Plant phenotyping platforms.

Summary.

High-Throughput Plant Phenotyping for plant nutrition and novel biologically active compounds: from seeds germination through whole plants to single cells resolution, with morphological and functional analysis.

Highly accurate non-destructive phenotyping techniques have attracted the interest of scientists and the industry as an efficient means for elucidating the effect of nutrition environmental factors and growth regulators on plant development. Non-destructive technique give us very detailed information about plant geometry and effect of each condition on whole plant shape.

However, for detailed investigation of the mechanism one need to use more deeper analysis with cellular and sub-cellular resolution.

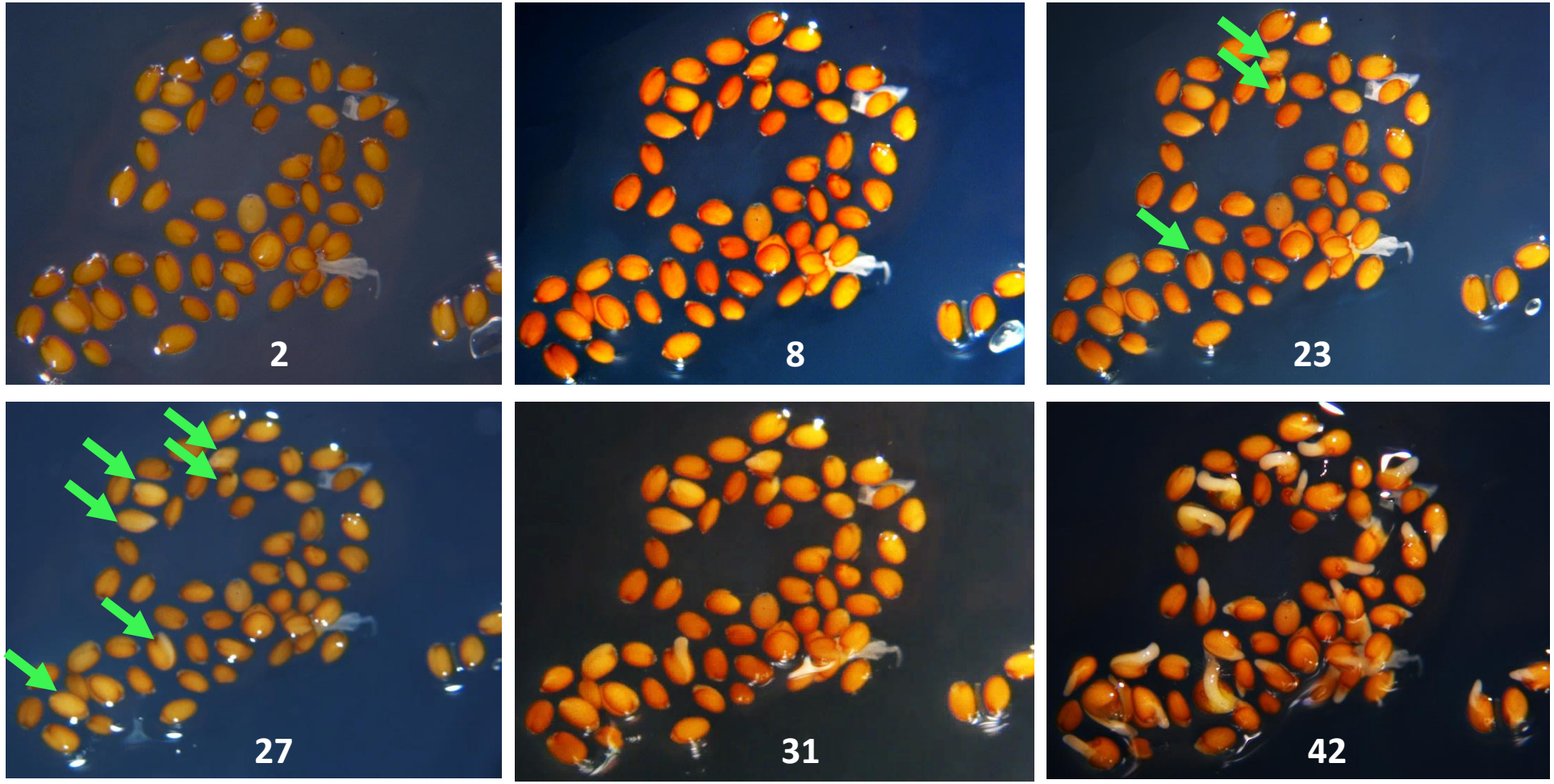
In this case non-destructive assay with cellular resolution possible only in the presence of some fluorescent markers and restricted only to specific lines.

Here we combine non-destructive plant phenotyping on whole plant level with deeper investigation of organ structure and molecular mechanism of plant responses on fixing material by using marker-free system.

Non-desctructive seedlings phenotyping in vitro.

- Growing plants in vitro opened researchers possibility to work under precisely controlled conditions in the term of plant nutrients and applications of bioactive substances.
- Direct observation of early stage of plant growth, and for model plant Arabidopsis even till maturity is possible.
- Non-desctructive phenotyping include quantitative analysis of seeds germination, kinetics of root, hypocotyl, cotyledon growth, quantification of the position of lateral organs.
- Seedlings were growth on vertical plates *in vitro*, have been scanned with high resolution and plant geometry have been quantified.
- For detailed investigation binocular can be used.
- Gene expression were quaintified by using fluorescent marker lines.

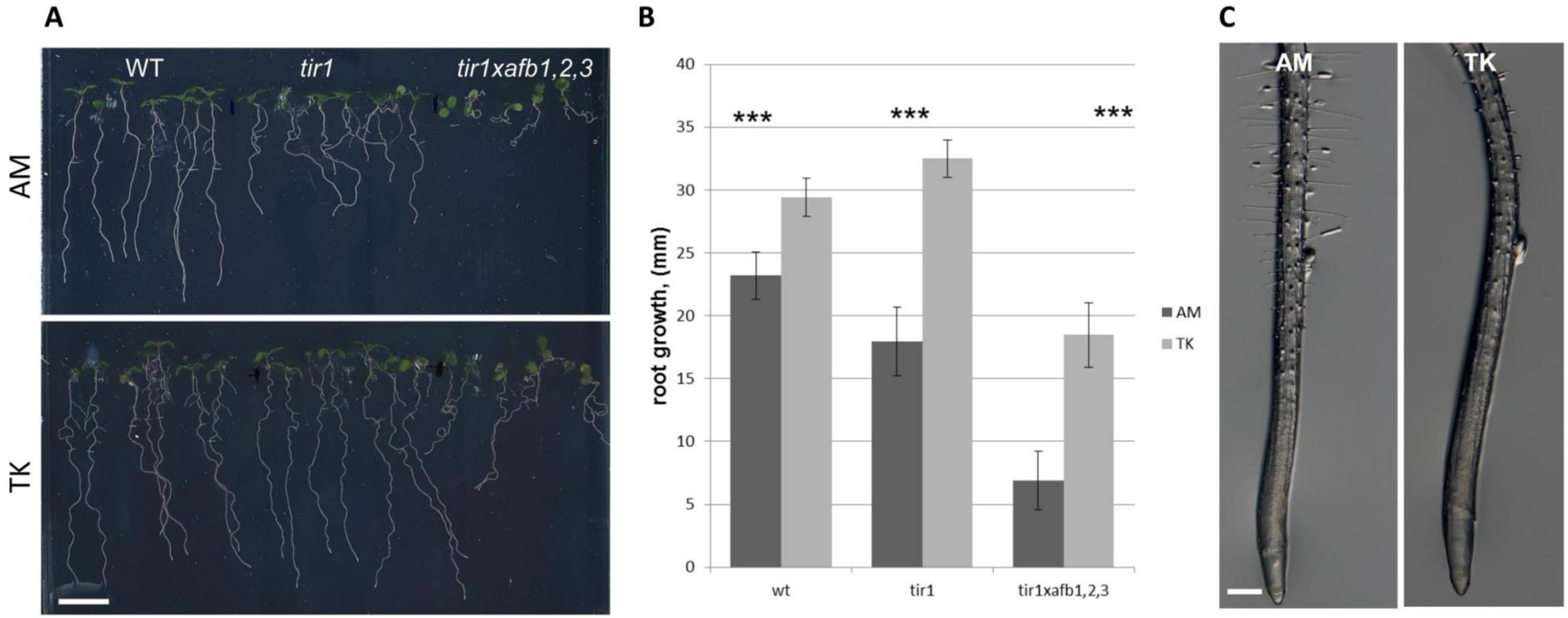
Seeds germination phenotyping



Seeds germination is important marker of the quality. Platform include investigation of dormancy broken and early stage of seedling growth. Seeds were sterilized and soaked on the medium. Images were taken at 2,8. 23, 27, 31 and 42 hours after soaking.

Germination ratio were count as % of the seeds with testa rupture at given time points. Seeds area were measured with Image J.

Example of usage of plant phenotyping for optimization of plant nutritions.



- Effect of nutritions on root development. (A) Plants of wt (Columbia), *tir1* and *tir1xafb1,2,3* mutants were cultured on AM medium for 4 days and were transferred on AM or TK medium thereafter. Pictures were taken after 3 additional days of growth. Scale bar - 1 cm; (B) - Quantification of root length (mm) on AM and TK medium. Error bar- SD; *** mean significant differences for P ≤0,001. (C) – details of root meristem. Scale bar - 100 µm.
- TK medium were optimized for nutrients contents.

Advantanges and limitations of non-destructive plant phenotyping.

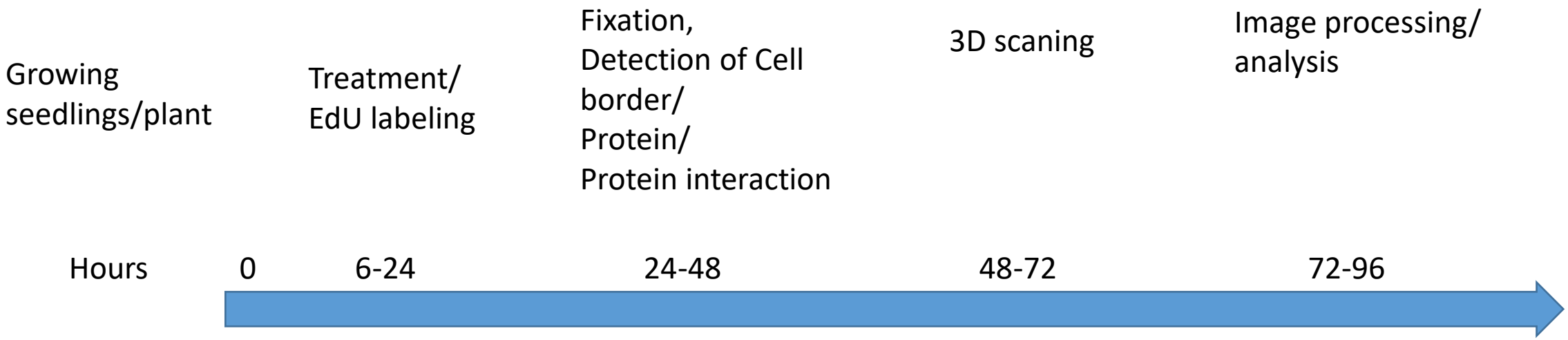
- **Main advantages**:
- Non-destructive.
- Rapid analysis.
- Growth conditions close to optimal.
- **Disadvantages:**
- Can not give a cellular resolution.
- Microscopic images require fluorescent marker what is limitation factor itself. One or maximal two markers can be used simultaneously.
- Cellular-resolution imaging for living plants can be apply to one organ simultaneously.

High-resolution plant phenotyping platform.

All limitations above prompt us to develop partially destructive high-resolution platform what allow to overcome majority of the limitations

Given the rapid development of plant genetic engineering, the lack of accurate methods for plant phenotyping with cell resolution limits our ability to analyze the genetics of quantitative traits and quantitatively related gene functions, expression, localization and proximity of a protein with a phenotype in subcellular resolution. Here we described an effective high-resolution phenotyping platform that allow to create a “passport” of each cell in plant organ which includes quantitative data on cell geometry, gene expression, chromatin status, cell cycle progression and protein-protein interactions. Usage the platform requires minimal time and experience and can be completed in 3-4 days, including a full analysis of whole organ. However, this method may require high-resolution microscopy and a high-performance computer. Below we describe its applications step by step with examples.

Pipeline.



- Pipeline step by step with time points. Whole procedure required 96 hours from starting of the treatments to final extractions of the results.
- To made platform semi-descructive, only part of the plants can be fixed what allow to further plant growth (only 2 mm root tips, for example).

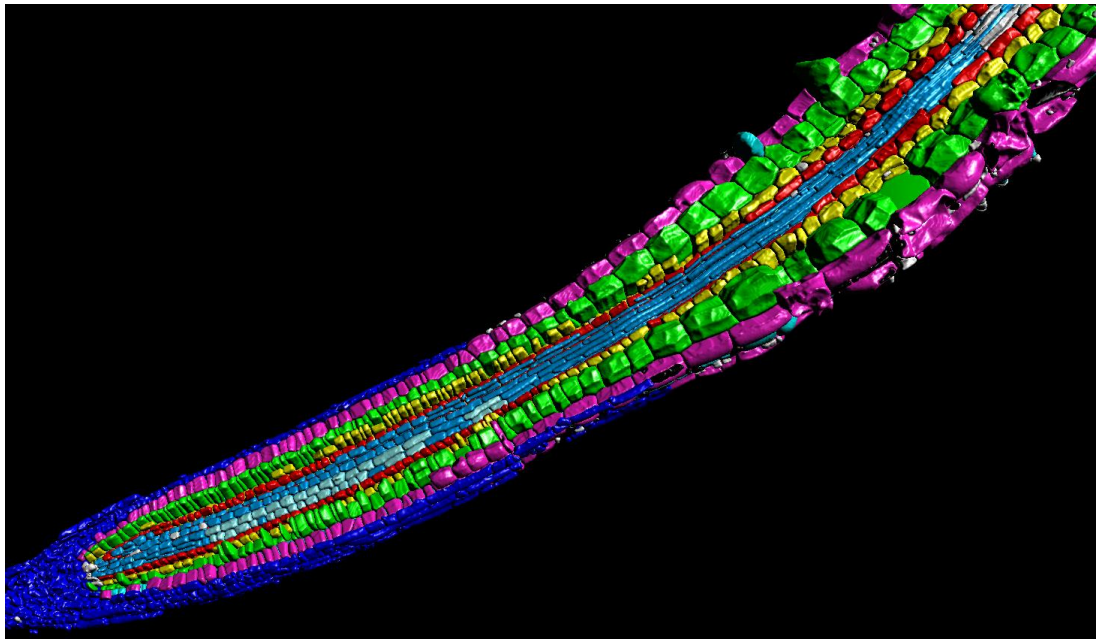
Labeling options.

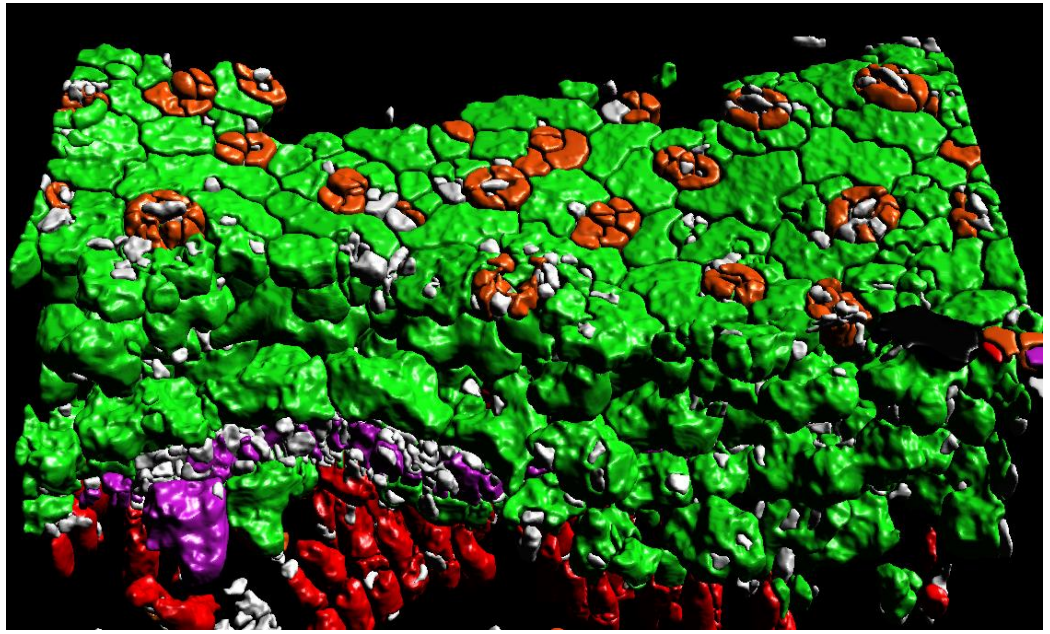
Labeling methods	Cell border labelling	Nuclei/EdU labelling	Protein localization/interactions
Extracted parameters	Cells geometry, 3D organ structure	Cells cycle kinetics, Chromatin status, Ploidy level	Gene expression *in situ* Protein activity Protein complex

All options can be combined to study cell geometry/cell cycle/gene expression simultaneously

3D structure allow to study cell to cell communications in whole organs.

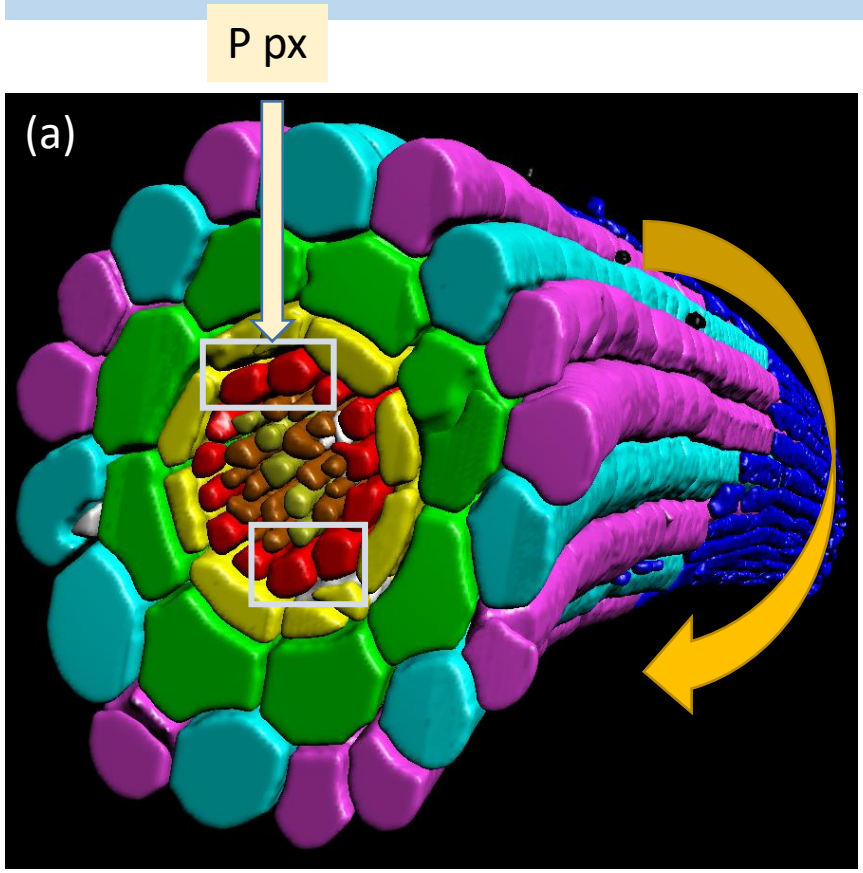
Example of the plant organs vizualizations.

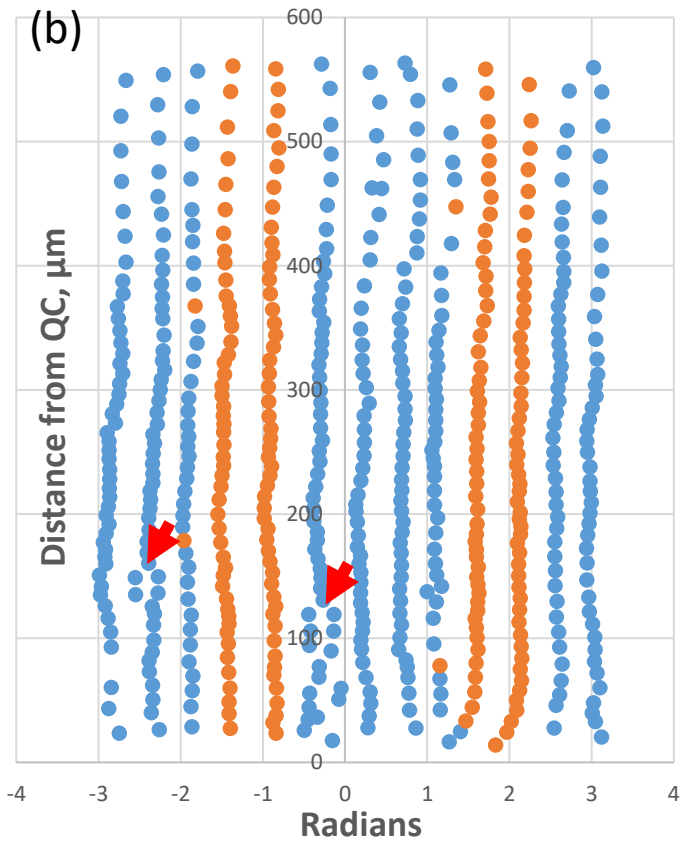


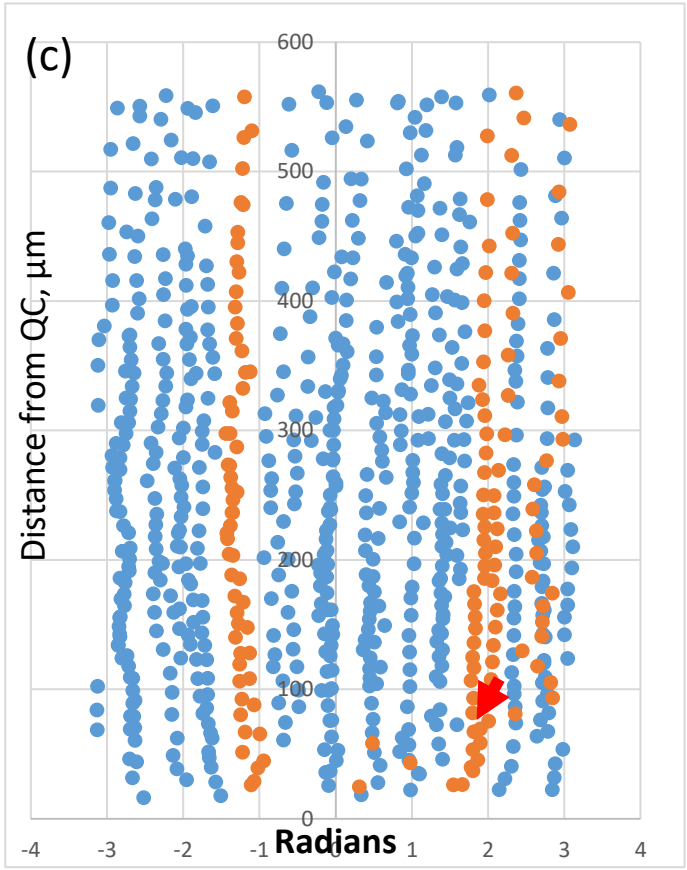


- Arabidopsis root structure.
- Tobacco leaf structure.

Cell „passportisation“ in the root.

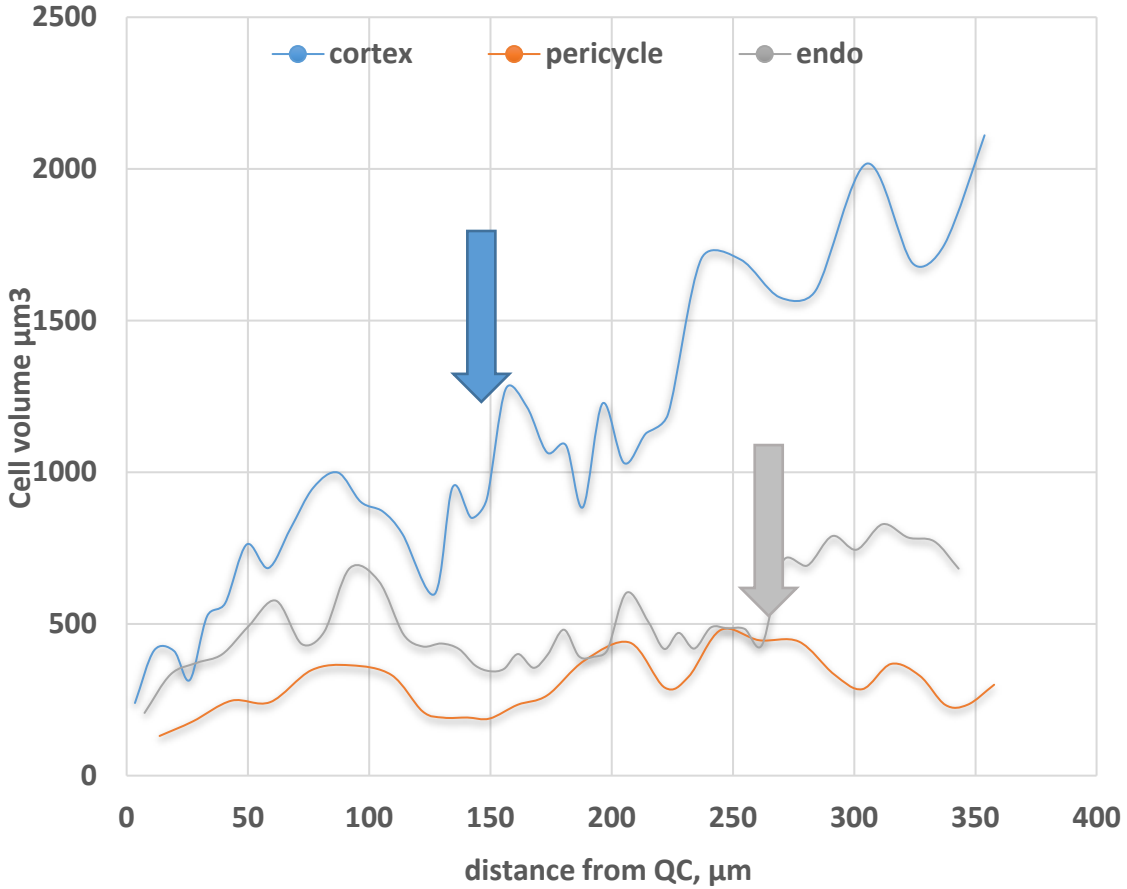


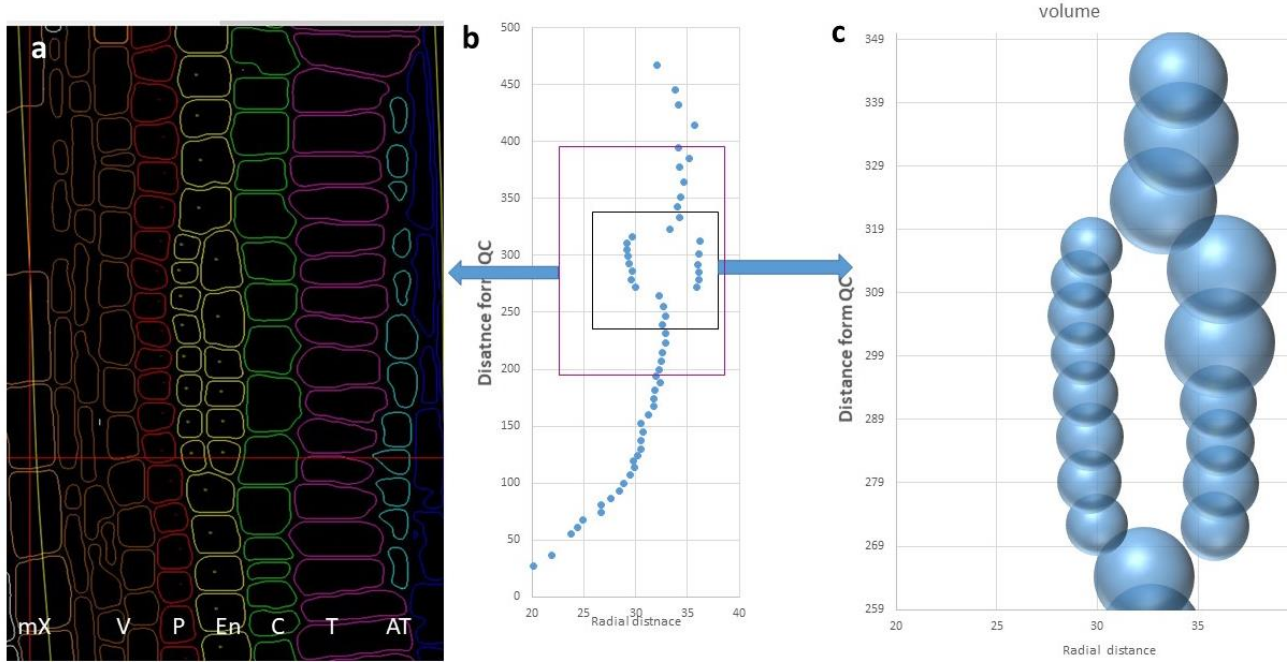




- Render and virtually opened along the arrow (unrolled) pericycle and vasculature layers.
- Each dot reprsent cell.
- Periclnal cell division in pericycle and xylem have been shown by red arrow.

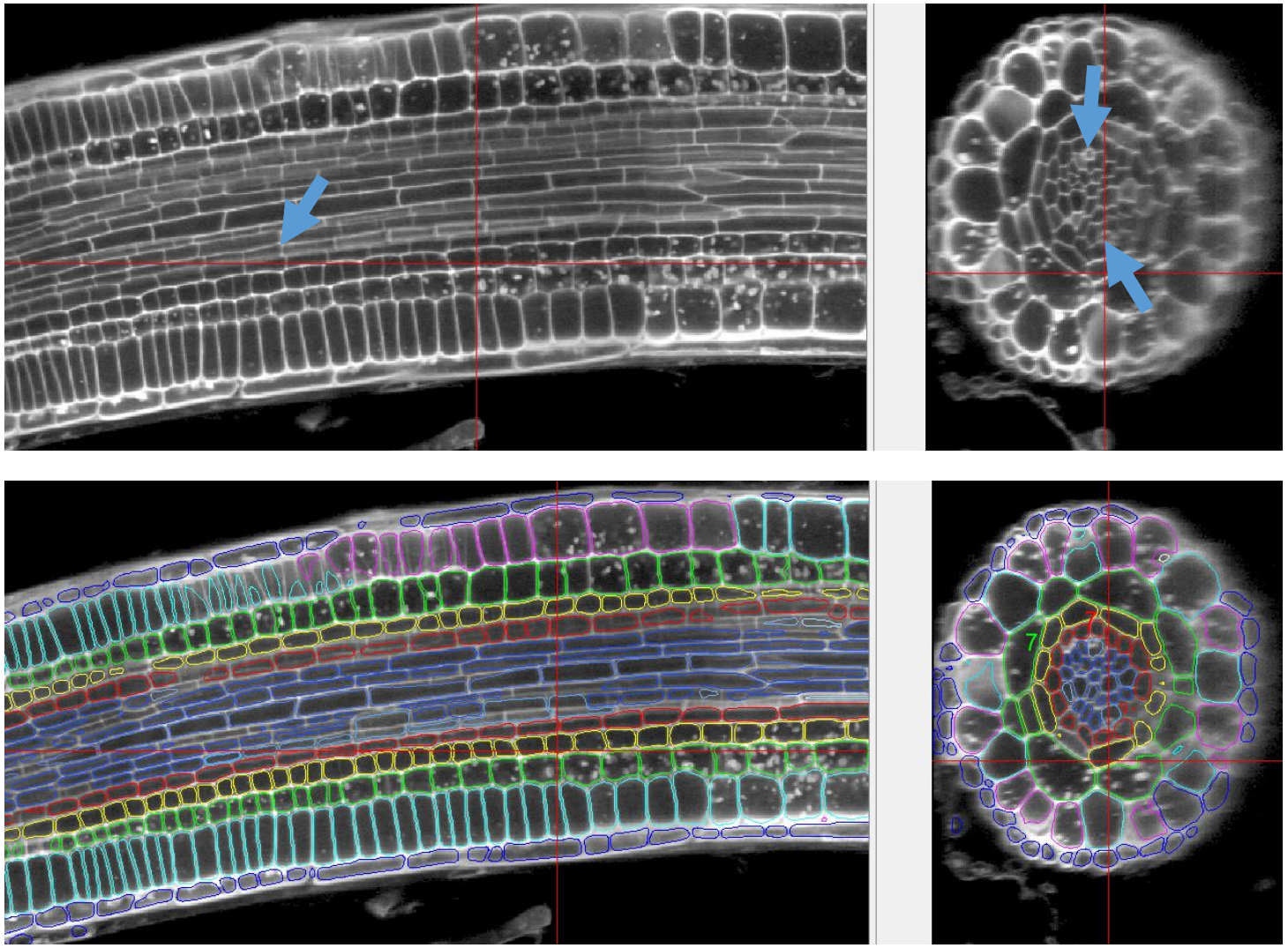
Cell „passportization“: volume evaluation.





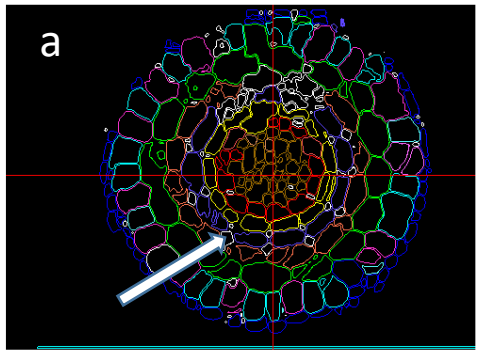
- Distribution of the cell volume along root axis (leaf panel) and quantitative analysis of periclinal cell division in the endodermis (right panel).
- Roots were staining for cell border, segmented and cell volume/positions were extracted.

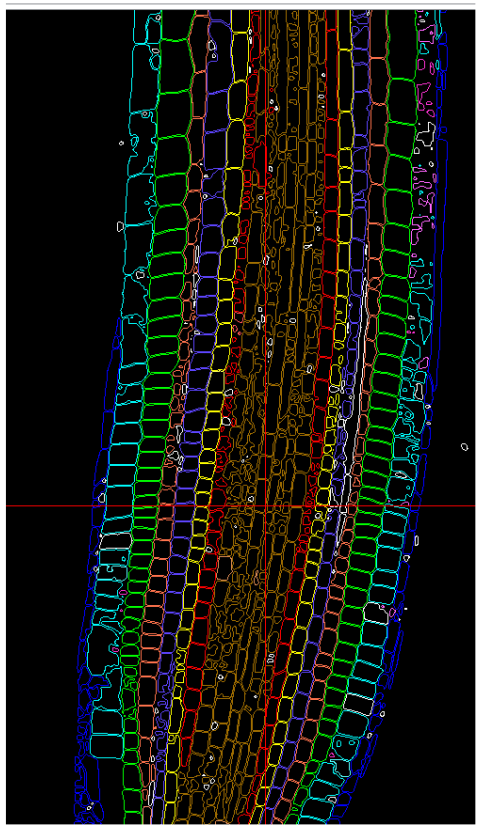
Quantificatitaive analysis of xylem/phloem structure

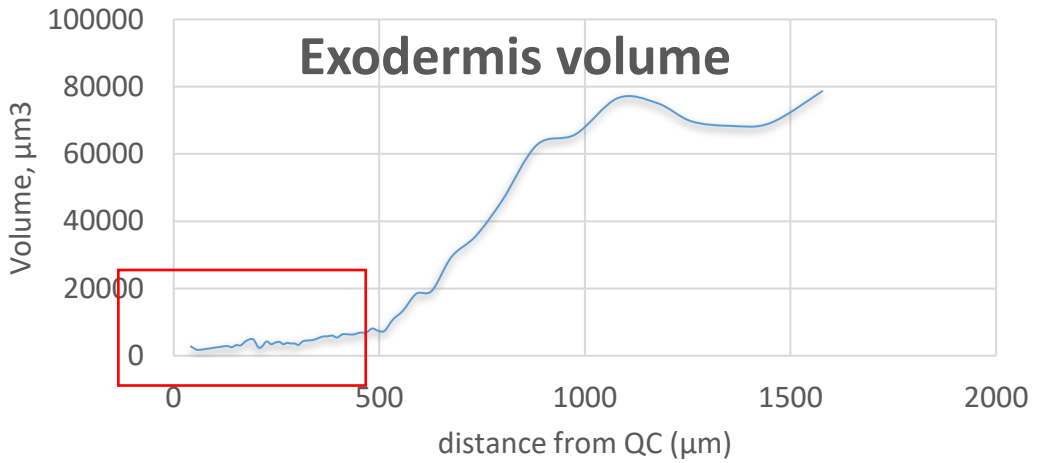


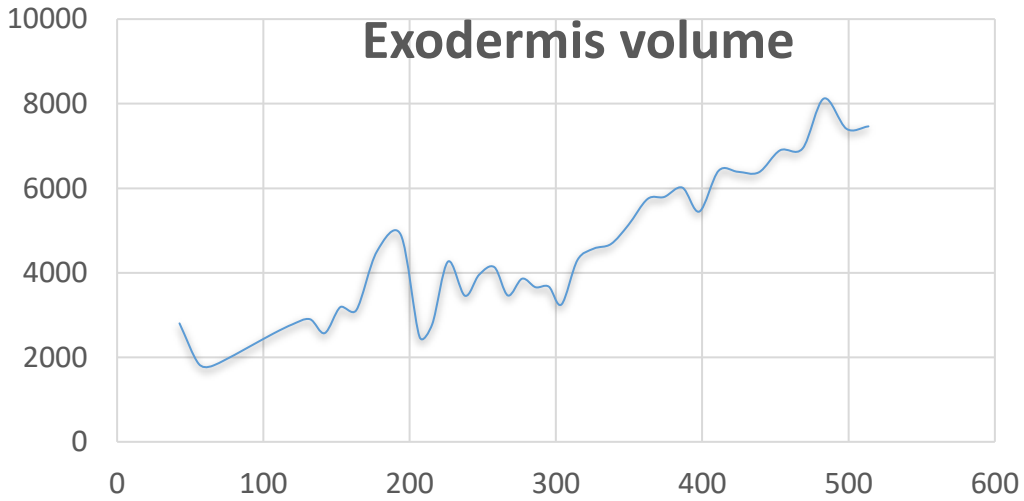
- Xylem/phloem is the most importnat part of the root what responsible for long-distance transport/communication between organs. That‘s why quantification of it appearance and structure is one of the most important task.
- The platform allow to all features of xylem and phloem cells in the root by extraction of it form segmented cells and further cell type recognition.
- Points of secondary cell wall formation (differentiation) shown by arrow.

Tobacco root analysis.





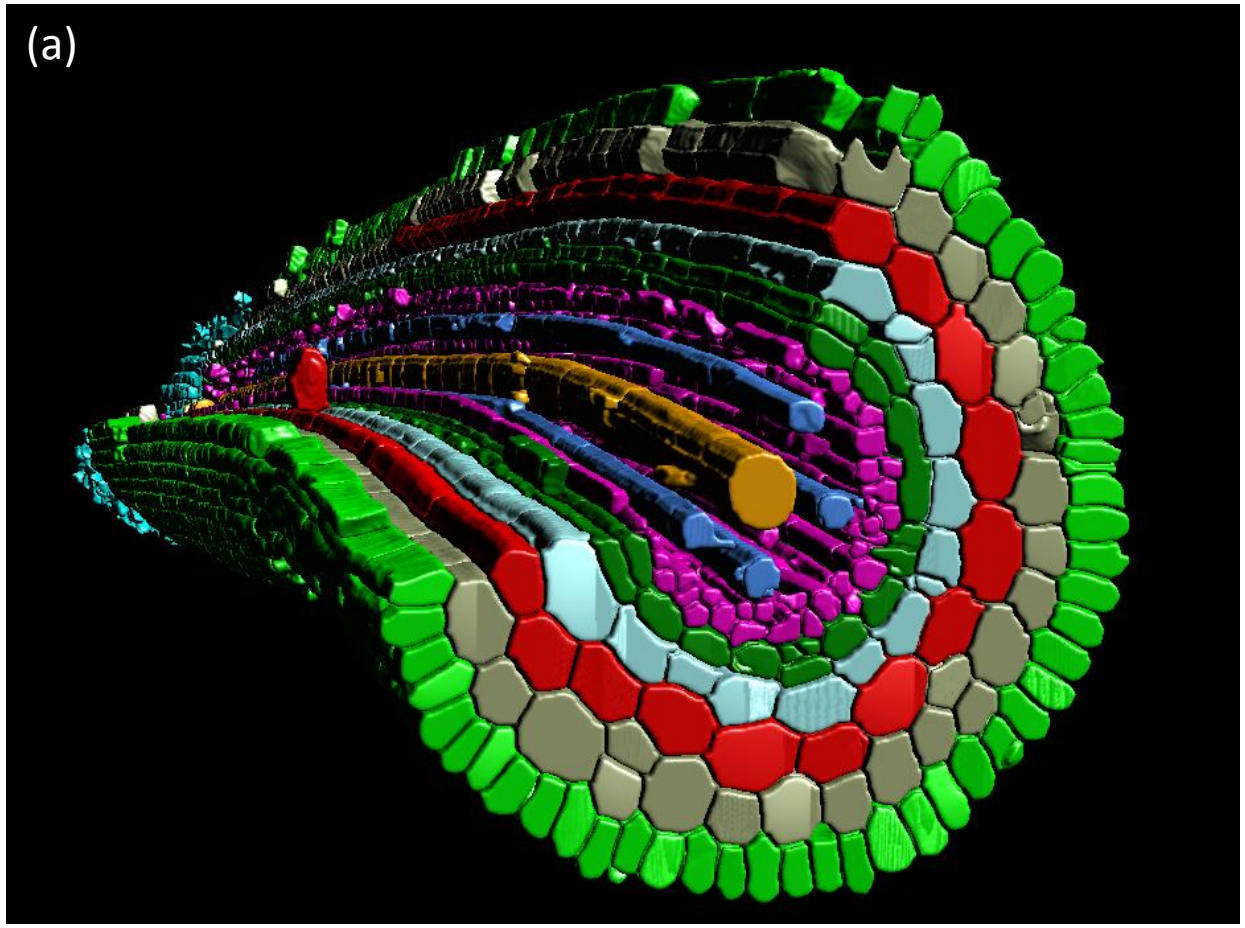


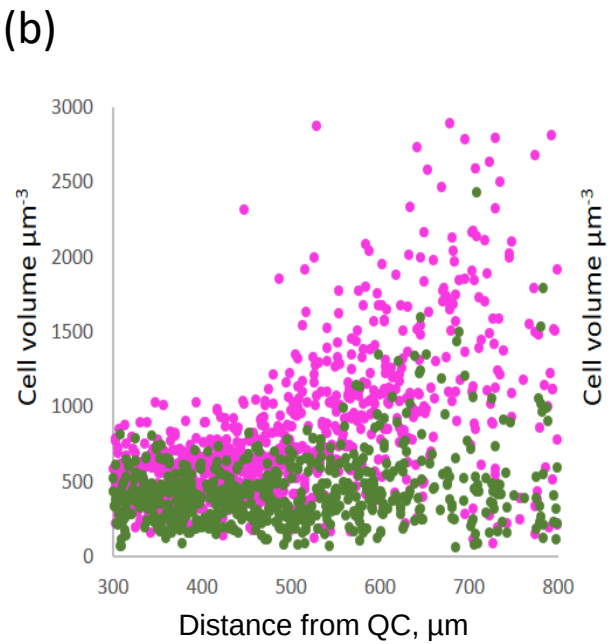


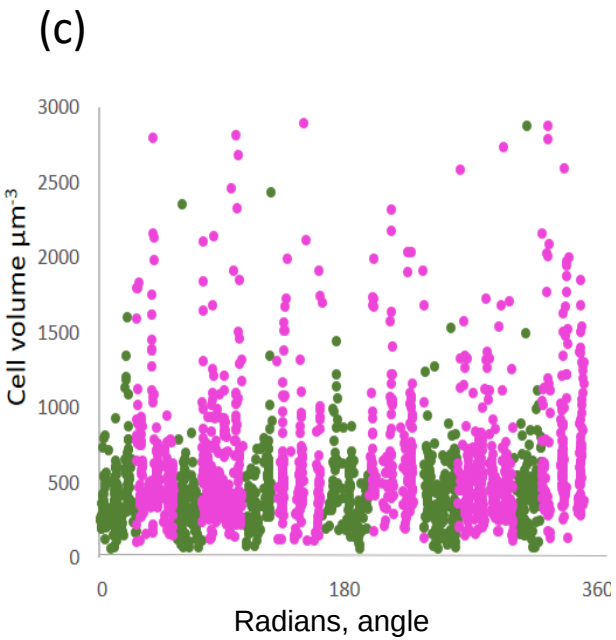
As another model organ with root diameter up to 250 µm we have used tobacco root. (a) . Segmented and layers assigned tobacco root. „Holes“ between cortex2 and cortex3 and between cortex 3 and endodermis shown by white arrow.

(b)- Evolution of cell volume of exodermis along the axis.Cell continusly increasing volume even in mersiteimc zone,

Passport of Setaria Italica root.

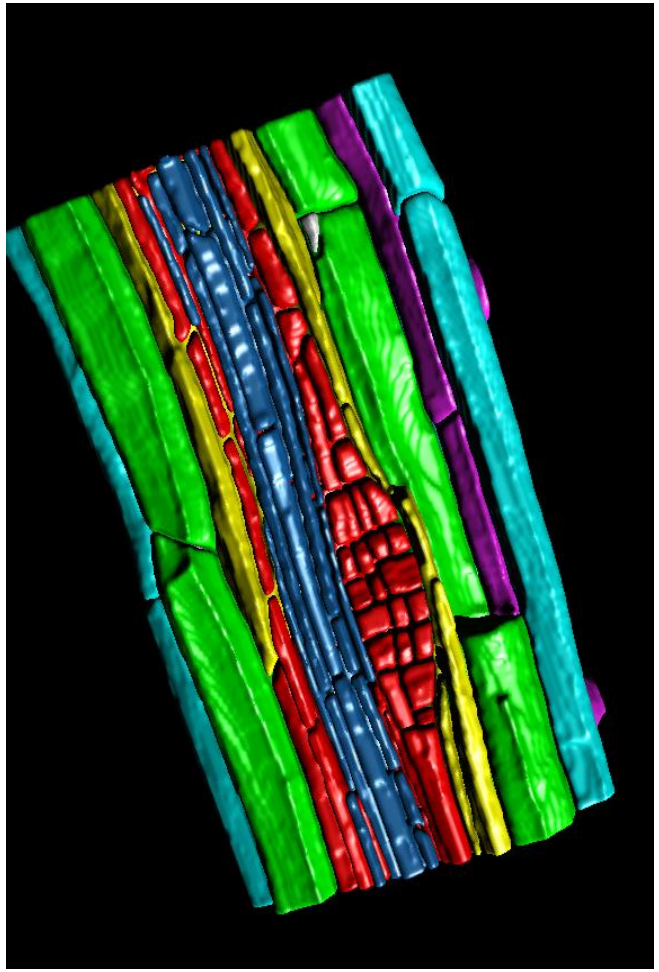


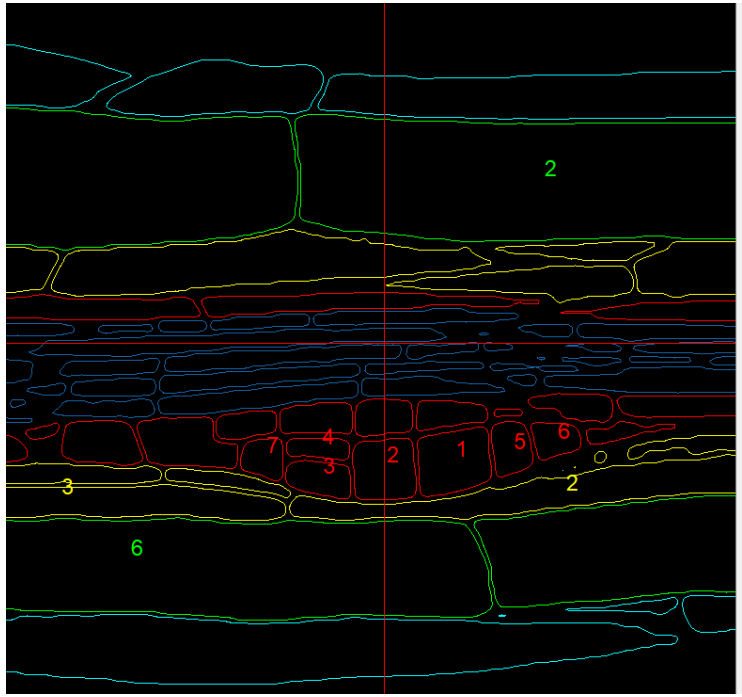


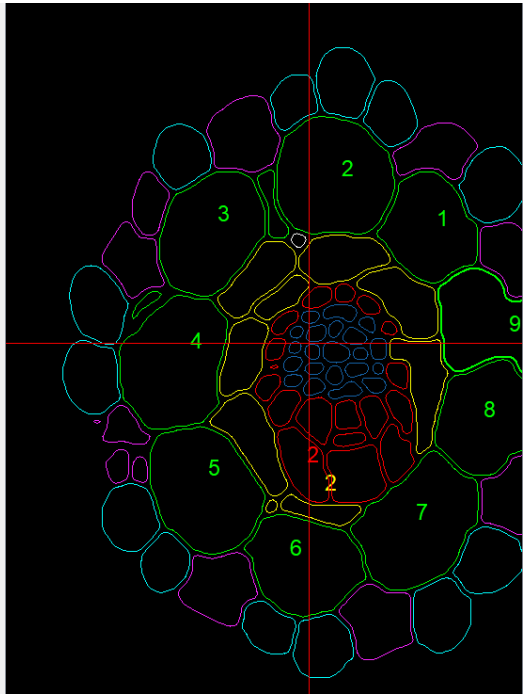


- Passport of the Millet root including labeling of all cell files and extract cell feautres. Assymetry in organs structure have been quantified (periclinal cell division in exodermis and endodermis ; assymetry of the pericycle in xylem/phloem pole, xylem and phloem structure).
- (a) – *Setaria italica* root render. (b) – pericycle cell profile. Two populations of pericycle cell (pink and dark green) were discernable and reflected directly the subtending vascular structure (right). Cells volume were pooled between 300 and 800 µm from the QC.

QuantiQuantitative analysis of lateral root structure.







Cell	volume (cube microns)	radial distance (r)
1	1398,92	19,2127
2	1325,51	20,4498
3	665,378	22,7166
4	387,482	16,6668

- Platform allows to study structure of lateral organs as well.
- Lateral root was segmented, layers labelling, and quantitative data were extracted. Cell volume of 4 cells were shown as an example. Pericycle/lateral root are in red.

Cell cycle events and root zonation passport.

Quantitative passportization:

- Cell cycle events
- Length of each domains for each cell file
- Cell cycle duration.

Five have been characterized:

Stem cell niche

Proliferation domain - cell cycle genes

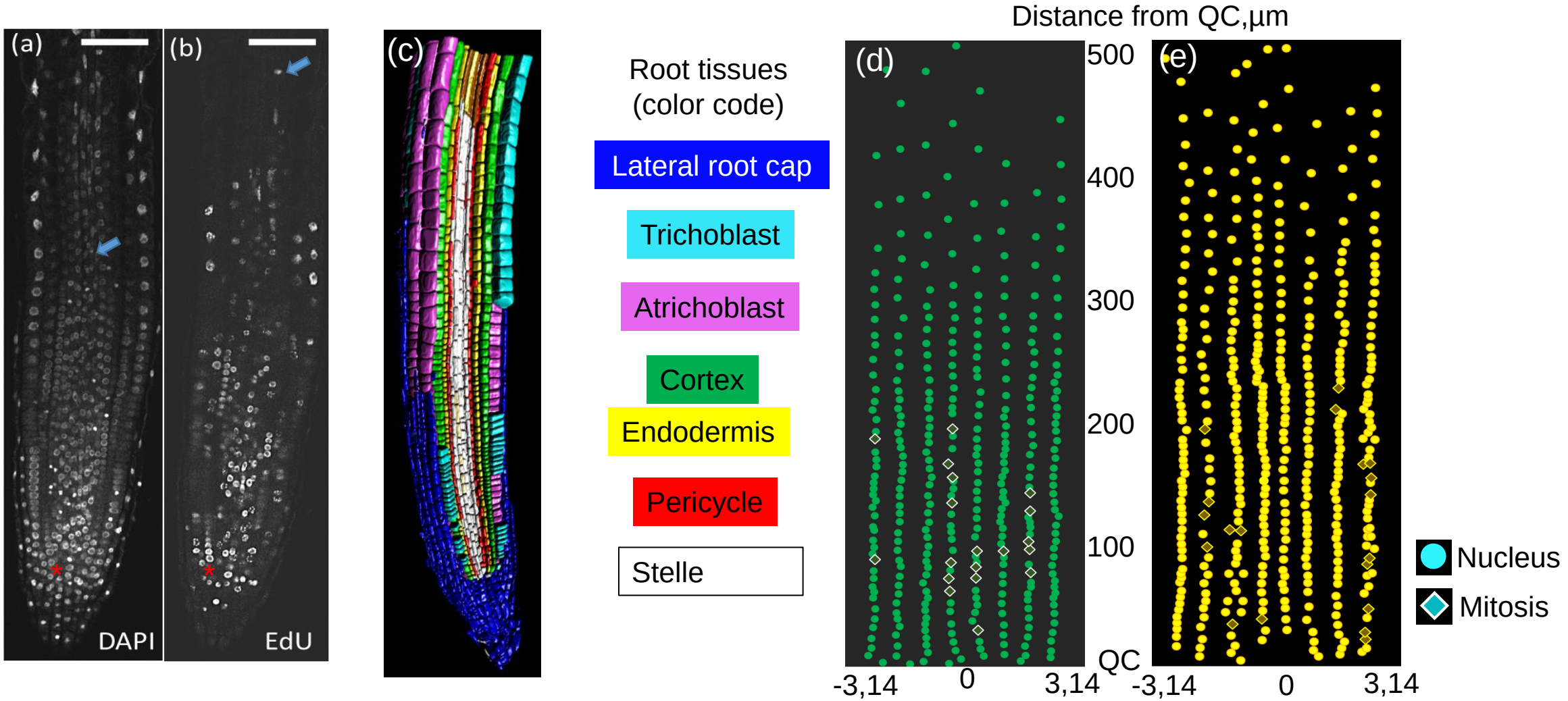
Transition domain – cell cycle/chromatin remodelling

Elongation domain – chromatin remodelling

Mature domain

Platform allow to functional anaylsis all these domains with quantitative characterization of each cells.

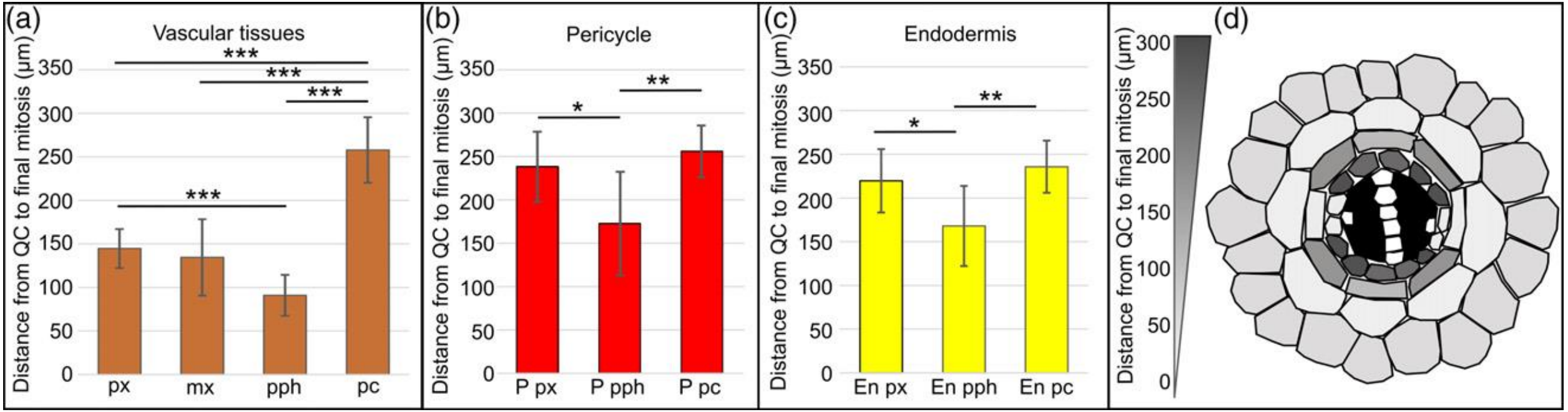
Cell cycle events visualization.



Annotation of mitosis and DNA replication in *A. thaliana.*

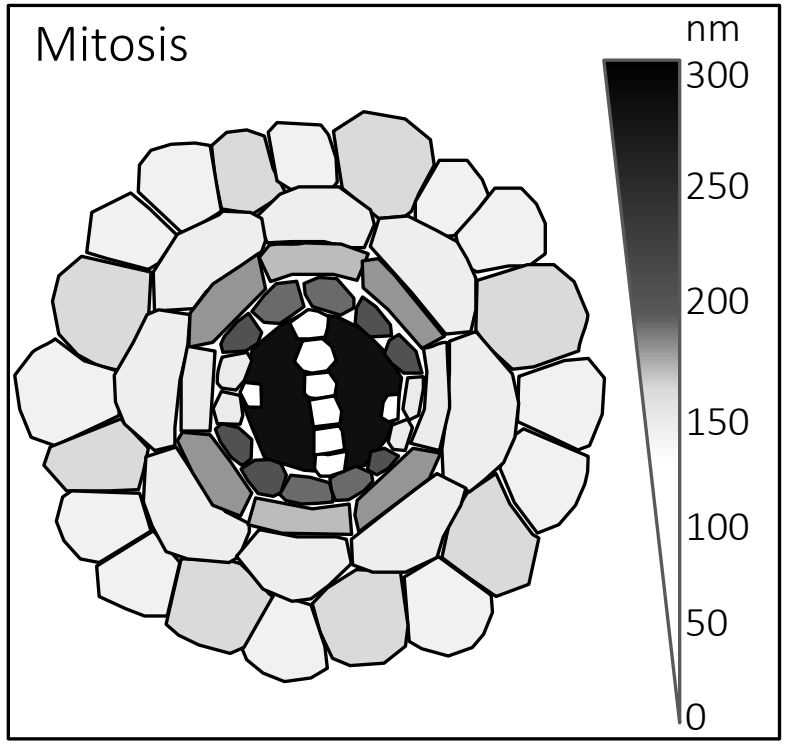
(a) – The root tip staining with DAPI for annotation of the nuclei positions and mitosis distributions. **(b)** – EdU-incorporated nuclei in the same root tip were annotated to study DNA replication events. Bars 50 µm. (c) -root with color code; **(d), (e)** – mitosis map of a virtually “unrolled” cortex (d) and endodermis (e) layers of *A. thaliana* root tip.

Quantification of the length of the proliferation domain in Arabidopsis RAM.



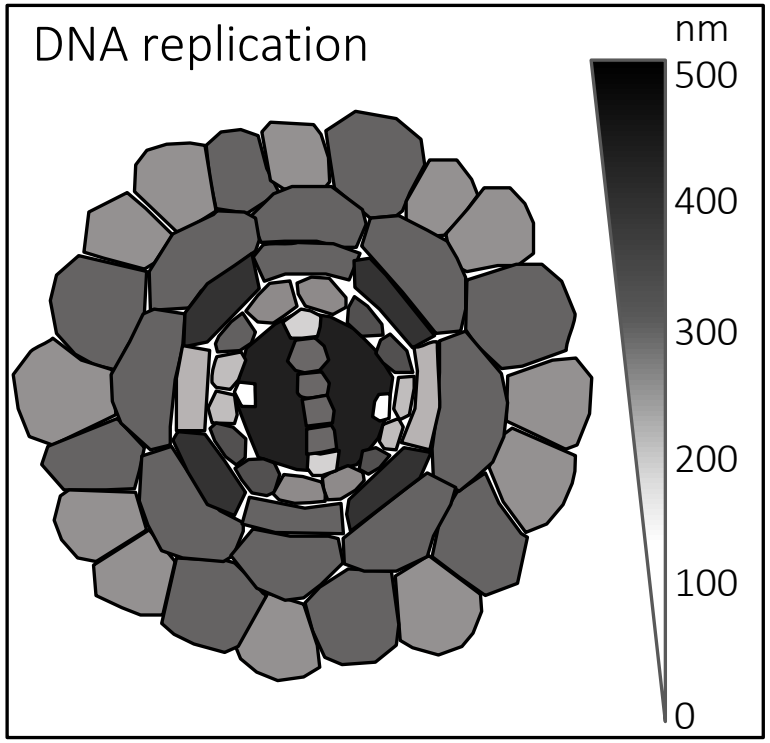
- Length of proliferation domains have been estimated as position of the last mitosis in each cell file/cell continuity. It was detected that zone length in endodermis, percicyle and vasculature have a diarch symmetry. From Lavrekha et al., 2017.

Determination of the proliferation and transition domains length in Arabidopsis RAM.



Lavrekha et al., 2017, Plant Journal

Proliferation zone length



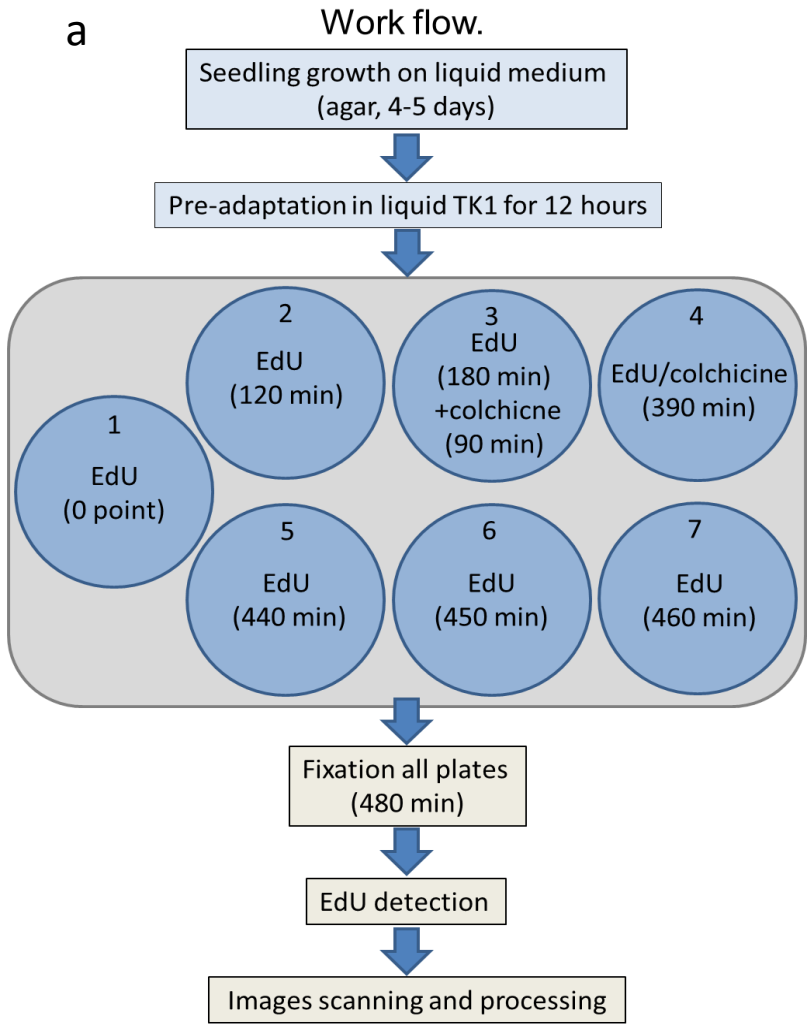
Not published

Proliferation +transition zone lenth

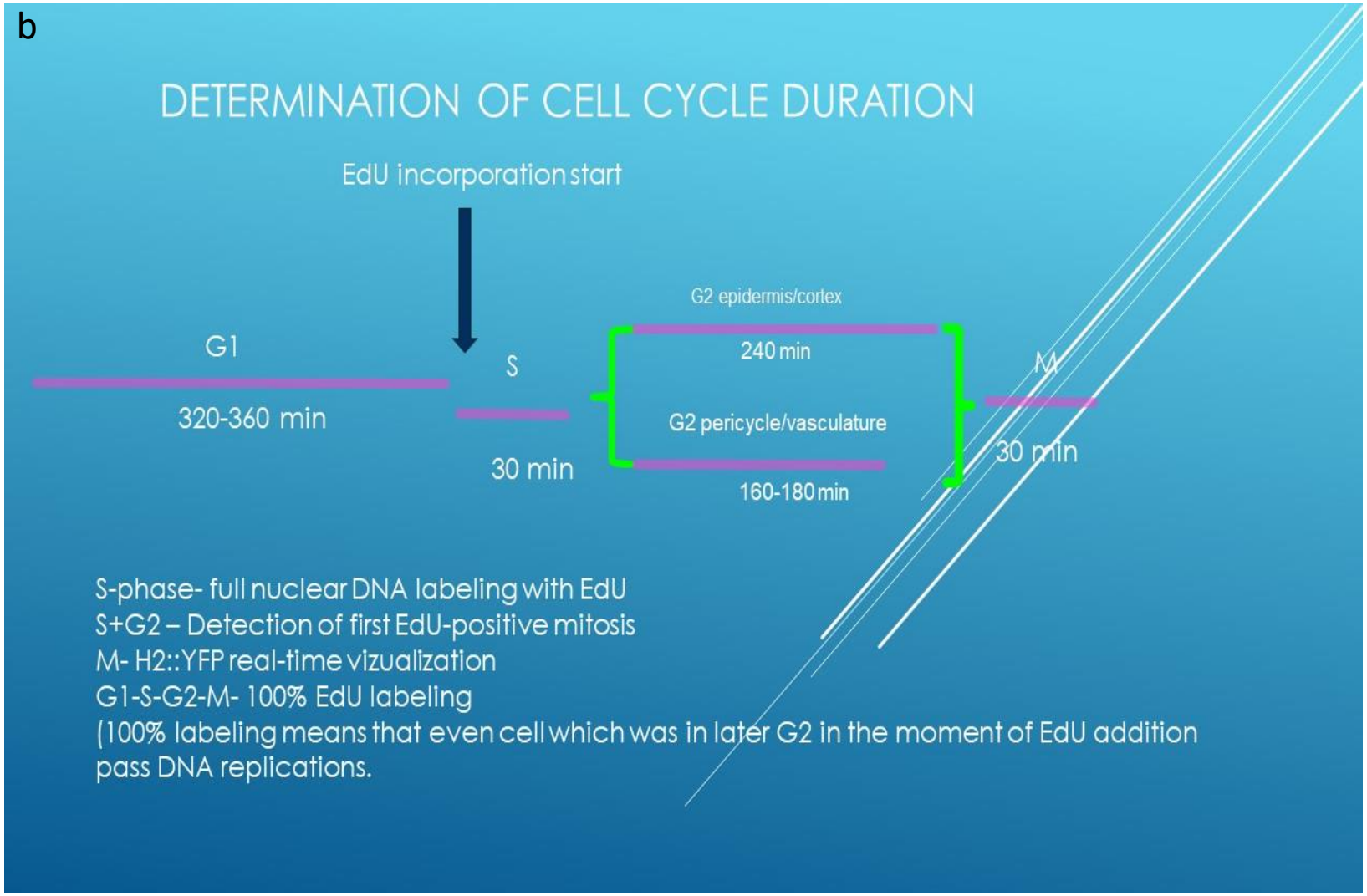
Transition domain were defined as domain in with slightly increase in cell volume/length and active DNA replication (enodocycle).

Determination of the cell cycle duration.

a

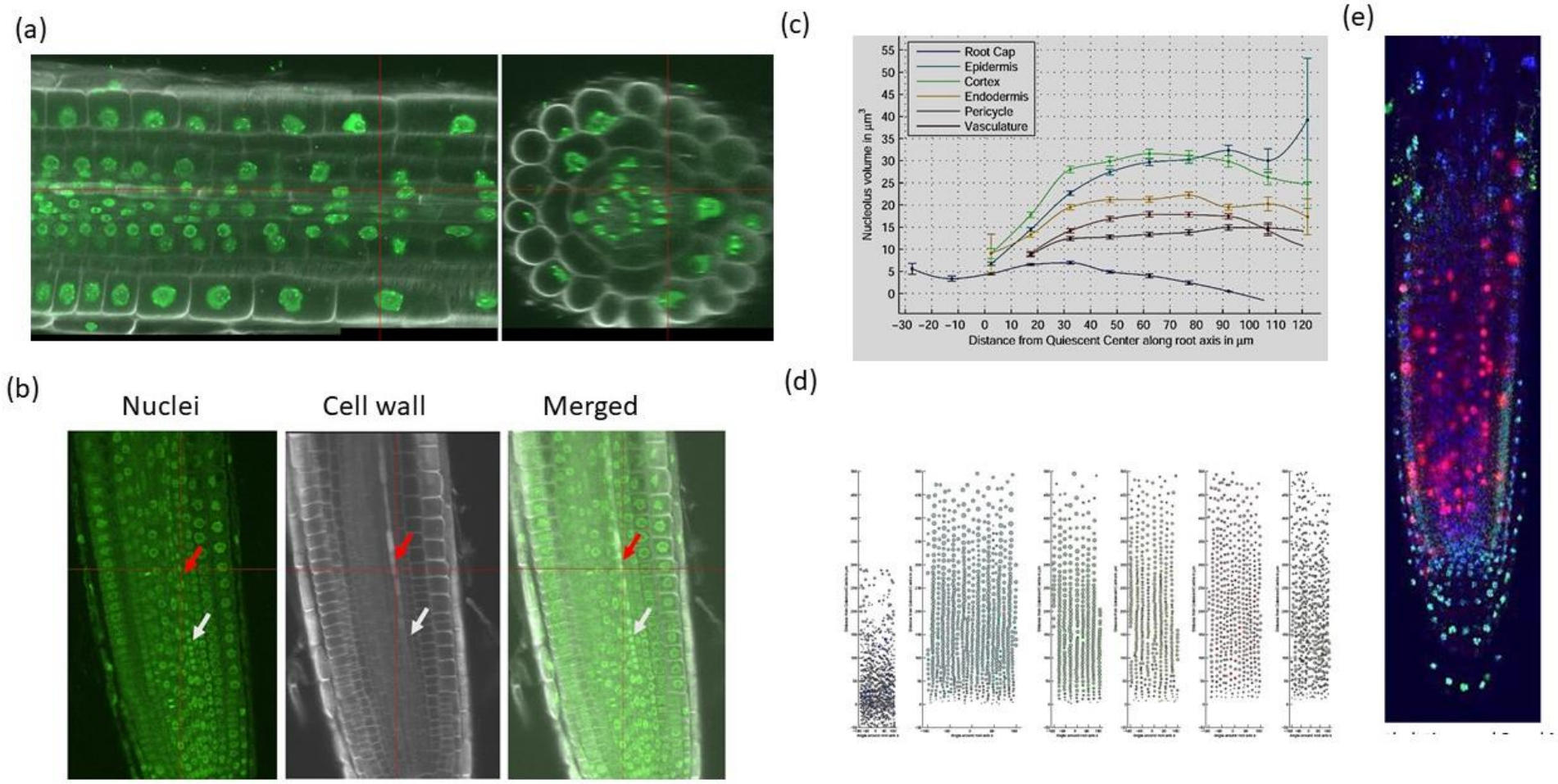


b



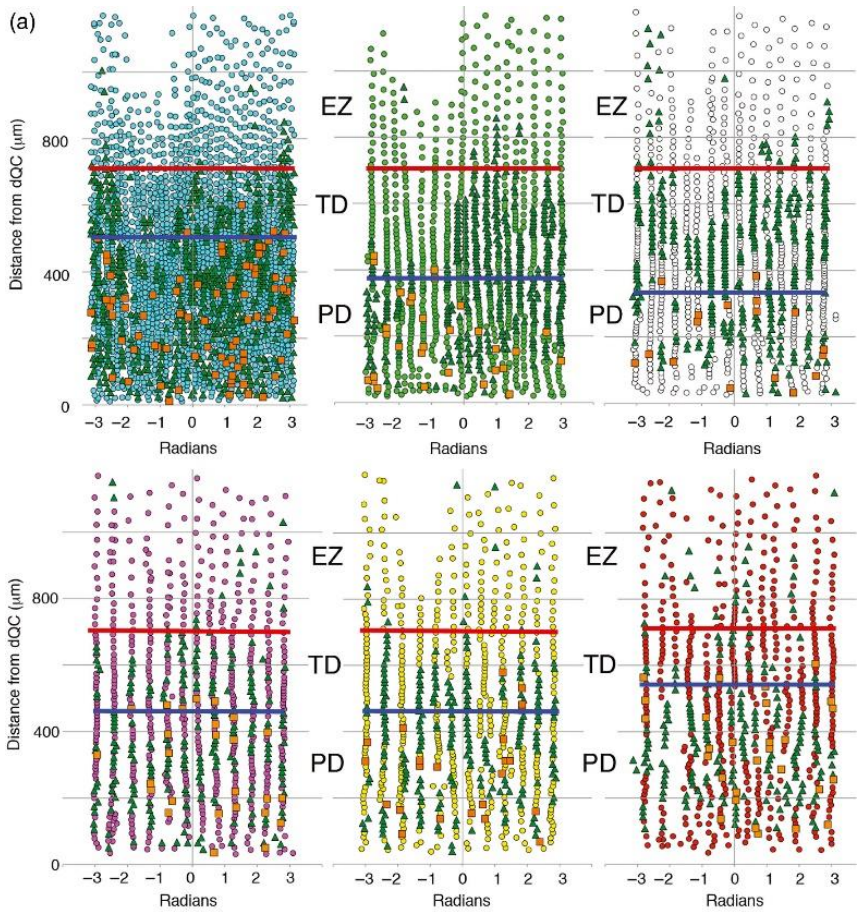
- Different EdU incubation time has been used as tool for estimation of duration of each stages of cell cycle. It was found that cell cycle duration (in particulary G2 duration) is different in the inner and outer cell layers. (a) – work-flow diagram (b) – summary of the finding

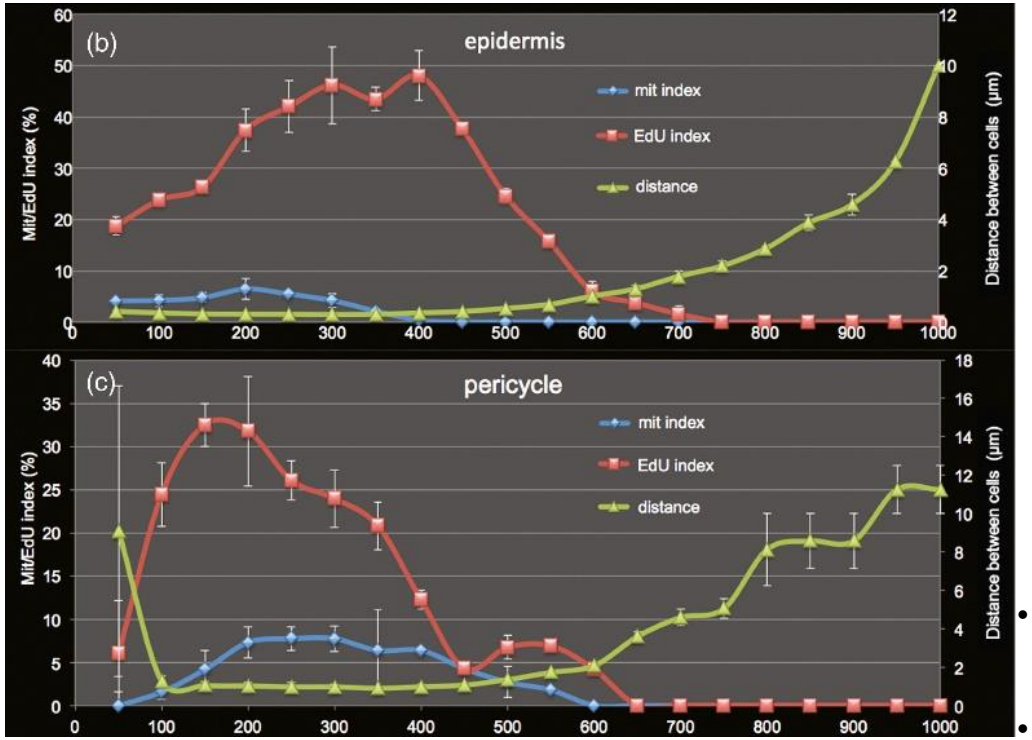
Analysis of chromatin structure in Arabidopsis root.



- (a)- cross-section of Arabidopsis root. Nuclei are in green; cell border are in white. Gradient in chromatin landscape from division to elongation zone were shown. T- trichoblast; C- cortex. Red arrow marked condense chromatin in phloem.
- (b) – chromatin condensation as a marker of phloem differentiation. Roots have been stained for nuclei (proridium iodide) and for cell wall (Calcofluor white) Cell differentiation in phloem were detected as decreasing in nucleoli size and chromatin condensation (white arrow). (c) – average nucleoli volume in different cell files at different distance from QC. (d)- distribution of nucleoli volume in the virtually un-rolled root. Diameter of the circle is proportional to the nucleoli volumes.
- (e) H3K9me2 localization (green) and EdU labelling (red) in Arabidopsis root. H3K9me2 is a marker of heterochromatin abundance.

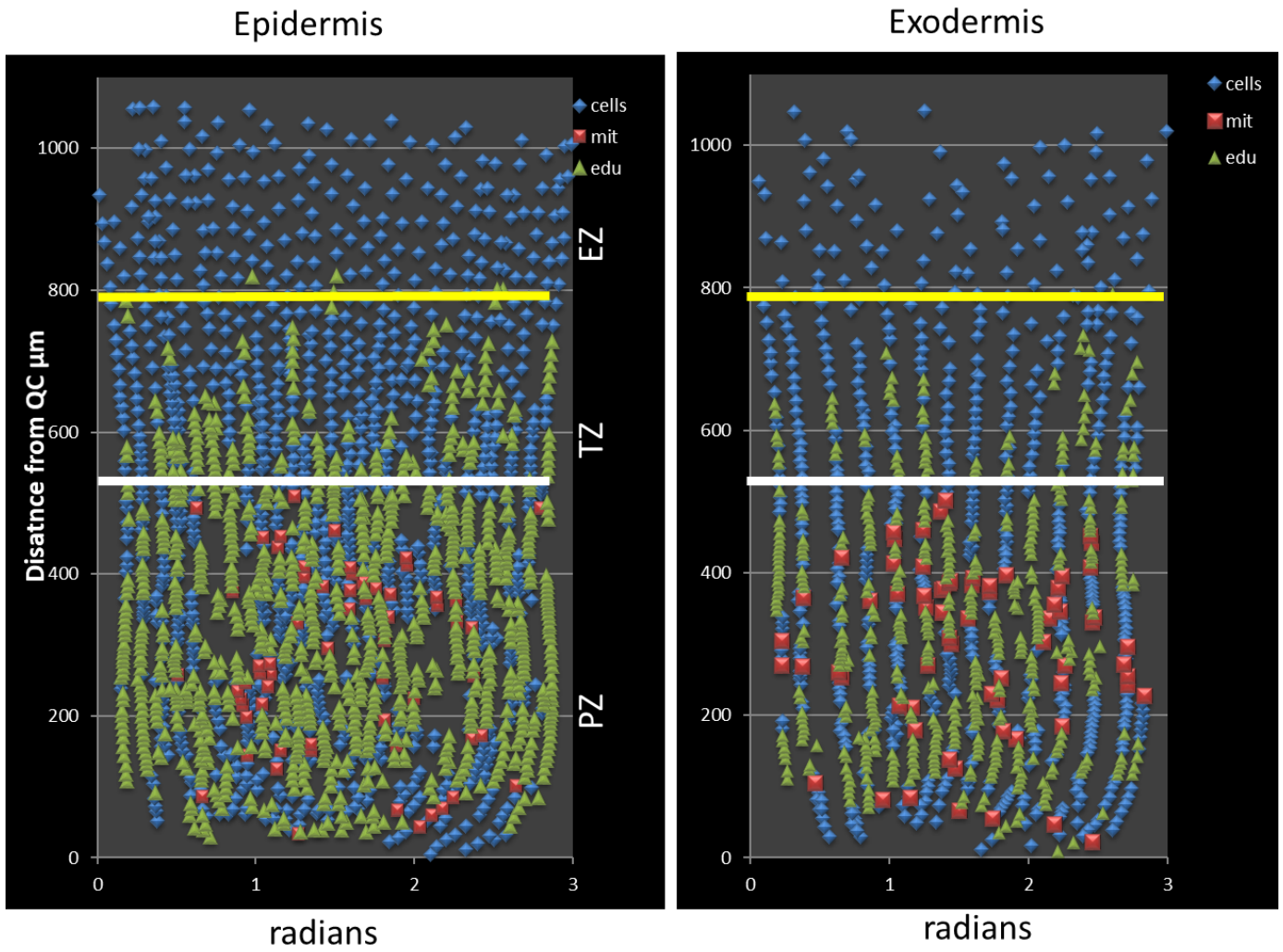
Cell cycle passport of tobacco root





- Longitudinal zonation pattern of tobacco roots.
- (a) Unrolled cell cylinders for epidermis, cortex1, cortex2, cortex3, endodermis and pericycle cells. Colors for cell types (circles) as in Figure 1. EdU-labeled cells are shown as triangles (dark green), mitotic cells as orange squares. In all cell types the EdU-labeled cells extend well beyond the region with mitotic cells. Rapid elongation starts after the region that contains more than 90% of the EdU-labeled cells. Based on the distribution of 90% of the mitotic and EdU-labeled cells, the root can be divided in a proliferation domain (PD), with DNA replication and mitosis), a transition domain (TD, mainly DNA replication) and an elongation zone (EZ). PD/TD borders: blue horizontal lines, TD/EZ borders: red horizontal lines. *X*-axis: radians (from −3.14 to 3.14), *Y*-axis: distance from the dQC in µm.
- (b) Averages of the mitotic and EdU-positive nuclei distributions in epidermis.
- (c) Averages of the mitotic and EdU-positive nuclei distributions in pericycle. In each root the number of mitotic and EdU-positive nuclei were calculated per each 50-µm section and normalized to the total cell number in the corresponding section.

Determination of root domain length in tomato.

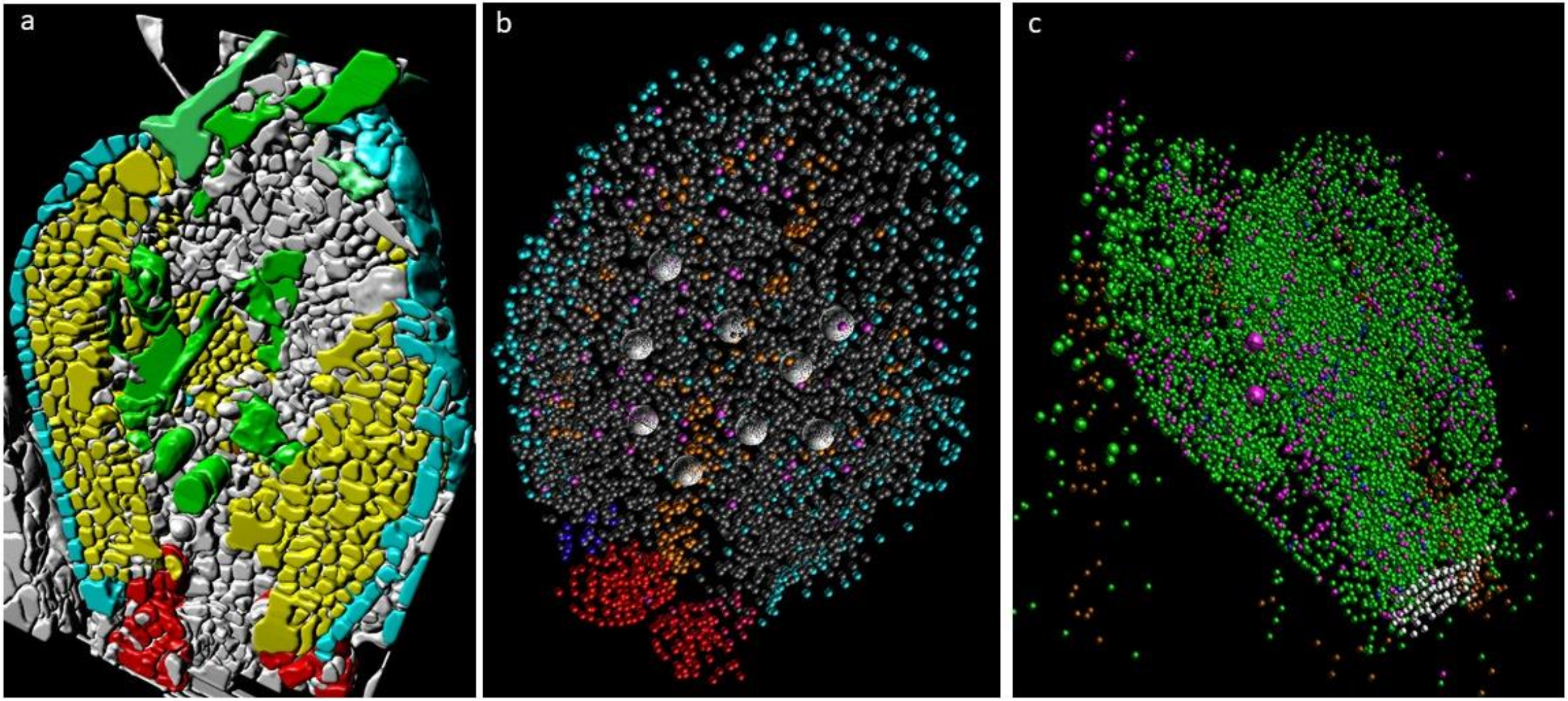


- Tomato seedlings have been incubated in the presence of EdU for 90 minutes, fixing, EdU has been detected and analysed. Cells are in blue; EdU positive cells are in green and mitotic cells are in red. Proliferation zone (PZ) is from QC till white line; transition zone (TZ) is between white and yellow lines.

Quantifications of leaf development.

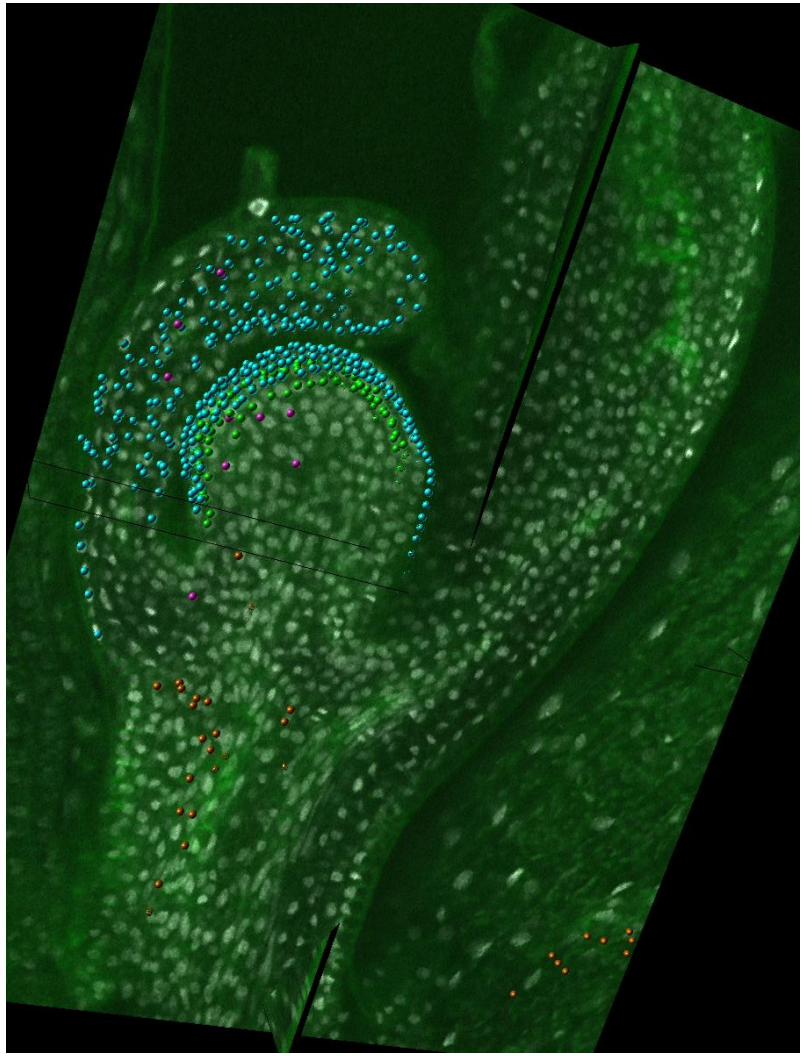
- Quantifications of leaf development including 2 possible options:
- 1. Cell geometry after staining of cell border.
- 2. Epigenetical cell status/gene expression and cell cycle analysis .
- Below we present some examples of quantitative leaf structure/cell cycle analysis after staining with calcoflour white (cell border) plus propidium iodide (nuclei).
- Quantitative analysis of cell cycle were done by detection of DNA replication and proliferation events.

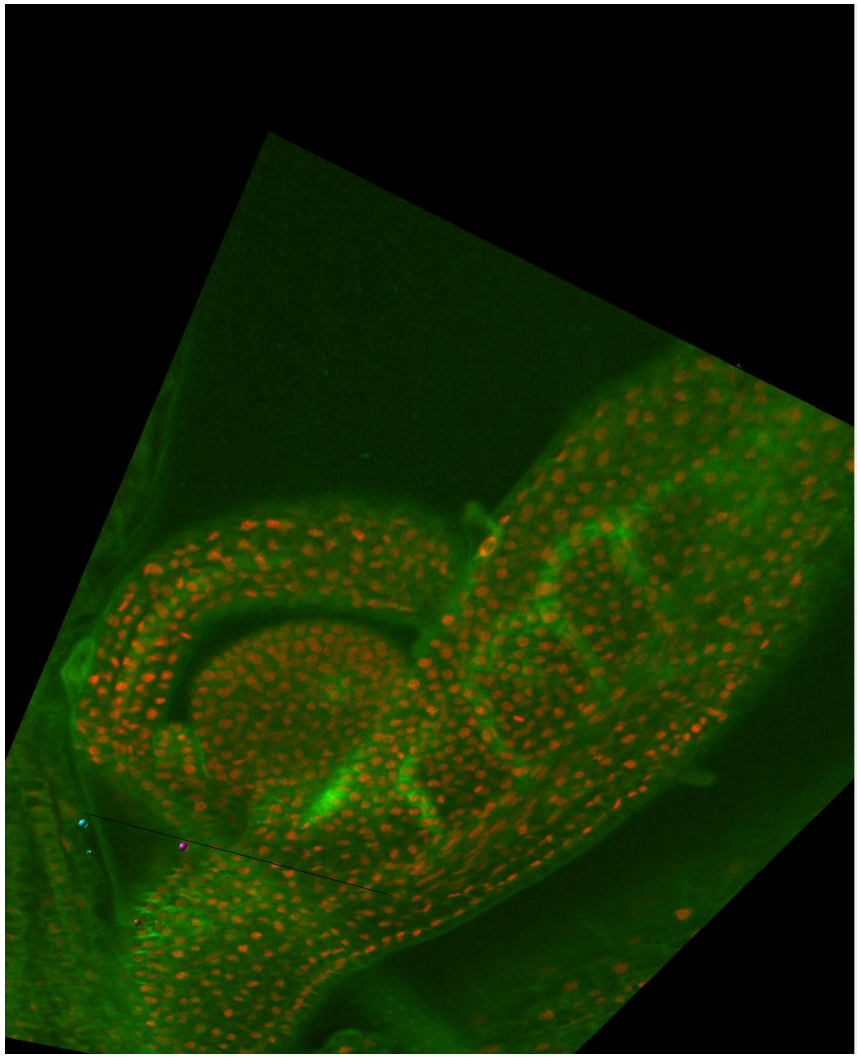
Vizualization of leaf development.



Passport of Arabidopsi leaf for cell geometry and cell cycle were created,
A- geometry; b- cell- chromatin; c- cell cycle.

Visualization of gene expression and cell cycle status in flowers.

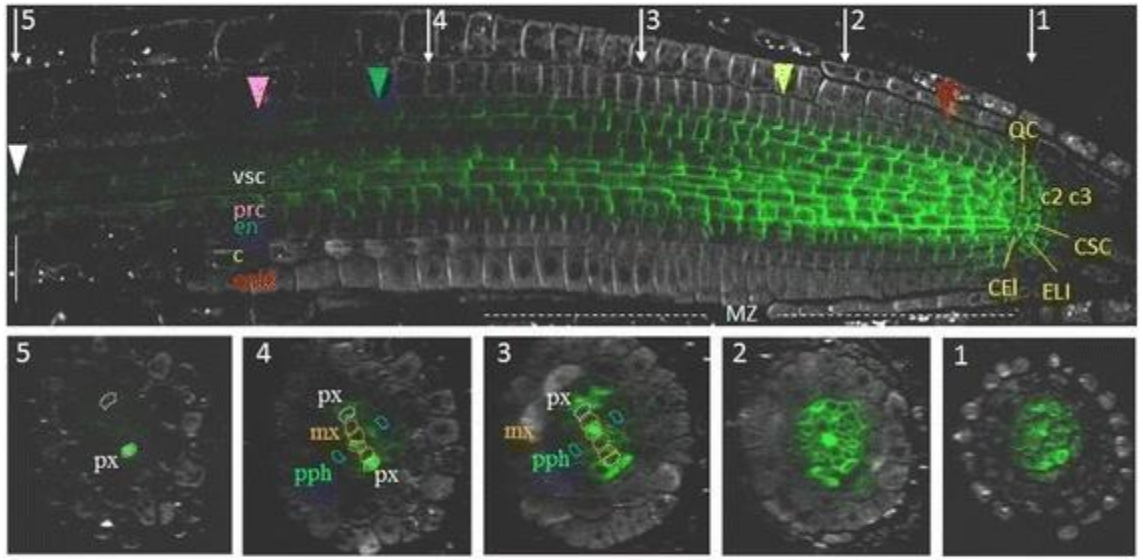




- Arabidposis stem/flowers were labelled with PIN1 antibody.
- Images analysis are in progress.

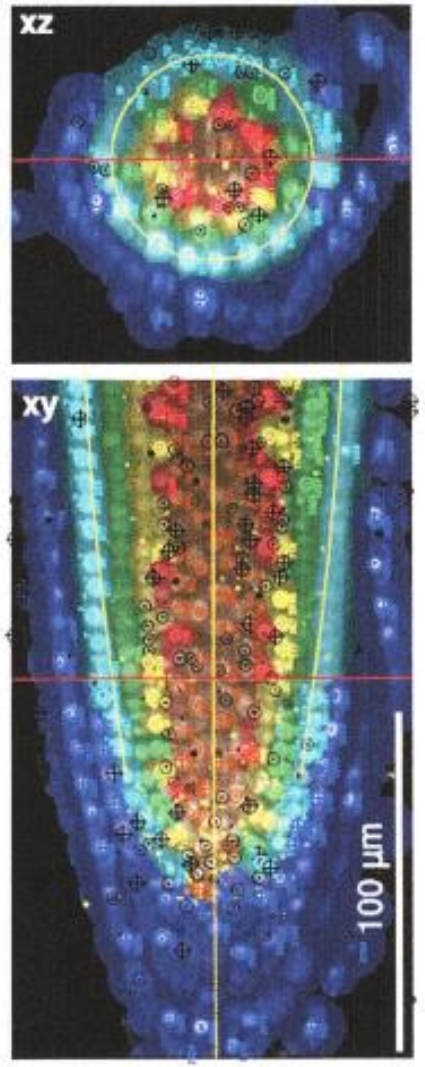
Analysis of gene expression and cell polarity in the root.

From: A detailed expression map of the PIN1 auxin transporter in *Arabidopsis thaliana* root



Whole-mount immunolocalisation of PIN1 in *A. thaliana* root tip. A longitudinal section (above) and five transverse sections (1–5) showing anti-PIN1 signal (green channel) and anti-PIN2 staining (white channel). CEI—cortex/endodermis initials, ELI—epidermis/lateral root cap initials, CSC—columella stem cell (columella initials), QC—the quiescent centre, c2—the second columella tier, c3—the third columella tier, epid—epidermis, c—cortex, en—endodermis, prc—pericycle, vsc—vasculature, px—protoxylem, pph—protophloem, mx—metaxylem. Coloured triangles—the end of the expression domain in the respective layer. White triangle —rootward PIN1 location in xylem elements in the elongation zone. MZ—the meristematic zone. Bars = 50 μm

Localization and Quaintification of protein-protein interaction in situ.



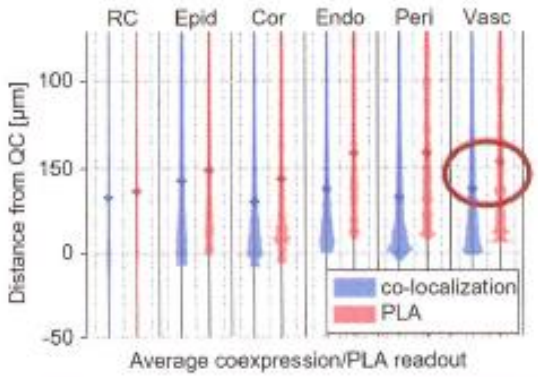


Fig. 1 *PLA-iRoCS in the Arabidopsis root apical meristem.* Left panel: Automatic annotation of nuclei superimposed on PLA signals (above, radial section; below, longitudinal section). Nuclei of cell layers are marked with circles and color coded as following: root cap, dark blue; epidermis, light blue; cortex, green; endodermis, yellow; pericycle, red; stele, brown. Right panel (above): Kite diagram showing normalized colocalization (blue) and PLA signal distribution (red) of two interacting target proteins as a function of distance from the quiescent center in two genotypes A and B. Each plot is the average of three experiments and the median signal is marked with a point. The root has been divided into its constituent cell layer for better visualization. Subtle differences between genotypes, in this case in the distribution of PLA signal in vascular cells, have been circled. Right panel (below): An overview of the steps which comprise the PLA-iRoCS pipeline

Conclusions/Platform applications.

- Platform allow researchers to investigate with cellular resolution: cell geometry (length, width, volume); cell cycle kinetics (map of cell cycle events and cell cycle duration); chromatin status (nuclei structure, histone modifications); gene expression (protein level and protein-protein interaction).
- Platform is marker-free.
- Platform applicable to almost all plant's organs (root, lateral root, stem, leaf, flowers).
- Platform can be applied to any plant species and organs.

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