Estimation of differential cell cycle kinetics in higher plant root meristem with cellular fate and positional resolution.

Taras Pasternak [1 *], Stefan Kircher[1] and Klaus Palme[1]

[1]Institute of Biology II/Molecular Plant Physiology, Centre for BioSystems Analysis, BIOSS Centre for Biological Signalling Studies University of Freiburg, 79104 Freiburg, Germany; * author for correspondence: taras.p.pasternak@gmail.com

Abstract

Plant root development is a complex spatial-temporal process that originates in the root apical meristem (RAM). To shape the organ's structure signaling within the different cells and the different cell files must be coordinated. Thereby, diverging kinetics of cell growth in these files needs to be integrated with differential cell growth and local differences in cell proliferation frequency. Understanding the local differences in cell cycle duration in the RAM is crucial to build a holistic view on the different regulatory processes and requires a quantitative estimation of the underlying mitotic cell cycle phases' timing at every cell file and every position. Unfortunately, so far precise methods for such analysis are missing.

This study presents a robust and straightforward pipeline to determine simultaneously the duration of the cell cycle's key stages in all cell layers of a plant's root. The technique combines marker-free experimental techniques based on detection of incorporation of 5-ethynyl-2′-deoxyuridine (EdU) and mitosis with a deep-resolution plant phenotyping platform to analyze all key cell cycle events' kinetics.

In the *Arabidopsis thaliana* L. RAM S-phase duration was found to be as short as 18-20 minutes in all cell files. The subsequent G2-phase duration however depends on the cell type/position and varies from 3.5 hours in the pericycle to more than 4.5 hours in the epidermis. Overall, S+G2+M duration in Arabidopsis is 4 hours in the pericycle and up to 5.5 hours in the epidermis. Endocycle duration was determined as the time required to achieve 100% EdU index in the transition zone and estimated to be in the range of 3-4 hours.

Besides Arabidopsis, we show that the presented technique is applicable also to root tips of other dicot and monocot plants (tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum* L.) and wheat (*Triticum aestivum* L.)).

Introduction

The duration of the mitotic cell cycle and the endocycle, including its phases, are essential characteristics for harmonized organ growth kinetics. Investigation of cell cycle duration in

plants was first done in 1951 (Brown, 1951) in pea (*Pisum sativum* L.) roots by quantification of the ratio of cells in a certain stage of the cell cycle to the total number of meristematic cells. The interphase duration was found to be 23 hours, prophase 2 hours, metaphase 25 minutes, anaphase 5 minutes, and telophase 22 minutes. Thereafter cell cycle duration was investigated by various different methods. Clowes (1961) as well as Van't Hof and Sparrow (1963) proposed a method based on H^3-thymidine incorporation into the replicating DNA. Van't Hof (1967) further modified this method by additional colchicine treatment in order to accumulate cells in mitosis. Using this approach, the entire cell cycle duration was found to be 24 hours in pea (*Pisum sativum* L.), sunflowers (*Helianthus annuus* L.), and bean (*Vicia faba* L.). Recent studies used 5-Ethynyl-2'-deoxyuridine (EdU) as non-radioactive, fluorescent alternative to H^3-thymidine incorporation (Buck, S. B. et al., 2008) enabling Hayashi et al. (2013) to determine a time window of 17 h for Arabidopsis cell cycle duration during root meristem proliferation. Some researchers performed estimation the duration of the different cell cycle phases G1, S, G2, M. Using the Van't Hof method, the S-phase duration in pea (*Pisum sativum* L.), sunflowers (*Helianthus annuus* L.) and bean (*Vicia faba* L.) was found to be 4.5 hours (i.e. 30% of total cell cycle length) (Van't Hof, 1969). More recently, a combination of EdU pulse labelling with flow cytometry (Mickelson-Young, L. et al., 2016) estimated S-phase duration in Arabidopsis to be 2-3 hours.

Besides the use of labeled nucleotide analogs, several other "kinematic" methods were developed to investigate cell cycle duration in the Arabidopsis root (Beemster and Baskin, 1998; Fiorani and Beemster, 2006). Newly developed stripflow software combined with kinematic measures gave a cell cycle duration in one of the root tissues (cortex) of 14,7±0,9 hours (Yang et al., 2017). A similar Rate of Cell Production (RCP) method (Ivanov and Dubrovsky, 1997) determined a cell cycle duration of 11-13 h for cortex cells of different lines of the *Pisum sativum*. Another recent approach to investigate cell cycle duration was the use of marker lines with a dual-color marker system (Yin K., et al., 2014). With this method cell cycle duration was found to be about 16 hours in root epidermis cells, including 3 hours S-phase. To our knowledge, these lines were developed for Arabidopsis preventing the use of these markers in other plants without time and costly procedures. To simplify procedures, a combination of markers was introduced in a single construct to simultaneously label all cell cycle stages and detect cell cycle kinetics in living roots (Desvoyes, B et al., 2020). However, similarly as in Yin K. et al. (2014), these lines are suitable for Arabidopsis only, do not distinguish between different cell types, and require costly efforts for mutant line analysis.

The kinematic method's main drawback is that it applies mainly to the outer cell layers (epidermis/cortex) and is based on the assumption that cell cycle duration is constant throughout the proliferation zone. It is well-known since 1961 (Clowes, 1961) that cell cycle duration depends on cell position and cell type. For example, it was reported that in *Convolvulus arvensis* cell cycle duration in the central cylinder and cortex were significantly different (21 h and 27 h, respectively), while in the quiescent center, it reaches even 420 h (Phillips Jr, H. L., & Torrey, J. G., 1972). However, these differences have not been addressed in most of the recently published methods. It was only recently shown that cell cycle duration can be regulated by H3 histone modification and was different in the proximal and distal zone of Arabidopsis root epidermis (Otero et al., 2016).

In summary, many fundamental questions about positional differences in the duration of the cell cycle phase in functionally distinct tissues and cell files in the root meristem remain unclear. Our working hypothesis is that different cell files may exhibit different cell cycle duration and kinetics to compensate for the differences in cell growth kinetics of the different cell lines in the context of a compact organ. To resolve this issue, the precise estimation of cell cycle kinetics in each cell in the frame of an organ coordinate system is required. Here we provide a simple and robust method for determination of S-phase, G2-phase, and M-phase duration in roots of higher plants and demonstrate its value. The proposed method does not require marker lines, allows to determine the duration of S, G2, and M stages in all cell files simultaneously and independently and its therefore generally applicable in plants. A combination of EdU incorporation and mitosis labeling with an accurate root coordinate system (iRoCS) (Schmidt et al., 2014) allowed us to generate precise maps of main cell cycle events in the root of Arabidopsis as well as several other plants.

Results

Divergence of cellular parameters in different tissues and cell files of the Arabidopsis RAM.

The Arabidopsis root apical meristem (RAM) has a radial structure built up by functionally distinct cell layers. In order to perform a high -resolution analysis of the Arabidopsis root we first used the iRoCS analysis pipeline for a detailed analysis of the geometry of all cells within this organ and the structures of the nuclei in these cells. We demonstrate a strong divergence between and within different cell types regarding cell volume dynamics and chromatin landscape (Figure 1). Interestingly, cortex cells exhibit a significant cell volume increase even in the proliferation domain, while for the pericycle and endodermis cells, this tendency is not visible (Figure 1b). The nuclear landscape (chromatin structure) differs significantly among

cells even in the proliferation domain with a strong divergence of the average volume of the nuclei at the end of the proliferation domain (71,5 µm^3, 50,6 µm^3 and 38,1 µm^3 for cortex, endodermis, and pericycle cells, respectively; compare Figure 1A and S6). Altogether, these data led us to conclude that cell cycle duration may differ in different cell files to compensate variations in cell sizes by balancing cell numbers and thereby keeping root integrity.

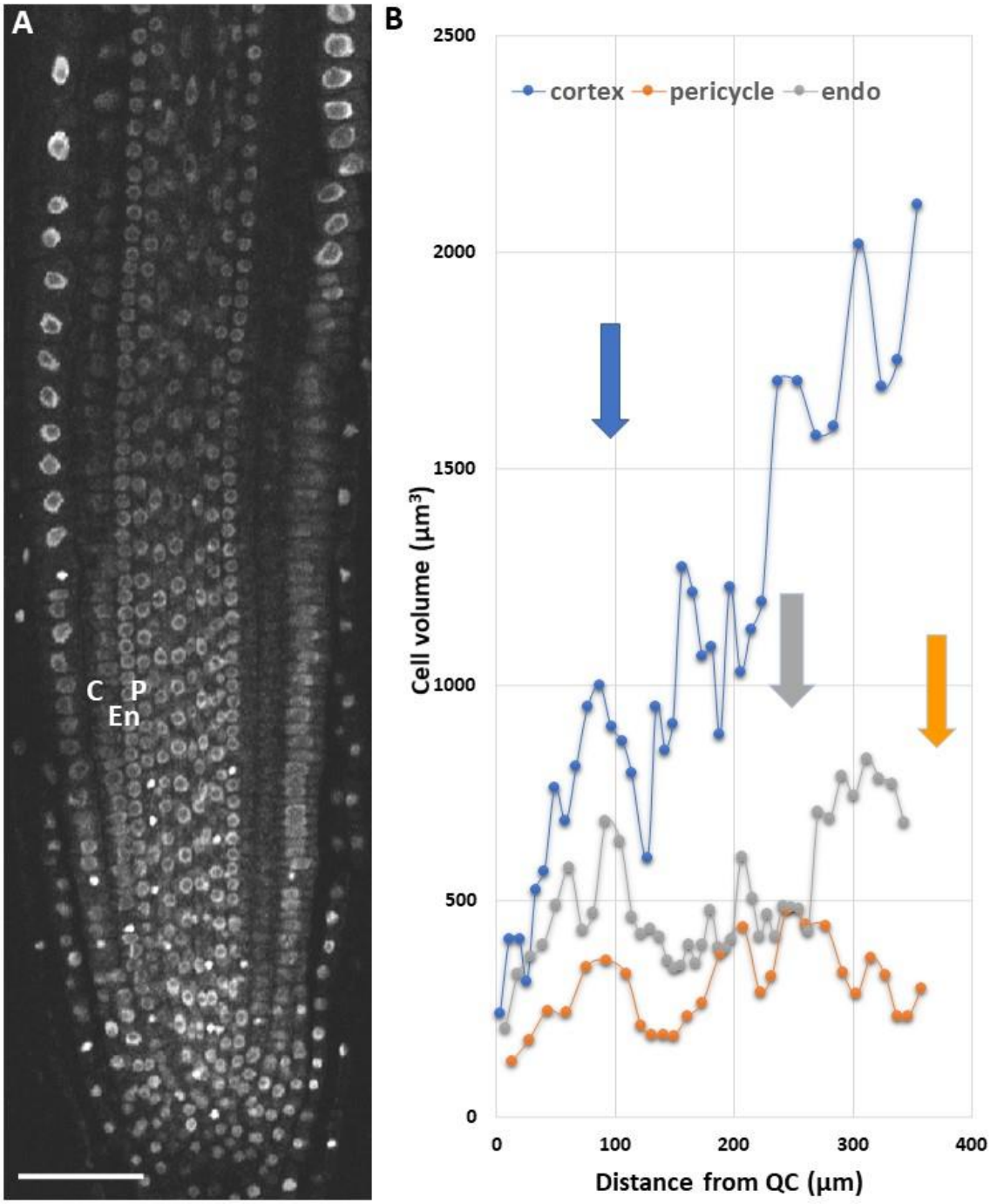

Figure 1. Divergence of cell geometries in the proliferation domain of Arabidopsis root. (A) Nuclear landscape after DAPI labelling. Roots were analysed five days after germination (DAG). C - cortex; En - endodermis; P- pericycle. (B) Volume of the cells in the cortex (blue), endodermis (grey) and pericycle (orange). Arrows mark the end of the proliferation domain,

which was determined as the position of the last mitosis in each cell file (Lavrekha et al., 2017). Scale bar: 50 µm.

Work-flow to determine cell cycle parameters.

To determine the duration of the different cell cycle phases we used the workflow presented in the Figure 2.

The main advantages of this systems are that seedlings of the same age are used under the same, carefully preadapted conditions. Potential variations were further minimized by simultaneous determination of all cell cycle stages in all root's cells in single seedling population by choose different EdU-incubation time for each stages. Experimentally seedlings were distributed to 6 individual vials, EdU was added at the given time points and samples were then fixed simultaneously at the end of the time course.

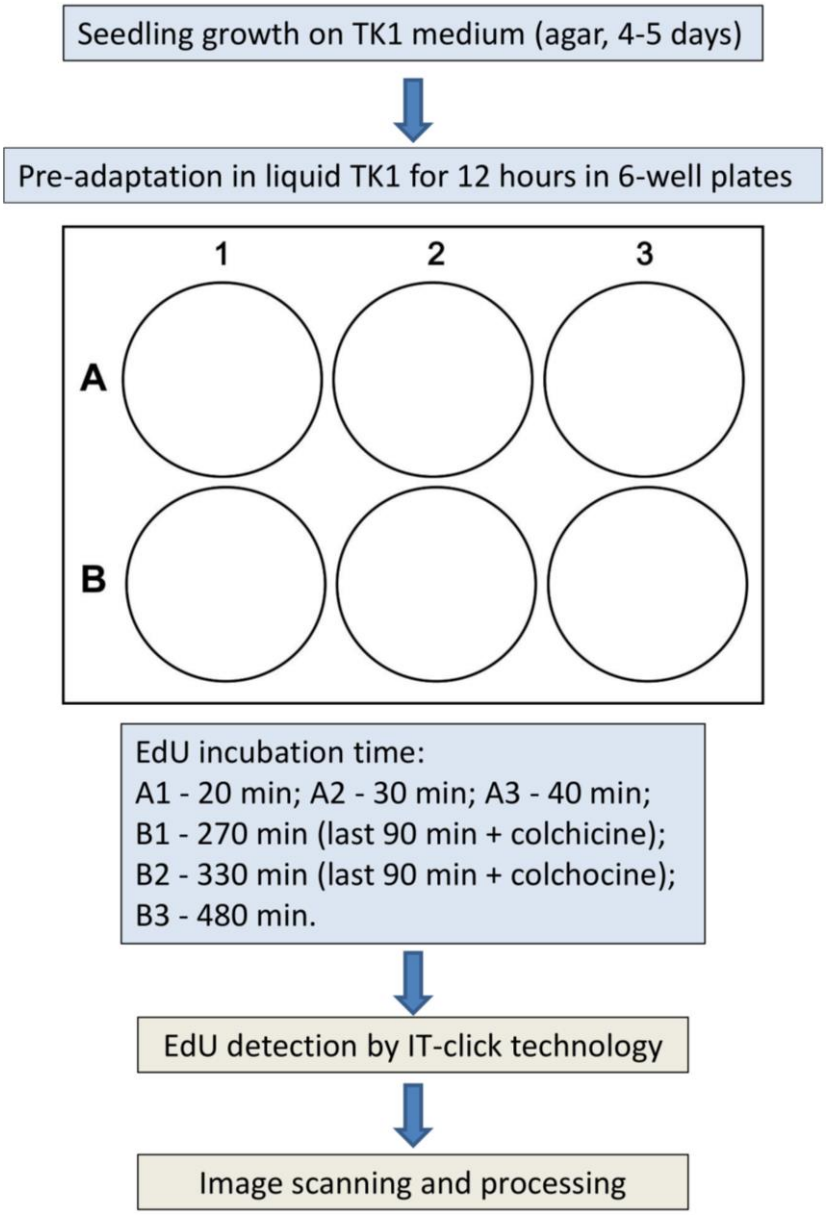

Figure 2. Workflow for estimation of cell cycle phases duration in Arabidopsis.

S-phase duration.

Using this workflow we determined the S-phase duration by estimating the minimal time required for full co-localization of DNA and EdU labeling. After incubation for 20 min, 30-40% of the total number of the nuclei showed complete EdU and DAPI co-localization (Figure 3). Interestingly during the 20 min incubation all cell files, even most central stele tissues in the Arabidopsis root tip showed many cells with DNA replication. From these data we conclude that the S-phase lasts about 20 min. We note that dense chromatin (hetero-chromatin) incorporated EdU (Figure 3, blue arrows) more faster as eu-chromatin, but these cells are mainly located in the transition domain when visible elongation are visible (post-mitotic domain) and therefore easily distinguishable.

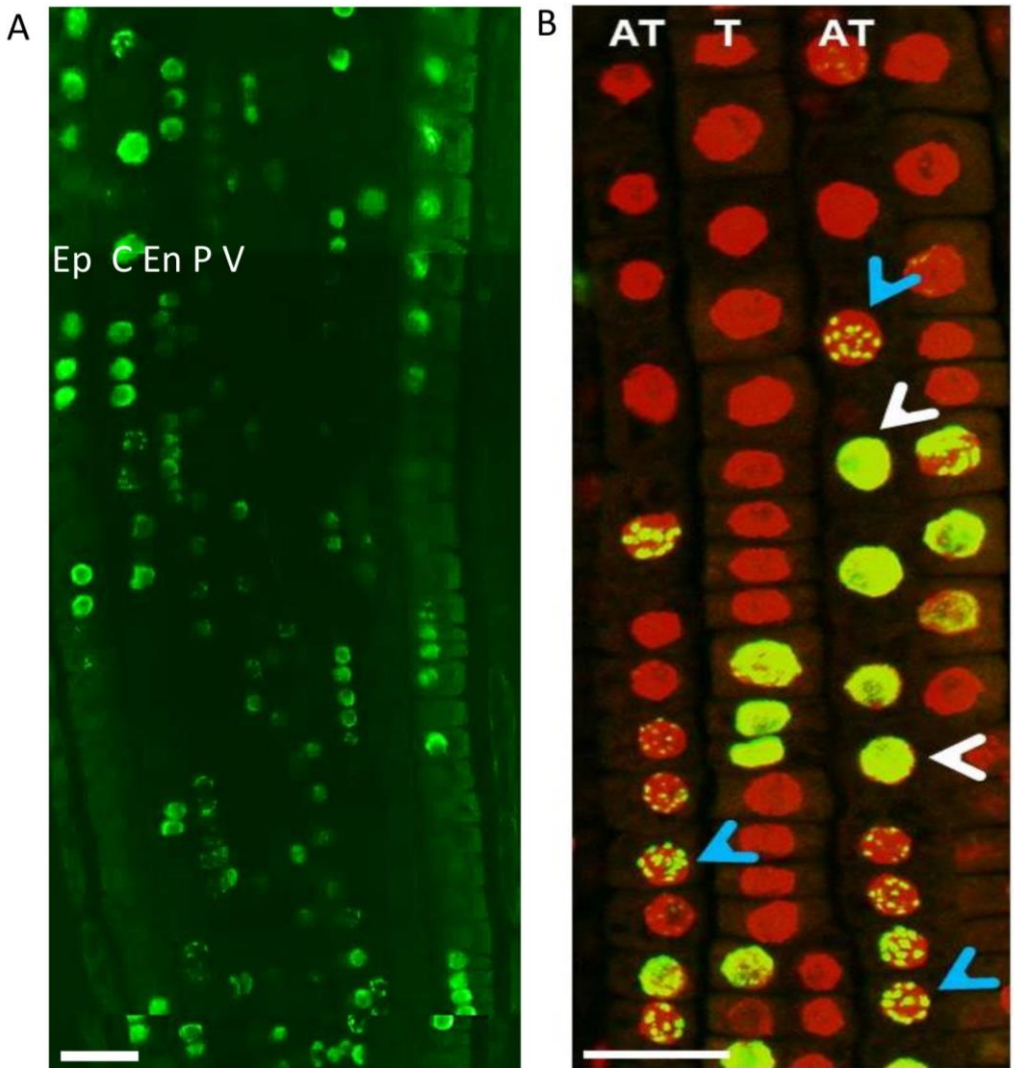

Figure 3. DNA replication in Arabidopsis root after 20 min. of the EdU incubation. (A) Maximum projection of the epidermal cell layer of a representative root is shown. DAPI staining is presented in red, EdU signals in green. White arrow heads point to examples of entire labelling, blue arrow heads mark partial labelling of only condensed DNA spots. The white asterisks mark visible cell elongation in the trichoblast (T) and atrichoblast (AT) cell files (onset of transition zone). (B) overview of the middle RAM. V - vasculature; P - pericycle; En-endodermis; C - Cortex; Ep - epidermis. EdU-positive nuclei are in green. Scale bar: 20 µm.

G2 duration.

To determine the G2 duration, we first analyzed the minimal time needed for appearance of EdU-positive cells' in the mitotic stage. For this purpose, EdU incubation was performed for 90, 180, 210, 240 and 270 minutes. EdU-positive cells passing through mitosis (i.e. pass DNA replication and G2 phase) in the pericycle and endodermis were detected after 240 minutes, but not after 210 minutes. The first EdU-positive cells in the cortex and epidermis were detected after 270 minutes. Based on this observation, we chose EdU incubation up to 270 minutes for detailed analysis and then quantified EdU-positive mitotic cells' ratio in each cell file. To prevent EdU-positive cells from passing through mitosis, colchicine was added after 180 minutes of EdU incubation.

This procedure allowed us to build a detailed map of mitosis distribution from a typical root (Figure 4A). This map was built on calculated average values of about 6000 individual cells obtained from three independent roots (Figure 4B). From these data we conclude that in the inner cell layers cells passing the (S+G2) phase between 180 and 270 minutes are distributed equally in the whole proliferation domain of endodermis, pericycle, and vasculature. However, in the cortex and epidermis, only few cells located close to the QC passed the (S+G2) phase during this time span, while more distally located cells obviously must have a longer G2 duration.

To further clarify G2 duration, we increased EdU incubation time to 330 minutes and added colchicine for the last 90 minutes. Results of a typical root analysis demonstrated that the number of EdU-positive cells entering in mitosis after that period increased to 80% in the cortex/epidermis, while in more inner cell layers, we still have 90-100% of EdU-positive mitosis (Figure S1).

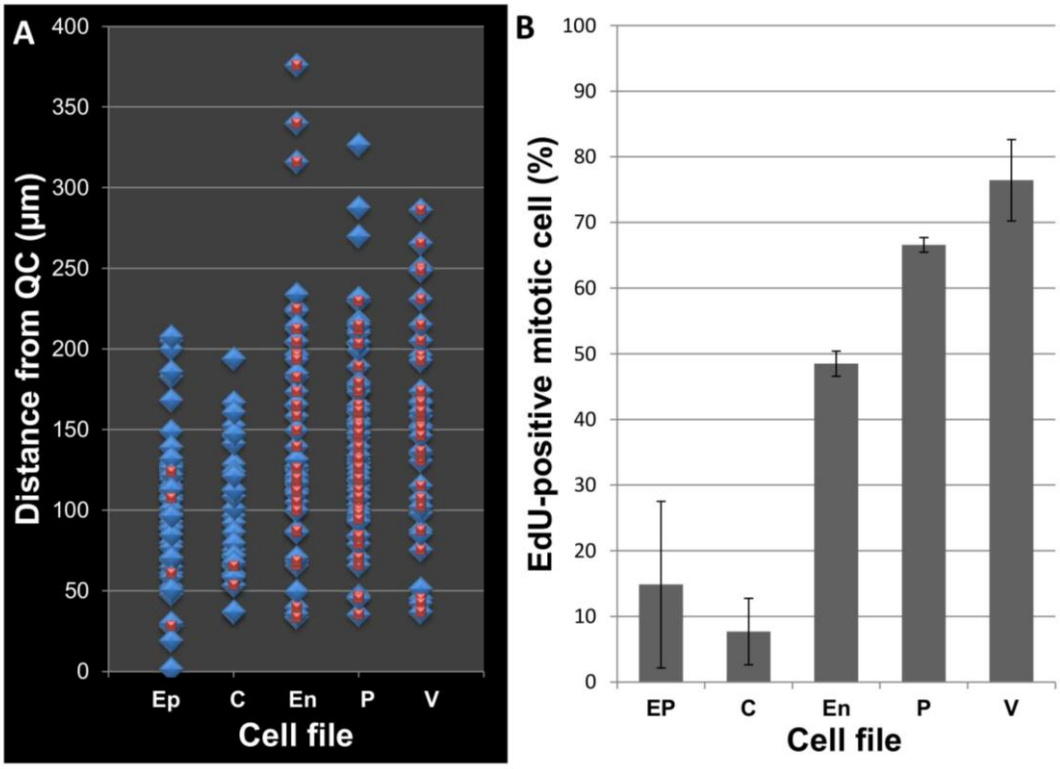

Figure 4. Vertical and horizontal gradients of G2 duration. (A) Mitosis distribution in different tissue layers in the of 4.5 days old Arabidopsis root tip. Seedlings were incubated in the presence of EdU for 180 minutes, subsequently colchicine was added for further 90 minutes. Seedlings were fixed immediately afterwards and subjected to analysis. Mitosis events only are depicted in blue; mitotic cells exhibiting EdU incorporation are marked blue plus red. (B) Average percent of EdU positive mitosis in different cell files. Ep - epidermis, C - cortex, En - endodermis, P - pericycle, V - vasculature.

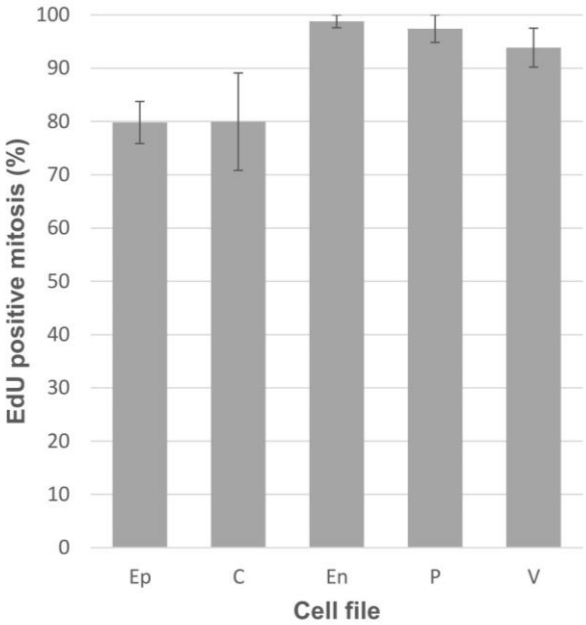

Figure S1. Average percent of EdU positive mitosis in different cell files. Five days old seedlings were incubated in the presence of EdU for 330 minutes, for the last 90 min colchicine

was added. Seedlings were fixed immediately afterwards and subjected to analysis. Ep - epidermis, C - cortex, En - endodermis, P - pericycle, V - vasculature.

Mitosis duration.

As an indirect estimation of mitosis duration (including all stages), we employed the value of the mitotic index, which reflects the relative duration of mitosis itself in relation to the whole cell cycle. We determined a mitotic index of 3-4% which means that the mitosis duration is noticeably short (about 20 - 25 minutes) and itself has only a limited contribution to total cell cycle duration.

Determination of entire cell cycle duration.

To investigate the entire cell cycle duration, we increased EdU incubation time to 8 hours. After this incubation period, gradients of EdU-positive cell frequency from inner to the outer cell layers still exist. In the cortex cells 85% EdU positive cells have been detected in the proliferation zone, while EdU-positive cell rate in the pericycle reaches 98% (Figure S1 for graphical illustration). In consequence, the entire cell cycle duration in the pericycle is less than 8 hours, while in the cortex and epidermis the process lasts up to 9 - 9.5 hours. Such differences may relate to different chromatin landscapes and nuclei sizes and even related to nucleoli size, which is quite different in different cell files, even in trichoblast and atrichoblast cell files (Figure S6). Unfortunately, it is exceedingly difficult to estimate the exact duration in outer cell layers. The duration was shorter in the more proximal zone in these layers and correlates with the smaller nuclei volume (figure S7) and cell volume (Figure 1). Changes in nuclei volume without a difference in the ploidy level may directly relate to chromatin status changes. These data are in accordance with observed differences in G2 duration and reflect differences in whole cell cycle as well. Interestingly, the EdU incorporation indices were significantly higher in the transition zone and reached 100% after about 5 hours of incubation (Figure S2). In consequence, we conclude that the duration of the endocycle is much shorter as the complete cell cycle in Arabidopsis.

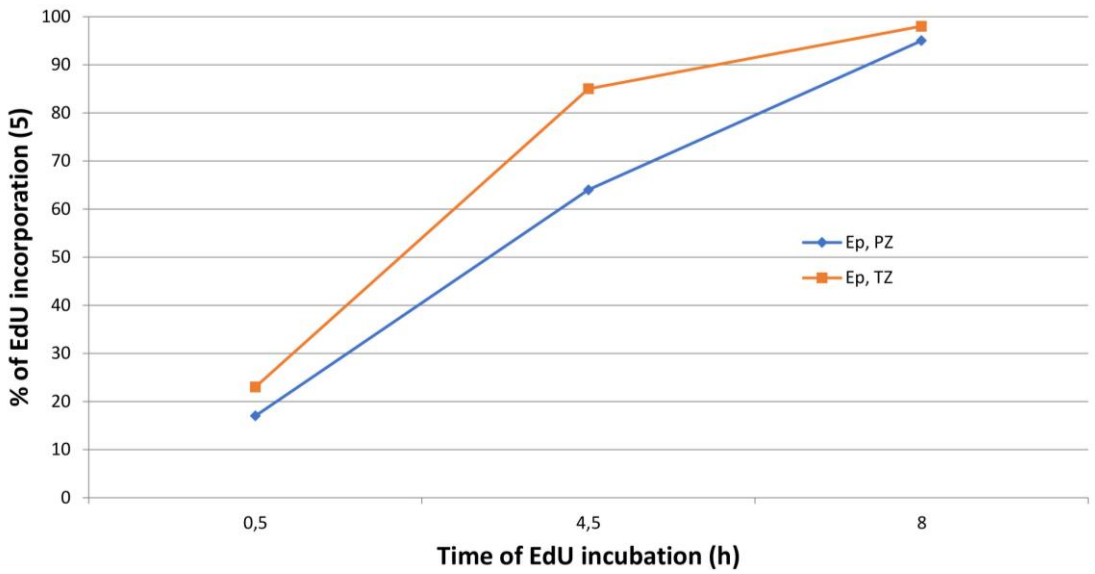

Figure S2. Ratio of EdU positive cell in the epidermis after 0.5; 4.5 and 8 h incubation in the presence of EdU. PZ- proliferation zone; TZ- transition zone (zone in which mitosis were not detected).

Divergence in cell cycle duration after formative cell divisions.

To further study potential differences in the cell cycle duration in cells with shared origin but different fate, we have investigated the ratio of cell production after tangential (in epidermis) and periclinal (in endodermis) cell division which leads to formation of daughter cells with different fates. In the epidermis tangential divisions lead to divergence of trichoblast and atrichoblast cells, while periclinal divisions in the endodermis generate the formation of middle cortex and endodermis cells (Baum et al., 2002).

Our results show that in both cases cell production exhibit different rates (Fig. 5), accompanied by differences in cell volume and orientation. Interestingly, divergence in cell production rates after "formative cell division" are increasing with increasing distance from the QC. For example, after periclinal cell division in the endodermis, we have observed a lower cell production (fewer cell numbers) in the more outer cell files for the same time period compared to the more inner ones. Additionally, EdU-positive mitosis was abundant more frequently (after shorter incubation time) in the cortex/epidermis cells with more proximal location (to QC), which means that the cells with less proximal location have a much longer G2 duration (Figure 3). In summary, these findings confirm that cell cycle duration can be dependent on cell fate and cell position.

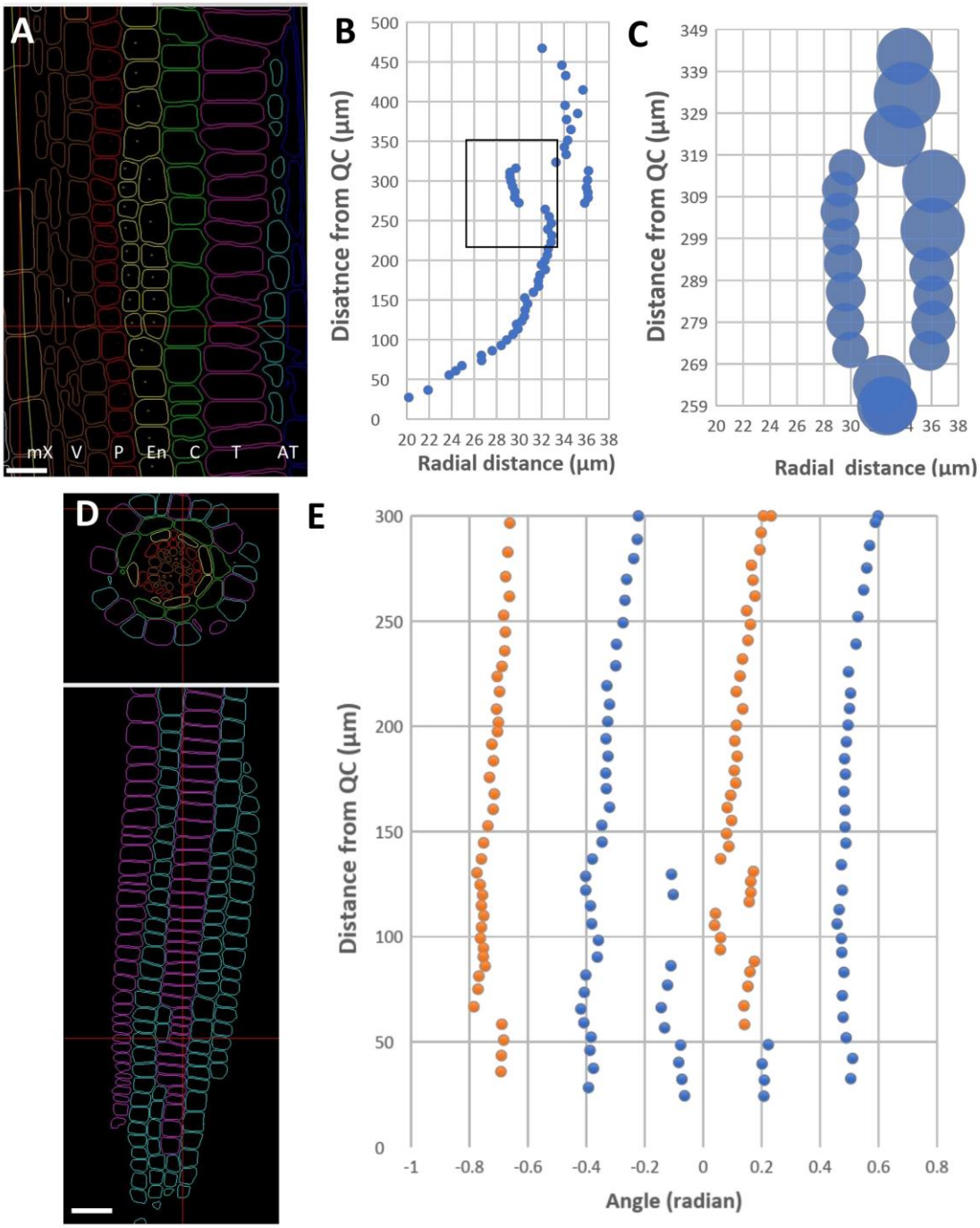

Figure 5. Differences in cell production rate after tangential and periclinal cell divisions. Roots of 6-7 days old seedlings were fixed, labelled for cell border detection, and segmented to analyse the precise tissue structures by iRoCS (Schmidt et al., 2014). Regions of interest related to cell divisions in tangential (A, B, C; trichoblast, atrichoblast) and periclinal (D, E; middle cortex, endodermis) orientation are shown. Relative differences in cell volume are demonstrated by the size of bubbles (panel C).

Cell cycle duration in other species.

In order to test the suitability of the presented approach in other species, we investigated, albeit in much less level of detail, roots of tomato, tobacco, and wheat (Figures S3-S5).

For S-phase duration we detected EdU incorporation with almost full DAPI co-localization after 20-35 minutes of incubation in these three species. EdU-positive mitotic events were detected in tomato and tobacco after 240 minutes of incubation. Interestingly, in tobacco and tomato, no obvious differences in nucleus/chromatin organization between outer and inner cell layers were detected. In consequence, in these species, remarkably similar G2 durations occur in all cell files analysed. Additionally, in tobacco and tomato, the duration of DNA replication and G2 phases are even shorter than Arabidopsis. Moreover, in the case of tomato, almost all cells became "EdU-positive" after 9-10 hours of incubation except in the stem cell niche where cells may less accessible for analysis due to enhanced density and attenuated stain accessibility (Figure S3).

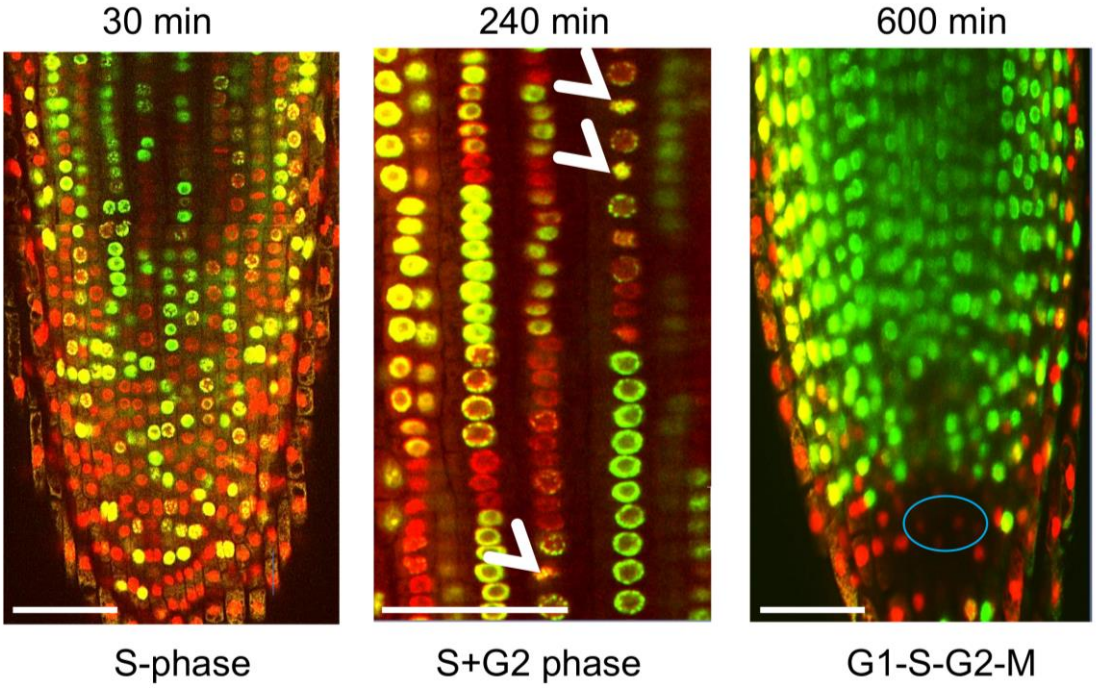

Figure S3. Cell cycle duration in tomato root. 4-5 days old seedlings were incubated with EdU for 30 min, 240 min, and 600 min. 30 minutes of incubation showed entire colocalization DAPI and EdU on cortex (2-3) focal plane; 240 min incubation led to the first appearance of EdU-positive mitosis (white arrows) and 600 min incubation to distinguish the stem cell niche. Nuclei are depicted in red; EdU positive nuclei are shown in green; the QC region (blue oval) is very dense and shows weak DAPI staining. Scale bar- 100 µm.

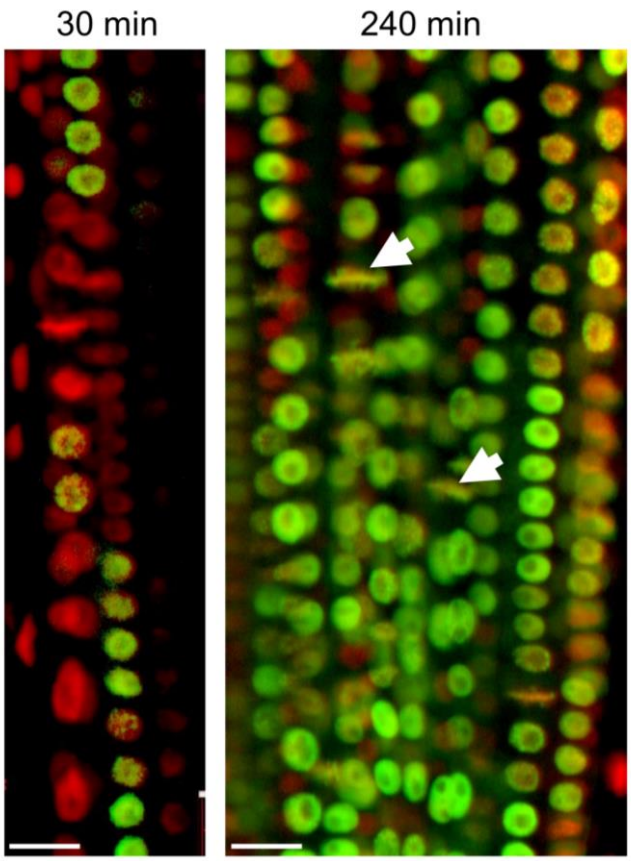

Figure S4: Cell cycle duration in tobacco. 4-5 days old seedlings were incubated with EdU for 30 min and 240 min. Arrow show selected mitotic plates. Nuclei are depicted in red; EdU positive nuclei are shown in green; Scale bar- 20 µm.

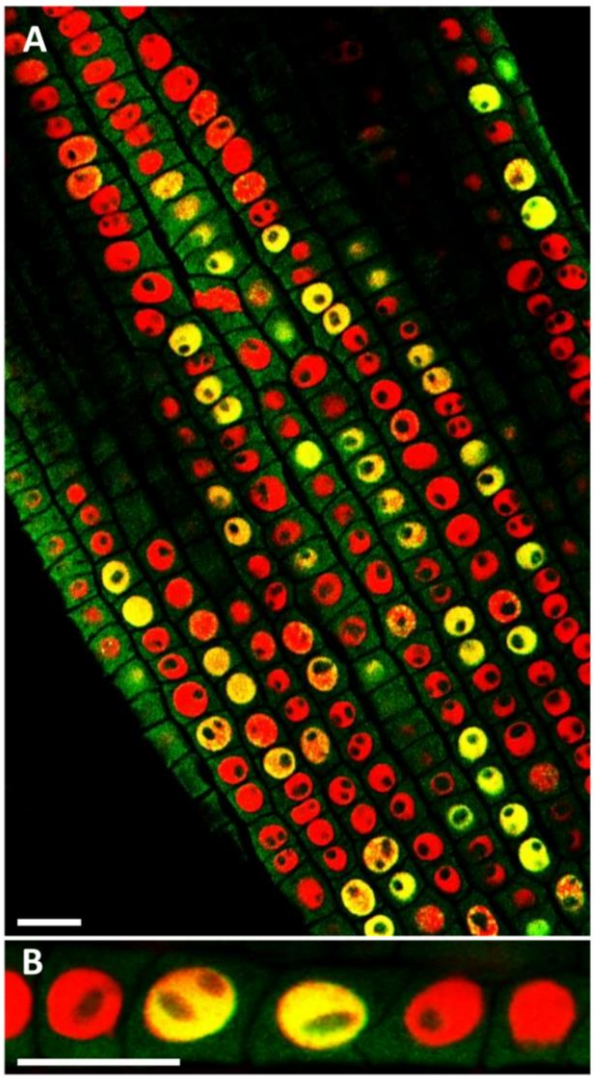

Figure S5: . EdU incorporation into wheat tissue. Four days old wheat seedlings were incubated with EdU for 35min. (A) – general view; (B) – close up view. Overlay images, DAPI stain of nuclei is red coloured, EdU signals are coded in green. Scale bar- 20 µm.

Discussion

Plant organ growth is regulated by a complex and dynamic set of multifactorial events that need multi-scale and multidisciplinary approaches to identify the different parameters for precise coordination between different cell types, individual cell position, rate of cell production, and cell growth. Even a simple organ like an Arabidopsis root possesses significant divergence of processes controlling cell geometry and chromatin status and which eventually control the subtle differences in cell cycle kinetics in the different files. However, most investigations ignore such important features mainly because of lack of efficiently applicable techniques and protocols for studying the cell cycle's kinetics in each cell separately and within the frame of whole organs.

EdU-incorporation as a marker to estimate cell cycle duration.

First, we studied the distribution and timing of EdU incorporation as a marker for DNA replication events. To do this, we incubated Arabidopsis seedlings in the presence of EdU for different time periods (Figure 2) and performed an analysis of the distribution of EdU-incorporating cells in the root. After 20 min of incubation, EdU-positive cells were detected in the proliferation domain as well as the transition zone, the domain where cells start to slightly elongate and do not divide anymore (Pasternak et al., 2017; Lavrekha et al., 2017) but undergo endo-reduplication.

Longer incubation time (4 hours) allows us to detect EdU-positive mitotic cells, which means that cells undergo DNA replication, G2 stage, and enter in mitosis. The distribution of EdU-positive mitosis over EdU-incubation time allows us to quantify S+G2+M duration.

Next, after 5 hours of incubation, almost all cells which undergo elongation became EdU -positive means that all cells in this zone passed G1-S transition and the entire cycle duration is around five hours (figure S2, S6).

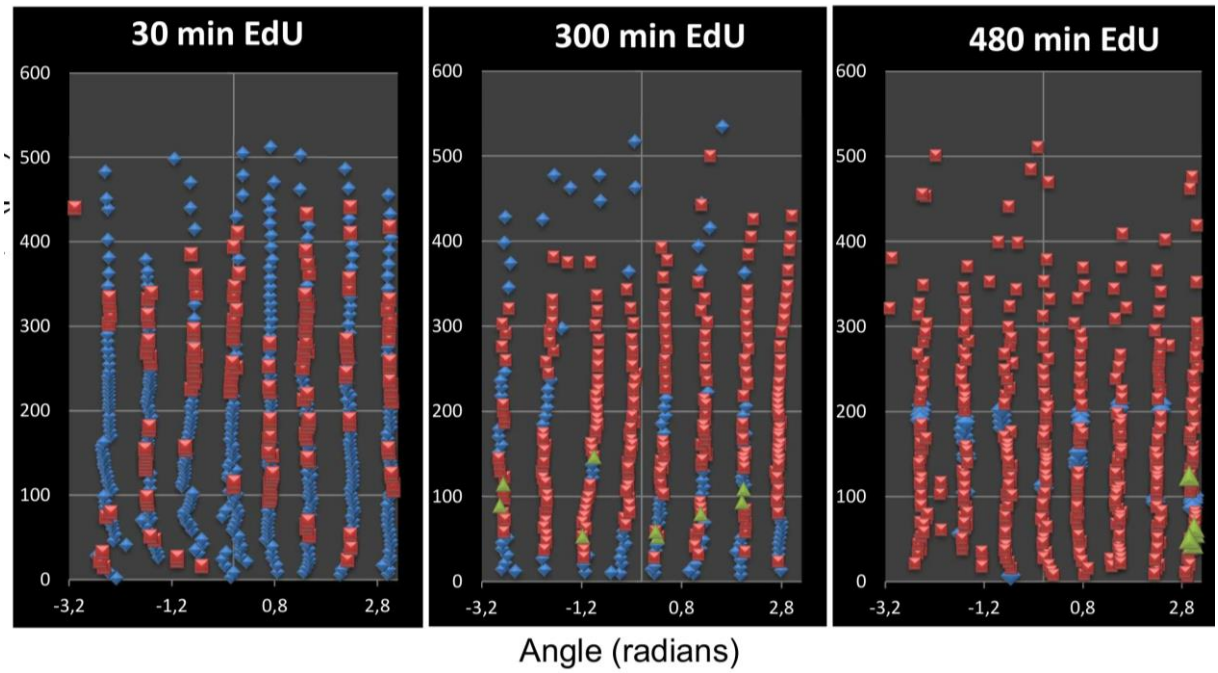

Figure S6. EdU incorporation into cortex cells after incubation for 0,5; 5 and 8h. Cells without EdU are depicted in blue, EdU-positive cells in red, and mitotic cells in green.

Cell culture based methods.

Classically cell culture based methods have been dominating the field of cell cycle analysis as they offer advantages of sampling nearly homogenous population of cells (Gould, 1984) but

unfortunately results obtained cannot be easily used for real plants because of the dependence of the cell cycle kinetics on cell-position within an organ. But still important features have been learned such as the relative duration of each cell cycle phase, elucidation of the rapid cycling of homogeneous cell populations with a G1:S:G2 ratio of 50:10:40 revealing that the S-phase represent 5-10% of cell cycle and that the G1 phase is roughly 20% longer than G2. This led to the concept that certain cell cycle genes represent the main hub for regulating root development but missing the role of cell file gradients or changes in chromatin organization as critical regulatory mechanism (Desvoyes et al., 2014); Otero et al., 2016).

Duration of DNA replication.

Many methods allow monitoring DNA replication and hence S-phase duration by using markers such as H^3-thymidine and more recently BrDU and EdU (Mickelson-Young, L.et al., 2016; Pereira P. et al., 2017). These studies claim that DNA replication duration spans about 2.5-3 hours based on estimation of the time after which fluorescence intensity reaches a maximum. However, if one considers that the simultaneous substitution of growth media without pre-adaptation with EdU may distort precise cell cycle progression and that cellular thymidine pools may be high, EdU will incorporate in parallel with H^3-thymidine leading with about 17 h to an overestimation of cell cycle duration (Hayashi et al., 2013). Therefore, new protocols need to take this into account and also consider the consequences of immediate medium exchange as proposed in the majority of published protocols (Van't Hof J. 1967; 1969) which may lead to a temporary extension of the cell cycle itself. Consequently, in our protocol, we propose root adaptation to the new medium to prevent potential disturbances in the cell cycle.

Molecular marker lines for quantitative analysis.

Another popular route is the use of molecular markers to investigate cell cycle dynamics (Yin K. et al., 2014). Despite their popularity and the fact that markers more or less precisely reflect cell cycle kinetics major pitfalls occur when markers like CyCB1.1::GFP are less well detectable in inner cell layers like pericycle and their expression reflects rather the G2 phase, but not the mitosis itself (Lavrekha et al., 2017). Another disadvantage limiting the general applicability is that all markers for cell cycle events are available only for a certain plant, in most cases only for Arabidopsis, and have to be generated de novo if other plants are to be studied. To overcome the limitations of these time-consuming transgenic procedures has been recently presented by Bhosale et al. (2018) using DNA staining for analysis of DNA replication. Based on this analysis a time window of only 30 minutes has been proposed for epidermis endocycle duration, which seems to be shorter as DNA replication time alone. Another protocol based on multicolor markers for each cell cycle stage and similar to Yin K (2014) was recently

applied to monitor Arabidopsis root cells cycle progression features (Desvoyes B. et al., 2020; Tosheva, K. L., et al., 2020). Although attractive and very useful, positional information on cell files, their distance from QC or periclinal divisions is not yet accessible by 2D analysis.

Positional cell cycle gradient analysis.

Roots exhibit continuous gradients of hormones, ROS accumulation, or cell size along their axis. Considering positional information is very important for understanding the cell cycle regulation mechanism in a whole organ (Wolpert L., 1989).

Our method combines and extends all of these features by simultaneously performing a detailed analysis of all cell cycle events in each cell file in 3D independently and simultaneously using chemical labeling, high-resolution confocal imaging, and subsequently rigorous, quantitative 3D image analysis. For this we first optimized application and timing of EdU incorporation as a marker for DNA replication by exposing Arabidopsis seedlings to EdU for different time periods (Figure 2) and analysed the distribution of EdU-incorporation in root cells. Then we applied consequently the iRoCS pipeline for 3D-high-resolution of the root organ. As shown previously this analysis is not limited to Arabidopsis for which it was first developed by can easily be also adopted to other plants as well (Pasternak et al., 2017). Using this pipeline, which resolves all root cells and root cell files simultaneously, we found already after 20 min exposure EdU positive cells in the proliferation domain as well as in the transition zone, the domain where cells stop to divide, start to slightly elongate (Pasternak et al., 2017; Lavrekha et al., 2017) but undergo endo-reduplication. Longer incubation times of up to 4 hours allowed to detect EdU-positive mitotic cells undergoing DNA replication, moving into G2 and entering into mitosis. By consequent changing the EdU-incubation time we could use the distribution of EdU-positive mitosis to quantify S+G2+M duration. After 5 hours EdU incubation for example, we found almost all cells which undergo elongation to be EdU-positive, which means that all cells in this zone passed G1-S transition. This allowed us to conclude that the entire cycle duration in this region is around five hours (Figure S2, S6). Overall, our method allowed a detailed analysis of all cell cycle events in each cell file independently and simultaneously. Previously, we demonstrated significant differences in cell geometry and sizes (Blein et al., 2018) among different cell layers what should eventually involve differences in cell cycle duration for harmonized organ growth. Here, we were able to describe significant differences in cell cycle duration in cells with different fate and show that cell cycle duration is much slower in the cortex and epidermis, accompanied by a progressive slowdown of their cell cycle as a function of QC's distance. Such differences may serve to compensate faster increases in cell volume in outer cell layers (Figure 1; see Blein et al., 2018). Finally, determination of the cell

cycle duration after tangential or periclinal cell divisions (Figure 5) resulted in altered cell fates and provided us with direct evidence for dynamic positionally driven regulatory mechanisms of the cell cycle duration.

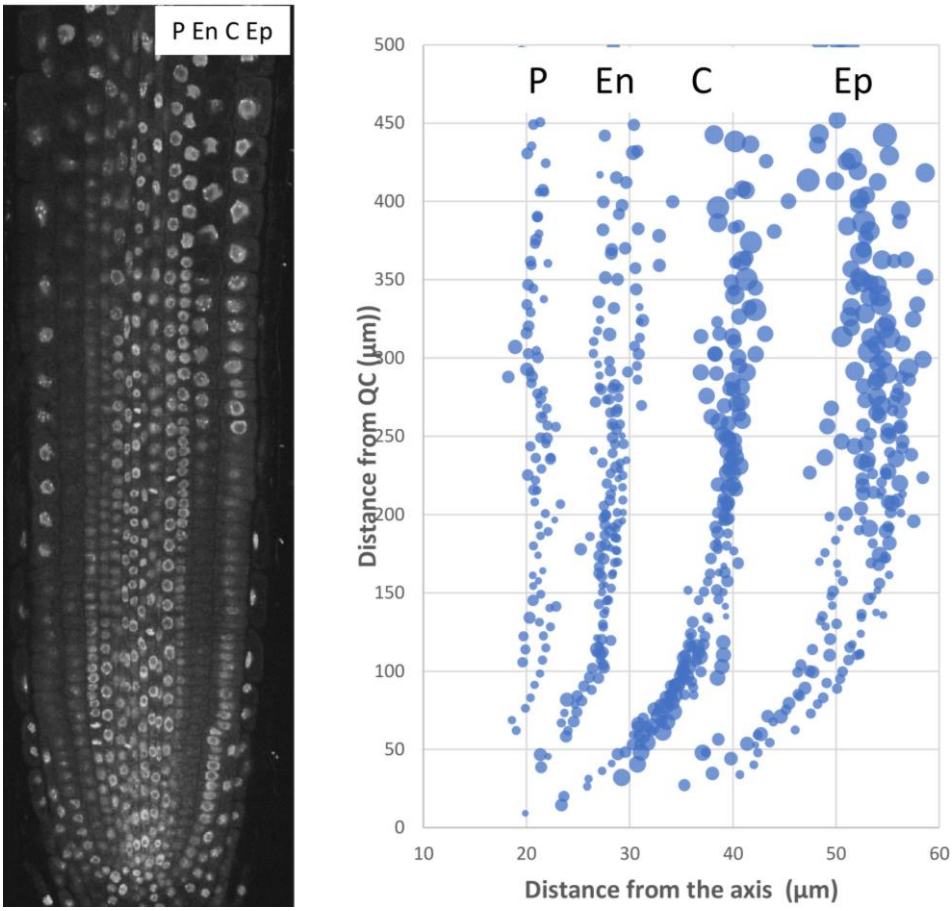

Figure S7. Analysis of nuclear volume is a function of the differences from QC in different cell files. The x-axis is the distance from the root axis; the y-axis is the distance from QC; Nuclei volume was proportional to the circle area.

Conclusions

Root growth is a dynamic process and occurs in a strictly coordinated manner. Consequently, dynamic changes in cell elongation, volume and cell proliferation of involved tissues must be strictly balanced. Here we present a simple and marker-free method and demonstrate its potential for rapid and simultaneous estimation of the cell cycle duration at all its stages in each cell file of plant roots. The method does not require significant mathematical calculation. Using this high-resolution pipeline we show that significant differences in cell volume kinetics, number of cells in distinct cell files, and their nuclear landscape exist even in the proliferation zone. These were datable not only in Arabidopsis but also in several other plant species. From our data we conclude that roots keep their structurally integrity at least partially by differences in the cell production ratio using distinct and different cell cycle duration times.

Materials and methods.

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heyhn. ecotype Col-0, *Nicotiana tabacum cv.* Samsun N.N., *Lycopersicum esculentum* L., and *Triticum aestivum* L. were used in experiments. Arabidopsis seeds sown on square Petri dishes containing TK1 medium (Pasternak et al., 2020). The plates were kept at room temperature for 4 h before being transferred to 4°C for 12 h. The plates were then transferred to 22°C under a 16 h / 8 h light/dark photoperiod with a light intensity of 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 4.5 days. The seedlings were transferred to a 6-well plate containing liquid TK1 medium. After a 12 h incubation in similar conditions as on agar medium growth conditions, 2.5 mM colchicine and 10 µM EdU (5-ethynyl-2'-deoxyuridine) were added at defined time points (Figure 2) and seedlings from all vials were fixed 8 h after onset of first EdU addition. Seeds of the *Nicotiana tabacum* Samsun NN and *Lycopersicum esculentum* L. were surface sterilized in 0.2% NaClO and sown on square Petri dishes containing T1 medium (supplemented with 2% sucrose) optimized for root growth (Pasternak *et al., 1999).* 4-5 days old seedlings have been used for labelling. Wheat seeds were soaked in water in Petri plates. 3-5 days old seedlings were transferred to liquid T1 medium for 16 h adaptation.

Pipeline for determination of cell cycle duration.

For determination of cell cycle duration 10 µM EdU were added to the seedlings in the liquid medium 12, 14, 16, 18.5 and 19.65 hours after starting of adaptation for each plate separately. Seedlings were fixed 20 h after onset of incubation. This timeline allowed us to apply different EdU-incubation times for either 8 h; 6 h; 4.5 h; 1.5 h or20 min. For determination of (G2+S) duration colchicine was added to the plate after 180 and 270 min EdU incubation. These plants were fixed after further incubation for 90 minutes (see Figure 2 for experiment design).

Staining.

After EdU incubation plants were fixed in microtubule stabilizing buffer containing 4% formaldehyde (MTSB) and EdU incorporation was detected as described previously (Pasternak et al., 2015).

For volume determination, roots were fixed and labeled with the modified Truernit et al. (2009) method. Scanning and analysis procedures were done essentially as described in Schmidt et al., 2014.

Confocal imaging.

DAPI/EdU-stained samples were recorded using a confocal laser scanning microscope (LSM 510 META NLO; Carl Zeiss, Oberkochen, Germany) with a LD LCI-Plan-Apochromat 25x/0.8 DIC Imm Corr objective. For DAPI excitation, a Chameleon laser adjusted to 740 nm excitation

was used and emission was detected with a band pass filter (BP 390-465 IR), Alternatively, all other suitable microscopes equipped with a diode 405 nm laser can be used. Serial optical sections were reconstituted into 3D image stacks with in-plane (x-y) voxel extents of 0.15 µm and 0.9 µm section spacing (z). Three to five overlapping images (tiles) were recorded for each root.

Image processing and analysis.

Images were converted to hdf5 format using the LOCI plugin for ImageJ (http://imagej.nih.gov/ij), then stitched together to obtain a root tip total length of 400 µm from the QC using xuvTools (Emmenlauer et al., 2009). 5-10 representative roots were chosen for detailed annotation. The DAPI and EdU channel images were processed with the iRoCS Toolbox (Schmidt et al., 2014) in the following way: nuclei were automatically detected using the "01-Detect Nuclei" plugin, then the epidermis was semi-automatically labelled using the "02- Label Epidermis" plugin. After the QC position was marked (Channel->New Annotation Channel), the nuclei were set in spherical coordinates using the "03-Attach iRoCS" plugin. Automatic classification of the nuclei to the corresponding cell types (epidermis, endodermis, cortex, pericycle, vasculature, root cap) was done using the "04-Assign Layers" plugin, which also enabled the automatic annotation of nuclei in mitotic state (option "Re-classify mitotic state"). All annotated roots were manually corrected for erroneous layer, mitosis, and EdU assignments.

Data analysis.

The data processed by iRoCS were exported to .csv files and analyzed with Microsoft Excel. Statistical analysis was done using Student's *t*-test. Roots were virtually divided to 50 µm sections. The DNA replication index (DRI) was calculated as the proportion of cells posing DNA replication during incubation time to all cells in the current section and was calculated for each 50 µm interval.

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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.

Author Contributions: Conceptualization: TP, SK, KP; methodology: TP, Sample preparation, scanning, image processing: TP; resources: KP; data curation: TP, SK, KP; writing TP, SK, KP. All authors have read and agreed to the published version of the manuscript.

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Table S1.Comparison of different methods for cell cycle duration estimation.

Method / Authors / Parameters extracted	H^3thymidine /colchicine Clowes 1961	H^3thymidine /colchicine Van‘t Hof 1967	EdU Hayashi et al.,2013	Kinematic Ivanov/Dybrovsky 1997	*In vivo* Method Yin et al., 2014	Stripflow Yang et al., 2017	„Plant cell cycle indicator“ Desvoyes et al., 2020	This paper
Cell type/subtype/position	yes	no	partially	only cortex	partially	only cortex	no	yes
Direct detection	yes	yes	yes	no	partially	no	yes	yes
Link to chromatin gradient	no	partially	no	no	yes	no	no	yes
Pre-adaptation	no	no	no	yes	yes	yes	yes	yes
Marker introduction to the mutant line	no	no	no	no	3-5 months	no	3-5 months	no
Cell cycle stages resolved	partially	partially	no	no	yes	no	yes	yes

Legends:

Cell type/subtype/position: information about cell type/subtype and cell position in organ.

Direct detection: detection of cell cycle stages in the living cells.

Link to chromatin status: link to histone methylation, nuclei structure in positional contents.

Marker introduction: time requires for the introduction of the marker to the mutant.

Table S2. Summary of cell cycle duration in Arabidopsis RAM.

Duration of the EdU incubation	Cell cycle stage	
20-30 min	DNA replication	Cell cycle/endocycle
3-5 hours	S-G2-M	
8-10 hours	S-G2-M-G1	Entire cycle duration
18-24 h	S-G2-M-G1	Status of the stem cell niche

Supplemental data.

Supplemental protocol.

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

Supplemental file 1: Example of segmentation for determination of cell volume.

Supplemental file 2: Example of the nuclei analysis.