetics by the "kinematic method."

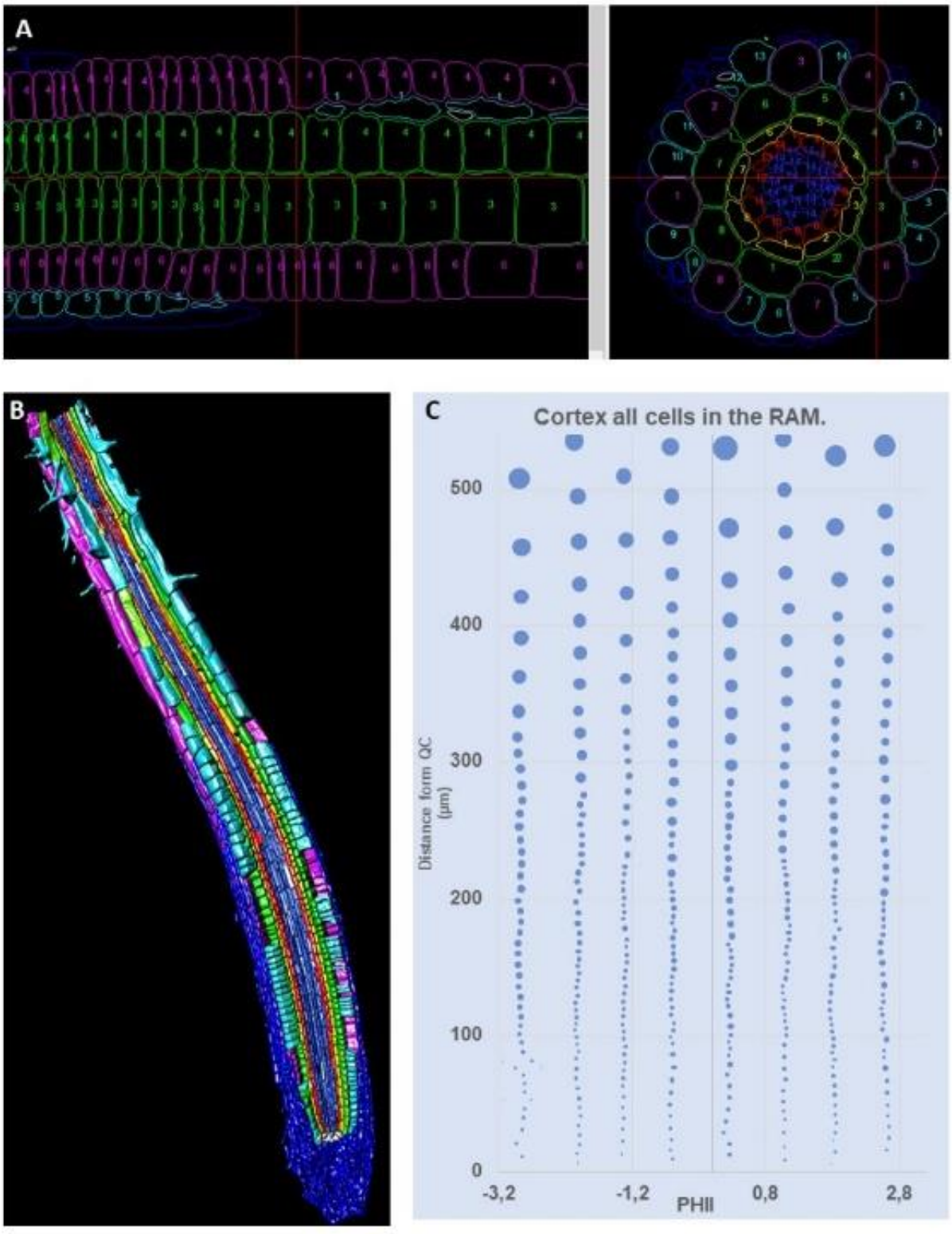
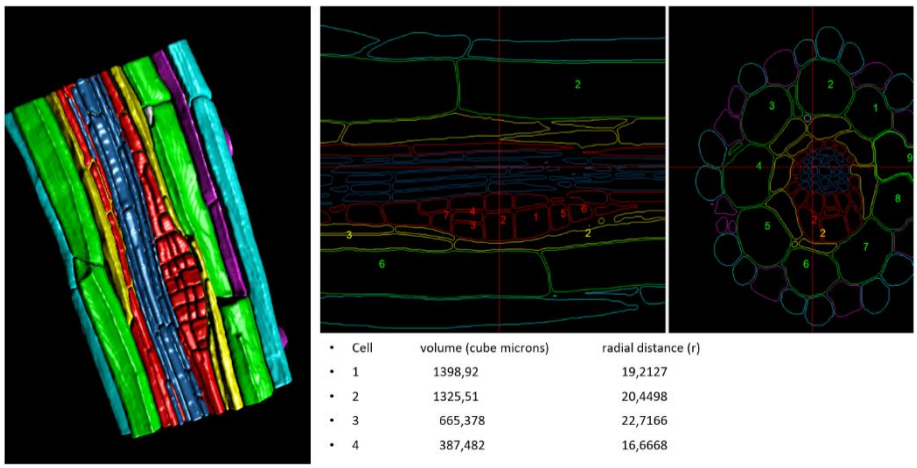


Figure 4. Extraction of geometrical data from the segmented root. (a) - yz and xy views of the root with layers labeled and cell file numbers. Root caps- dark-blue; Trichoblasts (T) - magenta; Atrichcoblasts (AT) - blue; cortex (C) - green; endodermis - yellow; pericycle - red; vasculature - dark blue. (b) – 3D-rendering; (c) – unrolled cortex layers.

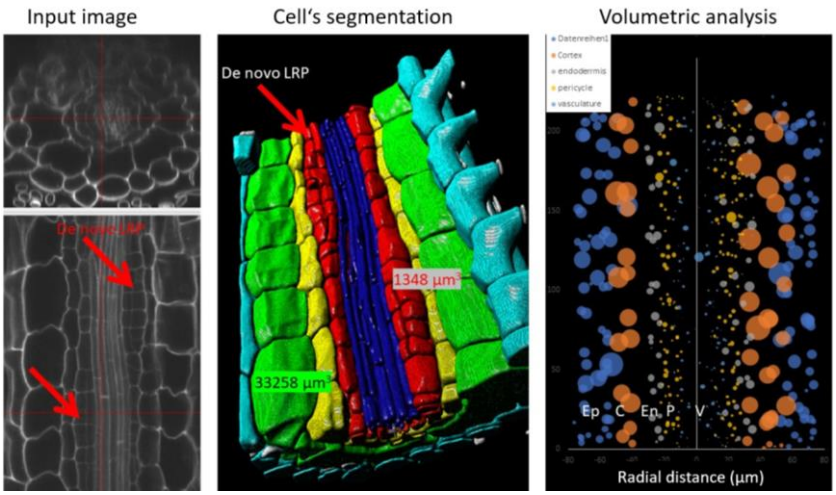
The root waving phenomena is causally related to unequal cell elongation/expansion in the outer cell layers (cortex/epidermis) (Thompson, M. V., & Holbrook, N. M., 2004), and requires a detailed characterization of the cells in cell files for understanding of the waving mechanism of root growth. Figures 3 and 4 (a) show that the epidermis consists of 8 trichoblast (T) layers (which produce root hairs) and 14 atrichoblast (AT) layers (which usually do not produce root hairs). Interestingly, the distribution of AT is clearly asymmetric, with 1 layer (2 cases) or 2 layers (6 cases) of AT cells between the T. This led to an unequal angle between the T and cortex cell files. Figure 4 (a) shows a segmented root with numbers of each continuity and coordinate system. Figure 3 (b) shows a 3D rendering and Figure 3 (c) shows an "unrolled" root. The asymmetry in the epidermis related to the presence of 2 or 1 AT layers between the T leads to asymmetry of the cortex cells, where the cortex cells touching one AT layer have a smaller size.

Quantitative analysis of lateral organs.

The lateral roots aid in anchoring the plant to the soil, while increasing water and nutrient uptake. In addition, lateral roots increase the surface area of the plant root system. Therefore, we obtained a full view of root system architecture (RSA) by testing the possibility of quantifying the kinetics of lateral root development in Arabidopsis in both natural and auxin-induced lateral root primordia (Figure S1 and S2). Here, the main advantage of the platform is that it enables researchers to see the changes in all cell files, which means that it can quantify the position of a lateral root on the plant body, the position of the founder cell, and any divergence in cell volume and orientation during lateral root formation from stage I to stage VIII.



Supplementary Figure 1. Lateral root was segmented, layers were labeled, and quantitative data were extracted. The cell volumes of 4 cells are shown as an example. The pericycle/lateral root is indicated in red.



Supplementary Figure 2. *De novo* auxin-induced lateral root primordia (LRP) in the early mature zone with inhibition of cell elongation.

Analysis of diverse plant species.

In addition to Arabidopsis roots, we tested applications of the protocol to larger species containing more cortex layers (tomato and tobacco) or more complicated inner cell layers (C4 plants with different xylem/phloem structure).

Protocol application for the thick roots.

The protocol allows quantitative analysis of roots of tobacco and millet with root diameters up to 300 µm. These roots contain up to 4 cortex layers, so they are quite similar to the inner layers of Arabidopsis or other C3 plants, but they are completely different from C4 roots (Figure 5, 6).

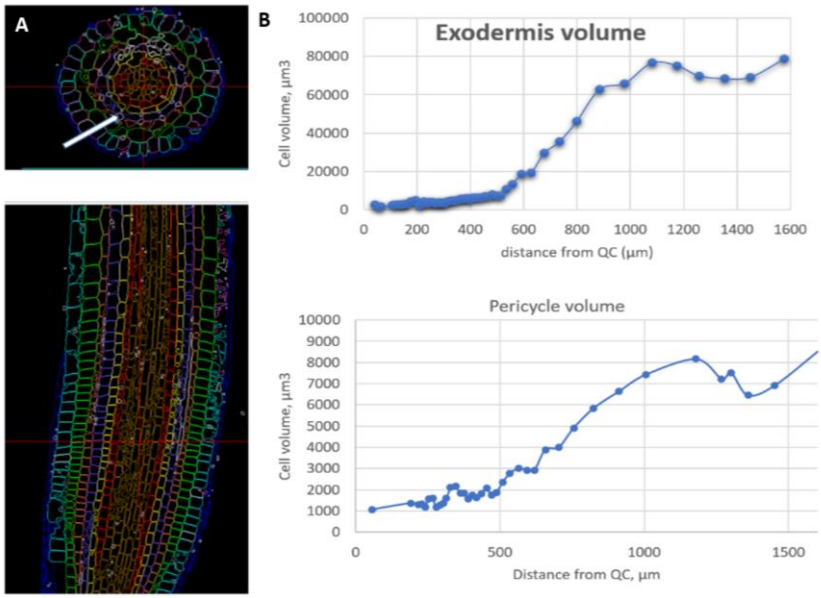


Figure 5. Tobacco root analysis. (a) Segmented and assigned tobacco root. Aerenchyma "holes" occurring between cortex 2 and cortex 3 and between cortex 3 and the endodermis are indicated by white arrows. (b) Evolution of the cell volume of the exodermis and pericycle along the axis. Note the 10 tenfold differences in the volume scale bars between the exodermis and pericycle.

As a further possible application, we compared the root structures of dicotyledonous and monocotyledonous plants (tobacco and foxtail millet, as model objects for both clades). Both species have a very similar outer layers structure (epidermis-exodermis, two cortex layers, and one endodermis layer), but very significant differences in the inner cell layers: monocot roots are characterized by 5–6 large metaxylem vessels and one central cylinder (Figure 6). In the case of millet, a quantitative analysis of 30000 cells up to 1.2 mm from the tip was possible.

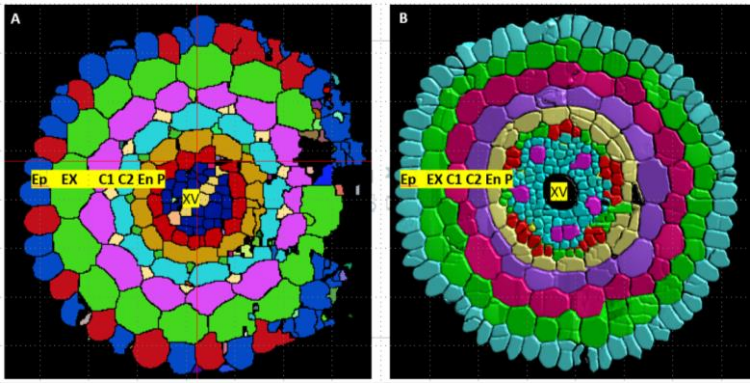


Figure 6. XY cross-section from 3D images of tobacco (A) and foxtail millet (B).
Ep- epidermis; Ex- exodermis; C1, C2- cortex 1 and 2; En- endodermis; P- pericycle; XV- xylem vessel. Both roots were quantitatively analysed, including the volume of the central and side-xylem vessels, and the asymmetric cell position.

Analysis of the geometry of the aboveground part of the plants.

Investigation of the cell geometry dynamics, and especially surface expansion, that accompany leaf primordium formation, can be one target of DRPPP. We can quantify all the geometrical parameters of each cell at all stages of leaf development and process up to 10 leaves simultaneously (68,000 cells in 7 to 8 day-old seedlings) with characterization of the cell position in the leaf, the shoot apical meristem (SAM) structure, and the trichome and stipule volumes.

The platform performs an automatic extraction of all phenotypic traits for an individual leaf and an individual cell in a 3D model. The most important parameters are the leaf area, the location and density of the stomata cells, the volume and surface area of the mesophyll cells, and the detailed structure of the vascular tissue. Simultaneous analysis of leaves of different ages (as shown in the example) allows observation of structural changes during leaf development. In conclusion, polysaccharide labeling allows investigation of quantitative plant organ geometry and provides equal labeling intensity to all cell files, independent of the cellulose content in the cell walls.

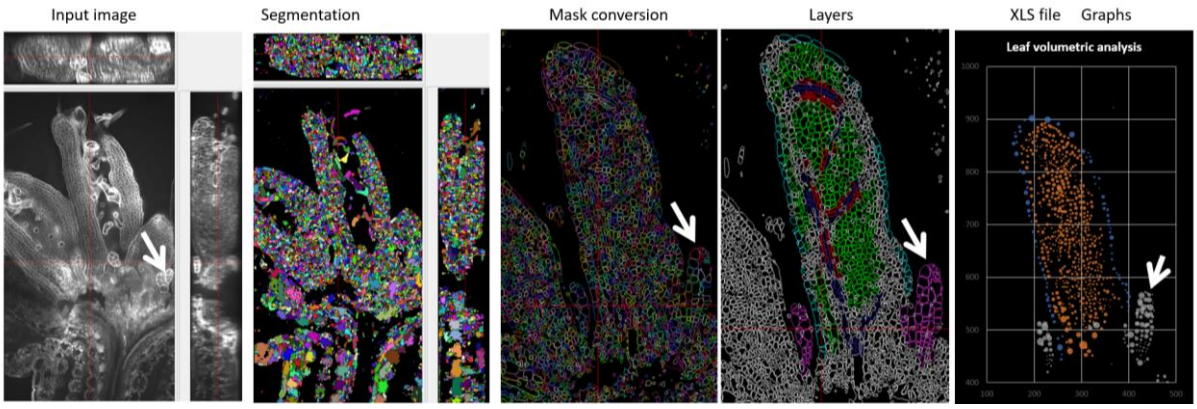


Figure 7. Example of geometric analysis of a 7–8-day-old Arabidopsis shoot. The shoot was segmented, converted to a mask, and the features of 68,000 cells were extracted and analyzed. One leaf is shown as an example. The bubble size indicates the volume. Stipule differentiation with increasing volume is visible (yellow arrow).

These data can be used to prepare a plant organ zonation model with the cell division position, endocycle, and cell differentiation for each cell layer in all plant organs, while also defining the boundary zone between each.

II. Functional plant organ analysis based on nuclear labeling.

In addition to cell border labeling, the platform also allows automatic detection of the nuclei, which in reflect position of each cell and the cell cycle status. When used in combination with EdU labeling, it allows mapping of DNA replication events as well.

Cell cycle analysis. The most straightforward demonstration of the platform is a quantitative analysis of Arabidopsis root apical meristem for cell cycle activities with 3D resolution. Two key cell cycle transitions have been detected: DNA replication (after a 90 min EdU incubation) and mitosis (after a 90 min colchicine incubation). Mapping of these events serves as the basis of functional root zonation: the border of the proliferation zone is the mean position of the last mitosis event in the corresponding cell file, while the border of the transition zone is defined as the starting point of rapid cell elongation and DNA reduplication. The platform can also be applied to investigate the cell cycle distribution and gene expression in the floral tissues (Supplemental Figure 3 and 4).

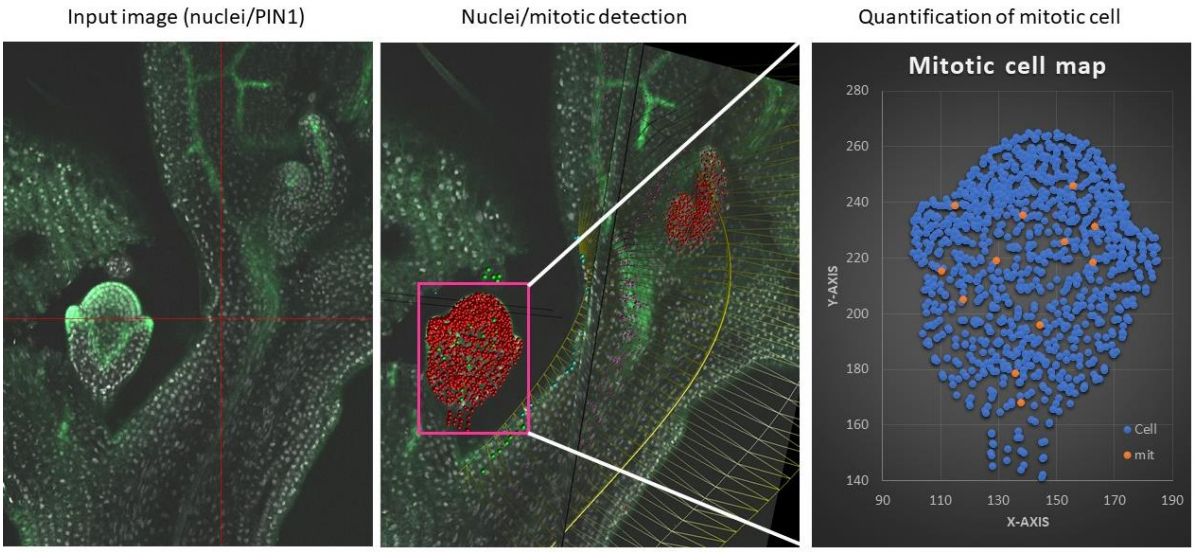


Figure S3. Quantitative analysis of the flowers development.

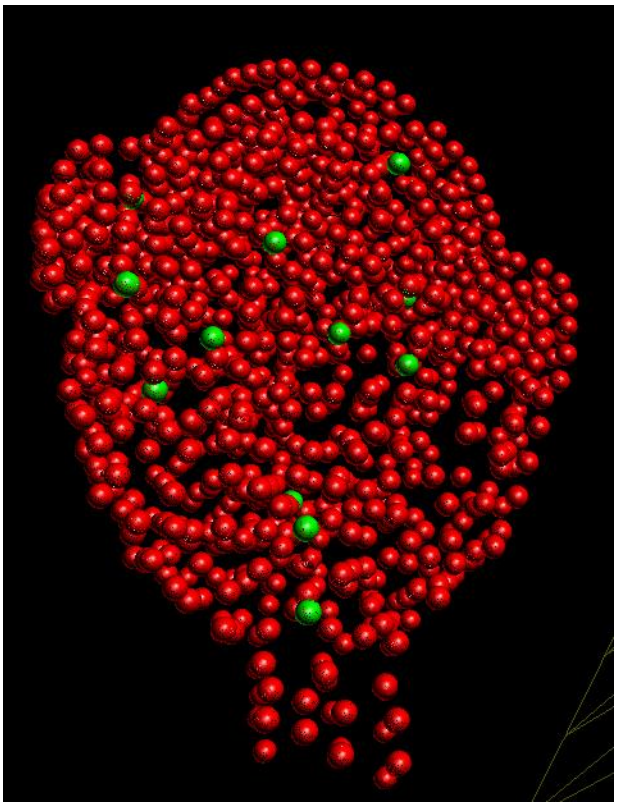


Figure S4. Details of the flower meristem.

Analysis of the cell cycle kinetics.

The cell cycle progression in the root apical meristem is crucial in regulating root growth and development.

That's why determining the cell cycle kinetics and location are essential for understanding the mechanism of root growth. Root meristem consists of proliferation zone, transition zone, and

elongation zone. In proliferation zone occurred de novo cell production, while in the transition zone, slight elongation accompanied by endocycle.

Detailed localization of all these parameters allows building functional root zonation (based on real cell cycle and elongation kinetics). root zonation

Figure 8 describes the example of functional root zonation in the epidermis of Arabidopsis root (functional root zonation means root zonation) based on real cell cycle events, contrary to geometrical 2D root zonation based on cell length).

The cell cycle duration (how rapidly new cells are produced) is another key parameter determining the kinetics of root growth. The platform also allows us to determine the kinetics of each stage of cell cycle progression. Another feature is the determination of such mysterious events as stem cell division, and it's distinguishing from an extension of stem cell niche by changes in post/mitotic cell fate. The rapid divergence in the cell and nuclei volumes in different cell files starting from QC suggests differences in chromatin structure which may cause differences in cell cycle progression. This led us to test our workflow for analysis of the cell cycle of individual cells within each cell file and in each cell. We demonstrated by using different EdU incubation times that our platform allows extraction of these data (Pasternak et al., 2021). Briefly, S+G2 duration is depicted as the time proportional to abundance of for EdU-positive and the whole cell cycle duration as the time required for EdU labeling of all cells. In addition, a more prolonged time incubation with EdU (18–28 h) allows detection of quiescence levels in the stem cell niche (Pasternak et al., 2021).

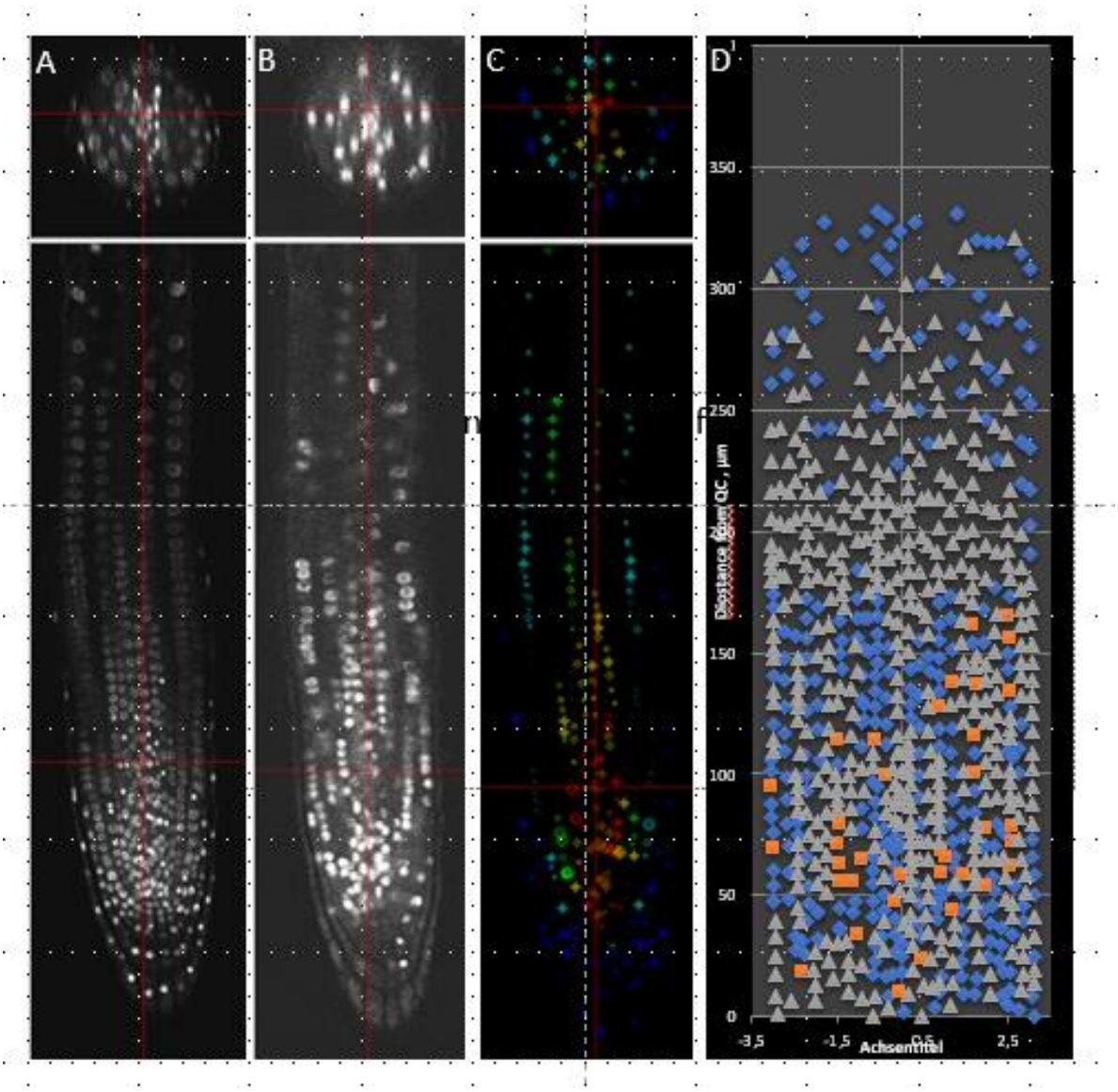


Figure 8. Quantitative analysis of cell cycle kinetics in Arabidopsis root. A - DAPI; B - EdU; C - nucleus detection; D - analysis of the cell cycle events in the epidermis.

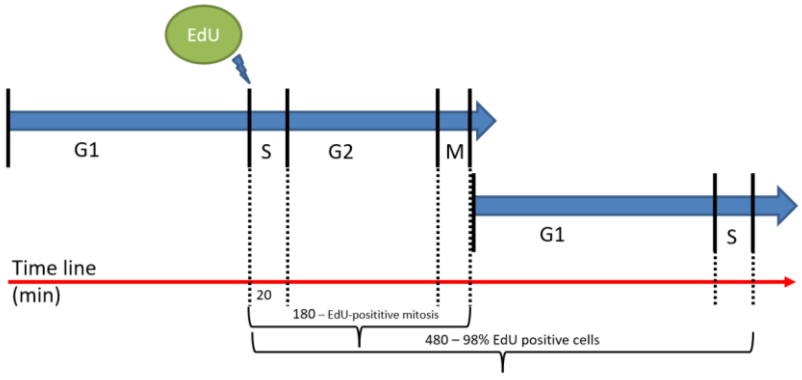


Figure 9. Cell cycle duration analysis.

Leaf/shoot functional analysis.

Leaves and shoots represent rapidly developing organ(s) with a complicated structure that mostly involves cell division orientation, cell polarity kinetics, and rapid divergence of cell fate. This platform can quantify all these different features. Figure 10 shows an example of quantitative analysis of the cell division orientation, PIN1 localization/polarity, and the induction of the first three leaves in 4-day-old Arabidopsis seedlings. All nuclei were automatically detected, and the cell fate was labeled manually (epidermis, mesophyll, and trichomes based on large nuclei and different orientations of the mitotic plates). Application of the platform workflow allows also to analyze leaf zonation. In the leaf, the exit from cell division starts from the leaf tip. In Arabidopsis leaves cells cease mitosis, but may continue DNA replication to reach 8C/16C. Therefore, the leaf can also be divided into a "proliferation zone" and a cell-type specific differentiating zone. The leaf has no mature zone (except several cells in the tips that show very compact chromatin). Detailed maps of all of these events can be created.

In addition, mapping of the DNA replication events allows us to map endocycle in the mesophyll cells of the cotyledon and quantify "ploidy level" after reduplication.

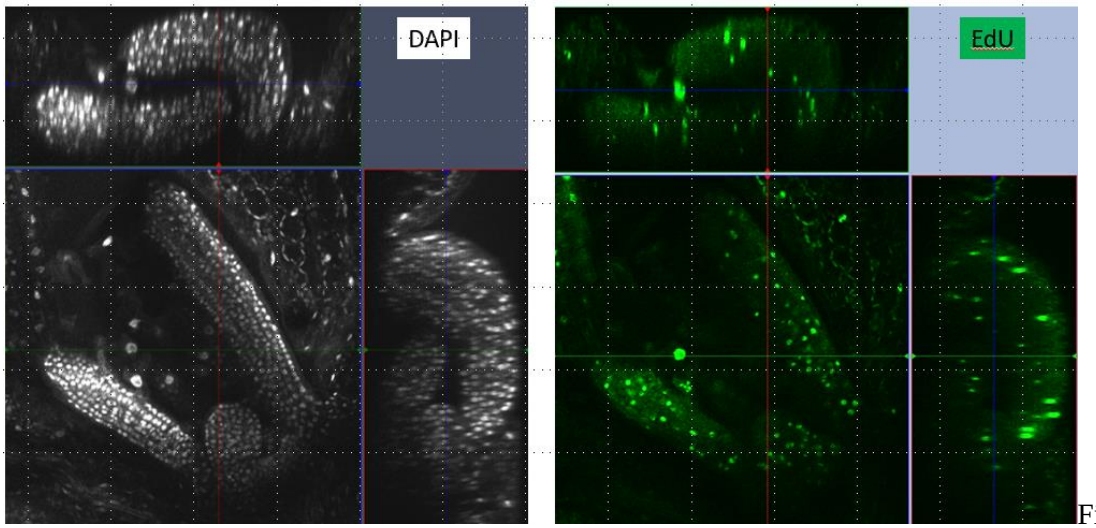


Figure 11.

Figure 11. Analysis of cell cycle kinetics and endocycle in Arabidopsis shoot. Seedlings were incubated with EdU for 45 minutes, fixed and EdU was detected with standard protocol. 3D scanning/reconstruction were done.

Analysis of the chromatin status.

The chromatin structures in plants are determined by DNA methylation and histone modifications. Chromatin organization contributes significantly to the regulation of the cell fate, cell cycle, and gene expression. For this reason, we included in the platform options for quantifying different aspects of epigenetic regulation *in situ* in the form of nucleus geometry (volume, nucleolus volume), histone methylation, chromatin density, and chromosome numbers. One of the most visible markers of the cell fate in Arabidopsis root is nuclear landscape (volume), which, in turn, is independent of chromosomes number. Chromatin condensation is an added versatile marker of phloem cell fate (sieve elements)

(panel (b)), as a heterochromatin map can be detected with a specific antibody. Examples of these options are presented in Figure 12.

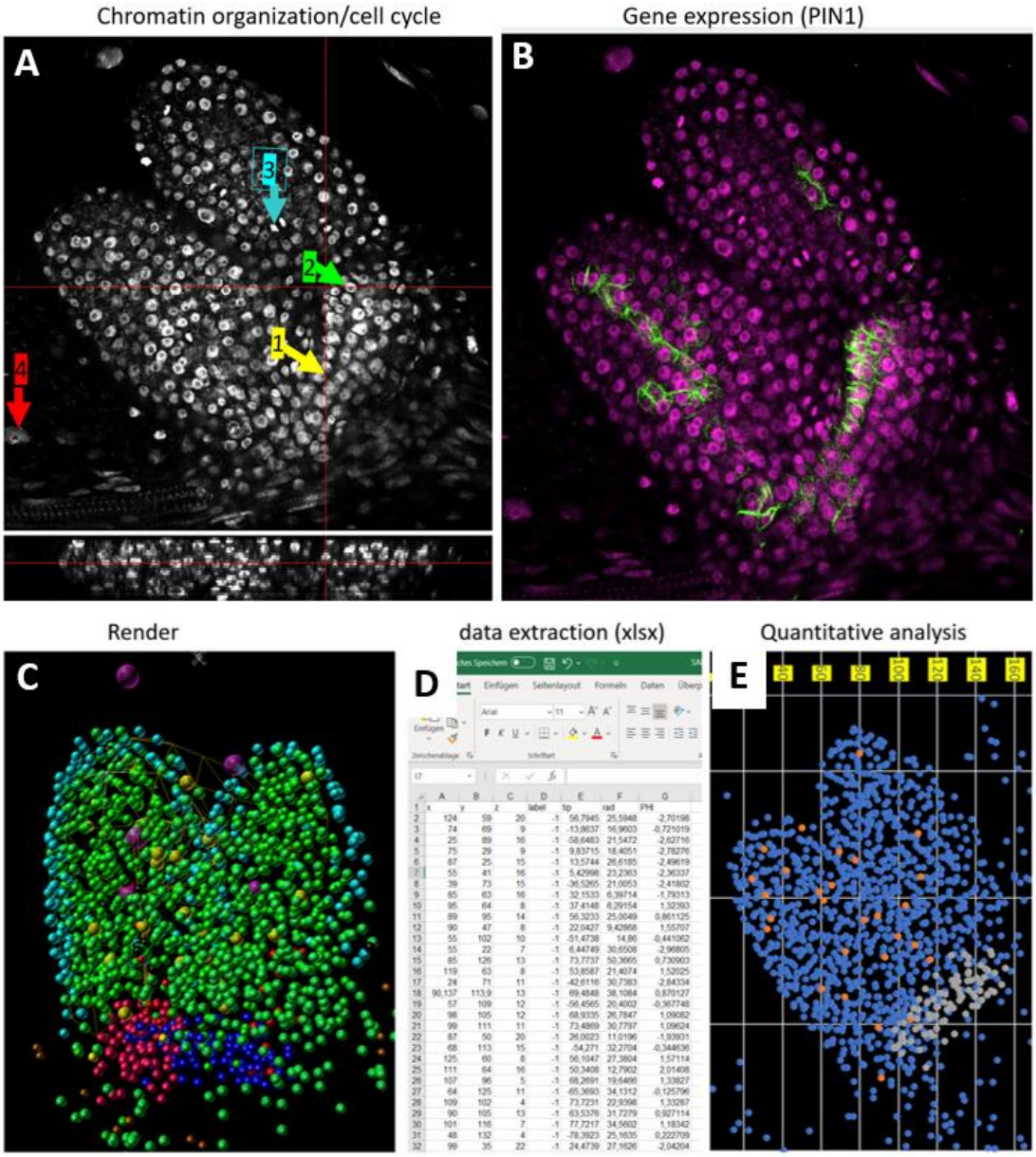


Figure 10. Quantitative analysis of shoot apical meristem structure and leaf formation in 4-day-old Arabidopsis seedlings. The seedlings were fixed, subjected to PIN1 immunolocalization, co-staining with DAPI, 3D scanning, and image analysis. (A) DAPI stained 3D leaf image; (B) PIN1 localization; (C) rendering of the nuclei after detection; (D) quantitative extraction of cell position in relation to SAM as the center; Reconstruction of mitosis position in the SAM.

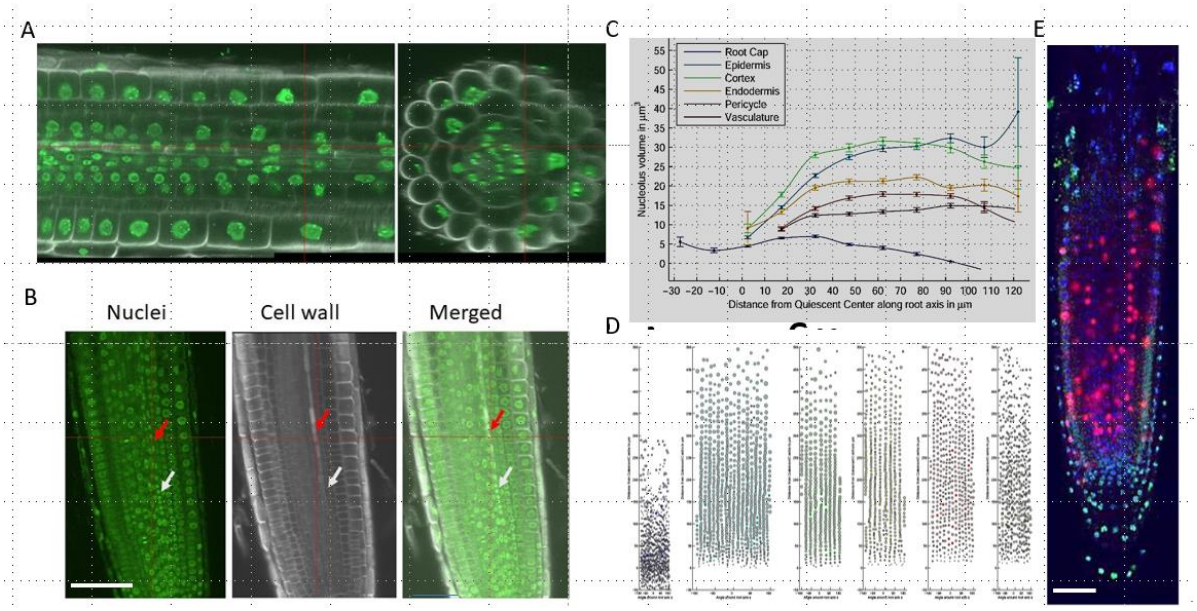


Figure 12. Chromatin analysis in Arabidopsis root.
(A) cross-section of Arabidopsis root. Nuclei are indicated in green; cell borders are in white. The gradient in the chromatin landscape from the division to elongation zone is shown. Trichoblast (T); Cortex (C). The red arrow indicates condensed chromatin in the phloem. (B) chromatin condensation as a marker of phloem differentiation. The roots have been stained for nuclei (propidium iodide) and for cell walls (Calcofluor white). Cell differentiation in the phloem was detected as a decrease in nucleolus size and chromatin condensation (white arrow). (C) average nucleolus volume in different cell files at different distances from the quiescent center (QC). (D) distribution of nucleolus volume in the virtually unrolled root. The circle diameter is proportional to the nucleolus volume. (E) H3K9me2 localization (green) and EdU labeling (red) in Arabidopsis root. H3K9me2 is a marker of heterochromatin abundance.

Protein localization and protein complex quantification *in situ*.

Apart from monitoring the cell cycle, the platform can be used to monitor expression of indiviudal protein as well as protein complexes using proximity for interactive labeling. Here the cell file–specific PIN1 localization is shown (Omelyanchuk et al., 2016) as well as complexes composed of different membrs of the PIN protein family (Pasternak et al., 2018). Quantitative analysis of proximity ligation assay (Pasternak et al., 2018) and demonstration of the the mechanism of NPA action (; Teale et al., 2021).

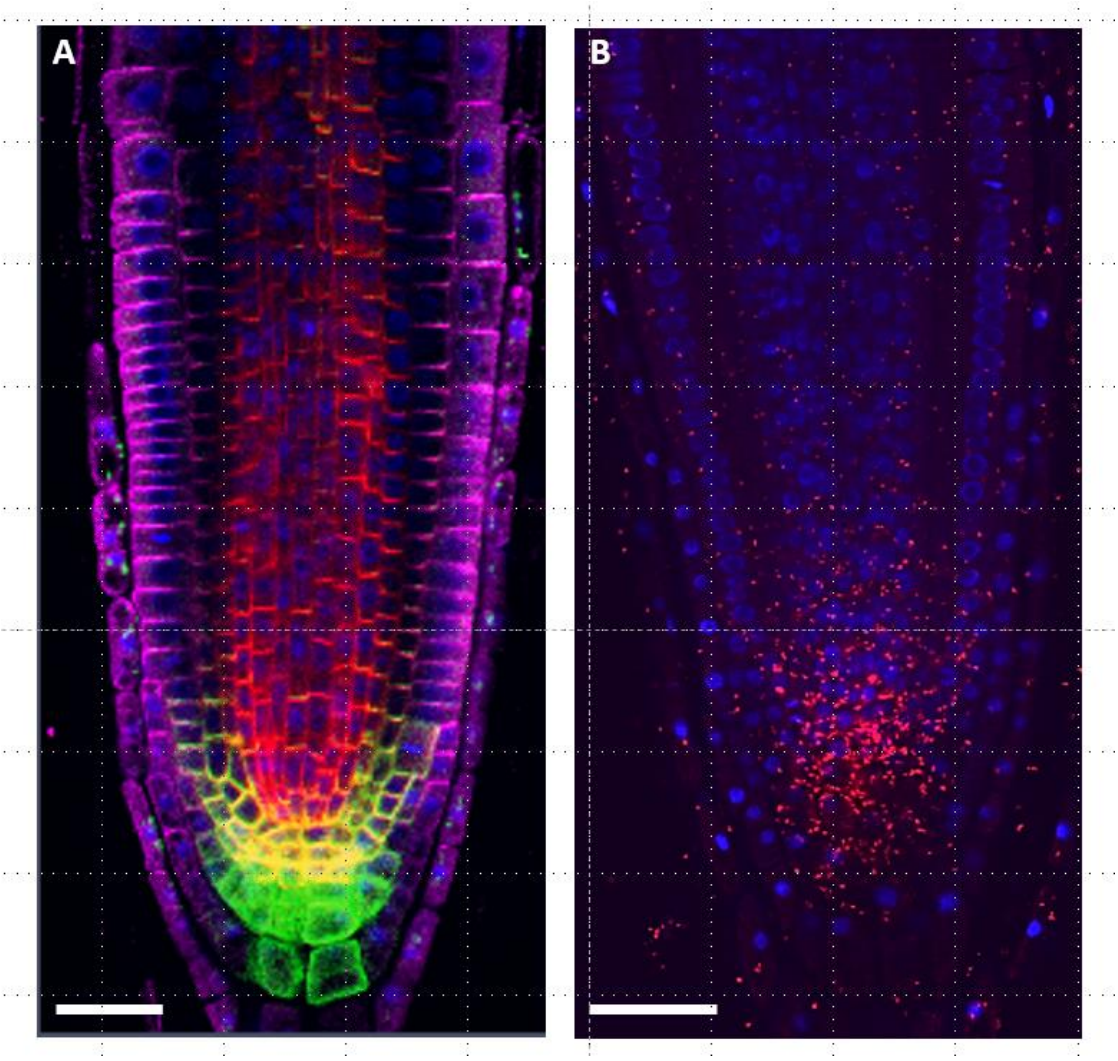


Figure 12. Proximity ligation assay: detection of PIN1 and PIN4 complex in Arabidopsis root. (A) – protein immunolocalization: PIN1 (red), PIN2 (magenta) and PIN4 (green) localization. (B) – Proximity ligation assay (PLA): PIN1:PIN4 complex (red). Nuclei are indicated in blue; scale bar = 20 μm.

Discussion

Phenotyping has long been viewed as the main bottleneck in breeding programs; however, the main focus of phenotyping was originally to characterize macroscopically the whole plant or plant organs (Fiorani, F., & Schurr, U., 2013). This phenotyping helps to identify the relationship between genes and complex traits, such as yield, plant height, root length, and root structure, but it does not help to understand the processes occurring at the cellular level that guide plant growth and development. Therefore, phenotyping at the cellular and subcellular levels, with the characterization of cell geometry and function *in situ*, is a key tool for developing mechanistic models for understanding genotype–environment interactions.

This phenotyping at the cellular and subcellular level can be attained by applying the DRPPP platform to reconstruct different organs at high 3D-resolution. This platform has been successfully applied to Arabidopsis and other plant species and explains their anatomical, morphogenetic, and functional traits.

The platform has four key parts that, in turn, represent different branches of science: plant growth (plant biology), sample preparation (physic/chemistry), imaging (microscopy), and image analysis (informatics). Only a combination of all these parts gives a satisfactory result. The preparation of samples and image generations are the most important steps because excellent sample preparation avoids many image analysis shortcomings.

We have described all the necessary details, from optimal growth conditions to the generation of the high-resolution images (Pasternak et al., 2015; Pasternak et al., 2020). The main advantage of our platform is that it is an endogenous marker-free system based on antibodies and other kinds of labeling. Most of the currently available image analysis protocols are based on the use of endogenous marker lines (see for example, Deshvoyes et al., 2020), which, on the one hand, have the advantages of saving time and allow the use of living samples. However, on the other hand, they do not allow the use of any mutants, especially if the mutants are in another genotypic background, and they do not allow the use of commercial cultivars that do not have endogenous markers.

The image analysis toolbox is the fourth part of the platform. The main advantages of this toolbox are the possibilities for simultaneous analysis of cell geometry, nuclei (cell cycle), and gene expression. Apart from this segmentation/nucleus detection, the signal quantification, simple cell positioning (layers recognition, cell position in organs, cell fate), and data extraction have primary importance. Our platform automatically detects layers (based on the previous training model), automatically detects coordinate systems, and automatically extracts all cell features using data conversion to a .csv file.

The platform has been applied thus far for the investigation of root zonation (Lavreha et al., 2017), new WOX5 function in the Arabidopsis root (Savina et al., 2020), protein complex formation (Teale et al., 2020), and cell cycle kinetics in Nicotiana tabacum root (Pasternak et al., 2017). With our platform, precise single-cell phenotypes can be identified in their original tissue context, providing new possibilities for direct *in situ* dissection of cell cycle kinetics and gene networks in the interaction with neighboring cells/tissues.

The wide application of DRPPP motivates further development of its key parts, where development of software for image analysis the most promising. Currently, in addition to iRoCS, the application of a MorphoGraphX is also possible (de Reuille PB et al., 2015; Wolny, A., et al., 2020); however, this software requires more computer resources and does not have a manual correction option. Moreover, the MorphograhX was not used for nuclei detection and did not have an automatic coordinate system for the root, and was not used for cell cycle detection.

During the development of DRPPP, we realized that only a few datasets were available to the plant biologist community for training cell segmentation and cell cycle analysis tasks. We have addressed this gap with a public release of sets of images and corresponding tutorials for each, as examples of application of our platform.

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Conflicts of Interest:

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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