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Marie-Cécile Caillaud *Editor*

Plant Cell Division

Methods and Protocols

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Preface

This volume of *Methods in Molecular Biology* aims to present a large panel of techniques for the study of Plant Cell Division. In its entirety, this book, entitled *Plant Cell Division*, captures basic experimental protocols that are commonly used to study plant cell division processes as well as more innovative procedures. I have gathered together chapters that I hope will be of great help for new students and postdocs entering the field, as well as for more established researchers. This book covers several different aspect of plant cell division and is split into five parts: "Cell Cultures for Cell Division Studies," "Cell Cycle Progression and Mitosis," "Imaging Plant Cell Division," "Cell Division and Morphogenesis," and "Cytokinesis."

The first part of the book, entitled "Cell Cultures for Cell Division Studies," presents original and classical cell line systems for the study of cell division in plants. Chapter 1 provides detailed protocols for the synchronization of emerging algal models for the study of mitosis and cell-cycle progression. In this chapter, variations around the main protocol are also described that enable researchers to follow nuclear and chloroplast divisions in several different algal species. The method described in Chapter 2 presents a protocol for the transient transformation of dividing tobacco BY-2 cells. In contrast with classical stable transformation methods (1), this procedure permits the analysis of transgenic, dividing BY-2 cells after just 2 days of co-culture with Agrobacterium. Other cell culture systems can be found throughout the book, notably in Chapters 6 and 17, in which Arabidopsis cell culture is used to isolate plant nuclei at defined steps of the cell cycle (Chapter 6) or to analyze membrane-lipid order during cell plate formation (Chapter 17). In Chapter 9, the authors provide advice on optimizing the synchrony of stably transformed BY-2 cells to conduct real-time imaging during cell division.

In the second part of the book, entitled "Cell Cycle Progression and Mitosis," Chapter 3 describes a three-step procedure for the identification of gene regulatory networks in Arabidopsis by in silico prediction, Yeast-1-Hybrid, and inducible Gene Profiling Assays. While this chapter is written to cover a broad subject area, it is particularly useful for cell division studies. Chapters 4–7 describe valuable methods to investigate the phenotype of loss- or gain-of-function mutants impaired in plant cell division. Chapter 4 focuses on the visualization of cell cycle progression in plant tissues using a dual-color marker system (2) which allows the in planta tracking of independent cell-cycle steps based on fluorescence combinations. Chapter 5 presents an easy-to-apply protocol for the visualization of mitotic figures and the morphology and organization of Arabidopsis nuclei. Chapter 6 describes a combination of pulse labeling and preparative sorting of nuclei isolated from fixed material, starting with an asynchronous population of nuclei and therefore avoiding artifacts sometimes associated with cell cycle synchronization procedures (3). Chapter 7 provides techniques for the preparation and analysis of plant metaphase chromosomes and includes guidance on how to grow plants in order to obtain good and healthy meristems containing abundant cell divisions.

Meiosis is the two-step series of cell divisions that makes sexual reproduction and genetic diversity possible (4). In this particular case, the mitotic rules of the cell cycle that ensure a strict alternation between replication and division must be prohibited to prevent replication events between the two cell divisions. Fluorescent in situ hybridization facilitates the direct observation of chromosome behavior and thereby serves as an indispensable tool in tracing individual chromosomes during this process (5–7). Chapter 8 explains how to perform FISH experiment on rice chromosomes that can easily be prepared either at mitotic prometaphase or during meiotic pachytene.

The third part of the book, entitled "Imaging Plant Cell Division," describes methods whereby key mitotic players can be visualized during cell division using fluorescent microscopy. Those chapters concern various different plant species and tissues materials and could also be adapted to visualize in vivo (or in fixed tissues) uncharacterized protein components important for mitosis. The plant nuclear envelope has the ability to organize microtubules and is proposed to act as a "microtubule organisation centre" for plant acentrosomal spindle organization (8–10). Chapter 9 describes how to use stably transformed, synchronized BY-2 suspension cells to visualize nuclear envelope dynamics during cell division. This chapter provides advice on optimizing synchrony but also on the in vivo subcellular localization of nuclear envelope-localized proteins using confocal microscopy.

The kinetochore, a fascinating protein complex that facilitates the attachment of spindle microtubules to pull sister chromatids apart during cell division (11), is poorly studied in plant. Kinetochore assembly is initiated by incorporation of the centromeric histone H3 into centromeric nucleosomes (12). Chapter 10 details indirect immunolabeling protocols to analyze the subcellular localization and distribution of cell cycle-specific proteins, in particular centromeric histone H3, which highlights kinetochore localization throughout mitosis (13).

The microtubule network undergoes significant spatial rearrangements during the steps of the cell cycle, especially at the G2/M phase transition and throughout mitosis. In somatic cells, cortical arrays are replaced at the commencement of mitosis by a densely packed ring of microtubules encircling the nucleus which defines the cytokinesis plane position (14). Subsequently, the mitotic spindle apparatus allows the separation of chromatids in two equal sets, while phragmoplast microtubules drive the growth of the centrifugally expanding cell plate, allowing the completion of plant cytokinesis (15). Mitosis can also be observed in physio-pathological conditions such as during the compatible interaction between the root-knot nematode *Meloidogyne incognita* and its host *Arabidopsis* (16-18). Chapter 11 describes how to observe dividing Giant Cells in vivo using confocal imaging. This technique, based on the removal of the hypertrophied plant cell material surrounding the Giant Cells, allows the experimenter to obtain better image resolution, which could not be achieved using classical imaging on whole tissues. Additional procedures to visualize cytoskeleton during plant cell division are described throughout the book. Chapter 2 describes how to transiently express actin markers in BY-2 dividing cells, while Chapter 16 presents protocols for live cell imaging of mitotic maize cells using microtubule markers as an example. In addition, Chapter 14 provides an original, integrated set of protocols including methods for the subcellular localization of cytoskeleton markers within Arabidopsis embryos.

Due to the absence of cellular mobility during plant development, the orientation of cell divisions determines the relative cell positions within the tissue and is therefore essential for morphogenesis (19). Cells in the Arabidopsis shoot apical meristem are small and divide frequently making them good candidates for studying the mechanisms of cell division during organogenesis. However, tracking these cells requires the acquisition over time of multiple

images on the same specimen, which can be arduous and time-consuming. In the section entitled "Cell Division and Morphogenesis," Chapter 12 addresses the question of how to perform real-time lineage analysis to study cell division orientation. This chapter provides instructions on how to prepare plants for live imaging, how to keep them alive and growing through multiple time points, and how to process the data obtained to extract cell boundary coordinates from three-dimensional images. Chapter 13 describes methods to analyze cell proliferation in the Arabidopsis meristem using pulse-chase labeling of newly replicated DNA in Arabidopsis. This technique allows the visualization of cell populations with slower division rates in long chase periods.

To study asymmetric cell division, which produces two daughter cells with different cellular fates, Arabidopsis early embryo remains one of the best models (20). Chapter 14 presents an integrated set of protocols to study three-dimensional cell division patterns in early-stage *Arabidopsis* embryos. The first protocol provides high-resolution three-dimensional images of cell walls and is suitable for the study of both embryo and maternal tissue development. A second alternative method provides information on the positioning of nuclei within cells, while a third procedure relies on fixed material, but is compatible with the additional detection of fluorescent-tagged protein markers.

Cytokinesis is the final stage of cell division during which a cell divides into two daughters through the assembly of new membranes and a new cell wall (21). Despite significant progress in the functional analysis of proteins essential for cytokinesis, the molecular mechanisms behind the completion of the cell division in plants remain poorly understood. Therefore, identification and characterization of new mutants with cytokinetic defects will continue to be an important tool for studying the molecular mechanisms of plant cell division. In the last part of this book, entitled "Cytokinesis," Chapter 15 describes the straightforward characterization of cytokinesis-defective mutants by the labeling of plant cell structures using fluorescent dyes. How the plant cell division site is determined and established during cell division is an essential question in plant morphogenesis. Chapter 16 describes techniques for observing and analyzing cell-division orientation and timing in maize using microtubule markers. This protocol includes suggestions on how to grow the plants, prepare the samples, set up time-lapses, and calculate division rates. Cytokinesis depends on the correct composition of membrane lipids, like sterols (22). The protocol included in Chapter 17 describes a very innovative procedure to quantify membrane-lipid order during plant cytokinesis. This protocol addresses how a lipid order-sensitive probe can be used to quantify the degree of membrane packing and establish the spatial and dynamic distribution of ordered domains in vivo during cell plate formation.

In conclusion, this volume of *Methods in Molecular Biology* has been designed to emphasize technology that can be applied to currently unanswered questions of plant cell division. The research community studying the fundamental process of plant cell division is still expanding. I hope this book will provide a valuable tool for the study of plant cell division at both the cellular and molecular levels, and also in the context of plant development. I wish to express my gratitude to all the contributing authors and their research groups who made this book a reality.

Lyon, France ***Marie-Cécile Caillaud***

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Part I

Cell Cultures for Cell Division Studies

Chapter 1

Synchronization of Green Algae by Light and Dark Regimes for Cell Cycle and Cell Division Studies

Monika Hlavová, Milada Vítová, and Kateřina Bišová

Abstract

A synchronous population of cells is one of the prerequisites for studying cell cycle processes such as DNA replication, nuclear and cellular division. Green algae dividing by multiple fission represent a unique single cell system enabling the preparation of highly synchronous cultures by application of a light–dark regime similar to what they experience in nature. This chapter provides detailed protocols for synchronization of different algal species by alternating light–dark cycles; all critical points are discussed extensively. Moreover, detailed information on basic analysis of cell cycle progression in such cultures is presented, including analyses of nuclear, cellular, and chloroplast divisions. Modifications of basic protocols that enable changes in cell cycle progression are also suggested so that nuclear or chloroplast divisions can be followed separately.

Key words Green algae, Multiple fission cell cycle, Synchronization, Illumination regime, Growth, DNA replication, Nuclear division, Cell division, Chloroplast division

1 Introduction

The history of green algal cell cycle studies goes as far back as the 1950s when the first study describing the establishment of a synchronous culture was published [1]. From that time, several algae from the genera *Chlorella*,*Chlamydomonas*, and *Scenedesmus* were selected as models for cell cycle studies. Although they are not evolutionary closely related, they share a common cell cycle pattern described as multiple fission. Multiple fission is a mechanism that allows multiple numbers of daughter cells to be formed upon division of a single mother cell. The number of daughter cells depends on the growth conditions and is always $2n$ where n is the number of divisions. Multiple fission can be achieved differently in different algal species, particularly because there exists at least two types of multiple fission cell cycles: the *Chlamydomonas* type and the *Scenedesmus* type (Fig. 1).

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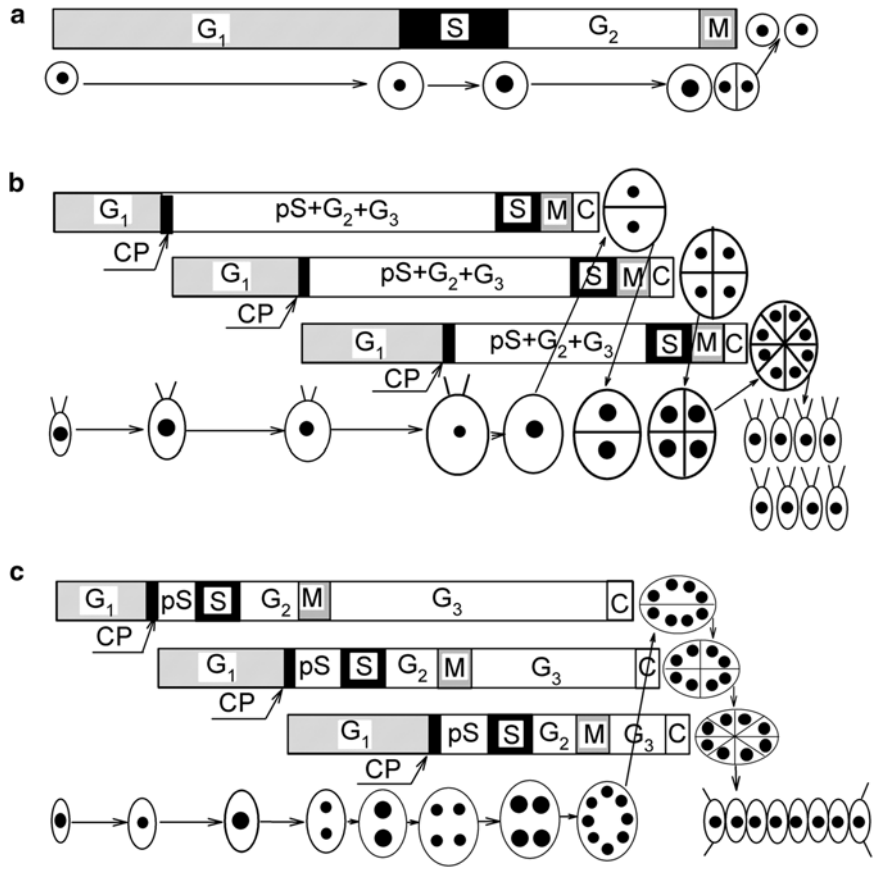

Fig. 1 Comparison of binary and multiple fission cell cycles. Diagrams comparing different types of cell cycle phases of the classical cell cycle model (**a**) and multiple fission found in the alga *Chlamydomonas* (**b**) and *Scenedesmus* (**c**) dividing into 8 daughter cells. Classical cell cycle model after Howard and Pelc [14], *Chlamydomonas* cell cycle model after Zachleder et al. [15], *Scenedesmus* cell cycle model after Šetlík and Zachleder [16]. Individual bars show the sequence of cell cycle phases during which growth and chromosome cycles take place. Whereas only single growth and the DNA replication–division sequence (DNA replication, nuclear division, cellular division) occurs during the cycle of cells dividing by binary fission (**a**), three partially overlapping growth and DNA replication–division sequences occur within a single cycle in cells dividing into 8 daughter cells (**b**, **c**). Three bars (**b**, **c**) illustrate the simultaneous course of different phases from three consecutively started sequences of growth and DNA replication–division. Schematic pictures of the cells indicate their increasing size during the cell cycle and the black spots inside illustrate the size and number of nuclei. Large black spots indicate the doubling of DNA. The small arrows pointing from the schematized cell pictures pinpoint the order of events as they occur within a single cell. G1: the phase during which critical cell size is attained. It can be called a pre-commitment period because it is terminated when the commitment point (CP) is reached. CP: the stage in the cell cycle at which the cell becomes committed to triggering and terminating the DNA replication–division sequence. pS: the pre-replication phase between the CP and the beginning of DNA replication. The processes required for the initiation of DNA replication are assumed to happen during this phase. S: the phase during which DNA replication takes place. G2: the phase between the termination of DNA replication and the start of mitosis. Processes leading to the initiation of mitosis are assumed to take place during this phase. M: the phase during which nuclear division occurs. G3: the phase between nuclear division and cell division. The processes leading to cellular division are assumed to take place during this phase. C: the phase during which cell cleavage occurs. (After Bišová and Zachleder [17], modified)

Although not the case for the very first report, protocols were soon developed that allowed algae to be synchronized by light and dark regimes. The treatment is akin to the natural conditions present in their habitats and the algal response is fast, reproducible, and applicable to many different species; the synchronized cultures can also be kept almost indefinitely. Synchronization by alternating light–dark periods represents a unique synchronization procedure since it does not require any specific inhibitors, mutants, or equipment. It provides a rapid means of preparing highly synchronous cultures so that 95–100 % of the cell population divides synchronously, usually three or four times within the period of three hours. The procedure is based on the ability of green algae to grow autotrophically in light and to perform DNA replication, nuclear and cellular divisions both in the light and in the dark. This means that growth and chromosomal (DNA replication, nuclear division) cycles that comprise the cell cycle [2] can be separated by dark treatment. Generally, the algae grow to a certain critical cell size, usually double the size of a daughter cell, and once this is achieved, they become competent to complete the cell cycle, e.g., they attain a commitment point. In fast growing cells, growth will continue after commitment and they will multiply their cell size fourfold or eightfold, or even more. However, if the cells are darkened at any time after attainment of the commitment point, they will divide in the dark and give rise to daughter cells (Fig. 2). Since cells in the dark cannot grow, they will "wait" until the start of illumination to restart growth. If illumination and darkening is repeated several times, most of the cells in the population will become synchronous, which can be easily demonstrated by showing that the size distribution of the population is very narrow (within a twofold range).

In the following sections, we discuss the synchronization procedure using light–dark regimes and the critical points required for successful synchronization. In principle, the higher the growth rate of the culture, the better is the synchrony that can be reached [3–5]. To this end, all factors affecting growth rate: light intensity, temperature and CO_2 supply should be optimized to give the best possible growth rate under the given conditions. We believe that any algal culture dividing by multiple fission can be synchronized as long as attention is given to the growth conditions and their optimization.

2 Materials

2.1 Organisms

Different algal strains can be obtained from various algal collections. A comprehensive collection of *Chlamydomonas* strains is available in the Chlamy Resource Center kept at University of

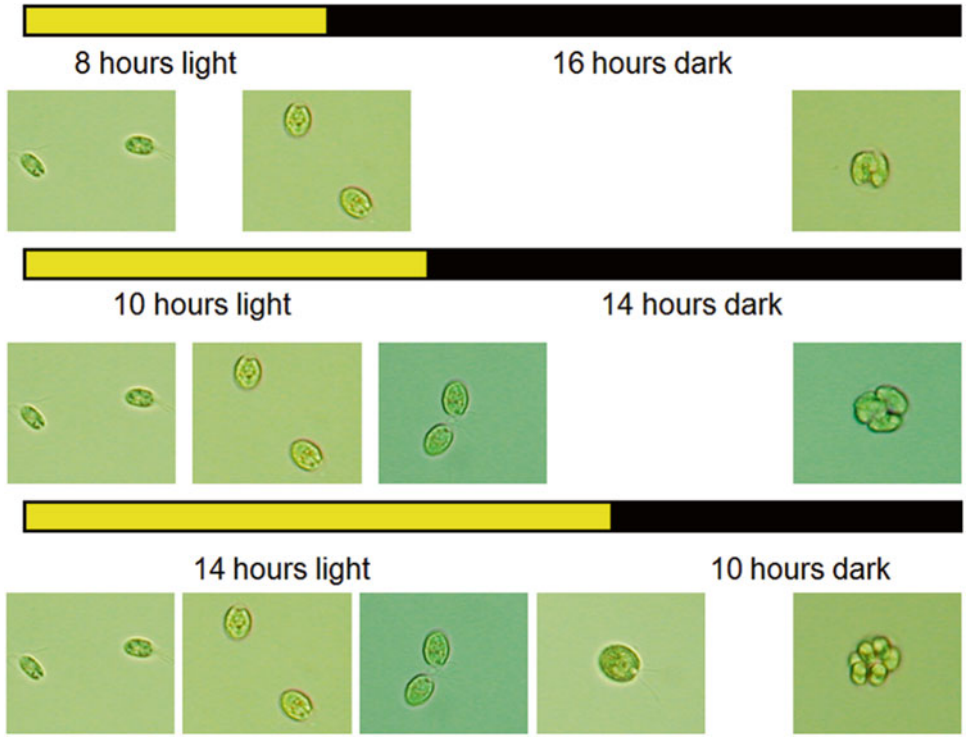

Fig. 2 Principle of light–dark synchronization. Schematic showing the effect of darkening *C. reinhardtii* cells at different hours after the beginning of the cell cycle. As long as the cells already attained commitment point they will divide in the dark into the different number of daughter cells based on cell size reached during the light period. The size of cells arising from different mother cells is very similar, giving rise to a narrow size distribution in the population. Iteration of the light–dark treatment will further narrow the population cell size distribution. The same rules are valid for other green algae dividing by multiple fission, e.g., *Scenedesmus* and *Chlorella*

Minnesota, USA (http://chlamycollection.org/). Other major collections include: SAG, Sammlung von Algenkulturen Göttingen, located in Göttingen, Germany (http://www.uni-goettingen.de/en/184982.html), UTEX collection kept at University of Texas Austin, USA (http://web.biosci.utexas.edu/utex/), CCAP, Culture Centre of Algae and Protozoa, UK (http://www.ccap.ac.uk/), CCALA, Culture Collection of Autotrophic Organisms, Trebon, Czech Republic (http://ccala.butbn.cas.cz/index.php) and RCC, Roscoff Culture Collection, Roscoff, France (http://roscoff-culture-collection.org/). A more detailed list of algal collections can be found at http://www.chlamy.org/resources.html.

2.2 Medium

For routine subculturing of *C. reinhardtii* strains, either *HS medium* (*see* **Note 1**) (500 mg/l NH_4Cl; 20 mg/l $CaCl_2{\cdot}2H_2O$; 200 mg/l $MgSO_4{\cdot}7H_2O$; 720 mg/l KH_2PO_4; 1340 mg/l K_2HPO_4 and the following microelements: 18 mg/l $C_{10}H_{12}N_2O_8FeNa$; 2.5 mg/l $Na_2MoO_4{\cdot}2H_2O$; 3.086 mg/l H_3BO_3; 1.180 mg/l

$MnSO_4 \cdot 4H_2O$; 1.404 mg/l $CoSO_4 \cdot 7H_2O$; 1.244 mg/l $CuSO_4 \cdot 5H_2O$; 1.430 mg/l $ZnSO_4 \cdot 7H_2O$; add 15 g/l agar for solid medium) or *TAP medium* (*see* **Note 2**) (400 mg/l NH_4Cl; 50 mg/l $CaCl_2 \cdot 2H_2O$; 100 mg/l $MgSO_4 \cdot 7H_2O$; 181.5 mg/l KH_2PO_4; 108 mg/l K_2HPO_4; 2420 mg/l Tris; 1 ml/l glacial acetic acid and the following microelements: 3.086 mg/l H_3BO_3; 1.18 mg/l $MnSO_4 \cdot 4H_2O$; 1.404 mg/l $CoSO_4 \cdot 7H_2O$; 1.244 mg/l $CuSO_4 \cdot 5H_2O$; 1.430 mg/l $ZnSO_4 \cdot 7H_2O$; 1.84 mg/l $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; 18 mg/l $C_{10}H_{12}N_2O_8FeNa$; 50 mg/l EDTA; add 15 g/l agar for solid medium) can be used.

For subculturing of *Scenedesmus quadricauda, ½ SS* (2020 mg/l KNO_3; 11 mg/l $CaCl_2 \cdot 6H_2O$; 988 mg/l $MgSO_4 \cdot 7H_2O$; 340 mg/l KH_2PO_4; 140 mg/l K_2HPO_4 and the following microelements: 3.086 mg/l H_3BO_3; 1.18 mg/l $MnSO_4 \cdot 4H_2O$; 1.404 mg/l $CoSO_4 \cdot 7H_2O$; 1.244 mg/l $CuSO_4 \cdot 5H_2O$; 1.430 mg/l $ZnSO_4 \cdot 7H_2O$; 1.84 mg/l $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; 18 mg/l $C_{10}H_{12}N_2O_8FeNa$; adjust pH by adding 1.5 ml/l 1 N NaOH after autoclaving; add 15 g/l agar for solid medium) is generally used.

For media preparation, 100× concentrated stock solutions of macroelements and 1000× concentrated stock solutions of microelements were used. All components were diluted in distilled water and autoclaved.

2.3 Growth Chamber and Growth Vessel

To synchronize cells, a growth chamber with stable temperature and switchable light source is required, and an optional source of CO_2 will significantly enhance synchronization quality. Optimal growth rates and synchronization can be reached by cultivation in a temperature controlled aquarium illuminated from one side by a high intensity light source (fluorescent tubes, LEDs) (*see* **Note 3**). The cultures are placed in cylindrical glass vessels of different volumes (300, 150, 50, and 10 ml) with a maximum diameter of the vessel being 25–30 mm (*see* **Note 4**). In order to reach the optimal growth rate, cultures are supplied with a source of CO_2, either by bubbling with a mixture of CO_2 (*see* **Note 5**) in air or by addition of sodium bicarbonate to 1 g/l (*see* **Note 6**) combined with bubbling by air or shaking.

2.4 Subculturing

For routine subculture, algal cultures should be transferred onto new plates every 3 weeks, and then allowed to grow for about a week on a light shelf (100 μmol/m^2/s, 25 °C, continuous light). Alternatively, cultures can be recovered from liquid nitrogen (*see* **Note 7**).

2.4.1 Storage in Liquid Nitrogen and Culture Recovery

1. Grow *Chlamydomonas* cells for 2–3 days to a minimal density of 10^6 cells/ml in either TAP or HS medium (for *Scenedesmus* use ½ SS medium) in a cylindrical vessel with continuous light at 30 °C and aeration with a mixture of air and CO_2 (2 % v/v). Check cell density by counting in a Bürker chamber.

2. Pellet 10 ml of cells by centrifuging for 3 min at 2500×*g* and resuspend them in 1 ml of HS medium (for *Scenedesmus* use ½ SS medium).
3. To a Nunc style cryotube, add 250 μl of appropriate growth medium containing 6 % (v/v) methanol (it is also possible to use DMSO at the same concentration) and add an equal volume (250 μl) of cells.
4. Place the cryotubes in a cooled isopropanol-containing Nalgene cryo 1 °C freezing container and put the cryo-container into a −80 °C freezer. Leave the cryo-container in the freezer for about 65–72 min.
5. Remove the cryotubes from the cryo-container and transfer them immediately to liquid nitrogen. Store under liquid nitrogen.
6. To thaw the frozen cultures put the cryotubes in a 35 °C water bath for 2 min with gentle shaking.
7. Transfer the cells to 10 ml of appropriate growth medium and cultivate them on a light shelf (100 μmol/m^2/s, 25 °C, continuous light) for 6–18 h before plating.

3 Methods

3.1 Basic Synchronization of Chlamydomonas-reinhardtii

1. Inoculate about a half-loop of a selected *Chlamydomonas* strain from an agar plate to 300 ml of fresh liquid HS medium in a glass cylindrical vessel.
2. Place the vessel into a temperate aquarium at 28–30 °C (*see* **Note 8**) illuminated with light of intensity 500 μmol/m^2/s (*see* **Note 3**) and aerate the liquid culture with 2 % (v/v) of CO_2 in the aeration mixture (*see* **Note 5**).
3. Set the light timer to 13 h of light and 11 h of darkness (*see* **Note 9**) under the conditions described above to obtain a synchronized culture, creating 8 daughter cells from one mother cell.
4. Grow the cells without dilution for two to three cell cycles (until culture density reaches approximately 10^6 cells/ml). Then maintain dilution to 10^6 cells/ml at the beginning of the light period to keep them synchronous; use the cells as soon as possible (*see* **Note 10**). Maintain this cell density during the entire synchronization process and thereafter during the experiments.
5. The entire synchronization process takes about 3 days under the conditions described above. However, the length of cultivation varies with the *Chlamydomonas* strain.

3.1.1 Analysis of Commitment Point Attainment

1. Take 1 ml samples hourly from the liquid synchronous culture, from the beginning of the light period until darkening.
2. Spread the sample immediately and quickly onto a plate containing HS medium solidified with 1.5 % agar. Carefully remove all remaining liquid using tissue paper (*see* **Note 11**).
3. Incubate the plate in the dark at 30 °C (or at other defined temperature according to the experimental protocol) for a minimum of 12 h (usually until 24 h from the beginning of the cell cycle).
4. Fix the samples on bottom-up plates by putting a drop of Lugol solution (5 % KI, 2.5 % I) onto the lid of a petri dish. The evaporating iodine will fix the cells. The fixed plates can be stored for a couple of days at 4 °C.
5. Using the light microscope, count the number of non-divided mother cells and mother cells that have divided into 2, 4, 8, or 16 daughters (*see* **Note 12**).
6. To obtain the commitment curves, plot the cumulative percentages of cells dividing into 2, 4, and 8 cells as a function of sampling time.

3.1.2 Analysis of Nuclear Division by Staining with SybrGreen

1. Pipette 1 ml of culture into a test tube.
2. Spin at 3000 × *g* for 3 min.
3. Wash three times with growth medium containing 0.01 % SDS to remove cell debris and contaminating bacteria.
4. Pipette 25 μl of suspension into a test tube, add 12.5 μl SybrGreen (1 % SybrGreen in TBE, pH 7.5) (2:1).
5. Mix well.
6. Leave in the dark at room temperature for 2–12 h (*see* **Note 13**).
7. Spin down and aspirate the supernatant.
8. Add PBS 1:1 and analyze by fluorescence microscopy using 460–490 nm excitation and 510–550 emission filters or their equivalent.

3.1.3 Analysis of Cellular/Chloroplast Division

1. Take 1 ml samples hourly over the entire experimental period.
2. Fix each sample immediately by adding 10 μl of Lugol solution.
3. Count the proportion of mother cells, dividing chloroplasts, sporangia and daughter cells using a light microscope.
4. To obtain cell division and daughter cell release curves, plot the cumulative percentages as a function of sampling time.

3.1.4 Analysis of Cell Growth by Measuring Changes in Cell Size

1. Take 1 ml samples hourly from a liquid synchronous culture over the entire experimental period.
2. Fix each sample immediately by adding 100 µl of 2.5 % glutaraldehyde to a final concentration 0.25 % (w/v).
3. Dilute 50–100 µl (according to cell concentration) of fixed cells with densities ranging from 10^6 to 10^7 cells/ml in 10 ml of electrolyte solution (0.9 % (w/v) NaCl).
4. Measure cell concentrations and cell size distributions using a Multisizer 3 (Beckman Coulter) or equivalent.

3.2 Basic Synchronization of *Scenedesmus quadricauda*

1. Inoculate about a half-loop of *S. quadricauda* from an agar plate into 300 ml of fresh liquid ½ SS medium in a glass cylindrical vessel.
2. Place the vessel into a temperate aquarium at 30 °C [4, 5] illuminated by light of intensity 560 µmol/m^2/s (*see* **Note 3**) and aerate the liquid culture with 2 % (v/v) CO_2 in the aeration mixture (*see* **Note 5**).
3. Set the light timer to 15 h of light and 9 h of darkness (*see* **Note 9**) under the conditions described above, to obtain a synchronized culture creating 8 daughter cells from each cell of the mother coenobium.
4. Grow the cells without dilution for three cell cycles and at the beginning of the next light period, dilute them to an initial density of 10^6 cells/ml (*see* **Note 10**). Maintain the cell density at this level over the entire synchronization process and thereafter during the experiment.
5. The entire synchronization process involves 3 days of cultivation under the conditions described above.

3.2.1 Analysis of Commitment Point Attainment

1. Take 10 ml samples hourly from the synchronous culture, from the beginning of the light period until darkening.
2. Cultivate the samples in a small aerated 15 ml glass test tube in the dark, at least until division is complete in the main experimental culture, or slightly longer (usually 24 h from the beginning of the cell cycle).
3. Take 1 ml samples from this dark incubated culture and proceed for nuclear staining with DAPI (Subheading 3.2.2).
4. Fix another 1 ml of the dark incubated culture with 10 µl of Lugol solution.
5. In the sample from **step 1**, count the number of non-divided mother cells and mother cells divided to 2, 4, 8, or 16 daughters using a light microscope.
6. To obtain the commitment curves, plot the cumulative percentages of cells dividing into 4 and 8 cells as a function of

sampling time. The number of cells dividing into 2 equals the number of mother cells with 2 nuclei from **step 3**.

3.2.2 Analysis of Nuclear Division by Staining with DAPI (4′,6-Diamidino-2-Phenylindole)

1. Pipette 1–1.5 ml of culture into a test tube.
2. Spin at 3000 × *g*, aspirate the supernatant leaving about 15–20 μl behind.
3. Freeze the sediment at –20 °C.
4. To the frozen sample add DAPI (5 μg/ml in 0.25 % (w/v) sucrose, 1 mM EDTA, 0.6 mM spermidine, 0.05 % (v/v) mercaptoethanol, 10 mM Tris–HCl, pH 7.6) (1:1) (*see* **Note 14**).
5. Mix well by vortexing.
6. Leave in the dark, at room temperature, for 20–30 min (*see* **Note 13**).
7. Spin down and aspirate the supernatant.
8. Add PBS 1:1 and analyze by fluorescence microscopy with 360–370 nm excitation and 420–460 emission filters or their equivalent.
9. Count the number of nuclei in mother cells and record the proportion of mother cells with different numbers of nuclei.
10. To obtain the nuclear division curves, plot the cumulative percentages of cells with 1, 2, 4, or 8 nuclei as a function of sampling time.

3.2.3 Analysis of Cellular/Chloroplast Division

1. Take 1 ml samples hourly over the entire experimental period.
2. Fix each sample immediately by adding 10 μl of Lugol solution.
3. Count the proportion of mother cells, dividing chloroplasts, sporangia and daughter cells, using a light microscope.
4. To obtain cell division and daughter cell release curves, plot the cumulative percentages as a function of sampling time.

3.2.4 Analysis of Cell Growth by Measuring Changes in Cell Size

1. Take 1 ml samples hourly from a liquid synchronous culture over the entire experimental period.
2. Fix each sample immediately by adding 100 μl of 2.5 % glutaraldehyde to a final concentration 0.25 % (w/v).
3. Dilute 50–100 μl (according to cell concentration) of fixed cells with densities ranging from 10^6 to 10^7 cells/ml in 10 ml of electrolyte solution (0.9 % (w/v) NaCl).
4. Measure cell concentrations and cell size distributions using a Multisizer 3 (Beckman Coulter) or equivalent.

3.3 Modifications of Basic Synchronization

The cell cycle of algae dividing by multiple fission, especially that of *S. quadricauda* and algae dividing by the same mechanism, is extremely complex. Therefore it is sometimes beneficial to simplify cell cycle progression by uncoupling cell and chloroplast division

cycles or nuclear and cell divisions. This can be achieved by using different specific inhibitors or different kinds of stress. Such treatments offer a tool to separately study the processes of nuclear, cell, and chloroplast divisions and are extremely useful as long as proper controls are employed.

3.3.1 Separation of Cell and Chloroplast Division Cycles

1. Follow the basic protocol for S. quadricauda synchronization up to **step 5**.
2. Add 5-fluorodeoxyuridine to a final concentration of 25 mg/l at the beginning of the cell cycle (*see* **Note 15**).
3. Cultivate cells in the light for 10 h (the time they undergo commitment into division into 8 daughter cells).
4. Follow growth and chloroplast DNA replication hourly by protocols 3.2.2 and 3.2.4.
5. Alternative step: Put cells into darkness after 10 h of light and follow chloroplast division by light microscopy (or fluorescence microscopy if you use tagged fluorescent protein) (*see* **Note 16**).
6. Alternative step: Leave cells in the light to follow cell growth, chloroplast DNA replication and nucleoid (chloroplast "nucleus") divisions further (*see* **Note 17**).
7. Alternative step: Remove the inhibitor by washing the culture with three culture volumes of fresh medium on a filtering unit or by three successive centrifugations and washes (*see* **Note 18**). Follow the fast nuclear and cellular divisions resembling the *Chlamydomonas* cell cycle using protocols 3.2.2 and 3.2.3.

3.3.2 Separation of Nuclear and Cell Divisions

1. Follow the basic protocol for *S. quadricauda* synchronization up to **step 5**.
2. Add cadmium to a final concentration of 25 μM at the beginning of the cell cycle (*see* **Note 19**).
3. Follow nuclear division in the absence of cellular division using protocol 3.2.2 (*see* **Note 20**).

4 Notes

1. We have been routinely using HS medium of slightly different composition to that described originally [6]. The medium contains a higher concentration of calcium (2×) and magnesium ions (10×). While this change in medium composition does not affect growth rate, we found that the increased concentrations improved separation of daughter cells from a single mother cell. For reasons of convenience, we have used one solution of microelements for all media (HS, TAP and ½ SS);

since the concentration of microelements is not limiting, this does not affect cell growth compared with *Chlamydomonas* medium prepared using Hutner's elements.

2. For synchronization experiments, HS medium should be used since it is the only mineral medium that permits solely autotrophic growth. This is important since in TAP medium, the daughter cells are able to grow, even in the dark, due to the presence of acetate as a carbon source. This intrinsically decreases cell synchrony that is obtainable in the culture.
3. Light intensity is one of the critical factors affecting synchrony. Limiting light will significantly affect growth rate and thus the timing of cell division. With increasing light intensity, growth rate increases and the length of the cell cycle shortens; in *C. reinhardtii,* at a light intensity of 250 μmol/m^2/s cycle length is 15 h. An increase in growth rate also leads to an increase in the number of daughter cells. At a low light intensity (28 μmol/m^2/s), *C. reinhardtii* mother cells each divided into two daughter cells, but at a high light intensity (250 μmol/m^2/s), 8 daughter cells were formed [7]. The situation is similar in *S. quadricauda* [4]. Thus, in order to attain high growth rates and high levels of synchrony, light intensities from 250 to 500 μmol/m^2/s are optimal. Cells can be synchronized at lower light intensities but then the light period will need to be prolonged and the protocol will need to be optimized for different light intensities. Also the obtainable synchrony of the culture could be lower.
4. The shape of the vessel is also important for attaining the highest light intensity and uniformity of illumination throughout the culture. Cylindrical vessels of diameter of 25–30 mm and variable length, or plan-parallel cuvettes with similar widths are optimal for this purpose, however, they are not always readily available. Erlenmeyer flasks can also be used provided it is understood that cells in any part of the flask should not be in a layer thicker than 20 mm otherwise light intensity should be increased. Any vessel used for cultivation should be mixed by shaking or optimally bubbled to prevent the cells from sedimenting.
5. The supply of CO_2 is another critical factor in achieving high growth rates that are critical in obtaining synchronous cultures. In principle, any concentration of CO_2 above that which is present in air will increase growth rate as long as no other limiting growth factor (low light intensity, low or high temperature) is present. A CO_2 concentration in the range of 1.5–2 % (v/v) seems to be a sufficient. Higher concentrations of CO_2 can be used since the CO_2 is dissolved in the medium

only to saturation, so any amount of CO_2 above that will leave the mixture.

6. Addition of sodium bicarbonate mimics the presence of CO_2 dissolved from the aeration mixture. It can be used if no other CO_2 supply is available. However, it should be kept in mind that at a concentration 1 g/l it can only support about one cell cycle of culture at a cell density 10^6 cell/ml so it would need to be added with each dilution of the culture.
7. Storage in liquid nitrogen is especially beneficial for maintaining collections of mutants not currently used since it prevents mutant strains from reversion, which could happen during prolonged routine sub-culturing.
8. Depending on growth conditions (light source, vessel, etc.) different optimal temperatures for *C. reinhardtii* were found, ranging from 35 °C [3] to 28 °C [8] Under our conditions, 28–30 °C was the most stable and reliable. Decreasing the temperature below optimal led to a prolongation of the cell cycle, similarly increasing the temperature eventually prolongs the cell cycle. The limiting temperature for *Chlamydomonas* is 39 °C, where cells are still able to grow but division is blocked completely [8].
9. The length of the light period is important for obtaining sufficient growth during the light-dependent G1 phase. If conditions of synchronization are not optimized for the particular cultivation set up, a suitable time for darkening the cells will need to be established. It should be chosen as the time when about 10 % of cells started their first protoplast fission. The length of the dark period is chosen to allow all cells of the population to release their daughters. A slight prolongation of the dark will not affect culture synchrony but will give the cells more time to finish daughter release. However, a prolonged dark incubation should be avoided, especially for *C. reinhardtii,* since cells in the dark may exhaust their energy reserves due to extensive usage of flagella for swimming. Prolonging the dark period is the simplest trick to shift the beginning of the cell cycle to a later time if required. The dark period can be also shortened as long as it is kept in mind that *Chlamydomonas* cells will completely divide and release after at least 6 h of darkness.
10. Too high a cell density will cause shadowing of the cells, and an uneven illumination of the culture will lead to an uneven growth rate, an uneven time of cell cycle duration, and thus to lower synchrony of the culture. Dilution of the culture can be done at the beginning of the light period, as suggested, or later in the cell cycle at 6–8 h. Dilution should be avoided when the culture is about to divide or is dividing since it will

affect this process. However, the culture can be again diluted once the division/s are complete, which is akin to dilution of newborn daughter cells at the beginning of the light period.

11. An alternative method is to take 10 ml samples hourly and cultivate them, in the dark, in a small aerated 15 ml glass test tube until the 10th hour from the beginning of the cell cycle and then spread them onto HS agar plates as described in **step 2**. This modification helps to overcome common problems with over-drying of the culture on the plate, which will affect their ability to divide. When spreading on the plate it is also crucial to avoid leaving any liquid since even a small amount of liquid will allow the newborn daughter cells to swim around leading to clumps of cells without a tractable relationship to their mother cells.
12. Cells that passed their commitment point for cell division form microcolonies of daughter cells. The number of daughter cells in a colony indicates the number of commitment points passed by a mother cell [9].
13. The length of incubation depends on the species and the stage of the cell cycle. Completion of staining should be checked by fluorescence microscopy before proceeding to the next step.
14. Freezing of samples and defrosting in the presence of dye helps the penetration of dye into the cells.
15. The inhibitor will block nuclear DNA replication and consequently nuclear and cellular divisions [10–12] while growth and chloroplast DNA duplication will follow normally.
16. In the dark, chloroplasts will eventually divide, while nuclear DNA, nuclear division, and cell divisions remain blocked.
17. If the culture is left in the light, chloroplast division will not occur; only growth will continue for at least 36 h.
18. The inhibitor specifically blocks thymidylate synthase [10–12] and its effect can also be abolished by addition of excess thymidine.
19. At this concentration, cadmium will specifically block cellular division [13] while nuclei will divide normally.
20. Alternatively, higher concentrations of cadmium (50 μM) will block all but the first nuclear division.

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Chapter 2

Plant Cell Division Analyzed by Transient *Agrobacterium-Mediated* Transformation of Tobacco BY-2 Cells

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Abstract

The continuing analysis of plant cell division will require additional protein localization studies. This is greatly aided by GFP-technology, but plant transformation and the maintenance of transgenic lines can present a significant technical bottleneck. In this chapter I describe a method for the *Agrobacterium-mediated* genetic transformation of tobacco BY-2 cells. The method allows for the microscopic analysis of fluorescence-tagged proteins in dividing cells in within 2 days after starting a coculture. This transient transformation procedure requires only standard laboratory equipment. It is hoped that this rapid method would aid researchers conducting live-cell localization studies in plant mitosis and cytokinesis.

Key words *Agrobacterium*, Transient transformation, Tobacco BY-2, Cell division, Green fluorescent protein, F-actin, Cytokinesis

1 Introduction

GFP (green fluorescent protein) technology has been invaluable in respect to analyzing the molecular dynamics of mitosis and cytokinesis. Membranes, the cytoskeleton, and DNA have been labeled during cell division and their behavior has been analyzed [1]. As more and more regulatory molecules become uncovered there is an increasing demand to localize and co-localize proteins during the process of cell division. The plant cell suspension line tobacco BY-2 has been especially useful in terms of analyzing protein localization and dynamics during cell division [2–4]. The tobacco BY-2 model has a number of benefits as a tool: it is easy to maintain and is fairly robust to handling variations; it is cycling fast and cell cycle synchronization protocols are available; most of its cells are clearly polarized; all cells form a preprophase band of microtubules before division; and—importantly—its cells are well suited for high-end microscopy. The latter is because the tobacco BY-2 line does not tend to form cell clumps, the cells are nearly colorless,

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nonfluorescent, and, in contrast to epidermal tissues from real plants, do not form a cuticle [5].

The method described in this chapter (coined the "TAMBY2 method") aims at transiently transforming dividing tobacco BY-2 cells through *Agrobacterium* [6]. Transient transformation systems for plants have been extremely popular during the last two decades [7–10]. Especially the method of infiltrating tobacco leaves (usually this employs *N. benthamiana*) with a dilute suspension of *Agrobacterium* has been adopted by laboratories all over the world. A major drawback of this method is that only interphase cells can be analyzed. Cell divisions cannot be found when searching for GFP-expression among epidermal cells. The TAMBY2 method on the contrary allows for analyzing transiently transformed cells engaging in mitosis and cytokinesis. A number of vectors and *Agrobacterium* strains have recently been shown to work sufficiently well in the TAMBY2 method [6].

The TAMBY2 method is based on producing a coculture of tobacco BY-2 with *Agrobacterium* in which both organisms are quickly proliferating. Importantly, the coculture is maintained on solidified culture medium (using the agar-substitute Phytagel). Previous testing suggested that this produces transformation rates superior over those obtained in liquid cocultures [6]. The method is also quick: after establishing the coculture through mixing *Agrobacterium* with tobacco BY-2 it requires only 2 days until GFP-expressing dividing cells can be observed.

Transient *Agrobacterium*-mediated transformation systems can produce artifacts. Protein overexpression as such can lead to protein mistargeting and to malfunction of the cellular machinery (e.g.) [11]. On the other hand there are a number of advantages. Transient *Agrobacterium*-mediated transformation is fast, allows for the quick subcellular localization of numerous proteins and their fragments, facilitates protein interaction studies, is capable of overcoming gene silencing mechanisms, etc. For every experimental purpose the researcher needs to balance these pros and cons. As other overexpression-based techniques, the TAMBY2 method yields cells with varying expression levels. Choosing cells with lower transgene expression for microscopic analysis (i.e., cells showing less fluorescence) may yield results that are more reliable.

For this method chapter I have chosen the transient CaMV35S-driven overexpression of the ABD2-GFP marker, a truncation of AtFimbrin1 coupled to GFP. Micrographs of F-actin in stably transformed tobacco BY-2 cells based on ABD2-GFP expression have been published and may serve the comparison [12]. The main challenge of the fast TAMBY2 method is to control the amount of active *Agrobacterium* in the coculture. It is recommended to check for *Agrobacterium* over-proliferation and for

attachment of *Agrobacterium* to tobacco BY-2 cells in the coculture by microscopic means. *Agrobacterium* cells of strain LBA4404 carrying the CaMV35S::ABD2-GFP construct published by Wang et al. 2008 are themselves highly GFP-fluorescent. The reason for this is unknown, but I speculate that this may arise from a hypothetical, cryptic promoter or activator in within the *Arabidopsis* ABD2 fragment. Whatever the reason for the fluorescence trait, I noticed that it helps to estimate the viability and proliferation rate of *Agrobacterium* in the coculture. It can also help to determine how many *Agrobacterium* cells have actually attached to the tobacco BY-2 cells. I hope that this strategy (i.e., the use of fluorescent *Agrobacterium* LBA4404 carrying the ABD2-GFP marker) will help researchers in remote laboratories to establish the TAMBY2 method.

2 Materials

2.1 Equipment

1. Photometer.
2. Plastic cuvettes (1 ml).
3. A shaker for tobacco BY-2 liquid cultures. The shaker was equipped for carrying Erlenmeyer flasks (culture volume 100 ml). The shaker was set to 150 rotations per minute (rpm).
4. A tobacco BY-2 incubation chamber, which was set to 25 °C and 65 % humidity. The chamber was used for the tobacco BY-2 shakers and for incubating the Phytagel plates containing the cocultures of tobacco BY-2 with *Agrobacterium*.
5. A temperature-adjustable shaker for liquid cultures of *Agrobacterium*. These were grown at 28 °C and 200 rpm.
6. A laser scanning confocal microscope (LSM). An attached mercury lamp is especially helpful when screening for transformed cells with the eyepieces.
7. Microcentrifuge.
8. Micropore tape (3 M Micropore; 1530-0). This was used to wrap plates containing tobacco BY-2/*Agrobacterium* cocultures.
9. A pair of strong scissors was used to cut-off micropipette tips.
10. Standard lab plasticware and adjustable pipettes and micropipettes are required.

2.2 Plasmid and Agrobacterium Strain

1. *Agrobacterium* LBA4404 (pBBR1MCSvirGN54D).
2. This *Agrobacterium* stock was previously transformed with vector ABD2-GFP in pCAMBIA1390 [6, 13]. The resulting strain is maintained in LB using 50 μg/ml Rifampicin, 75 μg/ml

Chloramphenicol, Kanamycin A, 50 μg/ml. The pCAMBIA1390 backbone confers Kanamycin resistance to bacteria. The pCAMBIA1390/ABD2-GFP plasmid allows overexpression of ABD2-GFP (green fluorescent protein coupled to the actin-binding domain 2 of AtFimbrin1) from the CaMV35S promoter.

2.3 Media and Chemicals

1. Pauls's medium (per liter): Murashige and Skoog (MS) without vitamins (4.3 g), 10 g sucrose. The pH was adjusted to 5.8 and the medium was autoclaved. Solid medium required addition of 5 g Phytagel per liter (Sigma, P8169) (*see* **Note 1**). No antibiotics were added to Paul's medium.
2. BY-2 medium (per liter): MS without vitamins (4.3 g), 30 g sucrose, 200 mg KH_2PO_4, 100 mg Inositol, 1 mg Thiamine, 0.2 mg 2,4-Dichlorophenoxyacetic acid (2,4-D). The pH was adjusted to 5.8 and autoclaved.
3. Luria-Bertani (LB) medium (per liter): 10 g NaCl, 10 g Tryptone, 5 g yeast extract, pH 7.0. The medium was autoclaved. Solid LB contained 1 % Kobe agar (Roth, Kobe I: 5210.2). Antibiotics were added after autoclaving (cooled to 60 °C in case of LB with agar).
4. Antibiotics: Rifampicin was dissolved in DMSO (dimethylsulfoxide) at 100 mg/ml. Chloramphenicol was dissolved in ethanol at 50 mg/ml. Kanamycin A was dissolved in H_2O at 100 mg/ml.

2.4 Plant Material

1. Tobacco *bright yellow-2* (BY-2) suspension culture. I employed a non-transgenic line obtained from John Innes Centre, UK.

3 Methods

1. A wild-type (i.e., non-transgenic) tobacco BY-2 culture is subcultured (5 ml per 100 ml fresh medium) and grown for 3 days.
2. A liquid culture of *Agrobacterium* harboring the desired vector (here: LBA4404 carrying the ABD2-GFP expression plasmid) is initiated 2 days later using LB medium including antibiotics and grown overnight (to saturation).
3. This overnight culture is diluted 1 in 20 on the next morning using fresh medium (including antibiotics). Grow for several hours. To confirm that the culture is growing well, check $O.D._{600}$ occasionally.
4. In the meantime, wash and concentrate the 3-day-old tobacco BY-2 culture. Use 50 ml of fresh culture and leave cells to sediment in a 50 ml tube (at 1 g; no centrifuging) (Fig. 1a). Decant medium and replace with Paul's medium. Leave cells

to sediment again. Finally remove supernatant to leave cells in a total volume of 10 ml. This equals a fivefold concentrated BY-2 culture (*see* **Note 2**).

5. Harvest *Agrobacterium* when having reached an $O.D._{600}$ of approximately 0.8 (*see* **Note 3**). This can be done in a microcentrifuge at 10,000 × *g* for 3 min at 18 °C using 1.5 ml reaction tubes. Pellet 1 ml *Agrobacterium* cells for each construct. Remove supernatant and replace with 30 µl Paul's medium. Redissolve *Agrobacterium* (*see* **Note 4**).
6. Prepare the coculture. For this add 1 ml of fivefold concentrated tobacco BY-2 cells (*see* **Note 5**) to 30 µl of redissolved *Agrobacterium*. This can be done in the reaction tube used for collecting *Agrobacterium*. Thoroughly but carefully mix *Agrobacterium* with tobacco BY-2. Do not vortex.
7. Now apply individual droplets of this mixture to solid Paul's plates (Fig. 1b). Seal with Micropore tape (*see* **Note 6**).

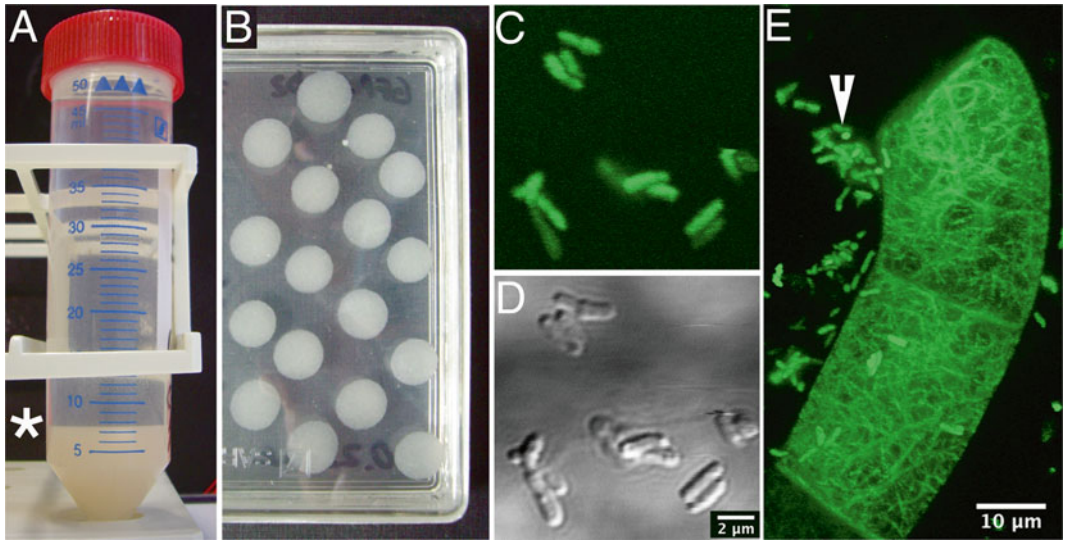

Fig. 1 Important steps and typical results when applying the TAMBY2 method. (**a**) After sedimenting 50 ml of a 3-day-old tobacco BY-2 culture a precipitate of about 7–8 ml tobacco BY-2 cells is obtained (*asterisk*). These cells are then washed and subsequently resuspended in 10 ml Paul's medium. (**b**) Single droplets of the coculture of tobacco BY-2 with *Agrobacterium* were applied to solid Paul's medium. Cultivation on solid medium boosts transfection. (**c**) The cells of LBA4404 carrying pCAMBIA1390/ABD2-GFP are themselves fluorescent. (**d**) Bright-field picture of the *Agrobacterium* cells seen in (**c**). (**e**) Typical result obtained after 2–3 days of incubation. The projection of a confocal *z*-stack shows a tobacco BY-2 cell with labeled F-actin. *Agrobacterium* is fluorescent as well (*arrowhead*) and some of its cells are directly attached to the plant cell. Scale bars: (**c**, **d**) 2 µm; (**e**) 10 µm

8. Incubate at 25 °C in the dark for 2 days.
9. After 2 days, scrape off a small amount of tobacco cells from the coculture plate (solid Paul's medium) and mix this with a drop of Paul's medium on a microscope slide. Apply cover slip and mount slide on confocal microscope.
10. Observe tobacco BY-2 cells (using the 63× oil objective) in bright-field. Observe *Agrobacterium*, some of which are attached to the BY-2 cell's surface.
11. Switch to FITC mode (using the mercury lamp). Observe fluorescent bacteria (Fig. 1c, d; *see* **Note 7**). Screen for tobacco BY-2 cells with labeled F-actin (Fig. 1e). Up to 12 % of tobacco BY-2 cells can show fluorescence. About 10 % of the tobacco BY-2 cells will be in cytokinesis [6].
12. Switch to LSM mode. It is easy to spot cells engaging in mitosis (Fig. 2a–f).
13. The steps described above line out the transient transformation technique. However, stably transformed BY-2 lines can be obtained after transferring the tobacco cells to solid BY-2 medium containing appropriate antibiotics [6].

4 Notes

1. Phytagel is not agar-based, but is a bacterial product. Phytagel does not solidify when reheated. Therefore, plates must be poured just after autoclaving (however, the medium can be kept at 60 °C for a few hours). After pouring, Paul's medium plates were kept with lid half open for 10 min. The plates were then closed and left to solidify.
2. When sedimenting tobacco BY-2 (50 ml cell suspension are sedimented in a 50 ml tube at 1 g; centrifuging is not required) white-yellow tobacco cells accumulate at the bottom of the tube while forming a clear supernatant. This takes about 15 min. When using a 3-day old tobacco BY-2 culture this loose cellular "pellet" typically has a volume of 7–8 ml (Fig. 1a). Now discard the supernatant and replace with Paul's medium. Leave cells to sediment again. Discard 40 ml of the supernatant. The tobacco BY-2 cells are now washed and five-fold concentrated and ready to use (however, repeated washing with Paul's medium was shown to increase transformation in some cases) [6].
3. It is essential for the method that *Agrobacterium* is in perfect physiological condition. This means that *Agrobacterium* LB-cultures should be growing fast. To make sure, check with photometer. A doubling-time of ≤2 h is to be expected.

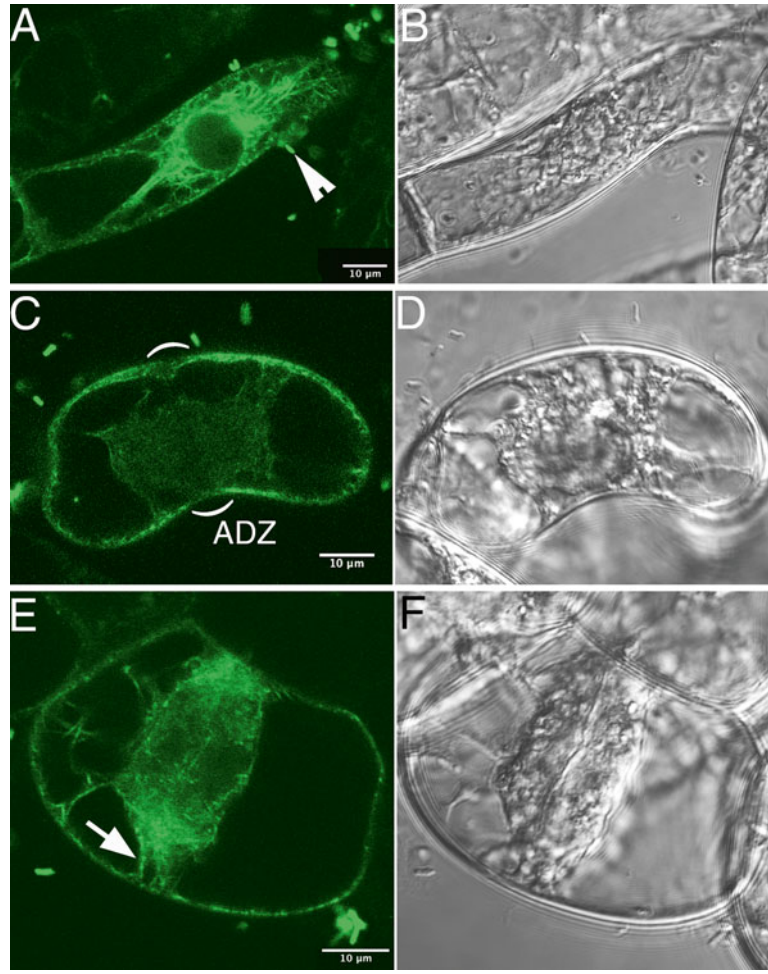

Fig. 2 Transient transformation with CaMV35S::ABD2-GFP yields labeled F-actin in dividing tobacco BY-2 cells. (**a**) An elongated tobacco BY-2 cell with attached *Agrobacterium* (*arrowhead*). The plant cell nucleus is surrounded by fluorescent F-actin and is suspended in the cell's center. Cortical F-actin appears to gather around the nucleus possibly indicating preprophase band formation. (**b**) Bright-field picture of (**a**). (**c**) A transformed cell photographed in metaphase. Note actin-depleted zone (ADZ) near the spindle equator (brackets indicate the region of decreased cortical F-actin density). (**d**) Bright-field picture of (**c**). (**e**) F-actin in cytokinesis. The phragmoplast and cell plate are labeled by ABD2-GFP. Note strands of F-actin connecting the phragmoplast with the cortex (*arrow*). (**f**) Bright-field picture of (**e**) shows growing cell plate. All fluorescent micrographs are single *z*-sections. Scale bars: 10 μm

4. It is convenient to manipulate the amount of bacteria supplied at this stage. Too much *Agrobacterium* in the coculture will inhibit tobacco BY-2 cell division. Experiment by centrifuging smaller amounts of *Agrobacterium* at this stage: try 0.5, 0.25

and 0.125 ml. Simply remove supernatant, redissolve cell pellet in 30 µl Paul's medium and add 1 ml of the fivefold concentrated tobacco BY-2 suspension, as in the standard procedure.

5. Cut off tips from 1000 µl pipette tips using a pair of scissors before pipetting tobacco BY-2.
6. Paul's medium plates are wrapped with Micropore tape twice.
7. In case of LBA4404 carrying the CaMV35S::ABD2-GFP construct, nearly 100 % of *Agrobacterium* cells should be fluorescent in the 2-day-old coculture.

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Part II

Cell Cycle and Mitosis

Chapter 3

Identifying Gene Regulatory Networks in Arabidopsis by In Silico Prediction, Yeast-1-Hybrid, and Inducible Gene Profiling Assays

Erin E. Sparks and Philip N. Benfey

Abstract

A system-wide understanding of gene regulation will provide deep insights into plant development and physiology. In this chapter we describe a threefold approach to identify the gene regulatory networks in *Arabidopsis thaliana* that function in a specific tissue or biological process. Since no single method is sufficient to establish comprehensive and high-confidence gene regulatory networks, we focus on the integration of three approaches. First, we describe an in silico prediction method of transcription factor–DNA binding, then an in vivo assay of transcription factor–DNA binding by yeast-1-hybrid and lastly the identification of co-expression clusters by transcription factor induction in planta. Each of these methods provides a unique tool to advance our understanding of gene regulation, and together provide a robust model for the generation of gene regulatory networks.

Key words Gene regulatory networks, Transcription factor–DNA binding, In silico prediction, Yeast-1-hybrid, Inducible gene expression

1 Introduction

Transcriptional regulation of gene expression is central to the growth and development of all living things. Defining the core network of transcriptional interactions at the heart of specific biological processes is key to understanding how living systems develop and respond to perturbation. Prior to the "omics" era, core networks were determined one component at a time and built from the bottom up. More recently, core networks have been defined using genome-wide approaches and determined from the top down. Gene regulatory networks (GRNs) defined using genome-wide methods can provide a systems-level view of key regulators within a specific tissue or biological process (reviewed in ref. [1]). Established GRNs then allow the generation of defined

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hypotheses about the tissue or biological process of interest. While there are many approaches to generate and analyze GRNs [2], here we focus specifically on the integration of three methods in Arabidopsis.

In most organisms, limited system-wide resources mean that no single approach is sufficient to generate comprehensive and high-confidence GRNs, therefore the utilization of multiple complementary approaches is encouraged. In this chapter, we outline an approach to first predict potential transcriptional interactions *in silico* from binding motif data. Second, we describe a yeast-1-hybrid assay to validate these predictions *in vivo*. Lastly, we outline an induction and profiling method *in planta* by which the predicted transcription factor–DNA interactions can be utilized to identify clusters of similarly regulated genes that are likely involved in the same processes.

The effectiveness of *in silico* transcription factor–DNA binding predictions depends strongly on the quality of transcription factor binding site information, and predictions often exhibit high false-positive and false-negative rates. In plants, true-positive *in silico* predictions are especially difficult to refine due to the movement of transcription factors between cells [3]. In Arabidopsis roots, for example, the expression of transcription factors is often broader than that of their targets [4]. The second approach outlined here, yeast-1-hybrid, has the advantage of determining interactions that can occur *in vivo*. However, yeast-1-hybrid has a high false-negative rate. False-negatives can arise from improper folding of the transcription factor in yeast, or as was recently suggested, nucleosome occupancy of the promoter [5]. Together these approaches enable large-scale prediction and *in vivo* validation of transcription factor–DNA binding interactions that are possible, but do not give insight into which interactions occur *in planta* under specific conditions. Interactions can be tested *in planta* by checking expression levels of downstream genes in transcription factor mutants and/or by chromatin-immunoprecipitation(ChIP)-qPCR assays. We do not highlight ChIP assays here, because this methodology has been outlined in a recent paper [6]. Finally, GRNs can be expanded from the validated interactions by temporal transcriptional profiling of plants with inducible expression of transcription factors. Co-expression clusters are indicative of genes that function in the same biological process and can be used to define transcriptional regulatory modules [7]. When applied in combination, these approaches overcome their individual limitations to generate high confidence GRNs. While this chapter is written in a general sense, it is particularly amenable to the study of cell division. A defined morphological readout such as cell division can help to define genes as a starting point for building GRNs, and also to elucidate time points for analysis after transcription factor induction [8].

2 Materials

2.1 Cloning Materials

1. Gateway compatible cloning primers for promoters and coding sequence/open-reading frames (CDS/ORFs).
2. Proofreading Taq polymerase.
3. Standard gel extraction kit.
4. Gateway cloning kits for promoters (5′topo) and CDS/ORFs (D-topo) and *E. coli* competent cells (Life Technologies).
5. Luria Broth (LB) with antibiotic agar plates and liquid media.

   For 500 mL of LB in H_2O, 5 g NaCl, 5 g Tryptone, 2.5 g Yeast Extract, 7.5 g Agar (for plates only).

   Autoclave LB on the liquid setting for 20 min and cool to 55 °C before adding antibiotic (1:1000 stock concentration at 50 mg/mL Kanamycin, 75 mg/mL Ampicillin or other). For plates, pour directly after adding antibiotic.
6. Standard plasmid mini-prep kit.
7. Entry vector-specific sequencing and colony PCR primers:
   (a) M13F 5′-TGT AAA ACG ACG GCC AGT-3′.
   (b) M13R 5′-CAG GAA ACA GCT ATG ACC-3′.
8. LR Clonase II Single Site Gateway (Life Technologies).
9. pMW2, pMW3, pDESTAD-2 μm Gateway-compatible destination vectors [9, 10].
10. Destination vector-specific sequencing and colony PCR primers:
    (a) pMW2:M13FW (same as entry vector-specific).
    (b) pMW2:HIS293RV 5′-GGG ACC ACC CTT TAA AGA GA-3′.
    (c) pMW3:1HIFW 5′-GTT CGG AGA TTA CCG AAT CAA-3′.
    (d) pMW3:LacZ592RV 5′-ATG CGC TCA GGT CAA ATT CAG A-3′.
    (e) pDESTAD-2 μm:AD 5′-CGC GTT TGG AAT CAC TAC AGG G-3′.
    (f) pDESTAD-2 μm:TERM 5′-GGA GAC TTG ACC AAA CCT CTG GCG-3′.
11. pENTR/5′topo or pENTR/p4p1 Gateway promoter vector.
12. pENTR/D-topo or pENTR/p221 Gateway CDS/ORF vector.
13. pENTR/p2p3 Gateway glucocorticoid receptor (GR) vector.
14. Gateway-compatible plant binary vector.
15. LR Clonase II Plus Multi-site Gateway (Life Technologies).

2.2 Yeast Materials

16. YM4271 and Yα1867 yeast strains.
17. 1 M 3-amino-1,2,4-triazol (3AT), filter sterilized, stored at 4 °C.
18. YPDA liquid media: For 1 L in H_2O, 10 g Bacto yeast extract, 20 g Bacto peptone.
    Add 950 mL of H_2O, autoclave on the liquid cycle 20 min.
    After cooling add: 50 mL 40 % glucose, 12 mL of 40 mM adenine hemisulfate.
19. Minimal liquid yeast media with nutrient dropout: For 1 L in H_2O, 1.7 g Yeast nitrogen base, 5 g Ammonium sulfate, Yeast dropout supplement (amount varies by the amino acid being dropped out); pH to 5.9 and autoclave on the liquid setting for 20 min.
20. 100 mm yeast plates:
    (a) YPDA-agar: Add 40 g Bacto agar to YPDA recipe.
    (b) Minimal yeast media-agar + –His–Ura dropout: Add 40 g Bacto agar to minimal media recipe.
    (c) Minimal yeast media-agar + –Trp dropout: Add 40 g Bacto agar to minimal media recipe.
21. 150 mm yeast plates:
    (a) YPDA-agar.
    (b) Minimal yeast media-agar + –His–Ura dropout.
    (c) Minimal yeast media-agar + –His–Ura dropout + 1 M 3AT for 20, 40 and 80 mM final 3AT concentrations. Add 1 M 3AT immediately before pouring plates.
22. Nitrocellulose Hybridization and Transfer Membranes
23. Restriction enzymes: NcoI, EcoRV, AflII, NheI, and XhoI.
24. TE/LiAc/H_2O (10 mL total-make fresh).
    (a) 1 mL 10× Tris-EDTA (TE), autoclaved.
    (b) 1 mL 1 M Lithium Acetate (LiAc), autoclaved.
    (c) 8 mL Sterile water.
25. TE/LiAc/PEG (10 mL total-make fresh).
    (a) 1 mL 10× Tris-EDTA (TE), autoclaved.
    (b) 1 mL 1 M Lithium Acetate (LiAc), autoclaved.
    (c) 8 mL 50 % PEG-3350, filter sterilized. This high percentage PEG will take several hours to solubilize. For best results, make a fresh 50 % PEG solution every few months. Evaporation will increase the PEG concentration and adversely affect transformation efficiency.
26. Sheared salmon sperm DNA.
27. Replica-plating apparatus for 150 mm plates.

28. Plate roller.
29. Clean, autoclaved squares of approximately 23 cm × 23 cm velvet (available at any fabric store and cut to size).
30. 1 L of Z-Buffer in H_2O:
    16.1 g $Na_2HPO_4 \cdot H_2O$.
    5.5 g $NaH_2PO_4 \cdot H_2O$.
    0.75 g KCl.
    0.246 g $MgSO_4 \cdot 7H_2O$.
    Adjust pH to 7.0 and can be stored at room temperature for about 1 year.
31. 4 % X-gal in dimethylformamide (DMF), store at −20 °C.
32. X-gal in Z-Buffer (make fresh).
    6 mL Z-Buffer.
    11 μL β-mercaptoethanol.
    100 μL 4 % X-gal in DMF.
33. 150 mm Whatman filter papers.
34. Digital camera (tripod optional).
35. QIAprep Spin Miniprep Kit for Yeast Plasmid Preps.
36. Acid-washed glass beads.
37. 96-Well and/or 384-well culture plates.
38. Omni Trays:
    (a) YPDA-agar.
    (b) Minimal yeast media-agar + −His−Ura dropout.
    (c) Minimal yeast media-agar + −Trp dropout.
    (d) Minimal yeast media-agar + −His−Ura−Trp dropout.
    (e) Minimal yeast media-agar + −His−Ura−Trp dropout + 3AT concentration (as above).
39. 96-Well or 384-well replicator.

2.3 Plant Growth and Induction Materials

1. 50 % Bleach + 0.1% Tween 20 for seed sterilization.
2. 1× Murashige–Skoog (MS), 1 % sucrose, 1 % agar plates.
3. Nylon mesh to facilitate seedling transfer.
4. 1× MS, 1 % sugar, 1 % agar + 10 μM Dexamethasone (DEX) plates (make plates fresh).
5. Primers for qRT-PCR of transcription factor targets of interest.
6. RNA-sequencing or microarray tools (can be sent to a core facility for processing).

3 Methods

3.1 In Silico Prediction of Protein–DNA Interactions

1. Select promoter sequence from the gene(s) of interest (*see* **Note 1**). Genes of interest might include those expressed in a specific tissue or with a known role in a biological process (e.g., cell division).
2. Obtain transcription factor binding site information from available databases (*see* **Note 2**).
3. Binding predictions can be determined using the Finding Individual Motif Occurrences (FIMO) package [11] that is part of the MEME suite [12].
4. FIMO can be run directly from the website (http://meme.nbcr.net/meme/cgi-bin/fimo.cgi) with user input promoter sequence and transcription factor binding site information (*see* **Note 3**).

3.2 Enhanced Yeast-1-Hybrid Assay (Adapted from Deplancke et al. [10] and Gaudinier et al. [13])

3.2.1 Cloning of Promoter Constructs

1. Design Gateway-compatible primers to clone promoter sequence(s) of interest (*see* **Notes 4** and **5**).
2. PCR amplify promoter sequences from genomic DNA using a proofreading Taq polymerase and sufficient extension time to amplify the full promoter length.
3. Gel extract the PCR band corresponding to the promoter size and clone into a Gateway entry vector (*see* **Note 5**).
4. Transform Gateway reaction into *E. coli*, plate for selection on LB + antibiotic plates, then perform colony PCR to check for the promoter product of interest. Colony PCR should utilize one vector-specific primer (M13F or M13R for entry vectors) and one promoter-specific primer.
5. For colonies that were confirmed by PCR, grow 2 mL LB + antibiotic liquid cultures, isolate the plasmid and perform Sanger sequencing to confirm the full promoter sequence. Initial sequencing can be obtained by vector-specific M13F and M13R. After initial confirmation, the full sequence should then be verified by designing primers to walk through the missing regions. Depending on the sequence quality and promoter length, the additional number of primers will vary.
6. Upon successful cloning and sequence confirmation, promoters are transferred by single site LR Clonase II reactions into two Gateway-compatible destination vectors, pMW2 and pMW3. Cloning into pMW2 generates a promoter-His growth reporter, and pMW3 generates a promoter-LacZ reporter (*see* **Note 6**).
7. Perform colony PCR with one vector-specific primer (M13F and HIS293RV for pMW2 and 1HIFW and LacZ592RV for pMW3) and one promoter-specific primer to confirm insertion into the destination vectors.

3.2.2 Transformation, Auto-activation, and Confirmation of Promoters in Yeast

Day 1

1. Under sterile conditions, streak yeast strain YM4271 from glycerol stock onto a full nutrient YPDA-agar plate and incubate at 30 °C (*see* **Note 7**).
2. Grow 5 mL cultures of pMW2-promoter and pMW3-promoter constructs, if plasmid is not currently available. High concentrations (>150 ng/μL) of plasmid are preferable for yeast transformation.

Day 2

3. Under sterile conditions, start a 5 mL culture of YM4271 in YPDA liquid media from the yeast plate, and grow in a shaking incubator at 30 °C. If many promoters are being transformed multiple cultures may be required. Return the YM4271 plate to the 30 °C incubator for the following day.
4. If necessary, plasmid prep pMW2-promoter and pMW3-promoter constructs.
5. Look within each promoter sequence for the presence of the following restriction enzyme sites: NcoI, EcoRV, AflII, NheI, and XhoI.
6. Set up pMW2-promoter and pMW3-promoter plasmids for overnight restriction digest to linearize promoter-reporter vectors with enzymes that are not found in the promoter sequence. For pMW2 choose from: AlfII, NheI, or XhoI. For pMW3 choose from: NcoI or EcoRV (*see* **Note 8**).
7. For each digestion, use 14 μL plasmid, 2 μL of buffer, 2 μL of BSA or H_2O, and 2 μL of enzyme. Incubate overnight at recommended temperature. Please refer to the enzyme manufacturer for specific instructions.

Day 3

8. In the morning, prepare sterile flask with 50 mL of YPDA liquid media per three promoters. For optimum growth, cultures should be less than 250 mL total. If more volume is required, grow additional cultures.
9. Sterile inoculate the flask in 500 μL increments from the 5 mL YM4271 culture from the previous day to reach a starting OD600 of 0.1–0.2. If the culture is not sufficient to reach the starting OD600, yeast from the plate may be used to supplement (*see* **Note 9**).
10. Place flasks into shaking incubator at 30 °C for 3–5 h.
11. While yeast is growing, inactivate the enzyme digestions per the manufacturer's instructions.

12. Prepare fresh sterile solutions of TE/LiAc/H_2O and TE/LiAc/PEG for yeast transformation.
13. Turn on a heat block to 95 °C, and a water bath to 42 °C.
14. Continue to periodically monitor the yeast cultures until they reach an OD600 of 0.4–0.6.
15. Once cultures reach desired density, pour cultures into sterile 250 mL centrifuge bottles.
16. Centrifuge at 1800 × *g* for 5 min at room temperature.
17. While cultures are spinning, boil sheared salmon sperm DNA in the prepared 95 °C heat block for 5–10 min. After boiling place on ice until ready to use.
18. Carefully and quickly pour off the YPDA media from the centrifuge bottles to maintain the yeast pellet.
19. Resuspend the pellet with 10 mL of sterile water and transfer to a sterile 15 mL conical tube.
20. Repeat centrifugation at 1800 × *g* for 5 min at room temperature.
21. Carefully and quickly pour off the water from the conical tube to maintain the yeast pellet.
22. Resuspend the pellet with 5 mL of TE/LiAc/H_2O
23. Repeat centrifugation at 1800 × *g* for 5 min at room temperature.
24. Carefully and quickly pour off the TE/LiAc/H_2O from the conical tube to maintain the yeast pellet.
25. Resuspend the pellet in 100 μL of TE/LiAc/H_2O and 10 μL of boiled, sheared salmon sperm DNA per promoter and mix well.
26. Combine the pMW2-promoter and pMW3-promoter digestions into one tube and add 100 μL of yeast to the linearized plasmids.
27. Add 600 μL of TE/LiAc/PEG to each promoter tube and mix by carefully pipetting up and down.
28. Incubate at 30 °C for at least 30 min.
29. Heat shock tubes in 42 °C water bath for exactly 20 min.
30. Centrifuge tubes at maximum speed in a tabletop microcentrifuge for 5 s.
31. Remove supernatant, and resuspend each yeast pellet with 150 μL of sterile water.
32. Spread onto individual minimal yeast media –His–Ura plates. These plates contain all of the amino acids for yeast to grow, except Histidine (His) and Uracil (Ura). Only yeast that has incorporated both plasmids, pMW2 providing Uracil and pMW3 providing Histidine, will grow under these conditions.

33. Incubate plates at 30 °C for 3–5 days.

Day 6–8

34. Patch 8 yeast colonies per promoter onto a 150 mm minimal media –His–Ura plate (Fig. 1).
35. Incubate plate at 30 °C for 2 days.

Day 8–10

36. Replicate plate (Fig. 2) onto the following 150 mm plates for auto-activation assays: one –His–Ura plate to maintain yeast colony growth, one 20 mM 3AT plate, one 40 mM 3AT plate, one 80 mM 3AT plate, and one YPDA plate with nitrocellulose filter. His-reporters display some level of auto-activation therefore a competitive inhibitor of His, 3AT, is used to negate the auto-activation and enable assay of interactions.
    (a) Under sterile conditions, place a clean, autoclaved velvet square onto the replica-plating tool.
    (b) Position patch plate yeast-side down over replica plating tool, and using the plate roller transfer yeast onto velvet.
    (c) Remove patch plate and set aside.
    (d) For each of the plates described above, place upside down over the yeast-containing velvet on the replica-plating tool and roll to transfer yeast onto these plates.
    (e) After all plates have received yeast, remove the velvet and replica clean the 3AT series. Replica cleaning is used to remove excess yeast and ensure all growth originates from yeast in direct contact with the 3AT competitive inhibitor.

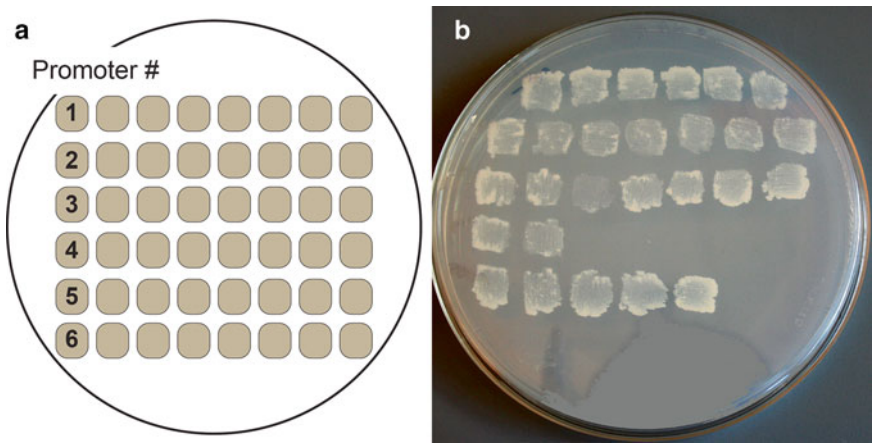

Fig. 1 Example patch plate for yeast colonies. (**a**) Template for patching yeast colonies up to six different promoters. (**b**) Bottom view of a patch plate with colonies from five promoters

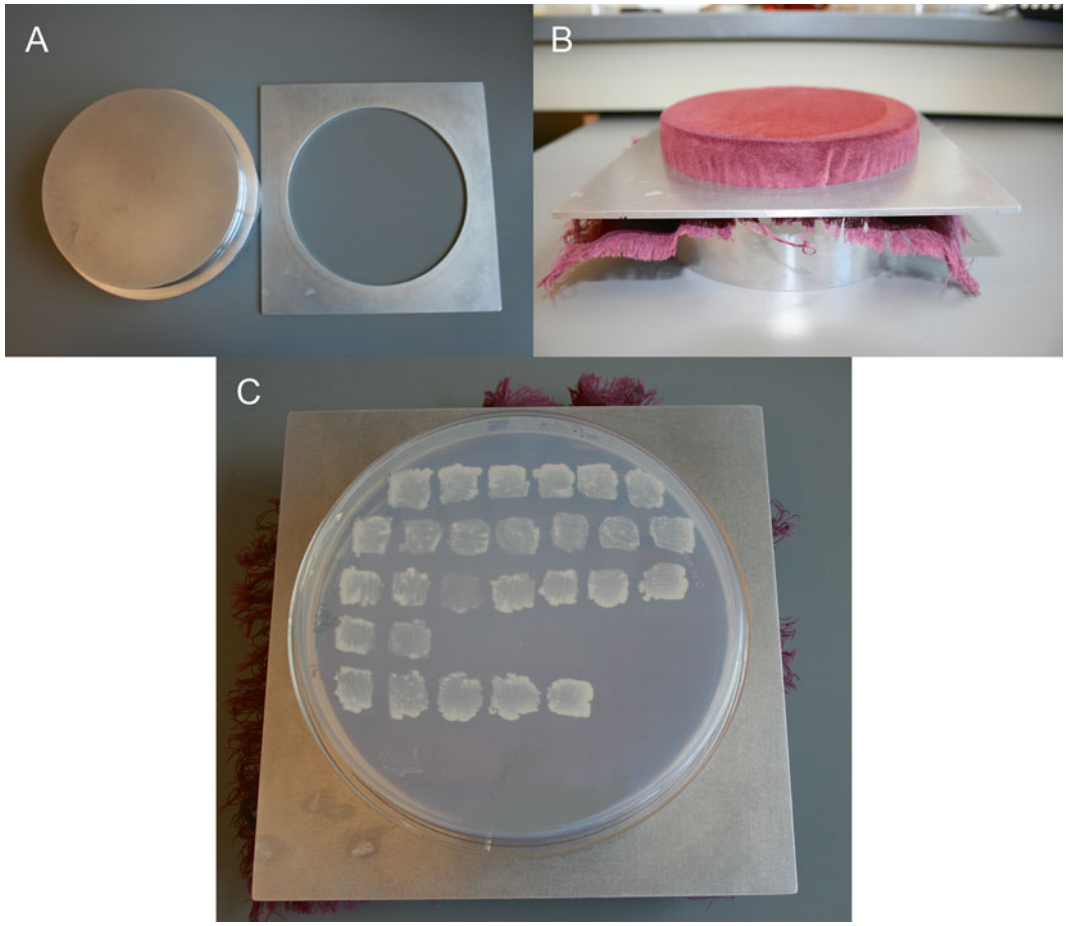

Fig. 2 Replica plating for yeast auto-activation assay. (**a**) Replica plating apparatus with central cylinder and metal collar. (**b**) Replica plating apparatus with velvet secured by the metal collar. (**c**) Patch plate is placed face-down over the velvet and yeast is transferred onto the velvet by rolling across the back of the plate

(f) To replica clean, place a fresh piece of velvet onto the replica-plating tool.

(g) Position the first 3AT plate over the velvet, and roll over the back of the plate to remove any excess yeast.

(h) Replace the velvet with a new square, and repeat for the remaining two 3AT plates.

37. Incubate plates protected from light at room temperature (*see* **Note 10**).

Day 10–12

38. Two days after replica plating, perform auto-activation assay for the LacZ-reporter.

39. Add two Whatman filter papers to an empty 150 mm plastic plate. The purpose of these filters is to absorb and maintain the X-gal liquid.
40. Add 6 mL of X-gal in Z-Buffer
41. Ensure the filters are completely saturated and no large bubbles are present.
42. Using forceps, gently remove the nitrocellulose filter from the YPDA plate, and dip into a liquid nitrogen bath for 10–20 s. Keep holding the filter with the forceps to maintain orientation.
43. Carefully remove the filter and place onto the saturated Whatman filters. Use forceps to adjust the nitrocellulose filter and ensure all yeast is in contact with liquid. Be careful as the individual yeast patches can run together with too much manipulation.
44. Incubate the X-gal assay at 37 °C for 24 h and take a picture at the following time intervals: 30 min, 1, 2, 3, 4, 6, and 24 h.
45. After 24 h, discard filter in accordance to local regulations for β-mercaptoethanol disposal.

Day 13–15

46. Five days after replica plating, take pictures of 3AT series to visualize auto-activation of the His-reporter.
47. Select transformants with minimum auto-activation for both LacZ and His. For the His-reporter make note of the minimum concentration of 3AT at which growth was inhibited; this information will be used in Subheading 3.2.5 to assay for transcription factor interactions.
48. Make glycerol stocks of yeast colonies from the replica plated –His–Ura plate: Add 500 μL of sterile H_2O to a sterile microfuge tube; use a pipette tip to transfer yeast from patch plate into this tube and vortex to resuspend. Add 500 μL of sterile 60 % glycerol, mix well and store at −80 °C.
49. To confirm the transgenic yeast, grow a 2 mL culture for each promoter colony in YPDA overnight at 30 °C.
50. The following day perform a yeast mini-prep using a QIAprep Spin Miniprep Kit with the following modifications (*see* **Note 11**):
    (a) Before addition of Buffer 2, add 250 μL of acid-washed glass beads to each sample and vortex well (*see* **Note 12**).
    (b) Elute DNA in 20 μL of sterile water.
51. Perform colony PCR as outlined in Subheading 3.2.1, **step 7** to confirm the presence of both reporter plasmids.

3.2.3 Cloning of Coding Sequence/Open-Reading Frame (CDS/ORF) Constructs

1. Design Gateway-compatible primers to clone CDS/ORF sequence(s) of the transcription factors identified in Subheading 3.1 or from literature (*see* **Note 13**).
2. Perform CDS/ORF amplification and cloning as described in Subheading 3.2.1, with the following considerations:
   (a) CDS/ORFs should be PCR amplified from a pool of cDNA generated from whole plant RNA. It is unclear whether plant introns can be successfully spliced in yeast, therefore genomic amplification is not advised.
   (b) CDS/ORFs should be inserted into the middle position Gateway vector (either pENTR/D-topo or pENTR-221).
3. Upon successful cloning and sequence confirmation, CDS/ORFs are transferred by single site LR Clonase II reactions into a Gateway-compatible destination vectors, pDESTAD-2 μm. Cloning into pDESTAD-2 μm generates a CDS/ORF-fusion to the Gal4 activation domain (AD) (*see* **Note 6**).
4. Perform colony PCR with one vector-specific primer (AD or TERM) and one CDS/ORF-specific primer to confirm insertion into the destination vector.

3.2.4 Transformation and Confirmation of CDS/ORFs in Yeast

Day 1

1. Under sterile conditions, streak yeast strain Yα1867 from glycerol stock onto a full nutrient YPDA-agar plate and incubate at 30 °C (*see* **Note 7**).

Day 2

2. Under sterile conditions, start a 5 mL culture of Yα1867 in YPDA liquid media from the yeast plate, and grow in a shaking incubator at 30 °C. Return the Yα1867 plate to the 30 °C incubator for the following day.
3. Grow 5 mL cultures of pDESTAD-CDS/ORF constructs, if plasmid is not currently available. Concentrations should be >100 ng/μL.

Day 3

4. Perform a yeast transformation as described in Subheading 3.2.2, **steps 8–33**, with the following modifications.
   (a) Starting OD600 should be as close to 0.2 as possible. If the culture is not sufficient to reach the starting OD600, yeast from the plate may be used to supplement (*see* **Note 14**).
   (b) When resuspending yeast for the final time, add 25 μL of TE/LiAc/H_2O and 2.5 μL of boiled, sheared salmon sperm DNA per CDS/ORF.

(c) Add 150 μL of TE/LiAc/PEG to each tube and mix by carefully pipetting up and down.

(d) After incubation and heat shock, spin down quickly, remove supernatant, and resuspend each yeast pellet with 10 μL of sterile water.

(e) Spot 5–7 μL of the transformation onto –Trp minimal media plates. This media contains all necessary amino acids for yeast growth except for Tryptophan (Trp), which is provided from the pDESTAD vector. It is not necessary to obtain individual colonies for CDS/ORF clones (*see* **Note 15**).

5. To confirm the transgenic yeast, perform yeast mini-prep and colony PCR as described in Subheading 3.2.2, **steps 49–51**.

3.2.5 Enhanced Yeast-1-Hybrid Assay by Mating

This protocol is designed for screening 23 transcription factors plus empty vector against a single promoter (Fig. 3). The protocol can be scaled up or down as desired (*see* **Note 16**).

Day 1

1. Prepare 96-well culture plate containing yeast transcription factors and pDESTAD-2 μm empty vector in quadruplicate or in duplicate with 300 μL of minimal media –Trp per well. Incubate in a 30 °C shaker.

Day 2

2. Inoculate 2 mL liquid –His–Ura cultures with promoter yeast glycerol stocks. Incubate in a 30 °C shaker.

Day 3

3. Spin down transcription factor plate at 1550 × *g* for 5 min.
4. Using a 96-well replicator, transfer transcription factor yeast cultures to an Omni Tray YPDA plate for bulking (*see* **Note 17**). Incubate at 30 °C.

Day 4

5. Spin down promoter cultures at 1550 × *g* for 5 min.
6. Remove ~1.5 mL of liquid from the cultures. Resuspend yeast and plate 550 μL onto Omni Tray –His–Ura plates to generate a yeast promoter lawn. Incubate at 30 °C.

Day 5

7. Transfer yeast transcription factors onto an Omni Tray –Trp plate for selection. One –Trp transcription factor plate is sufficient for three promoters. Incubate plates at 30 °C.

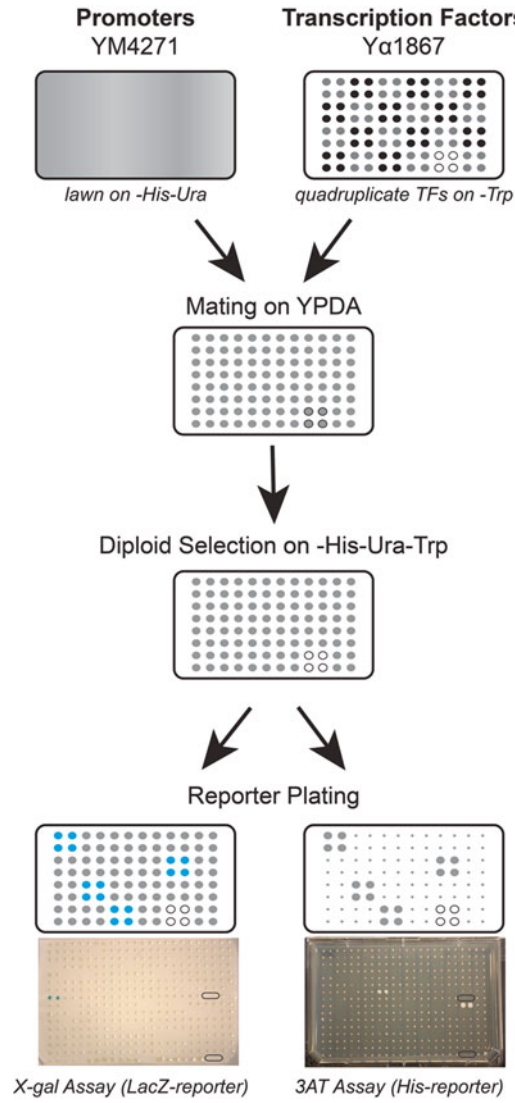

Fig. 3 Mating schematic for yeast-1-hybrid. Promoter-containing haploid YM4271 yeast strains are grown as a lawn on –His–Ura agar plates. Transcription factor-containing haploid Yα1867 yeast strains are grown in quadruplicate (as illustrated) or duplicate in 96-well (as illustrated) or 384-well format on –Trp agar plates. Ensure one of the replicates contains the pDESTAD-2 μm empty vector (*open white circles*). The two yeast strains are mated together on YPDA agar plates, and incubated for 2 days at 30 °C. Successfully mated yeast colonies are diploid-selected on –His–Ura–Trp agar plates, and incubated for 2 days at 30 °C. Diploid yeast are plated onto a nitrocellulose filter on YPDA and the 3AT concentration determined by auto-activation. After incubation for 2 days at room temperature, an X-gal assay is performed and blue colonies are recorded as LacZ-positive interactions. After incubation for 5 days at room temperature protected from light, His-positive interactions are recorded as growth in the absence of Histidine. Examples from a 384-well, duplicate transcription factor format are shown. Black circles indicate where pDESTAD-2 μm empty vector is located

8. Using a sterile pipette tip or tooth pick, patch promoter lawns to cover any portion of the plate that does not have yeast growing on it and return to 30 °C incubator.

Day 6

9. Using the 96-well replicator and included plastic guide, transfer from the promoter lawn onto an Omni Tray YPDA plate.
10. Using the 96-well replicator and position in the same plastic guide, add transcription factors from –Trp plate to the YPDA plates from the previous step and incubate at 30 °C.

Day 8

11. Using the 96-well replicator, transfer yeast from mating plates onto Omni Tray –His–Ura–Trp plates for diploid selection. Incubate at 30 °C.

Day 10

12. Replica plate diploid selected yeast from –His–Ura–Trp onto reporter plates: 1 YPDA + nitrocellulose filter, and 1 3AT – His–Ura–Trp with the concentration determined from auto-activation assay. Incubate at room temperature.

Day 12

13. X-gal assay as indicated in Subheading 3.2.2, **steps 38–45**.

Day 15

14. Take pictures of 3AT –His–Ura–Trp plates at 5 days after plating.
15. Positive interactions between transcription factors and promoters are called from pictures, and require the agreement of two independent individuals. LacZ-positive interactions are called based on the presence of blue color, which exceeds that of auto-activation in all colonies (i.e., background levels). His-positive interactions are called based on the mated clone growing larger than that of auto-activation in all colonies (i.e., background levels) (*see* **Note 18**). Three of the four clones must agree for a positive interaction to be determined. If performing assays in duplicate, both clones must agree for a positive interaction to be determined. Ensure that the negative control (empty pDESTAD-2 μm) positions do not show growth.

3.3 Induction Time Course to Identify Downstream Gene Clusters

1. Generate inducible expression lines for transcription factor(s) of interest by using the cloned fragments and cloning strategies indicated above (*see* **Note 19**).
2. Prepare multi-site Gateway reactions to include the 5′pENTR promoter construct, the CDS/ORF construct and a 3′pENTR

glucocorticoid receptor (GR) construct (*see* **Note 20**). Insert these pieces into any Gateway compatible plant binary vector using LR Clonase II Plus.

3. Transform confirmed constructs into plants with a loss of function mutation in the transcription factor of interest (if available) by standard floral dip [14]. These plants will then be pPRO:CDS:GR;*mutant* plants or pPRO:CDS:GR plants (*see* **Note 21**).
4. Surface sterilize seeds from pPRO:CDS:GR;*mutant* plants with 50 % bleach and 0.1 % Tween 20 for 5 min. Rinse three times with sterile water and plate onto nylon mesh in 1× MS, 1 % sucrose, 1 % agar square plates.
5. Stratify plates at 4 °C protected from light for 2 days then transfer to a growth chamber set at 22 °C and 16 h light, 8 h dark conditions. Position plates vertically in the growth chamber so that roots grow along the surface of the mesh-covered agar plate (Fig. 4).
6. Five days after exposure to light (~3 days post-germination), transfer seedlings on nylon mesh to 1× MS, 1 % sucrose, 1 % agar plates containing 10 μM DEX and 0 μM DEX to control for gene expression changes due to physical manipulation (*see* **Note 22**).

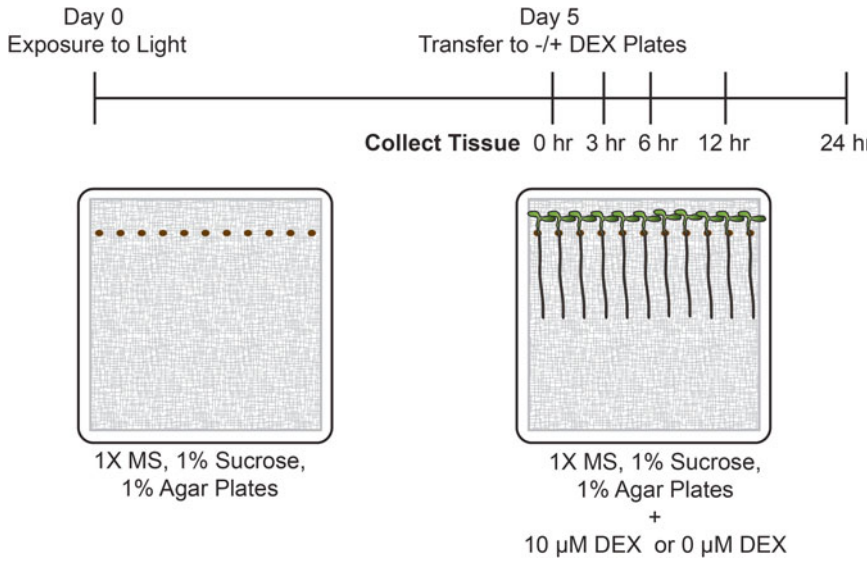

Fig. 4 Induction schematic for time course analysis. Seeds are plated on nylon mesh covered 1× MS, 1 % sucrose, 1 % agar plates and stratified for 2 days at 4 °C protected from light. Plates are placed vertically into a growth chamber to allow the roots to grow along the face of the nylon. At 5 days after exposure to light, mesh is transferred to plates containing 10 μM DEX or 0 μM DEX. The first time point of 0 h is collected immediately after transfer. Seedlings are collected at 3, 6, 12, and 24 h after transfer to –/+ DEX and stored at –80 °C until ready to process

7. Collect seedlings at 0, 3, 6, 12, and 24 h (time can be adjusted as needed). Snap freeze in liquid nitrogen and store at −80 °C until ready to process.
8. Extract RNA and generate cDNA from whole seedlings or specific tissue using standard techniques.
9. Perform qRT-PCR for gene target prediction to determine the timing of induction for the gene of interest using the 0 μM DEX as control for each time point.
10. After determining the optimal time points for induction of the gene of interest, generate genome-wide transcriptome profiling by RNA-sequencing or microarray.
11. Analyze the time course data by using a clustering algorithm to identify the genes that are regulated in a similar manner to the gene of interest. There are many clustering algorithms and approaches available in the literature. Often these cluster algorithms rely on a user-defined number of clusters and make a hard-call as to which cluster a gene (RNA-seq data) or probe (microarray data) belongs to. We highlight one approach, which has proved successful for us in the past [7]. This approach first uses fuzzy K-means clustering to provide a soft-call probability, and then filtering to remove low probability genes/probes. This data is then hierarchically clustered to ensure distinct expression clusters.
    (a) Fuzzy K-means can be implemented using the Fuzzy Analysis (FANNY) package for R (http://cran.r-project.org/doc/packages/cluster.pdf) with the distance between two genes/probes defined as $(1-r)/2$ where r is the Pearson correlation between the two genes/probes.
    (b) Different values for K (i.e., number of clusters) should be tested to determine which number of clusters captures the dominant patterns. We found that $K=60$ was appropriate for our data.
    (c) Fuzzy K-means analysis outputs a probability matrix for each gene or probe against each defined cluster. The output should be filtered to remove any genes or probes that do not have a m-value of >0.4.
    (d) Initial patterns determined from fuzzy K-means are hierarchically clustered (again using the cluster R package), where the distance between patterns is $1-r$ (r=Pearson correlation coefficient).
    (e) The hierarchical tree is cut at 0.1 ($r=0.9$) and patterns lying on the same branch are collapsed into a new pattern by taking the median profile for each gene/probe.

4 Notes

1. While the upstream 500–1500 bp contains "core promoter" elements necessary for transcription initiation [15], we consider the promoter to include 3000 bp upstream of the ATG or to the nearest gene. In our experience this expanded promoter definition enables the identification of additional regulatory interactions.
2. Several databases for *Arabidopsis thaliana* transcription factor binding information exist, however the most inclusive is the CIS-BP database [16], which includes interactions curated from the literature and additional information generated from protein binding microarrays. Other databases include AtcisDB [17] and Transfac [18]. Information for individual transcription factors has also been determined by SELEX [19].
3. Very few parameters can be modified for running FIMO. The main parameter is p-value cutoff; in our experience, a cutoff of $1E^{-3}$ broadly captures the majority of binding interactions found *in planta*, however it is worthwhile to adjust the p-value and view the output. A second parameter that can be modified in the motif input file is the background letter frequency. For Arabidopsis the frequencies are: A 0.32, C 0.18, G 0.18, T 0.32 [20].
4. If the promoter proves to be in a difficult region to clone, we often perform nested PCR [21] where a second set of "outer" primers are designed outside the promoter region of interest. The outer PCR product is then used as a template for the "inner" PCR for your promoter of interest.
5. Using Gateway-compatible cloning entry vectors simplifies cloning for downstream applications. Cloning entry vectors can be accomplished by two different methods; the first is using the 5′topo (promoters) and D-topo (CDS/ORFs) kits directly. This method requires a CACC sequence to be added at the 5′ end of the CDS/ORF cloning primer. Alternatively, corresponding att sites matching the p4p1 (promoter) or p221 (CDS/ORF) vector can be added to the primer sequence. For more information on Gateway cloning, please refer to the Life Technologies website. Incubate topo reactions overnight at 4 °C, and BP reactions overnight at 25 °C before transformation into *E. coli*.
6. For best results, the destination vector plasmid preps should be prepared fresh, within 2–4 weeks of clonase reaction.
7. When handling yeast, it is very important to prevent repeated freeze–thaw cycles. Always make back-up stocks to protect the

yeast collection. When making glycerol stocks it's best to use as much yeast as possible.

8. If all restriction sites are present in the promoter construct, it is possible to transform non-linearized vector although the transformation is less efficient. In this instance, transform 20 μL of non-linearized vector into YM4271.
9. Inoculating cultures with yeast grown overnight in liquid culture yields the best results for growth efficiency. However, if sufficient yeast was not obtained from the liquid culture, transformations can be supplemented from yeast on plates. To do this, add 1 mL of sterile water to a sterile microfuge tube. Using a sterile 1 mL pipette tip, gather yeast from the plate and swirl into water tube. Vortex tube to resuspend yeast and inoculate flasks using 100–200 μL increments.
10. Assays are performed at room temperature to obtain conditions closer to those occurring *in planta*.
11. Plasmid isolation from yeast is relatively inefficient, therefore while alternative mini-prep kits can be used, the most consistent results are found with the Qiagen Plasmid Mini kit.
12. To measure acid-washed glass beads, cut off a microfuge tube at the 250 μL mark. Tape this to the bottom of a p1000 pipette tip and then use to scoop and measure 250 μL of beads.
13. Several CDS/ORF clones are already available in the yeast-compatible pDESTAD-2 μm vector at the Arabidopsis Biological Resource Center (ABRC). It is worthwhile to check for available clones. Inclusion of the stop codon does not affect the yeast-1-hybrid assay, since the AD is a C-terminal fusion. However, if clones are to be used to for other analyses (e.g., reporter or induction constructs) it is important to not clone the last three nucleotides of the CDS/ORF.
14. The Yα1867 strain grows slower than the YM4271, therefore it is best to start with an OD600 of 0.2. It will take at least 3 h to reach the minimum OD600 of 0.4. If your culture grows faster than this, it is likely contaminated.
15. When spotting the transformed Yα1867, it is best to not pipette to the second stop. Often, this results in minute spray of yeast that can contaminate other transformants. It is better to lose some material, than to dispense all of the yeast out of the pipette tip.
16. The pDESTAD-2 μm vector has enabled high-sensitivity detection by mating and since mating allows the greatest throughput, that protocol is outlined here [13]. To scale up, transcription factors can be cultured in 384-well plates. Alternatively if only a few interactions are being tested, the direct transformation method can be used. For direct

transformation, the CDS/ORF transformation protocol is used with the modification of using the YM4271-containing promoter strain instead of Yα1867. The transformation is then spotted on –His–Ura–Trp plates and replicated plated to assay for interactions.

17. Ensure that the 96-well replicator is washed, sterilized and cooled before each replication. Pins are dipped in water, then three successive flame sterilizations with 100 % ethanol.
18. Calling positive interactions using the His-reporter is a particular challenge. It is important to look at the size of surrounding yeast colonies before making a determination. Yeast that are grown without neighbors tend to grow larger, therefore an edge effect is very prominent in this assay. Be sure that colonies are larger than others in a similar position before calling a positive interaction.
19. A unique resource has recently been cultivated from a consortium in Spain to facilitate this type of analysis. The "Transplanta" lines are a collection of plants expressing β-estradiol-inducible promoter driven transcription factors [22]. Currently there are 1697 lines, representing over 450 transcription factors, available at the Nottingham Arabidopsis Stock Center (NASC), and additional lines are being deposited as they are generated.
20. Ideally, a GR-fused CDS/ORF would also contain a fluorescent tag for *in planta* tracking of induction. Unfortunately, we have been unable to successfully generate a GR-GFP that folds properly *in planta*. An alternative strategy to monitor induction is utilizing a GAL4:UAS system with a two constructs: pPRO:GAL4-GR and UAS:CDS-GFP.
21. To generate transgenic lines, the first generation (plants that are dipped) are T0. The seeds from these plants are the T1 generation, and should be selected for the presence of the resistant marker found in the plant binary vector. Often the resistance is Basta, Hygromycin, or Kanamycin. T2 seeds are collected from resistant T1 plants, and ~60 seeds screened for proper segregation of the resistance marker. T2 populations should exhibit Mendelian segregation to confirm a single insertion site. Results from transgenic plants should always be confirmed in 3–5 independent transformants (T2 populations).
22. Plate-based DEX administration is particularly suited to root assays, as DEX uptake occurs through the roots under this conditions. For shoot analysis, the same assay will work, however the time frames may need to be adjusted to allow for transport of DEX to the shoot tissue. Alternatively, seedlings can be sprayed with a solution of 30 μM DEX with 0.01 % Tween 20. Since DEX uptake is more difficult to control with spraying [23], plate-based DEX administration is recommended when possible.

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Chapter 4

Cytrap Marker Systems for In Vivo Visualization of Cell Cycle Progression in *Arabidopsis*

Shiori Sugamata Aki and Masaaki Umeda

Abstract

This chapter focuses on visualization of cell cycle progression in plant tissues using the dual-color marker system "Cytrap." The Cytrap line carries a part of *Arabidopsis CDT1a* fused to the red fluorescent protein (RFP) gene, which monitors the cell cycle phases from S to late G2 or early mitosis, and the G2/M-specific cyclin B1 marker fused to the green fluorescent protein (GFP) gene. We introduce growth conditions of *Arabidopsis* roots, the setup for microscopic observation, and analysis of obtained images.

Key words Cell cycle, Real-time imaging, Fluorescent protein marker, Root, *Arabidopsis*

1 Introduction

Real-time imaging of cell cycle progression is crucial to understand various developmental processes. A combination of S- and M-phase markers, termed "Fucci," was developed for human and zebrafish cells [1, 2], while in plants, only G2/M-phase markers have been available [3]. This made it difficult to monitor cell cycle progression in tissues, restricting the studies on plant growth and development.

We generated a red fluorescent protein (RFP)-fusion gene carrying a part of *Arabidopsis CDT1a*, which encodes a factor involved in DNA replication (reviewed in [4]). This marker displayed the RFP fluorescence from S to late G2 or early mitosis [5]. We then combined this marker with the green fluorescent protein (GFP)-fused cyclin B1 marker, which has been widely used as a G2/M--specific reporter [3]; the resulting dual-color marker system was termed Cell-Cycle Tracking in Plant Cells (Cytrap) [5]. This system enabled us to identify each cell-cycle phase based on fluorescence combinations, *i.e.*, no fluorescence, either RFPor GFP, and both RFP and GFP fluorescence, representing G1, S/G2, G2/M, and late G2 (to early M), respectively. Here, we introduce the

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method to monitor cell cycle progression in the epidermis of *Arabidopsis* roots using the Cytrap system.

2 Materials

2.1 Plant Material

The *Arabidopsis* transgenic line (ecotype Col-0) carrying the Cytrap system, which consists of a part of *Arabidopsis CDT1a* fused to *RFP* and the cyclin B1 marker fused to *GFP*, is used for microscopic observation (*see* **Note 1**).

2.2 Seed Sterilization

1. 70 % Ethanol.
2. Sterilization solution: 0.05 % Triton X-100, 4 % sodium hypochlorite.
3. Sterilized water.
4. Microtube rotator.
5. Pipette.

2.3 Medium Preparation

1. Murashige and Skoog Plant Salt Mixture.
2. 2-(*N*-morpholino) ethanesulfonic acid (MES).
3. 1 N KOH.
4. Phytagel™ (Sigma Aldrich, St. Louis, MO, USA).
5. Iwaki coverglass chamber (1 well).
6. Medicine spoon with a flat back side.
7. Parafilm®.

2.4 Seed Sowing

1. Aluminum foil.

2.5 Cultivation

1. Plastic tray.
2. Plant growth chamber.

2.6 Time-Lapse Observation

Time-lapse imaging is performed with an Olympus FV1000 confocal microscope. Images are obtained and analyzed by FV10-ASW software including the Kalman filter (Olympus, Tokyo, Japan).

3 Methods

3.1 Seed Sterilization

1. Mix seeds with 1 mL of 70 % ethanol in a 1.5-mL microtube.
2. Rotate with a microtube rotator at room temperature for 5 min.
3. Remove 70 % ethanol with a pipette and add 1 mL of sterilization solution.

4. Rotate at room temperature for 5 min and remove sterilization solution with a pipette.
5. Add 1 mL of sterilized water.
6. Invert the tube a few times and remove sterilized water with a pipette.
7. Repeat **steps 5** and **6** twice.
8. Remove sterilized water with a pipette and add 1 mL of sterilized water (go to Seed Sowing).

3.2 Medium Preparation in a Coverglass Chamber

1. Dissolve 0.23 g of Murashige and Skoog Plant Salt Mixture in 90 mL of distilled water.
2. Add 0.05 g of MES and dissolve it.
3. Adjust pH to 5.8 with 1 N KOH.
4. Adjust the volume to 100 mL by adding distilled water.
5. Add 1 g of Phytagel™.
6. Sterilize in autoclave at 121 °C for 15 min.
7. After cooling down, pour 1.8 mL of the medium into a coverglass chamber (Fig. 1a).
8. Put a cover on the chamber immediately and solidify the medium for few minutes (*see* **Note 2**).
9. Remove 1 cm of the top part of the medium using the flat back side of the medicine spoon just before sowing the seeds (Fig. 1b).

3.3 Seed Sowing

1. Sow sterilized seeds along the edge between glass bottom and solidified medium (Fig. 2a; *see* **Note 3**).
2. Put the cover on the chamber and seal it with Parafilm® (Fig. 2b).
3. Cover the chamber with aluminum foil for shading, and keep at 4 °C for 2–3 days.

3.4 Cultivation

1. Tilt coverglass chambers upward at an 80-degree angle in a plastic tray (Fig. 3; *see* **Note 4**).
2. Place the tray in a plant growth chamber (continuous light, 22 °C) and incubate for 5 days.

3.5 Microscopic Observation

1. Epidermal cells in the meristematic region of roots (approximately 100–200 μm from the root tip) are subjected to time-lapse imaging (Fig. 4a; *see* **Note 5**). The wavelengths for GFP and RFP laser are 473 and 559 nm, respectively, and the laser strength is set to 4 %. GFP, RFP, and bright-field images are simultaneously obtained with the "line-scan mode"; (1) scan speed, (2) image size, (3) magnification, and (4) detector are

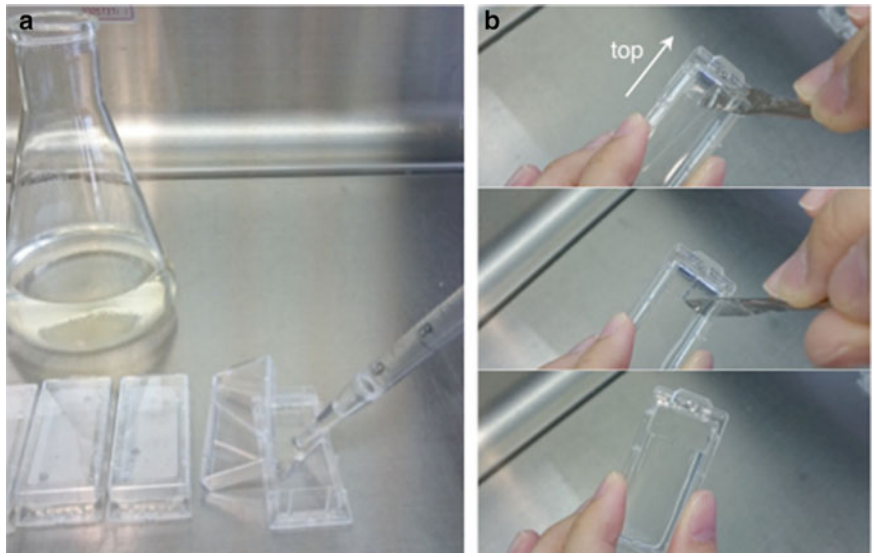

Fig. 1 Medium preparation in a coverglass chamber. The medium (1.8 mL) is poured into the chamber (**a**), and after it solidified, the top part (1 cm) of the medium is removed with the flat back side of a medicine spoon (**b**)

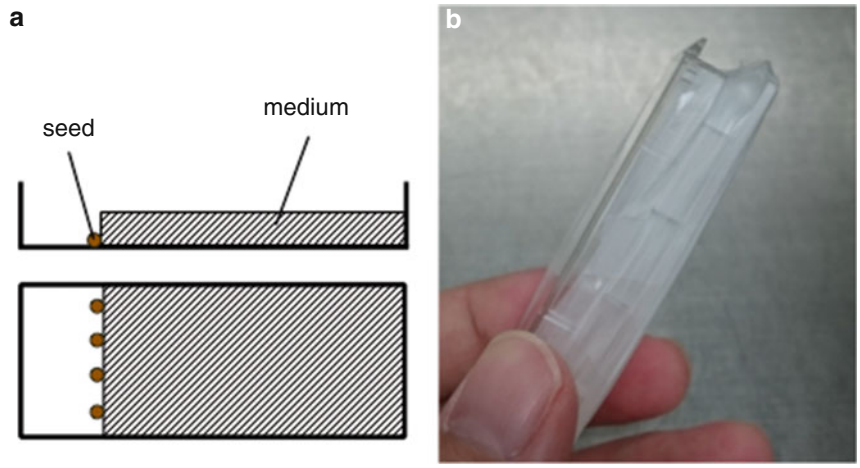

Fig. 2 Seed sowing. Four seeds are sowed along the edge between the glass bottom and the medium (**a**), and after putting the cover, the chamber is sealed with Parafilm® to prevent drying of the medium (**b**)

set to (1) 10.0 us/pixel, (2) 1600×768 pixel, (3) 20×3.0 zoom, and (4) 656 V (GFP), 708 V (FRP) and 95 V (bright field). Images are processed with a Kalman filter to reduce the noise.

2. Images are obtained every 10 min for 14 h. The three-dimensional electric stage allows simultaneous tracking at multiple points. Two visual fields are set to monitor meristematic cells of elongating roots during the 14-h observation (Fig. 4b, visual fields 1 and 2). Several *Z*-stack images are obtained

Fig. 3 Setup of coverglass chambers for cultivation. Chambers are placed in a plastic tray at an angle of 80° and incubated in a plant growth chamber

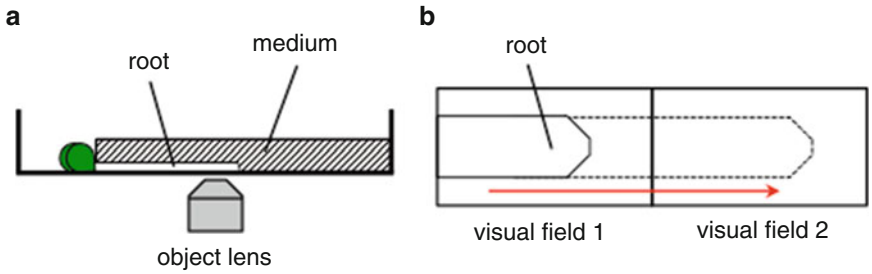

Fig. 4 Setup of visual fields. Microscopic observation is conducted on roots growing between the glass bottom and the medium (**a**). Two visual fields are set in the direction of root growth (**b**)

around the epidermal layer at 4 or 5 μm intervals. The example images are shown in Fig. 5.

3.6 Image Analysis

Fluorescent intensity of GFP and RFP in a particular cell is measured by FV10-ASW software and depicted in a graph sequentially over 14 h (*see* **Note 6**). To exclude the variation in fluorescent intensity derived from differences in cell size, only signals from nuclei are measured. The nuclear area is assigned using the "Ellipse" tool of the software.

4 Notes

1. The Cytrap line can be obtained from our laboratory.
2. Immediate covering of the chamber is important to prevent drying of the medium. To store the medium, seal the chamber with Parafilm® and keep at 4 °C. Use the medium within a month.
3. We usually sow four seeds per chamber and prepare several chambers because some roots do not enter the space between

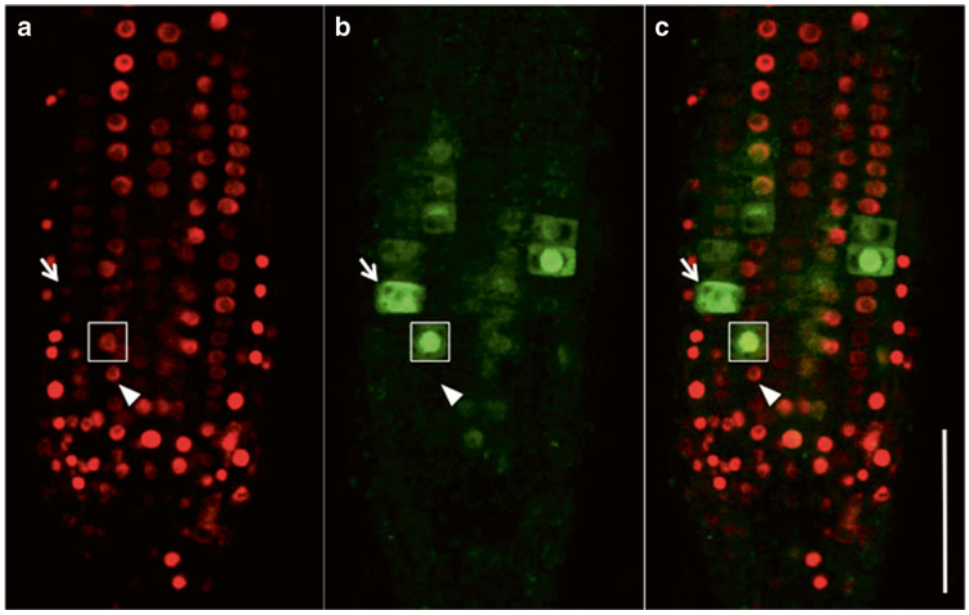

Fig. 5 Example images of microscopic observation. Images of RFP (**a**) and GFP fluorescence (**b**), and a merged image (**c**), are shown. Either RFP (*arrowhead*) or GFP (*arrow*), and both RFP and GFP fluorescence (*square*), represent S-G2, G2-M, and G2 (to early mitosis) phases, respectively. Bar = 50 μm

the glass bottom and the medium. Seeds should be in contact with the medium in order to grow roots between the bottom of the chamber and the medium.

4. Without tilting, roots do not grow in the space between the glass bottom of chambers and the medium.
5. Observe healthy roots that are longer than 1 cm.
6. Since the duration of the cell cycle is more than 14 h in the epidermis of *Arabidopsis* roots, two graphs need to be prepared to represent the whole cell cycle. To provide a timescale for each graph, 0 h is set as follows:

   Graph 1: when the equatorial plane is visualized by GFP signals.

   Graph 2: when the intensity of RFP signals for the first time exceeds twice the value detected 2 h earlier.

   Examples of a set of graphs are shown in [5].

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Chapter 5

Imaging Nuclear Morphology and Organization in Cleared Plant Tissues Treated with Cell Cycle Inhibitors

José Dijair Antonino de Souza Junior, Maria Fatima Grossi de Sa, Gilbert Engler, and Janice de Almeida Engler

Abstract

Synchronization of root cells through chemical treatment can generate a large number of cells blocked in specific cell cycle phases. In plants, this approach can be employed for cell suspension cultures and plant seedlings. To identify plant cells in the course of the cell cycle, especially during mitosis in meristematic tissues, chemical inhibitors can be used to block cell cycle progression. Herein, we present a simplified and easy-to-apply protocol to visualize mitotic figures, nuclei morphology, and organization in whole Arabidopsis root apexes. The procedure is based on tissue clearing, and fluorescent staining of nuclear DNA with DAPI. The protocol allows carrying out bulk analysis of nuclei and cell cycle phases in root cells and will be valuable to investigate mutants like overexpressing lines of genes disturbing the plant cell cycle.

Key words *Arabidopsis*, Roots, Cell cycle, Inhibitors, Nuclei

1 Introduction

Determining mitotic indexes in meristematic root cells has been used for many years as an important tool to determine root health and meristem activity [1]. We have used chemical compounds to induce cell cycle arrest in Arabidopsis root cells [2]. Here, we present a protocol based on nuclear staining and drug arrest to rapidly and efficiently monitor the cell cycle status of root cells.

The cell division cycle is a highly regulated process having the function to generate two daughter cells. Successful progression through the cell cycle requires four steps: the S phase, where DNA replication or duplication of the genetic material occurs, preceded by the G1 phase, and followed by the G2 phase and mitosis (M). The mitotic phase usually ends up into cytokinesis, or cell division. The ensuing daughter plant cell can either continue to divide, exit the mitotic cell cycle and enter the endocycle, or definitely leaving the cell cycle to undergo differentiation.

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Plant development engages rigid control and coordination of proliferative activity and growth in meristematic and differentiated tissues. A synchronous cell culture is characterized by a high fraction of cells proceeding to the same phase of the cell cycle at the same point. Whilst innate synchrony is observed in particular plant tissues which can be a resource of synchronized cells, induction of synchrony can be attained by the arrest of a cell population at a specific stage via addition of chemical agents. This strategy is most frequently used to achieve simple and highly reproducible synchrony of plant cells [2].

Potential toxicity prevents the use of high concentrations of chemical inhibitors although efficacy is a requirement. Synchrony progressively decreases once treatment is released. Therefore, a one-step treatment can be improved by combining different inhibitors in a two-step blocking method. The synchronized cells obtained after the first drug treatment can be further treated with a second drug to induce an arrest in the subsequent phase. For example, a mitotic index (MI) of 30–50 % is obtained in tobacco BY-2 cells after the release from a one-step aphidicolin block [3], while the MI is enhanced to 90 % by a treatment with propyzamide, an anti-tubulin drug, after the release from an aphidicolin block [4].

Classical squash preparations [5] of root meristems have been used for years to access mitotic figures but cellular root integrity is often lost. Whole-mount (WM) techniques have been reported to analyze nuclei in their cellular context of different Arabidopsis tissues (e.g. root meristems, endosperm or leaves) [6–9]. However, plant cells present an intricacy due to the existence of the cell wall as a barrier for efficient drug penetration, plus the presence of autofluorescence complicates data interpretation.

Herein, we describe a rapid nuclear staining method and an adapted clearing procedure employed to study the effect of different cell cycle inhibitors on the nuclear division behavior in Arabidopsis roots. When using thick tissue slices, the method can be extended to study nuclei in organs of species harboring a more complex multicellular organization. Our whole mount protocol allowed us to acquire insights into the cellular and nuclear organization of Arabidopsis root cells and permitted the identification of promising candidate cell cycle drugs to study mitotic events in transgenic roots displaying an altered regimen of cell cycle gene expression.

2 Materials

2.1 Equipment and Material

1. Petri dishes.
2. Microscope glass slides.
3. Coverslips.

4. Box to store mounted microscope slides.
5. Tweezers.
6. Hematology/Chemistry Mixer (Model 346, Fisher, USA).
7. Confocal Microscope (Leica SP8 equipped with a diode 405 nm UV laser).

2.2 Culture Media, Buffers, and Other Solutions

1. *4 % Formaldehyde*: Heat PBS pH 9.0 in the microwave until approximately 60 °C and add 4 g/40 ml of solid paraformaldehyde (8 % stock solution). Stir the solution until completely dissolved and after cooling, adjust to pH 6.9 with H_2SO_4 and complete the volume to 50 ml. *Note:* adjusting the pH with HCl leads to the generation of toxic products. Formaldehyde solution should be kept at −20 °C and diluted in PBS buffer when needed to 1 % formaldehyde working solution.
2. *Fixative buffer 1*: Phosphate-buffered saline (PBS), pH 7.2, containing 1 % formaldehyde supplemented with 10 % dimethyl sulfoxide (DMSO).
3. *Fixative buffer 2*: PBS containing 0.1 % tween 20 (PBT) and 1 % formaldehyde.
4. *PBT*: PBS, containing 0.1 % tween 20.
5. *DAPI staining solution*: 1 μg/ml DAPI in PBS supplemented with 0.05 % of Triton-X.
6. *Ethanol:xylene mixture*: Mix equal volumes of ethanol and xylene (1:1).
7. *PBS-formamide solution*: PBS containing 50 % formamide.
8. *Propidium iodide (PI) staining solution*: Dissolve PI in PBS in final concentration of 100 ng/ml.

2.3 Cell Cycle Inhibitors' Stock Solutions

1. *Hydroxyurea:* Stock solution of 1 M dissolved in water, and use a final concentration of 100 μM.
2. *Aphidicolin:* Stock solution of 10 mg/ml dissolved in DMSO, and use a final concentration of 10 μg/ml.
3. *Colchicine:* Stock solution of 25 mM, dissolved in DMSO, and use a final concentration of 5 mM.
4. *Oryzalin:* Stock solution of 20 mM, dissolved in DMSO, and use a final concentration of 10 μM.
5. *MG132:* Stock solution of 10 mM dissolved in DMSO, and use a final concentration of 100 μM.
6. *Nocodazole:* Stock solution of 10 mg/ml dissolved in DMS, and use a final concentration of 10 mM.

2.4 Plant Material

1. *Arabidopsis thaliana* (L.) Heynh. genotype Columbia (Col-0).

3 Methods

3.1 Plant Growth Conditions

1. Surface-sterilize seeds of *Arabidopsis thaliana* (L.) Heynh. genotype Columbia (Col-0) wild-type (WT) for 10 min in 5 % NaOCl.
2. Wash four times with 95 % ethanol, and dry under the hood overnight on a sterile filter in a Petri dish.
3. Seeds are then placed in sterile Petri dishes on 1 % Murashige and Skoog (MS) germination medium containing 1 % sucrose and 0.8 % plant cell culture-tested Phytagel.
4. Plantlets are grown vertically, to allow root growth along the surface, with a 16 h light:8 h darkness photoperiod at 21°C and 18 °C, respectively.

3.2 Plant Treatment with Cell Cycle Inhibitors

1. Pharmacological treatment with cell cycle inhibitors is performed on roots of *in vitro* grown Arabidopsis seedlings (Fig. 1).
2. At 4 days after germination seedlings are transferred to new plates supplemented with aphidicolin and kept for 16 h to allow synchronization in S-phase (Fig. 2). *Note:*Hydroxyurea can be used to synchronize root cells, but it is less efficient.

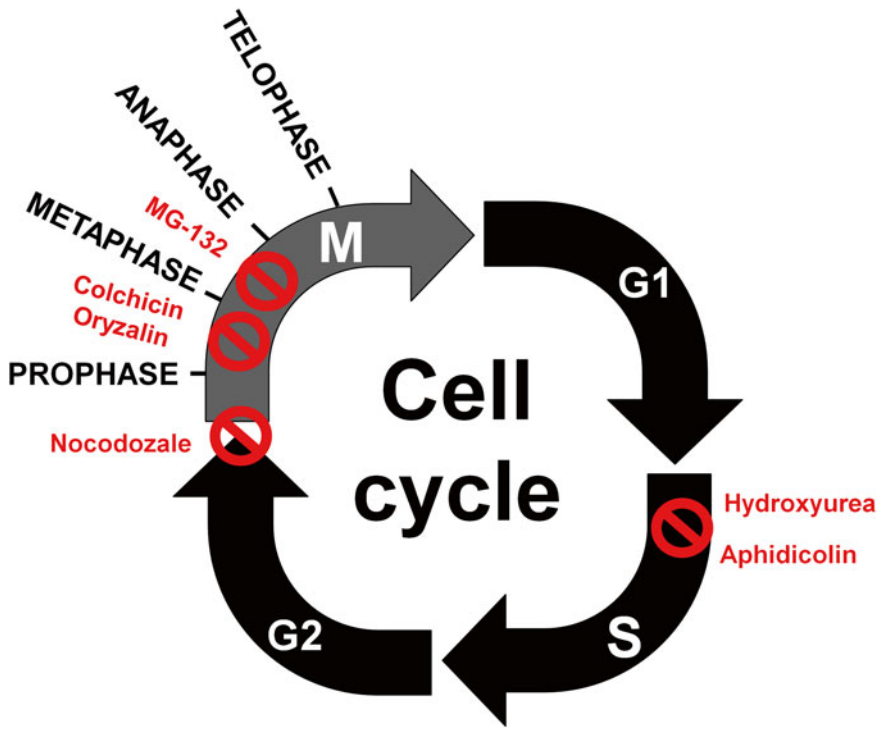

Fig. 1 Cell cycle scheme showing in which phases inhibitors actively blocks mitosis. Aphidicolin and hydroxyurea were first applied to synchronize root cells during the S phase of the cell cycle. Two hours after release, roots were treated with inhibitors at G2 and M phases to obtain mitotic figures visualized by confocal microscopy. M. Mitosis; G1. Gap 1; S. S-phase; G2. Gap 2

3. Thereafter, seedlings are moved to a new plate and kept from 1 to 2 h to release root cells from cell cycle arrest.
4. Finally, a drop of each of the chemical inhibitors listed below was added to the roots to block mitosis in root cells (a to d) and further incubated for at least 2 h to induce cell cycle arrest (Fig. 3):
   (a) 10 μM oryzalin.
   (b) 5 mM Colchicine.
   (c) 100 μM MG132.
   (d) 10 mM Nocodazole.
5. To fix control and inhibitor-treated roots, samples are transferred into fixative buffer 1 and incubated for 30 min at room temperature. *Note:* A similar fixation procedure can be applied for whole seedlings, or other Arabidopsis organs.
6. Samples are subsequently washed twice in PBS for 10 min to remove excessive fixative.

3.3 Rapid Nuclear Staining with DAPI

1. Fixed and PBS-rinsed samples are twice dehydrated with absolute methanol, and four times with absolute ethanol for 10 min each step.
2. Samples are washed five times with PBS for 10 min each, and are then stained with DAPI staining solution for 20 min.
3. DAPI-stained samples should be washed twice with PBS for 10 min each.
4. Stained samples are mounted in 90 % glycerol on microscope slides, cover-slipped, and sealed with nail polish to prevent solvent evaporation. Store at room temperature protected from the light.
5. DAPI-stained samples are subsequently imaged using a Leica SP8 confocal microscope using the 405 nm excitation light (Fig. 2).
6. Images are generated using the following parameters:

   Objective lens HC PL APO CS2 40X/1.30 OIL. Dye excitation was done with a 405 nm diode laser and fluorescence was collected between 431 and 532 nm. Samples were scanned at 400 Hz and standard 16× line averaged for image recording.

3.4 Clearing and Nuclear Staining Protocol

1. Fixed and PBS-rinsed whole plant material is subsequently twice dehydrated with absolute methanol, and three times with absolute ethanol for 10 min each step.
2. Samples are stored at –20 °C in absolute ethanol for 2 days to enhance tissue clearing.
3. Subsequently, samples are washed twice for 10 min with 100 % ethanol and incubated 40 min in ethanol:xylene mixture (1:1).

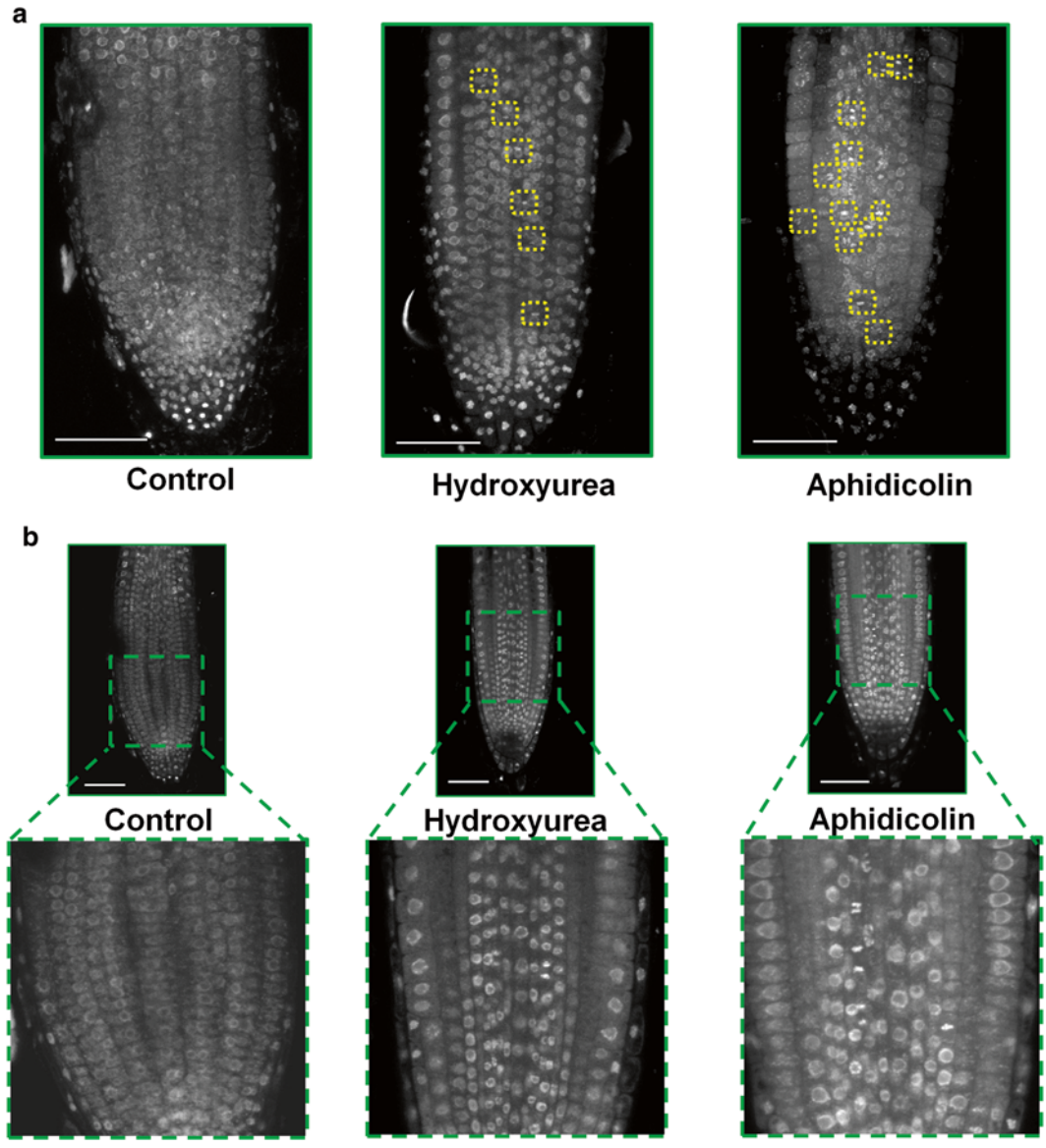

Fig. 2 Determining nuclear morphology and organization in intact roots by clearing and DAPI staining under cell cycle inhibitor treatments. Confocal single images and projections of serial optical sections of Arabidopsis whole roots treated with inhibitors of S-phase, hydroxyurea (HU), and aphidicolin. (**a**) Maximum brightness projection of DAPI-stained whole root treated with S-phase inhibitors. Aphidicolin is more efficient in synchronizing root apical meristem cells after 16 h of treatment and 2 h of cell cycle release compared to HU. (**b**) Single images of DAPI-stained whole roots showing in detail the mitotic nuclei obtained upon aphidicolin treatment. Control images devoid of mitotic figures correspond to roots not treated with inhibitors. Confocal *Z*-stacks were generated from approximately 150 images of 1 μm optical slice thickness and used for 3D visualization of nuclei. Image acquisition was performed using a Leica SP8 confocal microscope. Maximum brightness projections were created using the SP8 software to overview nuclear organization in whole tissues. The scale bar represents 50 μm

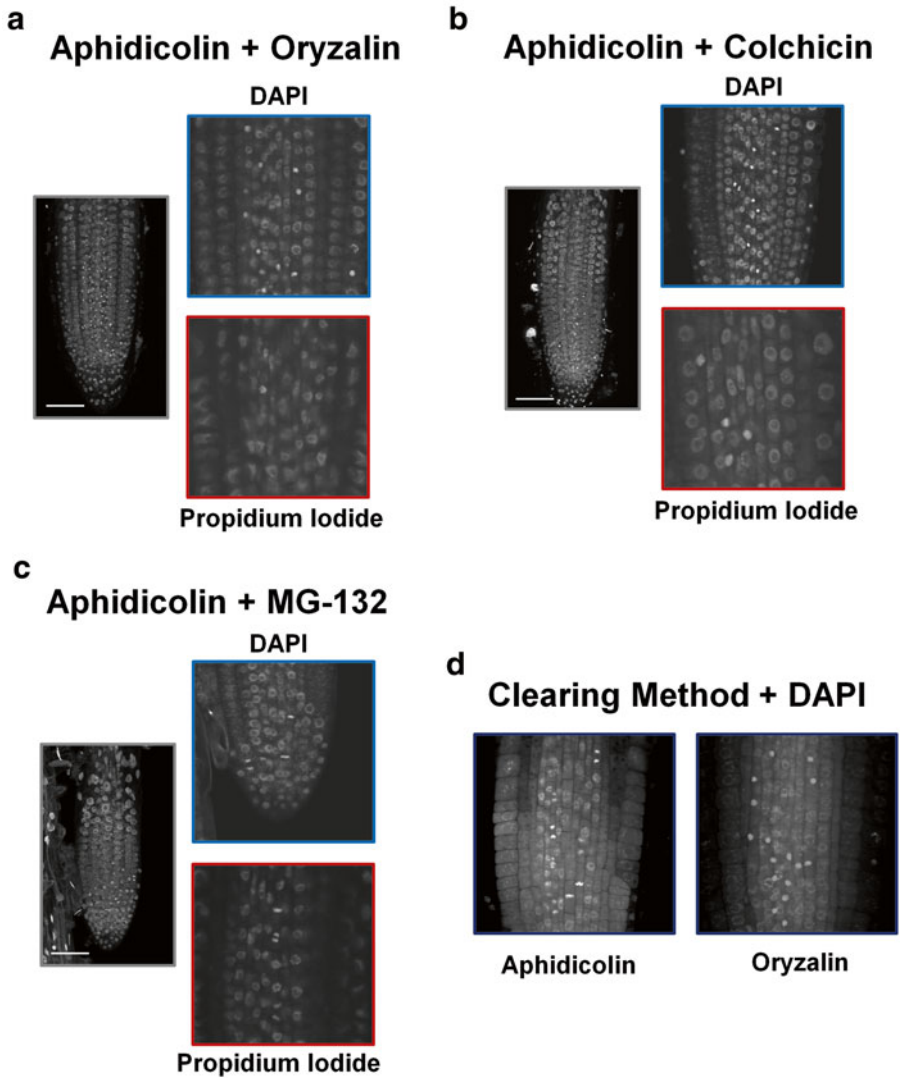

Fig. 3 Staining methods to evaluate nuclear cell cycle phase after inhibitor treatments. Two staining methods were presented herein: (1) a rapid DAPI staining method that allows fast nuclei evaluation in Arabidopsis roots. (2) Alternative simplified clearing procedure followed by propidium iodide (PI) or DAPI nuclear staining. (**a**) Images showing mitotic figures stained with DAPI or PI of roots blocked in prophase after cell cycle aphidicolin synchronization followed by oryzalin treatment. (**b**) Images showing mitotic figures stained with DAPI or PI of roots blocked in prophase after cell cycle aphidicolin synchronization followed by colchicine treatment. (**c**) Images showing mitotic figures stained with DAPI or PI of roots blocked in metaphase/anaphase after cell cycle aphidicolin synchronization followed by MG132 treatment. (**d**) Cleared Arabidopsis roots stained with DAPI show better nuclei and cytoplasm details. *Z*-stacks were generated from approximately 150 images of 1 μm optical slice thickness and used for 3D visualization of nuclei. Image acquisition was performed using a Leica SP8 confocal microscope. Maximum brightness projections were created using the SP8 software to overview nuclear organization in whole tissues. The scale bar represents 50 μm

4. Wash then samples twice with absolute ethanol and absolute methanol, for 5 min each.
5. Post-fix the samples for 5 min in fixative buffer 2.
6. Wash samples three times with PBT for 10 min each.
7. Incubated samples twice for 5 min with PBS containing 50 % formamide.
8. Subsequently, boil samples twice in a waterbath or thermomixer for 3 min following quenching on ice for 5 min.
9. Store samples overnight at room temperature.
10. Wash samples three times in PBS, for 15 min each.
11. Stain samples for 30 min at room temperature with either 1 μg/ml DAPI staining solution or 50 ng/ml (up to 100 ng/ml for larger samples) propidium iodide (PI) staining solution.
12. PI-stained samples should be washed twice with PBS at least for 2 h each.
13. Stained samples are mounted in 90 % glycerol on microscope slides, cover-slipped, and sealed with nail polish to avoid evaporation. Store at room temperature in slide boxes protected from the light.
14. Long period storage of samples (~30 days) improves tissue clearing.

3.5 Confocal Microscopy

1. Stained whole roots are observed under a confocal microscope (here, using an inverted Leica SP8 confocal microscope).
2. DAPI-stained samples are excited with a 405 nm diode laser and emission light is collected between 413 and 532 nm.
3. PI-stained samples are excited with a 552 nm OPSL laser and fluorescence emission is collected between 588 and 702 nm.
4. High-resolution optical sections are acquired using a HC PL APO CS2 40X/1.30 OIL objective lens allowing imaging to a depth of 200 μm within thick cleared root tissues. Samples are scanned at 400 Hz and standard 16× line averaged for image recording.
5. *Z*-stacks were generated consisting of approximately 150 images of 1 μm optical slice thickness and used for 3D visualization of nuclei. Maximum brightness projections were created using the SP8 software to overview nuclear organization in whole tissues (Fig. 2a).

4 Notes

1. The clearing method presented here is essentially based on the PI staining protocol published by [9], however it includes several simplifications such as reduction in washing and infiltration times. In addition, this method can be applied for thick

fixed plant tissue slices (>100μM) of diverse organs performed with a simple vibrotome.

2. Using the rapid DAPI staining protocol, efficient staining of nuclei was obtained to generate a general overview of all nuclei in complete samples, allowing the identification of mitotic events. DAPI staining of fully cleared samples not only resulted in a more detailed visualization of nuclear structures, but also offered a better cell and cytoplasm visibility due to the lack of fluorescence derived from the plant cell wall. PI staining on the other hand generated more background and showed to be more prone to incomplete clearing; a common problem when dealing with more compact tissues like root meristems. This is largely prevented when performing DAPI staining.
3. Cleared roots stained with DAPI besides staining the nuclei also leads to DNA staining of plastids and mitochondria giving more details on the overall cell structure and shape.
4. Roots after cell cycle aphidicolin synchronization followed by MG132 treatment blocked root cells in metaphase/anaphase and was the most efficient inhibitor to generated mitotic figures.
5. DAPI-stained samples pre-treated with aphidicolin followed by nocodazole treatment appeared similar as with aphidicolin treatment alone, therefore images are not presented.
6. Both methodologies presented here can be applied to intact seedlings as well as leaves, flowers, siliques and is compatible when using other plant species. In addition, these methods can be applied for thick fixed plant tissue slices (>100μm) of diverse organs performed with a simple vibrotome.
7. The use of inhibitors to block the cell cycle combined with the confocal visualization of nuclei when applying the methodology presented here can be of great help to study mutant lines affected in the plant cell cycle.

Practical Notes

8. Avoid sample drying during any step of the procedure to preserve tissue morphology.
9. Glycerol mounted slides containing samples can be stored at room temperature in the dark for more than a month still keeping the DAPI or PI fluorescence.(Steps 3.3.4 and 3.4.13)
10. It is not necessary to work under aseptic conditions throughout the whole protocol.
11. Time of fixation can be extended to O/N when processing large samples.(Step 3.2.5)
12. If you need to interrupt the protocol after fixation, keep samples in PBS at 4 °C, but for short time.(Step 3.2.6)
13. Vacuum infiltration of fixative and stains will improve and speed up infiltration within plant tissues.(Steps 3.2.5, 3.3.3 and 3.4.11)

14. The clearing method can be applied to large-sized plant material or other plant species when using thick tissue slices (100–500 μm mounted in 3–5 % agarose) made by a vibratome (e.g. Vibratome Series 3000; The Vibratome Company, St. Louis, MO, USA).
15. Staining samples with DAPI and PI simultaneously and evaluating images using different lasers can provide additional details on cell nuclear morphology.

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Chapter 6

Isolation of Plant Nuclei at Defined Cell Cycle Stages Using EdU Labeling and Flow Cytometry

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Abstract

5-Ethynyl-2′-deoxyuridine (EdU) is a nucleoside analog of thymidine that can be rapidly incorporated into replicating DNA in vivo and, subsequently, detected by using "click" chemistry to couple its terminal alkyne group to fluorescent azides such as Alexa Fluor 488. Recently, EdU incorporation followed by coupling with a fluorophore has been used to visualize newly synthesized DNA in a wide range of plant species. One particularly useful application is in flow cytometry, where two-parameter sorting can be employed to analyze different phases of the cell cycle, as defined both by total DNA content and the amount of EdU pulse-labeled DNA. This approach allows analysis of the cell cycle without the need for synchronous cell populations, which can be difficult to obtain in many plant systems. The approach presented here, which was developed for fixed, EdU-labeled nuclei, can be used to prepare analytical profiles as well as to make highly purified preparations of G1, S, or G2/M phase nuclei for molecular or biochemical analysis. We present protocols for EdU pulse labeling, tissue fixation and harvesting, nuclei preparation, and flow sorting. Although developed for Arabidopsis suspension cells and maize root tips, these protocols should be modifiable to many other plant systems.

Key words 5-Ethynyl-2′-deoxyuridine, Flow cytometry, Nuclei sorting, Arabidopsis, Maize, Percoll gradient, Cell cycle, DNA replication, Chromatin

1 Introduction

To investigate cell cycle-related processes, it is critical to be able to separate cells in distinct stages of the cell cycle for analysis. Mechanisms governing key cell cycle processes like chromosome segregation and DNA replication can then be studied. To do this, cells preparing to enter replication or go through cell division must be distinguished from actively replicating cells with the same DNA content. To this end, many types of nucleoside precursors have been used as labels for DNA synthesis over the years. Currently, the most efficient and versatile DNA precursor for

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fluorescent labeling is 5-ethynyl-2′-deoxyuridine (EdU), a nucleoside analog of thymidine. EdU, which has become the new standard for labeling newly replicating DNA, has been used to accomplish a wide range of experimental goals in diverse plant species (reviewed in [1]). Darzynkiewicz et al. [2] reviewed the benefits of using EdU in place of other precursors such as 5-bromo-2′-deoxyuridine (BrdU). In particular, eliminating the denaturation step that is necessary for antibody detection of BrdU helps maintain DNA and protein structural integrity and enhances structural preservation [2].

After EdU is incorporated into DNA, the terminal alkyne group can be coupled to a fluorescent azide such as Alexa Fluor 488 (Alexa-488) [3] through a process called copper(I)-catalyzed azide-alkyne cycloaddition or "click chemistry" [4]. This reaction is quite robust, and can be carried out on fixed or unfixed biological material. Labeled material can be used for flow cytometry, microscopy, fluorescence in situ hybridization (FISH), and immunochemistry in many plant tissues [5]. However, the few studies that have combined EdU labeling with flow cytometry were analytical in nature, and did not use flow sorting to enrich for plant nuclei at specific stages in the cell cycle. We have developed a method that uses flow sorting to prepare pure populations of unlabeled G1 and G2/M nuclei separate from EdU/Alexa-488-labeled, S-phase nuclei. This approach for staging nuclei allows us to start with an asynchronous population of nuclei and avoid artifacts sometimes associated with cell cycle synchronization procedures, such as sucrose starvation or incubation with nucleotide synthesis inhibitors [6].

Flow cytometric analysis of plant nuclei was originally made possible by the development of the widely used tissue chopping method introduced by Galbraith and colleagues [7]. They found that chopping with a razor blade mechanically releases nuclei from plant cells with a minimum of shear stress and structural disruption. The resulting homogenate may include a large amount of cellular debris, but when debris is reduced by filtration; such preparations can be analyzed on a flow cytometer. Modifying this original method, we found that nuclei can be released from fixed, frozen plant cells or tissues using a commercial blender or food processor by gentle blending in short bursts in cold cell lysis buffer. This modification allows us to dramatically scale up the amount of material we can process for flow sorting. Different plant species and tissues yield different levels of unwanted cellular debris during this process, and the nuclei isolation procedures must be optimized carefully. High levels of debris in a sample can greatly reduce the efficiency of sorting.

Several plant studies have described flow sorting of fresh or fixed nuclei based on DNA content [8–10]. Plant protoplasts have also been sorted based on their expression of GFP or other

fluorescent proteins [11–14]. However, protoplast preparations are not typically used for experiments where DNA content needs to be accurately estimated, because the DNA signal is often confounded by nonspecific binding of DNA fluorochromes, autofluorescence from plant pigments, and the non-symmetric position of nuclei within plant cells, which can alter light scattering properties during sorting [15].

The protocol presented here highlights the combination of EdU pulse labeling and preparative sorting of nuclei isolated from fixed plant cells or tissues. By adding a second parameter in addition to DNA content for the flow sorting—e.g. the fluorescence of Alexa-488 coupled to EdU as a measure of DNA synthesis, we can distinguish nuclei in different stages of the cell cycle with unlabeled G1 and G2/M nuclei separated from the arc of EdU-labeled, S-phase nuclei (Fig. 2 panels c and g). Importantly, G1 and G2/M nuclei with 2C and 4C DNA content, respectively, are readily resolved from nuclei in very early or very late S phase, which have similar DNA content. We discuss flow cytometry parameters and conditions for sorting plant nuclei using Arabidopsis suspension cells or maizeroot tips as starting material. Arabidopsis suspension cells provide a relatively homogeneous model system, while maize roots offer an opportunity to explore developmental regulation in the context of a whole tissue [1]. By describing methods of preparing and sorting nuclei from these two very different plant systems, our hope is to show how the protocols described here can be adapted to other plant systems.

2 Materials

All chemicals are reagent grade, unless otherwise noted.

2.1 Plant Material and Growth

2.1.1 Maize Seedlings

1. Plant material: *Zea mays* cv B73 seeds. The total number of seeds required should be determined empirically based on the germination rate and the amount of material required for the downstream analysis. A single experiment typically requires 400–800 maize seedlings.
2. 150 mL 10 % bleach containing 1–2 drops of Tween 20.
3. Supplies: magnetic stir plate, stir bar, large plastic or glass container, small fish tank bubbler, Magenta™ boxes, paper towels.
4. Equipment: growth chamber.

2.1.2 Arabidopsis Cells

1. Plant material: Arabidopsis suspension cells (Col-0, ecotype Columbia).
2. 2,4-Dichlorophenoxyacetic acid (2,4-D): 100 mg in 10 mL of 95 % ethanol, store at −20 °C.

3. Arabidopsis culture medium: 3.2 g/L of Gamborg's B5 basal medium with minimal organics, 3 mM MES, 3 % sucrose, 1.1 mg/L 2,4-D, pH 5.8, autoclave and store at 4 °C.
4. Supplies: 250-mL baffled flasks, serological pipettes.
5. Equipment: laminar flow hood, refrigerated console shaker with a light bank.

2.2 Labeling Newly Replicated DNA In Vivo and Harvesting Tissue

1. 5-Ethynyl-2′-deoxyuridine (EdU) (Life Technologies): 40 mM solution in dimethyl sulfoxide (DMSO), store at −80 °C.
2. 1× Phosphate buffered saline (PBS).
3. 16 % Paraformaldehyde (EM Grade).
4. 2 M Glycine, filter-sterilized.
5. Liquid nitrogen.

2.2.1 Maize Seedlings

1. Supplies: #10 scalpel, fine tipped forceps, Petri dishes, 2-mL microcentrifuge tubes.
2. Equipment: bench-top orbital shaker, vacuum pump attached to a desiccator.

2.2.2 Arabidopsis Cells

1. Supplies: 50-mL conical tubes.
2. Equipment: refrigerated swinging bucket centrifuge.

2.3 Isolation of Nuclei

1. Cell lysis buffer (CLB): 15 mM Tris (pH 7.5), 2 mM Na_2EDTA (pH 8), 80 mM KCl, 20 mM NaCl, 0.1 % Triton X-100. Adjust pH to 7.5 then add 15 mM 2-mercaptoethanol (buffer modified from LB01 in [15]).
2. Complete or Complete Mini protease inhibitor cocktail tablets (Roche).
3. Supplies: Miracloth, small plastic funnel.
4. Equipment: commercial blender (like Cuisinart, model SPB-7) or food processor (like Cuisinart mini-prep).

2.4 Percoll Gradient Purification (Optional)

This optional procedure is modified from [16], and is only necessary when the percentage of nuclei is very low in relation to the percentage of unwanted debris in the sample.

1. Gradient buffer (5×): 2.5 M hexylene glycol, 25 mM PIPES-KOH (pH 7.0), 50 mM $MgCl_2$, 25 mM 2-mercaptoethanol, and 5 % Triton X-100. 5× gradient buffer can be prepared as a stock solution without 2-mercaptoethanol and stored at 4 °C. Add 2-mercaptoethanol immediately before use.
2. Gradient buffer (1×): 0.5 M hexylene glycol, 5 mM PIPES-KOH (pH 7.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 1 % Triton X-100. Dilute to 1× from the 5× stock buffer and add 2-mercaptoethanol immediately before use.

3. Extraction buffer (1×): 2.0 M hexylene glycol, 20 mM PIPES-KOH (pH 7.0), 10 mM $MgCl_2$, and 5 mM 2-mercaptoethanol.
4. 30 % Percoll: 100 % Percoll diluted in 5× gradient buffer and sterile H_2O to achieve a final concentration of 1× gradient buffer.
5. 80 % Percoll: 100 % Percoll diluted in 5× gradient buffer and sterile H_2O to achieve a final concentration of 1× gradient buffer.
6. Supplies: 50-mL glass Corex tubes with screw cap.

2.5 Clicking EdU to Alexa Fluor 488

1. Click-iT®EdU Alexa Fluor® 488 Imaging Kit (Life Technologies).
2. Modified CLB: 15 mM Tris (pH 7.5), 80 mM KCl, 20 mM NaCl, 0.1 % Triton X-100, pH 7.5.
3. DAPI stock solution: 1 mg/mL in sterile H_2O.
4. CLB containing 2 μg/mL DAPI.
5. Equipment: refrigerated microcentrifuge.

2.6 Flow Cytometric Sorting

1. 1× Sodium Chloride-Tris-EDTA (STE) buffer: 10 mM Tris–HCl (pH 7.5), 1 mM EDTA (pH 8), 100 mM NaCl, pH 7.5.
2. Laser alignment beads suitable for the flow sorter and particle size to be analyzed. For the BD InFlux 3–3.4 μm and 8.1–12 μm Ultra Rainbow Fluorescent Beads (Spherotech) are one option.
3. Nuclei collection buffer suitable for the downstream application (*see* **Note 14** and Table 1).
4. CellTrics® 20 μm nylon mesh filters (Partec).

Table 1

Collection buffers and post-sort nuclei handling

Downstream application	Collection buffer	Immediate post-processing	Method reference(s)
DNA-IP or other DNA application	STE buffer	Freeze in STE or immediately process	[18]
ChIP	2× Extraction buffer 2	Centrifuge, extract chromatin	[19]
Microscopy/FISH	2× CLB without 2-mercaptoethanol	Store protected from light at 4 °C	[1], Bass et al. in preparation
Sort reanalysis	CLB-DAPI	None	–

5. Supplies: 5-mL round-bottom polypropylene tubes, microscope slide.
6. Equipment: fluorescence microscope, flow sorter equipped with a 355 nm UV laser and a 488 nm sapphire laser.

3 Methods

3.1 Plant Material and Growth

3.1.1 Maize Seedlings

1. Imbibe the maize seeds overnight in constantly moving water with aeration. To do this, set up a large container with a stirbar and a fishtank bubbler, and fill it with diH_2O.
2. Surface-sterilize the seeds by adding the 10 % bleach with Tween 20 and gently shake for 10 min. Rinse with sterile H_2O three times.
3. Germinate the seeds in autoclaved magenta boxes containing several layers of paper towel dampened with sterile H_2O. Place 10–12 seeds per box and place the boxes in a growth chamber set to 28 °C with continuous dim light (~500 lux) for 3 days.

3.1.2 Arabidopsis Cells

1. Arabidopsis suspension cells are grown in 250-mL baffled flasks containing 50 mL medium in a refrigerated console shaker set to 23 °C and 160 rpm with constant light (~2000 lux). Propagate the cells every 7 days by transferring a 6 mL aliquot to a flask with 50 mL fresh, pre-warmed medium. To maximize EdU incorporation, cells have to be in the logarithmic phase of the growth curve [8]. To achieve this, 25 mL of 7-day cells are diluted 1:1 in an equal volume of fresh, pre-warmed medium and grown for 16 h before adding EdU.

3.2 Labeling Newly Replicated DNA In Vivo and Harvesting Tissue

3.2.1 Maize Seedlings

1. Place the 3-day-old seedlings in a large container with 100–300 mL sterile H_2O. Swirl to rinse and pour off the water.
2. Depending on the number of seedlings, add 100–300 mL of sterile H_2O containing a final concentration of 25 μM EdU, making sure the roots are covered in the water. Incubate the seedlings for the desired pulse-labeling time (*see* **Note 1**) with gentle shaking (65 rpm) on a bench-top orbital shaker at room temperature in the dark.
3. Pour off the EdU solution and rinse the seedlings three times with sterile H_2O, leaving some of the last rinse in the container to keep the roots moist.
4. Prepare a solution of 20 mL 1 % paraformaldehyde in 1× PBS.
5. Dissect the desired root tip segment in a Petri dish using a #10 scalpel and fine-tipped forceps. Transfer the cut segments to a small beaker or tube containing 20 mL of 1 % paraformaldehyde and fix for 15 min, with the first 5 min under vacuum. The length of time cutting a batch of root segments should be minimized as much as possible.

6. Add 1.33 mL of 2 M glycine (final concentration 0.125 M) to quench the fixation, and incubate for 5 min under vacuum.
7. Wash the root segments three times with ~20 mL 1× PBS and remove the liquid completely with a pipet after the final wash. Transfer the roots to a 2-mL tube and snap-freeze in liquid nitrogen. Store at −70 °C.

3.2.2 Arabidopsis Cells

1. Add 12.5 μL of 40 mM EdU (final concentration 10 μM) to each flask containing 50 mL of cell culture in logarithmic phase (the 1:1 diluted culture, grown for 16 h).
2. Allow cells to incorporate EdU for the chosen amount of time (*see* **Note 1**) by incubating in the console shaker.
3. Add 3.33 mL of 16 % paraformaldehyde to each flask (final concentration 1 %) and place in the console shaker for 10 min.
4. Add 3.55 mL of 2 M glycine (final concentration 0.125 M) to quench fixation, and incubate for 5 min.
5. Pour the contents of each flask into a 50-mL conical tube.
6. Centrifuge at 200 × *g* for 3 min and remove the supernatant.
7. To wash the cells, dilute to 50 mL with 1× PBS and gently invert the tube.
8. Centrifuge at 200 × *g* for 3 min and remove the supernatant.
9. Repeat **steps 7** and **8** for a total of three washes.
10. After the final wash, pipette off the supernatant and remove the residual PBS by pressing the tip of a pipette against the bottom of the conical tube and slowly aspiring until the cells are dry and there is no visible liquid.
11. Snap freeze the tube in liquid nitrogen and store at −70 °C.

3.3 Isolation of Nuclei

Nuclei are isolated from fixed, frozen cells or tissues by chopping the frozen pellet in a blender or food processor in cold CLB. All steps should be done on ice or at 4 °C. Additionally, the nuclei must be re-suspended very well after every centrifugation to ensure that large clumps of aggregates and debris do not form (*see* **Note 3**).

1. Set up a double layer of Miracloth in the top of a small plastic funnel. Place the blender or food processor inside a cold room or fridge before starting. Set a large refrigerated swinging bucket centrifuge to 400 × *g* at 4 °C and a refrigerated micro-centrifuge to 200 × *g* at 4 °C.
2. Make fresh CLB and keep on ice. If using, dissolve a Complete or Complete Mini protease inhibitor cocktail tablet in the water used to make the buffer (*see* **Note 13**). Add an appropriate amount of CLB to the blender or food processor (*see* **Note 2**).
3. Take the sample tube out of the −70 °C freezer, and tap the tube until the cell or tissue pellet is released from the bottom

of the tube. Two frozen pellets can be chopped together in large-scale experiments. If the pellet does not release from the tube, a small amount of CLB can be added and forceps inserted to break the pellet.

4. Transfer the frozen pellet to the blender or food processor. Grind the pellet on a low speed (low or chop) for 15–30 s at a time with 15–30 s breaks. Do this two to six times depending on the length of the chop and the tissue and species used.
5. Allow the cellular homogenate to incubate in the blender or food processor for 5 min at 4 °C to facilitate nuclei release from the cells.
6. Place the Miracloth plus funnel over a 50-mL conical tube or small beaker on ice. Moisten the Miracloth by adding 5–10 mL CLB and discard the flow-through.
7. Swirl to mix the cellular homogenate and filter through the Miracloth into the tube or beaker. Allow the homogenate to filter through for several minutes, then gently press on the Miracloth to remove excess buffer.
8. Dispense the filtered homogenate into 2–4 pre-chilled 50-mL conical tubes (each containing 15–20 mL) and centrifuge at 400 × *g* for 5 min at 4 °C. Pipette off the supernatant being careful not to disturb the white nuclei pellet.
9. Proceed immediately to the optional Percoll gradient purification (Subheading 3.4) or to the click reaction (Subheading 3.5).

3.4 Percoll Gradient Purification (Optional)

Several sample preparation conditions may need to be tested and analyzed on the flow cytometer to determine the optimal set up for each sample type. Preliminary flow cytometer results can determine the relative percent of the sample that is nuclei compared to small or large debris, and further improvements should be made to the blending conditions or filtering if possible. If the nuclei isolation cannot be improved further, you can consider using a density gradient purification step to remove some debris after nuclei isolation. An optional Percoll gradient purification protocol (adapted from [16]) is presented here (*see* **Note 4**).

1. Prepare 15 mL of 1× gradient buffer and 30 mL 1× extraction buffer per gradient. Also prepare 7 mL 80 % Percoll and 19 mL 30 % Percoll per gradient and keep all buffers on ice.
2. The number of gradients needed per sample must be empirically determined. Use a serological pipette with a bulb or an electric pipettor at low speed to layer each gradient in a glass Corex tube. First, add 12 mL of the 30 % Percoll layer, then sub-layer 6 mL of 80 % Percoll, and put on ice (see diagram in Fig. 1).

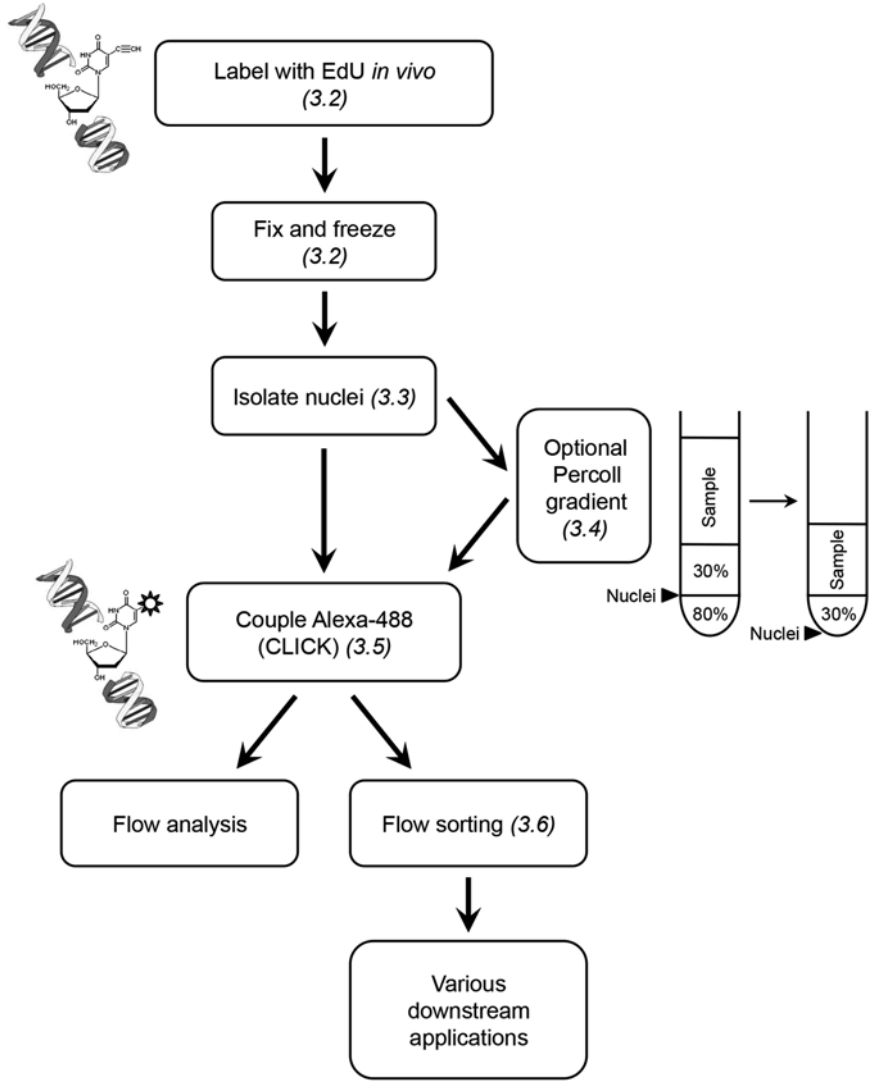

Fig. 1 Flow chart of the protocol to EdU pulse label and prepare nuclei for flow sorting

3. Re-suspend the nuclei pellet in each 50-mL conical tube with 2 mL 1× extraction buffer. To do this, gently pipet up and down at least 50 times for each tube. Combine the nuclei into one tube and bring the final volume up to 30 mL in 1× extraction buffer.
4. Using a slow, constant speed, add the re-suspended nuclei to the top of the gradient. Do this by putting the pipette against the side of the tube and slowly releasing the sample until there is about half an inch in depth, and then the sample can be added in the center of tube.

5. Centrifuge at 1500 × *g* for 30 min at 4 °C.
6. Take off the top layer of gradient buffer with a glass pipette. Do not allow back flow, which could disrupt the gradient.
7. Use a glass pasture pipette to carefully remove the white nuclei layer, and transfer to another cold 50-mL glass Corex tube.
8. Re-suspend the nuclei well in 1× gradient buffer, to a total volume of 10 mL. Then slowly underlay with 6 mL of 30 % Percoll.
9. Centrifuge at 1500 × *g* for 10 min at 4 °C. Remove the supernatant (buffer and Percoll), the nuclei are pelleted at the bottom of the tube.
10. Wash the nuclei pellet with 3–5 mL of 1× extraction buffer. To do so, re-suspend the nuclei well, centrifuge at 1500 × *g* for 10 min at 4 °C and remove the supernatant.
11. Proceed immediately to the click reaction (Subheading 3.5).

3.5 Clicking EdU to Alexa Fluor 488

Before the click reaction, the nuclei must be washed in a buffer without 2-mercaptoethanol and Na_2EDTA, so the reaction can proceed efficiently.

1. Wash each 50-mL sample tube with 2 mL Modified CLB and re-suspend the white nuclei pellet very well by pipetting 50 times for each tube. Transfer the re-suspended nuclei into two 2-mL microcentrifuge tubes.
2. Centrifuge at 200 × *g* for 5 min at 4 °C to pellet nuclei. Remove the supernatant by pipetting.
3. Prepare the Click-iT® reaction cocktail according to the kit instructions (*see* **Note 5**).
4. Add the Click-iT® reaction cocktail and re-suspend the nuclei well. Depending on the amount of reaction cocktail used, split the sample between sufficient 2-mL tubes to maintain ~1 mL in each tube. Incubate 30 min at room temperature in the dark.
5. Centrifuge at 200 × *g* for 5 min at 4 °C and remove the supernatant.
6. Depending on the amount of reaction cocktail used, add twice that volume of CLB to wash out the reaction cocktail, and re-suspend the nuclei very well. Centrifuge as before, and remove the supernatant.
7. Re-suspend the nuclei pellet in an appropriate volume of CLB-DAPI (*see* **Note 6**) depending on nuclei yield. Pipet the sample gently ~100 times, but try to avoid making bubbles.
8. Check a small aliquot of the nuclei on a fluorescence microscope to verify nuclei quality and DAPI and Alexa-488 fluorescence (DAPI needs at least 5 min for staining).

9. Just before flow cytometric analysis and sorting, filter the nuclei suspension through a 20-μm nylon mesh filter into a 5-mL round-bottomed polypropylene tube to remove large debris.

3.6 Flow Cytometric Sorting

Flow sorting may take place in consultation with a flow-core facility that will have specific recommendations and settings. Settings for an InFlux (BD Biosciences, originally Cytopeia) are described here, but these settings should be considered as suggested values only. The specific settings will need to be determined empirically based on the experimental goals, sample type, and flow sorter.

1. Turn on and prepare the flow sorter. A 70-μm nozzle tip is used with 16 psi sheath pressure. This gives stable droplet formation with a droplet delay of 17.6, and a piezo amplitude of 1–3 V. However, machine settings change some from day to day, and over the life of the instrument.
2. Using a forward angle light scatter (FSC) trigger (*see* **Note 7**), prepare the software to measure FSC, 90° angle side scatter (SSC), and emission at 460/50 nm for DAPI fluorescence and at 530/40 nm for Alexa-488 fluorescence.
3. Begin running a sample tube containing the nuclei preparation and adjust the sample pressure to achieve an event rate of 3000–5000 events/s (*see* **Note 8**).
4. First, create dotplots of FSC vs. SSC, FSC vs. DAPI fluorescence, and SSC vs. DAPI fluorescence (Fig. 2 panels a and e). Additionally, a FSC threshold or cutoff should be set empirically to minimize the amount of small debris in the analysis, without excluding the particles of interest. Apply the photomultiplier tube (PMT) voltage settings so that the measured events are centered in the plot. However, because plant tissue homogenates are inherently noisy, and the nuclei may be a small percentage of the total particles (*see* **Notes 3** and **4**), you may need to reduce the FSC or SSC voltages so that the measured events are centered more in the bottom third of the plot.
5. In the FSC vs. DAPI fluorescence and SSC vs. DAPI fluorescence plots, distinct populations should be visible for 2C, 4C and larger ploidy nuclei on the DAPI axis. Create a univariate histogram of DAPI fluorescence to see this clearly (Fig. 2 panels b and f). The coefficient of variation (CV) should be calculated for the G1 peak, which ideally should be below 5 % (*see* **Note 9**).
6. Next, create a dotplot of Alexa-488 fluorescence (530/40 nm—Height) vs. DAPI fluorescence (460/50 nm—Height). Apply the PMT voltage settings so that an arc of Alexa-488-labeled nuclei in S phase can be seen above the unlabeled G1

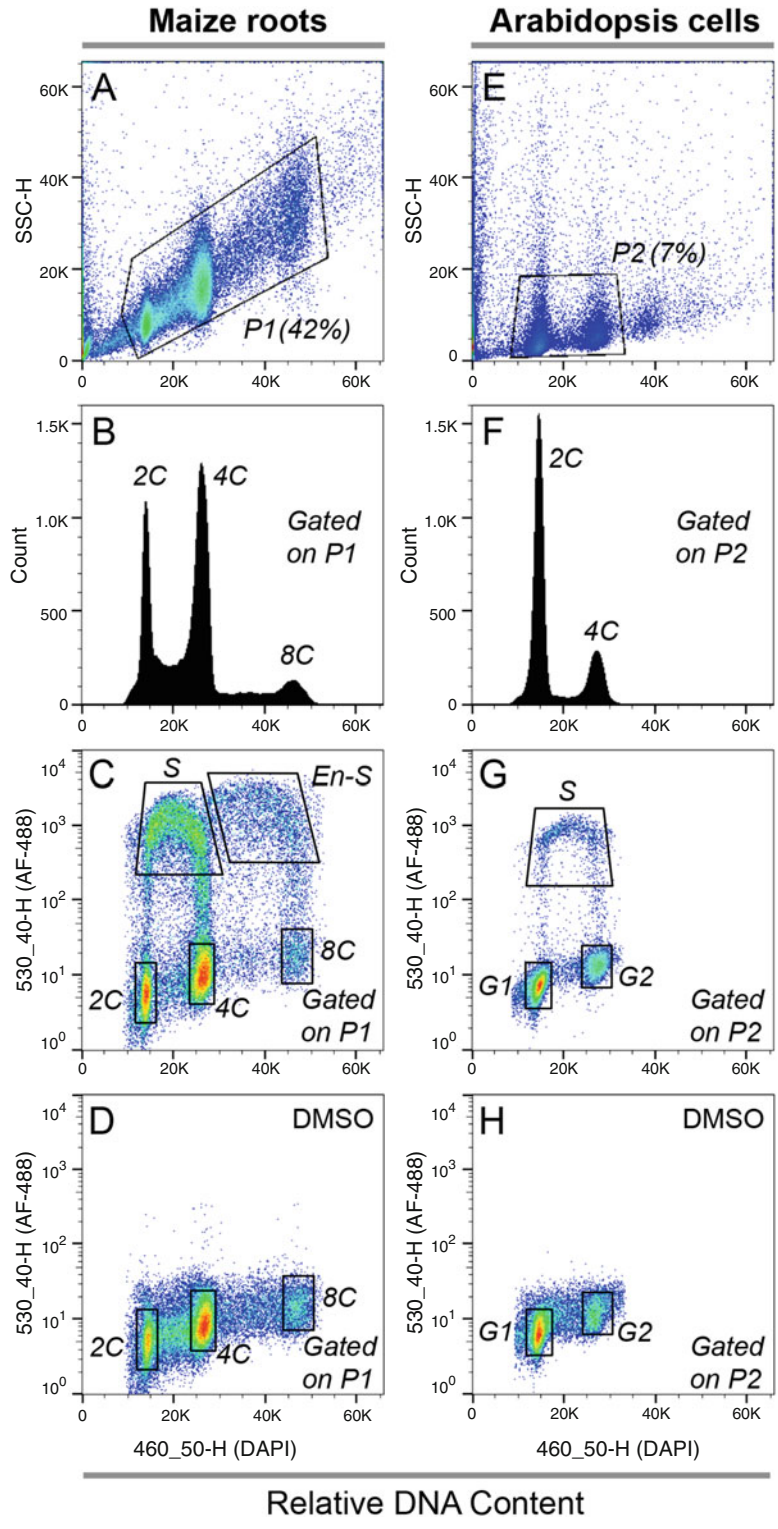

Fig. 2 Flow cytometric analysis and sorting of nuclei prepared from maize roots and Arabidopsis cells. (**a–d**) Intact maize roots were pulse-labeled with EdU for 20 min. The first 3 mm of the root tip was then dissected, fixed, and frozen. (**e–h**) Arabidopsis suspension cells were pulse-labeled with EdU for 30 min, followed by

and G2/M nuclei. Depending on the tissue type used, there may be multiple arcs for several rounds of endoreduplication (*see* Fig. 2 panels c and g).

7. Place sorting gates to divide the cell cycle populations as desired (*see* **Note 10**).
8. Set the sort mode based on the experimental needs and abundance of the nuclei to be sorted. Single-droplet purity mode can be used to achieve a sort efficiency of 85–95 %.
9. Validate the sort parameters and gate placement by sorting 100 nuclei onto a microscope slide and observing and counting them on a fluorescence microscope. It is also important to reanalyze a small amount of sorted nuclei to further confirm sorting parameters and to determine the sort purity (*see* **Note 12**).
10. The amount of nuclei to be sorted for an experiment should be empirically determined for each plant species and downstream application (*see* **Note 13**). Nuclei preparations are often split between several round-bottomed 5-mL tubes that must be kept on ice and protected from light while sorting. Use the cooling function on the flow sorter to keep both the sample tube and the nuclei collection tubes chilled.
11. Depending on the downstream application, the collection buffer for sorted nuclei and handling after sorting will vary (*see* **Note 14** and Table 1).

4 Notes

1. *EdUpulse length*. Depending on the experimental goals, the length of the EdU pulse should be selected carefully. In many cases, knowing the approximate length of S phase in the material under study will allow a more informed decision on pulse length. We have found that a 5 min EdU pulse is detectable by

Fig. 2 (continued) fixation and freezing. Nuclei were prepared and analyzed by flow cytometry. (**a, e**) Typical bivariate plots of side scatter (SSC) and relative DNA content (measured by DAPI fluorescence in the 460 ± 50 nm detection range). Nuclei are gated on SSC to exclude small and large cellular debris as well as damaged nuclei from further analysis and sorting. The SSC gate can be drawn to include endocycling nuclei, as depicted for maize roots in panel (**a**), or exclude endocycling and anueploid nuclei, as depicted for Arabidopsis cells in panel (**e**). (**b, f**) The corresponding univariate histograms of relative DNA content, which only include events within the parent SSC gates (P1 in panel (**a**), or P2 in panel (**e**)). (**c, g**) Bivariate plots of EdU incorporation (measured by Alexa-488 fluorescence in the 530 ± 40 nm detection range) and relative DNA content show nuclei that are actively replicating their DNA as an arc above the G1 and G2/M populations. Sorting gates can be placed to purify nuclei from different cell cycle phases, including 2C/G1, 4C/G2, 8C, S, and endocycling S (En-S). (**d, h**) Samples were prepared as described above, except roots or cells were treated with DMSO in place of the EdU. Mock-labeled control samples are useful for appropriate sort gate placement

flow cytometric analysis, but a 10–15 min pulse is more useful to achieve the separation of the S-phase arc needed for sorting. For many purposes longer pulses (30 min–1 h) are more practical, but the appropriate time depends on many factors, including the desired cell cycle population, percentage of cells in S phase in the tissue, genome size, sorting yield, sensitivity of the analysis, and the downstream application.

2. *Blending optimization.* The amount of CLB will depend on the specific blender or food processor, and the position of the blades. We routinely use ~80 mL CLB in a large Cuisinart blender for Arabidopsis cell pellets, and ~35 mL CLB in a Cuisinart mini-prep food processor for maize roots. Use one of the lowest settings (low or chop) for short intervals with resting periods to allow the liquid to collect back in the bottom of the jar. For maize root tips, we use 5–6 15-s intervals with 15-s rests. For Arabidopsis cell pellets, we use two 30-s intervals with a 30-s rest.
3. *Nuclei handling.* In some plant nuclei samples, the percentage of the target population will be small. This percentage will decrease further if the tissues are blended too much, nuclei isolation conditions are not optimal, or there are large aggregate clumps. It is extremely important to re-suspend the nuclei well at each and every step during sample preparation to avoid forming these clumps. Large aggregate clumps will reduce the yield of nuclei that can be sorted, and can clog the instrument sample line or nozzle tip. Following every centrifugation, pipet gently up and down at least 50 times. Additionally, some tissues and cell types result in more debris and small particles than others. If the nuclei preparation cannot be improved further by technical optimization, consider using a Percoll gradient purification step (*see* **Note 4**).
4. *When to use a Percoll density gradient.* In a large-scale experiment, sorting a sample with a small percentage of target nuclei can be very time-consuming and, for this reason, can become the limiting step. Particularly for Arabidopsis cells, the main issue is often the presence of debris particles that are smaller than nuclei, but not so much smaller that they can be effectively excluded by setting a threshold cutoff on the flow sorter. The debris particles are counted as events, which can slow down the sort significantly. To remove some of the debris before sorting, a further Percoll gradient purification step can be performed.
5. *Click-iT® reaction cocktail.* The amount of Click-iT® reaction cocktail to use for a particular starting tissue amount should be empirically determined. As a starting place, we recommend 0.5 mL of reaction cocktail for a 250-mL flask of 7-day split

Arabidopsis cells, and 2 mL cocktail for the roots from ~250 maize seedlings.

6. *Final sample volume*. The amount of CLB-DAPI used for the final re-suspension of nuclei is variable and can depend on the efficiency of nuclei release, any losses that occurred during handling (especially if Percoll gradient purification is used), and the optimal sample rate of the flow sorter (*see* **Note 8**).
7. *Event triggering*. Events can be triggered on either FSC or SSC. Some find that triggering on SSC in plant preparations can be less noisy [12]. In our experience with plant nuclei preparations from fixed tissue, there was not much benefit from triggering on SSC, and therefore chose to trigger on the traditional FSC. Some instruments may allow triggering on UV fluorescence, depending on the specific configuration of the lasers, which may be advantageous for samples that have a large amount of non-fluorescent debris.
8. *Event rate and sort rate*. The optimal event rate will depend on the type of flow sorter, the sheath pressure, and quality of the sample preparation. The relative "clumpiness" of the sample will increase the possibility of clogs in the sample line or nozzle, and can decrease the sort efficiency (number of sorted events/(sort aborts + sorted events) × 100). We typically use an event rate between 3000 and 5000 events/s. The sort rate, or rate that nuclei from each sort gate will accumulate, will vary depending on the percentage of the targeted population.
9. *Sample quality control*. Ideally, the CV of the G1 peak should be below 5 % [17]. However, we have experienced peaks with a CV of ~5–6.5 %, that still have good sort purity upon reanalysis, and yield good downstream results. Given that the end goal of our approach is to sort different cell cycle populations for further analysis, this is likely not an issue. Slightly larger CVs may be unavoidable because of the large-scale sample preparations needed for sorting. If the CV of the G1 peak is wider than expected, it is likely caused by poor sample quality. Checking the nuclei preparation on a fluorescence microscope will help identify if the sample has large aggregate clumps or if nuclei appear damaged from over-blending, or over-centrifugation. The plot of SSC vs. DAPI fluorescence is also useful to determine if the nuclei have irregular light scattering properties, which can indicate damaged or misshapen nuclei (*see* Fig. 2 panels a and e).
10. *Sort gates*. If necessary, hierarchical gates can be used to eliminate cellular debris as well as damaged or misshapen nuclei based on SSC (*see* Fig. 2 panel a and e). Then gating of the cell cycle is done only on the subsequent daughter population. The SSC gate can be drawn to include endocycling nuclei, as is

depicted for maize roots in panel a, or to exclude endocycling and anueploid nuclei, as is depicted for Arabidopsis cells in panel e. Sort gates can be set in various ways depending on the experimental goals (for example, *see* Fig. 2 panels c and g). Negative controls should also be used to verify the gating strategy is appropriate (*see* **Note 11** and Fig. 2 panels d and h).

11. *Negative controls for sort gating*. When optimizing the EdU pulse label, it is also important to run negative control samples for each treatment and label time. In the case of a short label, where a weaker signal is expected, negative controls are critical for setting sort gates. Usually, the most useful control for gating is a sample that is mock-labeled with DMSO, and then the click reaction and all other procedures are done in parallel to the experimental sample (Fig. 2 panels d and h).
12. *Sort reanalysis*. It is important to reanalyze a small amount of the nuclei to determine the sort purity and to ensure that sort settings and gates are appropriate. For reanalysis purposes, sort at least 12,000–15,000 nuclei into a small amount (~200 μL) of CLB-DAPI. In our experience, DAPI is more susceptible to bleaching than Alexa-488, however re-staining in CLB-DAPI alleviates most of the DAPI bleaching. Save data files to analyze the percentage of nuclei that fall back in the original sort gate. Some population spreading is inherent due to the fact that nuclei may not be exactly round and will pass through the laser beam the second time in different orientations.
13. *Sort yield and timeframe*. The number of nuclei sorted (sort yield) will vary based on losses during nuclei isolation and other purification steps, sorting parameters, and the size and number of gates. The number of nuclei needed for a downstream application will also vary. Preliminary sorts are required to determine the sort yield and timeframe. For longer sorts, the nuclei preparation can be done the day before a sample is run on the machine, as long as a Complete protease inhibitor cocktail tablet is used in the CLB buffer. The sample should be stored overnight at 4 °C and protected from light. **Steps 7–9** of Subheading 3.5 should be completed the morning of the sort.
14. *Nuclei handling aftersorting*. Depending on the volume of nuclei to be sorted, add 200 μL–1 mL of collection buffer (*see* Table 1 for buffer options for various downstream applications) to a 2-mL collection tube. Other collection tubes and plate formats may also be used, if the flow sorter allows. The sorted droplets of sheath fluid (STE buffer) containing the nuclei will dilute the collection buffer, necessitating the use of a 2× buffer in some cases. For applications where a large quantity of nuclei are needed, multiple tubes of sorted nuclei may need to be combined and centrifuged to remove excess buffer

(850 × *g* for 10 min at 4 °C) before proceeding. The nuclei pellet will likely be invisible at this stage, so leave ~0.5 mL of supernatant over the pellet. If the volume of sorted nuclei is sufficiently small, the sample can be stored at 4 °C (for microscopy), frozen at −70 °C (for DNA applications), or immediately processed to extract DNA or chromatin (*see* Table 1).

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Chapter 7

Preparation and Fluorescent Analysis of Plant Metaphase Chromosomes

Trude Schwarzacher

Abstract

Good preparations are essential for informative analysis of both somatic and meiotic chromosomes, cytogenetics, and cell divisions. Fluorescent chromosome staining allows even small chromosomes to be visualized and counted, showing their morphology. Aneuploidies and polyploidies can be established for species, populations, or individuals while changes occurring in breeding lines during hybridization or tissue culture and transformation protocols can be assessed. The process of division can be followed during mitosis and meiosis including pairing and chiasma distribution, as well as DNA organization and structure during the evolution of chromosomes can be studied. This chapter presents protocols for pretreatment and fixation of material, including tips of how to grow plants to get good and healthy meristem with many divisions. The chromosome preparation technique is described using proteolytic enzymes, but acids can be used instead. Chromosome slide preparations are suitable for fluorochrome staining for fast screening (described in the chapter) or fluorescent in situ hybridization (*see* Schwarzacher and Heslop-Harrison, In situ hybridization. BIOS Scientific Publishers, Oxford, 2000).

Key words Chromosome, Heterochromatin, Metaphase, Meiosis, Proteolytic enzyme, Fluorochromes, DAPI

1 Introduction

Chromosome number and their shapes and sizes are important characters of species and have been investigated since the early days of microscopy [1]. Initially, transmitted light microscopy was used and chromosomes were stained with carmine, orcein, or Feulgen [2] resulting in simple uniformly stained chromosomes showing the typical constrictions at the centromeres and secondary constrictions at the nucleolus-organizing regions (NORs) [3]. If specific pretreatments are used, characteristic bands, e.g. Giemsa C-banding for heterochromatin, are produced [1, 4]. Later, fluorescence microscopy and fluorochromes (*see* Table 1; Figs. 1 and 2) have been introduced that bind directly to DNA, either to AT-rich or GC-rich DNA sequences or uniformly ([5]; *see* Table 1). Classic

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Table 1

Characteristics of fluorochromes and fluorescent filter set specifications

Fluorochrome	Characteristics	Color	Filter set specifications		
			Approximate excitation range	Dichroic mirror	Approximate emission range
DAPI	AT-selective binding	Blue	350–375 nm (UV)	400 nm	435–485 nm or above 400 nm
Chromomycin A3 (CA3 or CMA)[a]	GC-selective binding	Yellow-green	430–480 nm (blue)	510 nm	540–590 nm or above 520 nm
YOYO-1	Total DNA	Green	480–500 nm (green)	505 nm	505–525 nm
TOTO-1	Total DNA	Red	490–520 nm	530 nm	530–550 nm
Propidium iodide (PI)[b]	Total DNA	Red	510–550 nm (green)	565 nm	590–630 nm

Different manufacturers use slightly different filters in predesigned cubes that they specify for the common fluorochromes in use, but they should fall within the ranges given

[a]CA3 will appear green in filter sets that cut of emission above 560 nm, but yellow if a wider band or long-pass filter is used. Normally the filter set specified for Fluorescein (FITC) can be used

[b]PI can also be excited with other filter sets of lower wavelength range allowing visualization of two fluorochromes simultaneously, but it will be less strong

stains include DAPI, Chromomycin A3, and propidium iodide, but many new, strong, and stable fluorochromes are regularly introduced. For example, the dimeric cyanine nucleic acid stains TOTO and YOYO from Molecular Probes are some of the high-affinity nucleic acid stains available and single molecules and DNA fibres can be stained (*see* e.g. [6]), but they also produce characteristic bands on chromosomes (*see* Fig. 2d). In many species, repetitive sequences have the same GC/AT ratio as euchromatin and are not distinguished (*see* examples in Fig. 1). NORs often show Chromomycin A3-positive and DAPI-negative staining due to the high content of the repetitive 45S rDNA genes that are GC-rich as genes in general (*see* Fig. 2a, b). In many species, bulk differences in DNA composition (AT:GC ratio) mean stains can characterize heterochromatin in more detail and can produce bands (*see* Fig. 2c, d), potentially defining several classes of heterochromatin (e.g. [7]).

Both banded or uniform, colorimetric or fluorochrome-stained chromosomes have been used extensively to establish karyotypes of many plant species and to describe evolutionary relationsships. In particular, chromosome base numbers in polyploidy series, interspecies diversity, and chromosome rearrangements have been determined [7–11]. The simple staining techniques are still of value for initial cytogenetic screening when studying a new species complex, and when ploidy level or aneuploidies need to be established for tissue culture, mutant, or hybrid and backcross lines. In

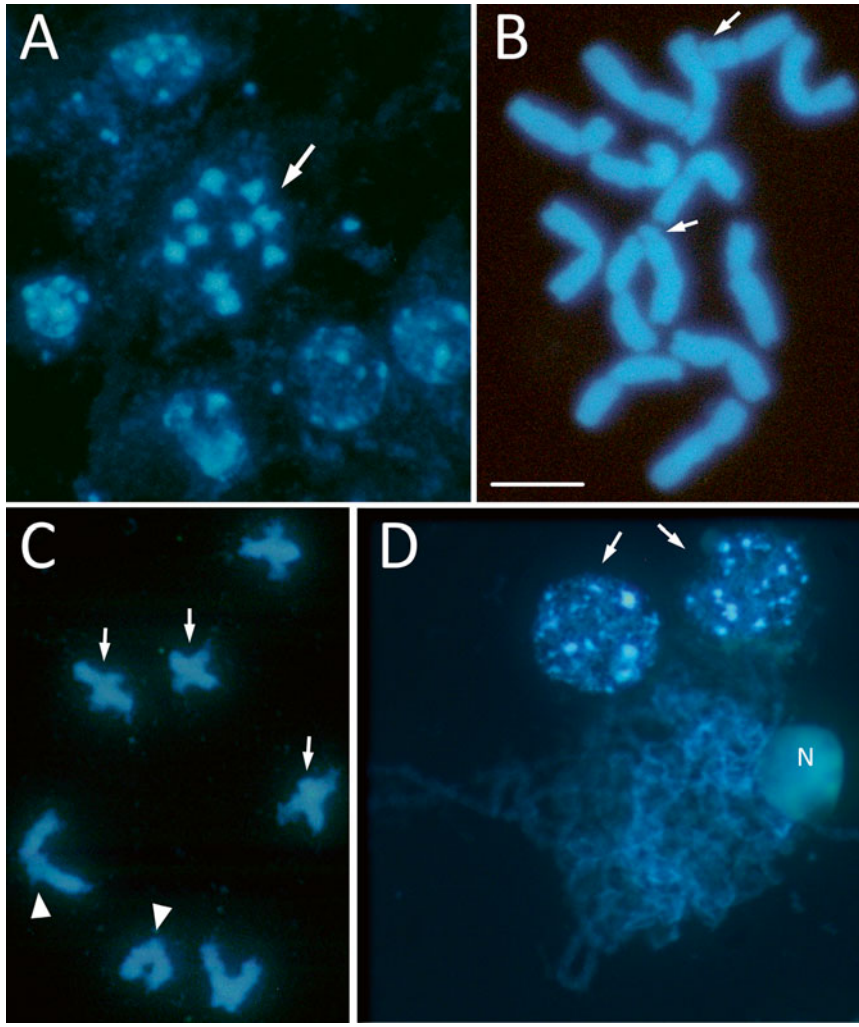

Fig. 1 Chromosomes stained with DAPI. (**A**) *Arabidopsis thaliana* (2*n*=10) flower bud meristem metaphase chromosomes (*arrow*) and four interphase nuclei with several strong chromocentres stained brightly. (**B**) *Secale cereale* (2*n*=14) root tip metaphase with much larger chromosomes showing clear gaps at the primary constriction, the centromere and the NORs of chromosome 1R (*arrows*). (**C**) Meiotic pollen mother cell of *Rosa canina* (2*n*=10) at metaphase I showing seven bivalents with each one proximal (*arrow*) or distal (*arrowhead*) chiasma. (**D**) Male meiotic prophase of diploid *Musa acuminata* (2*n*=22) showing thicker paired and thinner unpaired chromosomes in the middle; the opaque fluorescing round body is the nucleolus (N) while two somatic interphases with strong positively fluorescing chromocentres are near the top (*arrows*). Bar represents 2 μm in (**A**) and (**C**) and 5 μm in (**B**) and (**D**)

many karyotype studies, the more advanced molecular cytogenetic technique of in situ hybridization is used to analyse chromosome complements, locate the 5S and 45S rDNA genes, and localize satellite and dispersed repeats (*see* e.g. [3, 12] and references therein). Fluorochrome staining following chromosome preparation allows many more preparations to be examined compared to in situ hybridization and hence is advantageous for screening aneuploid lines or cytotaxonomy.

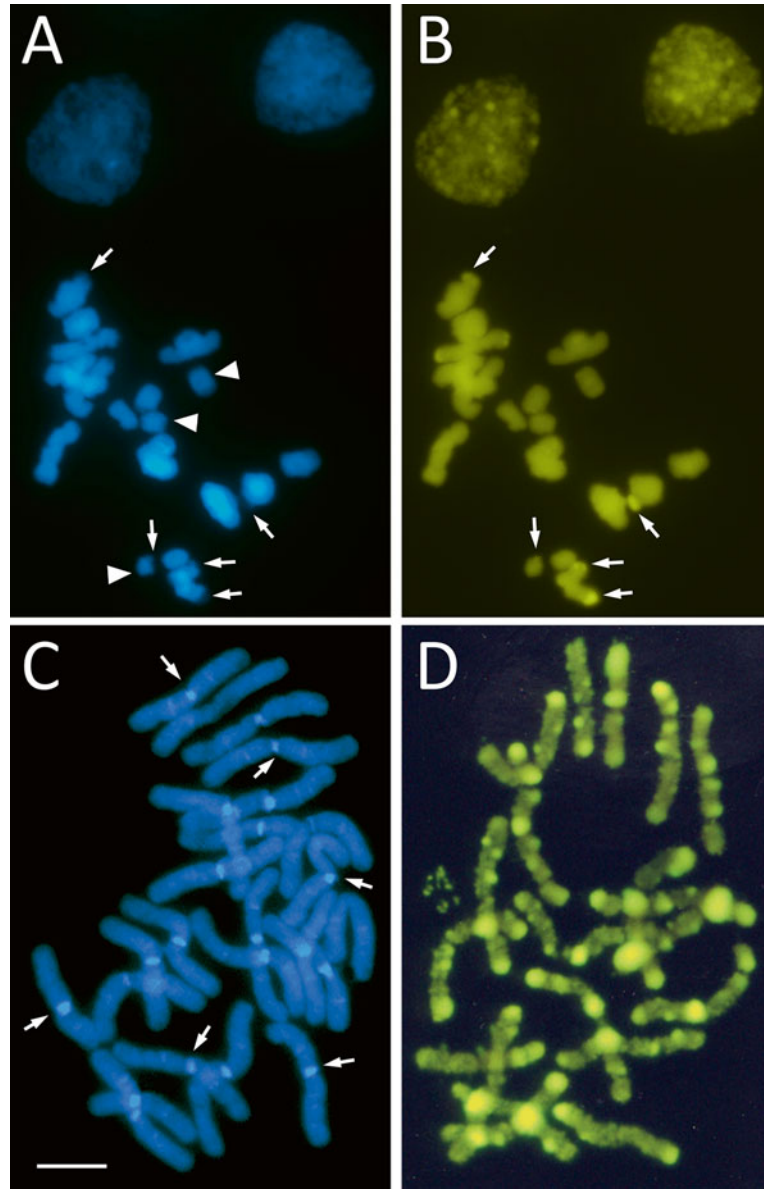

Fig. 2 Fluorescent chromosome banding. (**A**, **B**) The same metaphase I of meiosis of *Camellia japonica* × *C. saluenensis* ($2n = 30$) showing bivalents and some univalents (*arrowheads*) after staining with DAPI (**A**) and Chromomycin A3 (**B**). Several terminal bands are positive with Chromomycin, but negative with DAPI (*arrows*) and most likely correspond to 45S rDNA sites (Image curtsey of Kazumi Furukawa). (**C**, **D**) Root tip metaphases of *Pinus elliotii* ($2n = 24$) stained with DAPI (**c**) showing strong staining at the centromeres (some marked by *arrows*) and with YOYO-1 (**D**) showing several strong intercalary and terminal bands (Images courtesy of Pat Heslop-Harrison). Bar represents 5 μm in (**A**) and (**B**) and 10 μm in (**C**) and (**D**)

In this chapter, I describe the basic methods of chromosome preparations including the treatment of material before fixation. As most chromosome analyses rely on metaphases, dividing tissue is essential and is best obtained from healthy, disease-free, and rapidly growing plants. Root tips from young seedlings, freshly appearing roots at the edge of pots of older plants, or hydroponic cultures are all suitable. Great care needs to be taken when germinating seeds and watering young seedlings. The quality of water is important and it should not contain chlorine, heavy metal ions, or other water purification media or toxins, but it should have some salts and buffering capacity. Meristematic cells from young shoots, leaves or emerging buds or liquid tissue culture cells can also be used, but finding dividing cells within callus tissue is difficult.

In order to achieve good chromosome preparations with many metaphases, the material is pretreated with metaphase arresting agents before fixation. This step not only accumulates metaphases and condense the chromosomes, but importantly destroys the spindle microtubules allowing better spreading of the chromosomes. The spindle microtubule inhibitor colchicine gives heavily condensed chromosomes and is preferred for chromosome counting, while others such as ice water or 8-hydroxyquinoline give more extended chromosomes [12]. Alternative reagents, including spindle poisons used as herbicides, are also effective [13]. For meiotic studies, spindle arresting agents are not used so they do not interfere with pairing and disjunction (*see* Fig. 1c, d).

Most protocols use ethanol/acetic acid fixation that preserves chromosomes well while removing some of the chromatin proteins that would otherwise hinder the access to the DNA packaged within the chromosome. After fixation, material is either hydrolysed with acid [2] or digested with proteolytic enzymes [14] to soften the tissue, remove cell walls and allow spreading of chromosomes. Staining material with orcein or acetocarmine before spreading gives instant chromosome preparations and is often sufficient for chromosome counts. For small chromosomes or when much cytoplasm is present, fluorochromes are recommended as the unbound dye is essentially non-fluorescent and its fluorescence is a magnitude greater on binding to DNA (e.g. *Arabidopsisthaliana*, see Fig. 1a). Chromosome preparations as described here can also be used subsequently for fluorescent in situ hybridization (see [12]).

2 Materials

2.1 Preparation of Plant Material

1. Seeds or growing plants in pots.
2. Depending on material.
   (a) Petri dishes and filter paper.

(b) Plant pots of suitable size for material.

(c) Sterilization agent: 1 % (w/v) bleach in water.

(d) Soil or vermiculate.

(e) Agar minimal medium: e.g. Murashige and Skoog without sugar.

(f) Plant nutrient: commercial "complete" plant fertilizers (e.g. Phostrogen) made up in water to 1/10 the strength recommended.

3. Drinking water or bottled water (not distilled nor deionized) for germination and watering small plants.

2.2 Fixation of Material

1. Small tubes: 5–10 ml with tight caps (e.g. bijou tubes, freezer vials, or microcentrifuge "Eppendorf" tubes; *see* **Note 1**).
2. Forceps and scissors; forceps for handling unfixed material must be very clean and particularly not have residue of fixative (see Note 1).
3. Metaphase arresting agent: choose one of the following (*see* **Note 2**):

   (a) Ice water at 0 °C (typically for seedlings of temperate plants, e.g. grasses): fill a small tube with bottled water or distilled water, shake vigorously to aerate or bubble air through, keep at –20 °C until freezing, shake again and store in an bucket of ice (*see* **Note 3**).

   (b) 2 mM 8-hydroxyquinoline (for many species particular with small chromosomes): Make 500 ml or 1 l. Powder might take several hours to dissolve; stir, but do not heat. Store in dark at 4 °C (e.g. wrapped in foil in a refrigerator) for up to 1 year.

   (c) 0.05–0.1 % (v/w) colchicine (for most plants): prepare a 0.05–1 % (v/w) solution in water; store in dark at 4 °C for up to 1 year.

   (d) α-Bromo-naphthalene (for most plants): Overlay 10–20 ml with 3× volume of distilled water and shake vigorously to make saturated water; allow to settle for a few minutes and take upper water phase for use.
4. Fixative: three parts 96 % or 100 % ethanol, one part glacial acetic acid; prepared fresh, less than 1 h before use.
5. Optional 70 % ethanol.

2.3 Chromosome Preparation

1. 10× Enzyme buffer (pH 4.6): 40 ml 100 mM citric acid and 60 ml 100 mM tri-sodium citrate. Can be stored at 4 °C for a few days. Dilute to 1× with distilled water for use.
2. Enzyme solution: 2–4 % (w/v) cellulase (e.g. Sigma C1184; final concentration 10–20 U/ml), 0.2 %(w/v) "Onozuka" RS

cellulase (final concentration 10 U/ml), 3 % (v/v) pectinase (e.g. Sigma P4716 from *Aspergillus niger*; solution in 40 % glycerol, final concentration 15–20 U/ml). Make up in 1× enzyme buffer. Store in 2–5 ml aliquots at –20 °C (*see* **Note 4**).

3. 5–10 ml 45 % (v/v) and 60 % (v/v) acetic acid in distilled water.
4. Optional: acetic orcein or aceto-carmine (*see* **Note 5**).
5. Petri dishes.
6. Fine forceps and dissecting needle.
7. Microscope slides: normally 25 mm × 75 mm, either pre-cleaned (e.g. Super frost) or cleaned with strong acid (*see* **Note 6**).
8. Coverslips: 18 × 18 mm No. 1 (*see* **Note 7**).
9. Dissecting microscope.
10. Phase-contrast microscope.
11. Dry ice or liquid nitrogen (*see* **Note 8**).

2.4 Staining and Mounting Slides

1. McIlvaine's buffer (pH 7.0): 41 ml of 200 mM Na_2HPO_4 and 9 ml 100 mM citric acid.
2. Optional: Phosphate buffered saline (PBS): buy ready mixed concentrate or tablet, or prepare a 10× stock solution of 1.3 M NaCl, 70 mM Na_2HPO_4 and 30 mM NaH_2PO_4, adjust pH to 7.4 with 1 M NaOH or HCl and autoclave. Dilute to 1× before use (*see* **Note 9**).
3. Fluorochrome solution: choose as required (*see* **Notes 10** and **11**).
   (a) DAPI (4′,6-diamidino-2-phenylindole). Prepare 100 µg/ml stock solution in water. Aliquot and store at –20 °C (it is stable for years). Prepare a working solution of 1–2 µg/ml by dilution in McIlvaine's buffer, aliquot 0.5–1 ml and store at –20 °C.
   (b) CMA (Chromomycin A_3): 0.5 mg/ml in McIlvaine's buffer diluted 1:1 with 5 mM $MgCl_2$. Add the buffer slowly to the powder without stirring and leave at 4 °C overnight. Store at 4 °C (*see* **Note 12**).
   (c) PI (propidium iodide). Prepare a 100 µg/ml stock solution in water. Aliquot 50 µl or 100 µl in 1.5 ml microcentrifuge tubes and store at –20 °C. Dilute before use with 1× PBS to 0.1–5 µg/ml (*see* **Note 13**).
   (d) TOTO-1, YOYO-1 or derivatives (Molecular Probes); normally supplied as 1 mM stock solution in dimethylsulfoxide (DMSO) that needs diluting in PBS 10,000- to 100,000-fold (*see* **Note 14**).

4. Antifade mounting medium for fluorescence microscopy: e.g. Vectashield (Vector Laboratories), Slow Fade and ProLong (Life technologies), Fluorguard, Fluorshield and Fluoromount (Sigma) or Citifluor AF1 (Agar Scientific or Citifluor, Leicester).
5. Plastic coverslips cut to size from autoclavable bags.
6. Glass coverslips: No. 0 24 × 30 or 24 × 40mm (*see* **Note 15**).

2.5 Microscopy

1. Epifluorescent microscope with camera; in most cases this will now be an electronic (CMOS or CCD) camera (*see* **Note 16**).
2. Image analyzing program, e.g. Adobe Photoshop, NIH imaging or microscope software.

3 Methods

3.1 Plant Material Preparation

1. Depending on species and availability of material choose one of the options below to generate new growth and meristem formation for collecting somatic divisions. For meiosis, anthers from developing flower buds are best.

   (a) Root tips can be obtained with one of the following methods (*see* **Note 17**).

- Germinating seed or young seedlings (*see* **Note 18**).
- Put seeds onto filter paper saturated with drinking or bottled water, typically at 20–25 °C in the dark and leave until roots are about 10–20 mm long. Small seeds can be germinated under sterile conditions on agar minimal medium (*see* Subheading 2.1) in Petri dishes. Ensure the germinating seeds do not have any exposure to fixative fumes.
- Newly grown roots at the edge of plant pots.
- Plant seedlings in small pots with soil or vermiculate and wait until roots appear at the edge. Or repot established plants to generate new root growth. In some cases, applying compost on the surface around the stem is effective.

   Hydroponic culture.

- Suspend plantlets or bulbs cleaned from soil above aerated plant nutrient or place on a gravel bed.

   (b) Alternatively, flower buds, anthers, carpels, or leaf or apical meristems can be used. Cut plants back if necessary to generate new growth.

   (c) For meiotic tissue, collect anthers at different developmental stages.

2. Make sure that seeds and plants are moist and at the correct temperature and light regime. Water regularly, but do not over water (*see* **Note 19**).

3.2 Fixation of Material

Use small glass or plastic containers, fill typically two-thirds with solution (metaphase arresting agent and fixative as appropriate) and allow a generous amount of solutions for the specimen to be fixed, typically two times as much. Use clean forceps or pipettes to transfer material (*see* **Notes 1** and **17**).

1. To accumulate metaphases, treat excised root tips (5–20 mm long) or other material with one of the metaphase arresting agents as follows (*see* **Notes 2** and **20**):
   (a) Aerated ice water for 24 h (*see* **Note 3**):
   (b) 8-Hydroxyquinoline for 1–2 h at room temperature, then 1–2 h at 4 °C.
   (c) Colchicine for 3–6 h at room temperature or 10–24 h at 4 °C.
   (d) α-Bromonaphthalene saturated water for 2–6 h at room temperature.
2. For meiotic analysis, anthers are collected without pretreatment (*see* **Note 21**).
3. Blot material and transfer to fixative (*see* **Note 22**).
4. Fix for at least 16 h at room temperature. If fixed material is to be kept (up to several months), leave for 2 h at room temperature and then transfer to new fixative or 70 % ethanol and store at −20 °C.

3.3 Chromosome Preparation

Material is carefully transferred by forceps or, if small roots or buds are used, with a pipette. Alternatively, fluids can be removed with a pipette and replaced with the next required solution.

1. Wash 2–10 root tips or buds two or three times for 10 min in 2–5 ml enzyme buffer (*see* Subheading 2.3) in a small Petri dish to remove the fixative (until they sink). Make sure roots have intact tips and cut away any access material (e.g. outer layer of buds and only 1 cm of root is needed). Remove any dirt or soil remains.
2. Transfer material into 1–2 ml enzyme solution and digest at 37 °C until the material is soft, usually 45–90 min (*see* **Notes 4** and **23**).
3. Wash material in enzyme buffer for at least 15 min. Material can be stored at 4° for up to 24 h.
4. Transfer enough material for one preparation (typically one root, small bud, or anther) into 45 % acetic acid in an embryo dish, separate slide, or small Petri dish for 1–5 min (*see* **Note 24**).
5. Make chromosome preparations on clean microscope slides. Under the stereo microscope, in 1 drop (10–30 µl) of 60 % acetic acid, dissect the meristematic tissue or pollen mother cells by removing as much of the other tissue as possible, e.g.

remove the root cap or cut the anther and tease out the cells in the remaining material (*see* **Note 25**).

6. Apply a small coverslip to the material without trapping air bubbles. Blot away access liquid. Carefully disperse the material between glass slide and coverslip by tapping the coverslip gently with a needle or flat back of a pencil. Fold a filter paper in half around the slide, put it on a flat surface and then squash the cells, using the thumb to press as hard as possible onto the filter paper above the area of the coverslip (*see* **Note 26**).
7. Check the slide under a phase-contrast microscope. If not sufficiently flat squash again (*see* **Note 27**).
8. Place the spread slide onto dry ice for 5–10 min, then flick off the coverslip with a razor blade immediately before it warms up.
9. Allow the slide to air-dry. Screen slides to choose suitable preparations and store desiccated for up to 3 months at 4 °C or –20 °C (*see* **Note 28**).

3.4 Staining and Mounting Slides

1. Put 100–200 µl of fluorochrome solution (*see* Subheading 2.4) on the marked area of the preparation on each slide. Cover with a coverslip (either plastic cut from autoclavable bags or a large glass coverslip). Incubate at room temperature for 30 min in the dark avoiding bright light (*see* **Notes 29** and **30**).
2. Remove coverslips and wash slides briefly in fluorochrome buffer and shake excess solution off without letting the slide dry out.
3. Add one or two drops of antifade solution to each preparation and cover with a large, thin coverslip avoiding bubbles (*see* **Note 15**).
4. Allow to settle for a few minutes (or leave for hours as convenient) and then gently, but firmly, squeeze excess antifade from the slide between several sheets of filter paper.
5. Store slides flat at 4 °C until examination by fluorescent microscopy (*see* **Note 31**).

3.5 Microscopy

This section is only a brief guide and does not replace microscope manuals or books. Most advanced fluorescent microscopes and digital camera systems are increasingly operated through a computer interface that allows you to change objectives and filter blocks automatically as well as capturing and processing images. Detailed operation manuals are normally supplied.

1. Warm the slides to room temperature avoiding condensation on the coverslip.
2. Make sure that antifade is squeezed from underneath the coverslip and that the coverslip is clean.

3. Switch on the fluorescence lamp and wait until it is stabilized.
4. Add a drop of fluorescence immersion oil to the middle of the coverslip. We recommend using oil objectives for both low (20×) and high power (*see* **Note 32**).
5. Carefully load the slide onto the stage. Best is to leave the stage at the current height, but swing the objectives to the side. Carefully, while watching, swing in the 20× or 25× objective into place. The slide can be easily broken if it is not loaded properly, or the stage is too high.
6. Turn the filter wheel to the correct filter position (*see* **Note 33** and Table 1).
7. Open instrument and microscope shutters and make sure that the optical path switch levers (camera versus eyepiece) are in the correct position.
8. Focus images by using the manual or automatic focus button.
9. Scan slides using the stage controls.
10. When you find a good metaphase change to a higher power objective (63× or 100×). Note that particular UV excitation fades all fluorochromes and should not be used for more than a few seconds before photography, other than with low power objectives.
11. Take images following your microscope instructions and operation software guidance. Check histograms of pixel brightnesses to ensure you use the full range of levels available without clipping of bright areas. Make sure you save raw images before image manipulation (*see* **Note 34**).
12. Use image analysis to enhance weak signal and overlay images from different fluorochrome if necessary. Only use those function that treat all pixels in the image equally. Save images frequently in programme format and TIF, avoid formats that loos information (e.g. jpg) (*see* **Note 34**).
13. Slides can be de- or re-stained after photography (*see* **Note 35**).

4 Notes

1. It is important to have clean tools and containers during germination of seeds and pretreatment, to work away from fixatives and other toxins and to use airtight lids to exclude fumes, to avoid low metapahse index.
2. Different species and material, e.g. thickness of roots or size of buds, respond differently to metaphase arresting agents. Type

and concentrations need be established by trial and error. We prefer aerated ice water for small grained cereals and hydroxyquinoline for all other species and approximate conditions are given, but colchicine or α-bromonaphthalene can also be tried. Alternative reagents, including spindle poisons used a herbicides, are also effective ([13], and *see* **Notes 20** and **21**).

3. In order to make sure the ice water is at 0 °C, both aerated water and ice need to be present. Make sure that the bucket with ice is also partly liquid to allow for a tight seal around the tubes and not trapped air that might be warmer than 0 °C. For long storage and treatment keep the ice bucket in the cold room.
4. Enzyme concentrations are often given in percentage, but activity units are more important and so we give a range here. Batches of the same enzyme product can differ in strength and composition, while distributors may change isolation protocols or suppliers. Onozuka cellulase is a very pure enzyme and has a constant activity per weight. But purified enzymes (typically protoplast grades) in general give less good results than crude enzyme grades that contain a mixture of proteolytic enzymes. If enzymes are not available, hydrolysis in 1 M HCl for 10 min at 60 °C can be used. Enzyme mixtures can be reused several times: after use, centrifuge in a microcentrifuge, transfer the supernatant to a new tube, mark for reuse and freeze (not recommended for screening lines of similar material since cells may remain in the solution). Digestion time might need to be slightly increased after each round of use.
5. Dissolve 1 % orcein or carmine in 45 % acetic acid. For root tips, the addition of a part 1 N HCl to 10 part standard solution is recommended and gentle heating of the tissues in a drop of the mixture to assist the softening and separation of cells [2].
6. Microscope slides: good quality slides are needed, where no marks are left from the manufacturing process. Some come pre-cleaned, some charged or coated. To clean them yourself, put into 6 N HCl overnight and rinse in several changes of water, dip into distilled water and air dry.
7. 18 × 18 mm glass coverslips of medium thickness are used. Larger coverslips are not recommended, as well spread metaphases are often near the periphery of the coverslip; slightly larger coverslips need then to be used for staining and visualization (e.g. 24 × 30 mm or 24 × 40 mm) so that all cells can be probed and examined. The coverslips should be free of dirt, but should not be cleaned with alcohol or acid; otherwise material will stick to the coverslip and will be lost when the coverslip is removed (Subheading 3.3, **step 8**).

8. If dry ice or liquid nitrogen are not available, slides can be frozen on a metal plate in a −80 °C freezer.
9. Several formulations are available for PBS with the NaCl concentration either 138 mM or 145 mM and some include 2.7 mM KCl, but this is not necessary for this application.
10. Choice of fluorochrome depends on the type of question you want to answer, size of chromosomes and microscope availability. *See* Table 1 and Figs. 1 and 2 for examples.
11. Most fluorochromes are potential carcinogens. To avoid weighing out the powder, order small quantities and use the whole vial to make the stock solution.
12. Chromomycin A3 fluorescence is more stable and stronger with older solutions. Adding 4 % paraformaldehyde to avoid bacterial contamination is recommended by some authors (e.g. [15]).
13. PI does not keep in diluted form and even after a few hours fluorescence reduces quickly. Sometimes restaining is required after slides have been stored; but it can be used as advantage when overstaining has occurs as just a short rinse with water reduces PI strength.
14. It is best to first prepare a working stock solution of 1 μM and then dilute further to 2–10 nM. Test a quite wide range of dilutions before staining the best slides (*see* also **Note 29**).
15. Thin coverslips No. 0 (0.1 mm) are thinner than the most widely used specification (No. 1, 0.13–0.16 mm) and are essential for high-power fluorescent microscopy using oil objectives. Choose a size that is larger than the specimen area and used for making the preparations (*see* **Note** 7).
16. Main microscope manufacturers are Zeiss, Leica, Nikon, and Olympus. I have worked with all of them and cannot recommend one over the other. It is best to get several demonstrated and choose according to offers made and importantly after sale services. Also consider what you want to use the microscope for, routine fluorochrome staining for chromosome counts and ploidy checks or also high resolution in situ hybridization using several fluorochromes at once. The former can be done with a bottom-end microscope, while for the later the top of the range including camera and software are recommended. An important factor will be the location of the microscope on a stable bench in a room that can be darkened to see faint signal.
17. It is very important not to expose seedlings, roots, and plants during germination and growth induction and later during metaphase-arrest to chemicals and fumes, particularly fixatives (e.g. in a cold room also used for chemical storage) and to use

clean lab ware with tight lids (disposable plastic is ideal), clean forceps, and distilled water or bottled water (*see* also **Note 1**).

18. Some seeds are difficult to germinate and might need priming by moving between 4 and 25 °C every 3–14 days. Seed suppliers can also give advice about germination of difficult species. If seeds are prone to infection sterilize in diluted bleach by submerging for 5–10 min and then rinsing well until the smell has disappeared.
19. It is important to check seeds germinating in Petri dishes, so they have the correct amount of water; plants in pots can be put on a gravel bed and should be watered the day before collecting roots and not just prior to collection. For all material, make sure they are not stressed by lack of water or roots by lack of oxygen due to stagnated water created by overwatering. Root tips from germinating seeds, and plants grown in controlled conditions, often show waves of cell division that may follow internal or environmental hythms (e.g. light) or correlate with root length. At certain times, there may be no divisions at all, so it may be helpful to make several collections.
20. Representative times are given. For best results fix material after different times of treatment, experiment with different reagents and check the mitotic index by making chromosome preparations. Treating material for too long in arresting agents, particularly colchicine, results in over-condensation of the metaphase chromosomes which might be desirable for counting chromosomes, but not where spatial resolution along chromosomes is wanted.
21. Meiosis is best analyzed in male pollen mother cells as more divisions are available. It is critical to check a few anthers before making large fixations to assess the correct stage. Meiocytes undergo meiotic division in synchronously and therefore different sizes of anthers need collecting, at different times of day, for analysis of pachytene or metaphase I. As a norm anthers are normally whitish or light green and at meiosis before sepals or petals develop. In some species meiosis can take place a long time before flowering and anthesis, e.g. in Crocus where it happens in autumn for both autumn and spring flowering types. Some species will need a cold period for vernalization before flowering is induced.
22. Fixative should not be contaminated with water, so careful blotting or an extra rinse in fixative is advised.
23. Sometimes the ratio of cellulase to pectinase needs to be adjusted or different enzymes such as macerozymes, visco-

zyme, or pectolyase can be included. For meiotic tissue we recommend to add cytohelicase (2–5 %) to the enzyme mixture [16] which helps to remove the cell wall and cytoplasm. The enzyme digestion step needs to be adjusted to the material and species used, by changing the time of digestion; aim at a digestion time of 45–90 min; otherwise change the strength of enzyme. Ideally, cell walls should be weakened, so that the cells can be separated easily, and chromosome spreads are clean of cytoplasm. In most cases, the meristematic cells will be digested faster than the non-dividing tissue. The material should remain intact to handle, otherwise the dividing cells are lost into the medium. Once transferred to buffer, digestion will continue slowly; if roots are quite hard, they can be left overnight in buffer and will soften further. If material has been fixed for several weeks, the material becomes harder and needs longer digestion.

24. Acid disperses the material and so when the material is very soft it is better treated with 45 and then 60 % directly on the final slide. If material is hard it can be treated in acid for longer. Larger roots can be divided to make several slides.
25. The teasing out of good cells can be compared to extruding a sausage from its skin.
26. It is absolutely essential that the coverslip does not move sidewards creating a "noodling" effect. So tap and press vertically only. The folded filterpaper will help.
27. If there is much cytoplasm and cells are not squashed properly, a drop of acetic acid can be added to the side of the coverslip and the slide warmed over an alcohol burner before squashing again. Be careful that the solution does not get to hot or boil; just touchable to the finger is fine.
28. Slides need to fully dry out before storing and if used immediately they benefit for 1–2 h at 60–80 °C.
29. Fluorochrome concentrations might need adjustment, but note that DAPI has two modes of binding to DNA: in the minor groove, which occurs at high stain concentrations; and, at lower DAPI concentrations, the alternative of intercalation between AT-nucleotide tracts in the DNA [17]. The latter binding gives the greater increase in fluorescence compared to the unbound molecule. Hence, increasing stain concentration has sometimes the unusual effect of reducing signal, and weak signal may mean too high stain concentration. PI is not stable in diluted form (*see* **Note 13**).
30. For quick examination with DAPI, slides can be viewed directly without rinsing and mounting.

31. Fluorochromes stabilize with storage, e.g. ChromomycinA3; so we normally wait 2–3 days before examination and photography.
32. 20× or 25× oil objectives are expensive, but very bright and convenient for scanning. Using non-oil low power objectives is tricky when high power lenses demand oil are used at the same time. Make sure you use immersion oil for fluorescence. High temperatures or moisture can make oils opaque. Also do not mix different types of oils.
33. Filter set specifications are summarized in Table 1, but manufacturers will also have tables and several choices of differently priced filter sets. Often fluorochromes can be excited in a wide range of filters and testing several before buying or simple try and error with what is available is recommended.
34. Store images in lossless forms and compatible with different programmes (e.g. TIF format as well as the programme format); use JPG format only for incorporation into presentations or for sending via e-mail. Make sure you use short file names that do not loose vital information in case of computer and disk failure. Make frequent back-ups.
35. Remove the coverslip as follows:
    (a) With immersion oil on the coverslip, warm slide to 37 °C, gently but firmly pull off the coverslip being careful not to get oil on the slide surface and rinse the slide in 70 % ethanol.
    (b) Without oil, help the coverslip to fall off in 70 % ethanol. Transfer slides through a 70, 90, and 96 % ethanol series (2 min each) and air-dry.

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Chapter 8

Fluorescence In Situ Hybridization on Rice Chromosomes

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Abstract

Fluorescence in situ hybridization (FISH) has become one of the most important technologies applied in plant molecular cytogenetic research. FISH technique has been not only well applied in physical mapping and genomic studies, but also served as an indispensable tool in tracing the individual chromosome during cell division. This chapter provides protocols for basic FISH analysis using rice as a model, which can also be adapted to other model plant species.

Key words Rice, Mitosis, Meiosis, Chromosome, FISH

1 Introduction

Fluorescent in situ hybridization (FISH) is a cytogenetic technique based on the principle using the labeled DNA probes to bind to the complementary DNA. FISH technique was developed in the early 1980s [1, 2]. Procedures of FISH mainly include four individual parts: target DNA preparation, probe labeling, hybridization, and signal detection. The first two parts, target DNA preparation and probe labeling, are the most vital steps for FISH and determine the final results. Target DNA may be chromosomes, interphase nuclei, DNA fibers, or tissue sections, but for plant, the target DNA mainly refers to chromosomes. Probes are usually prepared by culturing bacteria that contains the desired cloned DNA sequence. Sometimes, the DNA PCR products or isolated genomic DNA can also be used. These desired probes were usually labeled by nick translation to incorporate biotinylated or digoxigenin-modified nucleoside triphosphates. In the past decades, accompany with the progress on both molecular and genomic studies, FISH has become one of the most important techniques for plant molecular cytogenetic study.

Rice is the staple food for more than half of the world's population. It also serves as the best plant model species for cytogenetic research [3]. As the cytological target, the rice chromosomes can

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easily be prepared at either mitotic prometaphase or meiotic pachytene due to the improvement of chromosome preparation techniques. Moreover, along with the completion of rice genome sequencing, a lot of tandem repetitive sequences have been identified, including 45S rDNA, 5S rDNA, CentO (a centromere-specific repeat), and Os48 (a subtelomeric repeat) [4, 5]. These labeled repetitive sequences may generate very specific FISH signals on different chromosomes. In addition, many rice chromosome-arm-specific markers based on rice bacterial artificial chromosomes (BACs) have been developed [6, 7]. All these markers are very convenient to identify the individual rice chromosome.

In this chapter, we present protocols for performing a complete FISH procedure using rice as a model, including the preparation of chromosomes, probe labeling, hybridization of probes to chromosomes, and FISH signal detection, which have been successfully used in the past years (Fig. 1).

2 Materials

2.1 Preparation of Chromosome Spreads

1. Carnoy's fixative: mix three volumes of absolute ethanol and one volume of glacial acetic acid.
2. 2 mM 8-hydrooxyquinoline: dissolve 8-hydrooxyquinoline in deionized water.
3. Digestion solution: dissolve 2 % cellulose, 1 % pectinase in deionized water.
4. 1 % Acetocarmine solution: dissolve acetocarmine in 45 % acetic acid in a flask. Boil for 8 h on a hot plate with an attached reflux column, and then cool rapidly by placing the flash on ice immediately. Store at room temperature until use.
5. Counterstaining solution: dissolve 4′,6-diamidino-phenylindole (DAPI) in deionized water at 1 mg/ml to make a stock solution. Dispense in aliquots and store at −20 °C. For use, add 10 μl of the DAPI stock solution to 1 ml of Vectashield anti-fade solution (Vector Laboratories, Burlingame, CA).

2.2 Probe Preparation

1. 10× Nick-translation buffer: prepare a solution of the buffer containing 0.5 M Tris–HCl (pH 7.5) and 50 mM $MgCl_2$.
2. dNTPs (A, C, G): mix 0.5 mM dATP, 0.5 mM dCTP, and 0.5 mM dGTP.
3. 0.5 mM Digoxigenin-dUTP or biotin-dUTP (*see* **Note 1**).
4. 1 % Sheared salmon sperm DNA: dissolve sheared salmon sperm DNA in deionized water.
5. 50 % Dextran sulfate: dissolve dextran sulfate in deionized water.

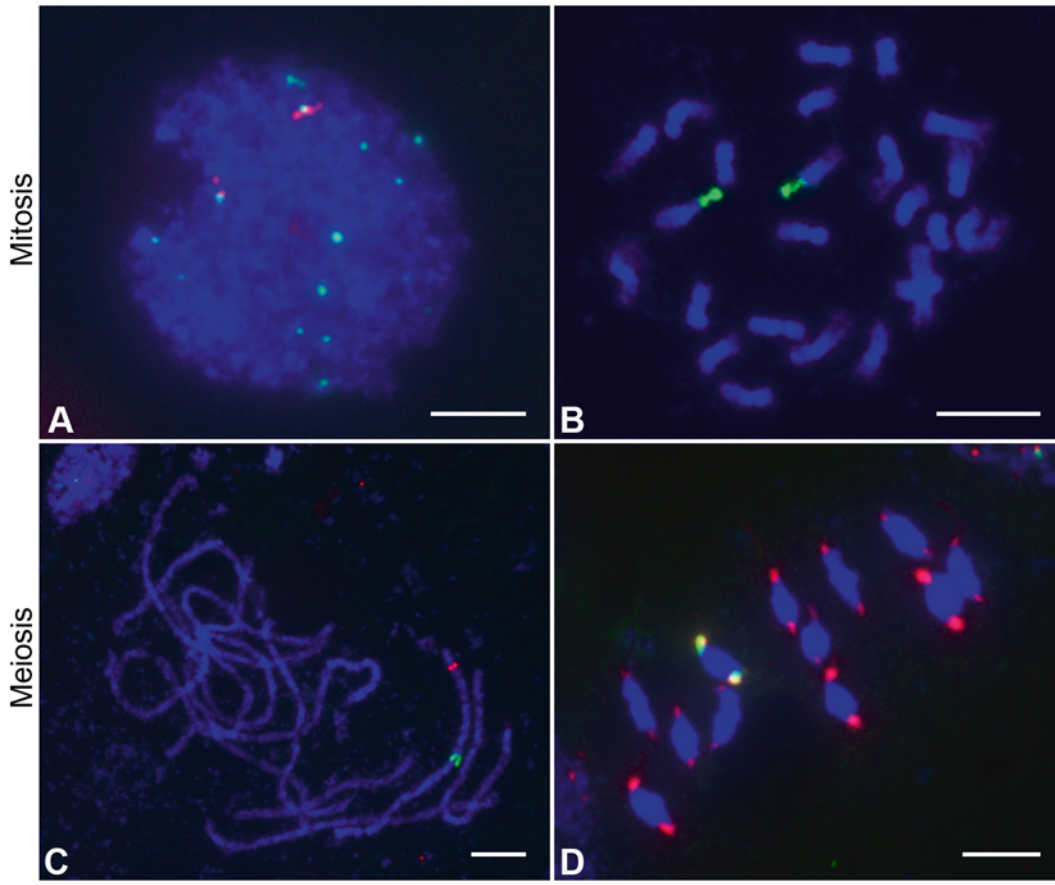

Fig. 1 FISH detection of different rice probes on both mitotic and meiotic chromosomes. (**a**) Mitotic interphase nuclei probed with 5S rDNA (*red*) and telomeric sequence (*green*). (**b**) Mitotic prometaphase chromosomes probed with 45S rDNA (*green*). (**c**) Meiotic pachytene chromosomes probed with OSJNBa0088I16 (*red*) and 5S rDNA (*green*) located near the end and close to the centromere, respectively, of the short arm of chromosome 11. (**d**) Meiotic metaphase I chromosomes probed with the centromeric repeat CentO (*red*) and 5S rDNA (*green*). The *red* signals were labeled with Digoxigenin-dUTP and detected with anti-Digoxigenin-Rodanmin antibody. The *green* signals were labeled with Biotin-dUTP and detected with anti-Biotin-FITC antibody. Chromosomes are stained with DAPI and are in *blue*. Bars, 5 μm

2.3 Hybridization, Washing, and Immunodetection

1. Alcohol series: 70 and 90 % absolute ethanol in deionized water and 100 % absolute ethanol.
2. 20× SSC: 3.0 M NaCl, 0.3 M sodium citrate, pH 7.0. Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate ($Na_3(C_6H_5O_7)\cdot 2H_2O$) in deionized water. Adjust the pH to 7.0. Make up to 1 l with deionized water. Sterilize by autoclaving and store at room temperature.
3. 10× PBS: 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 7.4. Dissolve 80 g of NaCl, 2 g of KCl, 14.2 g of Na_2HPO_4, and 2.7 g of KH_2PO_4 in deionized water. Adjust the pH to 7.4. Make up to 1 l with deionized water. Sterilize by autoclaving and store at room temperature.

4. 5× TNB: 0.5 M Tris–HCl pH 7.5, 0.75 M NaCl, 2.5 % blocking reagent (Boehringer Ingelheim, Ingelheim am Rhein, Germany). To prepare 10 ml of 5× TNB, mix 5 ml of 1 M Tris–HCl (pH 7.4), 0.4383 g of NaCl, and 0.25 g of the blocking reagent. Make up to 10 ml with deionized water and store at –20 °C until use.
5. Denaturing solution: To prepare 10 ml of denaturing solution, mix 7 ml of deionized formamide, 1 ml of 20× SSC, and 2 ml of deionized water. Store at 4 °C until use.
6. Stop solution (0.2 M EDTA): dissolve EDTA in deionized water.
7. Detection solution with antibody: Prepare 100 µl detection solution and 1 µl antibody for each slide by mixing 80 µl deionized water, 20 µl of 5× TNB buffer, and 1 µl of the antibody. Prepare fresh just before use.

3 Methods

3.1 Preparing Chromosomes for FISH

3.1.1 The Mitotic Chromosome Preparations

1. Spread rice seeds on moist filter paper at 37 °C for germination.
2. Harvest fresh root tips at 1–2 cm long from the germinating seeds.
3. Pretreat the excised root tips with 2 mM 8-hydrooxyquinoline at 20 °C for 2 h.
4. Fix the root tips in Carnoy's fixative solution at room temperature and keep in it until use.
5. Wash the root tips in distilled water for 10–15 min with 2–3 changes.
6. Put root tips in the digestion solution at 37 °C for 60 min.
7. Wash the root tips in distilled water for 10–15 min with 2–3 changes.
8. Fix the excised root tips again with the same fixative solution.
9. Make squashes in a precooled slides and flame dried (*see* **Note 2**).

3.1.2 The Meiotic Pachytene Chromosome Preparations

1. Harvest young panicles of rice at meiosis stage (*see* **Note3**).
2. Fix the young panicles in Carnoy's fixative solution (*see* Subheading 2.1) at room temperature overnight. After that, the tissue can be stored in the fixative at –20 °C for a couple of months until use.
3. Take out a spikelet at the appropriate meiosis stage (*see* **Note 4**) from the fixed panicle above. Put the spikelet on a slide and dissect out all six anthers using a pair of fine forceps in one hand and a needle in the other hand. Cut the anthers in half with the needle and add a small drop of acetocarmine on top of the anthers (*see* **Note 5**).

4. Use the needle to squash the anthers quickly and completely on the slide (*see* **Note 6**), and then cover them with a 22 × 22 mm coverslip.
5. Heat the slide containing the anthers gently and evenly over a small flame of an alcohol lamp until near-boiling. At that time, both cytoplasm and chromosomes will be strongly stained, and the cells will be resistant to swelling.
6. Put a drop of 45 % acetic acid at the left side of the coverslip and draw it across under the coverslip with a piece of filter paper on the right side. Repeat this step until the red color of the solution under the coverslip goes away.
7. Heat the slide gently again. Put the slide over a filter paper with the coverslip down and press the slide firmly with two fingers.

3.2 Probe Labeling

1. Prepare the following reaction:

   10× nick-translation buffer, 5 μl.

   0.5 mM dNTP(A,C,G) solution, 5 μl.

   0.5 mM Digoxigenin-dUTP or Biotin-dUTP, 5 μl.

   Probe DNA, 1 μg.

   DNA Polymerase I, 12 units.

   DNase I, about 1 unit (*see* **Note 7**).
2. Make up the total volume to 50 μl with deionized water. Mix carefully after each step of adding solutions and spin down shortly in a tabletop centrifuge at the end.
3. Incubate the reaction mixture at 15 °C in a water bath for 2 h.
4. Add 5 μl of stop buffer in each tube to stop the reactions.

3.3 Hybridization of the Probe to Chromosomes

1. Soak the slide with coverslip on in liquid nitrogen, and then remove the coverslip with a razor blade. Dry the slide by taking it through an ethanol series (70, 90 and 100 %, 5 min each wash) (*see* **Note 8**).
2. Prepare the following hybridization mixture:

   Deionized formamide, 10 μl.

   50 % dextran sulfate, 4 μl.

   Sheared salmon sperm DNA (10 mg/ml), 2 μl.

   20× SSC, 2 μl.

   Probe DNA, 2 μl.
3. Denature the mixture at 80 °C for 5 min by placing on a heating block and then immediately chill the mixture on ice. Spin down the solution shortly in a tabletop centrifuge.

4. Add 100 μl of denaturing solution on the dried slides. Place 24 × 32 mm coverslips on the slides, and put them on a plate (metal or glass) in an 80 °C oven for 2.5 min.
5. Remove the coverslips by swinging the slides, and take the slides immediately through a cold ethanol series (70, 90, and 100 %, 5 min each at –20 °C) and air dry the slides.
6. Prepare the wet chamber (e.g., a plastic box with lid). Place a few layers of paper towels on the bottom of the box and a plastic holder on top of the paper towels. Pour a thin layer of water onto the paper towels.
7. For each slide, apply 20 μl of hybridization mixture, and then cover the slide with a 24 × 32 mm coverslip. Seal the coverslip with rubber cement and place the slides with coverslips on in the wet chamber.
8. Incubate slides overnight (at least 6 h) in a wet chamber at 37 °C.

3.4 Detection of FISH Signals

1. Use forceps to peel the rubber cement from the slide and dip the slides in a staining jar containing 2× SSC. Shake the staining jar slowly until the coverslips fallout from the slides.
2. Wash the slides using the following steps:

   2× SSC, 42 °C, 10 min.

   2× SSC, room temperature, 5 min.

   1× PBS, room temperature, 5 min (*see* **Note 9**).
3. Prepare the detection solution with antibody during washing.
4. After the 1× PBS wash, drain (but do not dry) the slides with a paper towel. For each slide, add 100 μl of the detection solution with 1 μl antibody. Place a 24 × 32 mm coverslip on the slide and put the slides in the same wet chamber as used before. Incubate at 37 °C for 30 min.
5. Remove the coverslips by tilting the slides or dipping them in a beaker with 1× PBS.
6. Wash the slides in 1× PBS at room temperature for 15 min with three changes.
7. Drain the slides on a paper towel. Add 10 μl of the Vectashield solution with DAPI for each slide, and place a 24 × 32 mm coverslip on the slide.
8. Put the slides over a paper towel with the coverslip side down and allow the viscous DAPI solution to spread evenly, minimizing air bubbles.
9. Check the in situ hybridization results using a fluorescence microscope. FITC-labeled probes will emit yellow-green light. Rhodamine-labeled probes will emit red light.

4 Notes

1. The 0.5 mM Digoxigenin-dUTP or Biotin-dUTP is a mixture including 0.166 mM Digoxigenin or Biotin-conjugated dUTP and 0.333 mM dTTP. A 67 % saving on the amount of labeled dUTP is required for the experiments.
2. The morphology of the chromosomes in most of the cell on the slides should be determined under a phase-contrast microscope. If the chromosome morphology does not change, the chromosomes on the slides will be used for the following hybridization experiment.
3. Normally, the most suitable rice panicles for meiotic chromosomes preparation are that the panicle in length is about 5–10 cm and the span between the blade-sheath junctions of the flag leaf and the leaf below is around 3 cm.
4. Overall, in a rice panicle, the upper spikelets developed earlier than those in the lower part. However, on specific branches, the uppermost spikelet is the oldest one, while the next spikelet below it is the youngest one.
5. The Fe^{3+} ions facilitate differential staining of heterochromatins and euchromatins, particularly on pachytene chromosomes. If a slide is only to be used for phase-contrast microscopy observations, a trace amount of $FeCl_3$ and $Fe(OH)_3$ can be added to the Carnoy's fixative and 1 % acetocarmine solution, respectively.
6. If this step is too long, over-staining may occur.
7. The size of the final nick translation product is most critical part for the labeling. When the size of labeled probe is around 200–600 bp detected by gel electrophoresis, the best results of in situ hybridization will be obtained. As the enzymatic activities of DNase I may be affected during storage or handing, the optimal dosage of DNase I may need to be determined experimentally every time to obtain the desired probe fragment size.
8. After the ethanol series treatment, the dried slides should be checked again under a phase-contrast microscope to make sure the chromosomes are still in good shape.
9. The temperature and time of the 2× SSC wash can be increased to reduce background, if necessary.

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Part III

Imaging Plant Cell Division

Chapter 9

Dynamics of the Plant Nuclear Envelope During Cell Division

David E. Evans and Katja Graumann

Abstract

The use of suspension cultures synchronised by aphidicolin provides a method to study cell division in living plant cells. This chapter describes the use of this technique in tobacco suspension cultures expressing nuclear and nuclear envelope proteins that have been fused to fluorescent proteins. The protocol provides advice on optimizing synchrony and on real-time imaging by confocal microscopy.

Key words Plant cell culture, Mitosis, Cell synchrony, Cell division, Stable expression, Transient expression, Nuclear envelope, Nuclear envelope markers, *Nicotiana tabacum*, Tobacco BY-2 cells

1 Introduction

The methods in this section permit observation of the behaviour of fluorescently labeled nuclear and nuclear envelope markers in living, dividing cells. To do this, stable transformants are generated of a model plant cell culture—tobacco bright yellow 2 (BY-2). Synchronization of these cells with aphidicolin rapidly increases the number of dividing cells that can be observed at any one point—with a mitotic index of up to 70 % ([1]; Fig. 1). Techniques for transforming and synchronizing BY-2 cells have been developed by a number of laboratories [2]. The protocols described here have been used over a number of years and by a number of people in our laboratory and shown to be effective [3–5]; however, several steps are critical to obtaining high levels of synchrony. The general health of the cultures and the steps to wash out aphidicolin appear to be of greatest importance. At the point where aphidicolin is washed out of the cultures, ensuring the flow of wash buffer is steady and preventing the cells from drying out are both critical.

Stable transformation of BY-2 cells with histone H2B labeled with a fluorescent protein permits visualization of chromatin in live cells ([6]; Fig. 2a). Use of a DNA stain (Hoechst or DRAQ5 for

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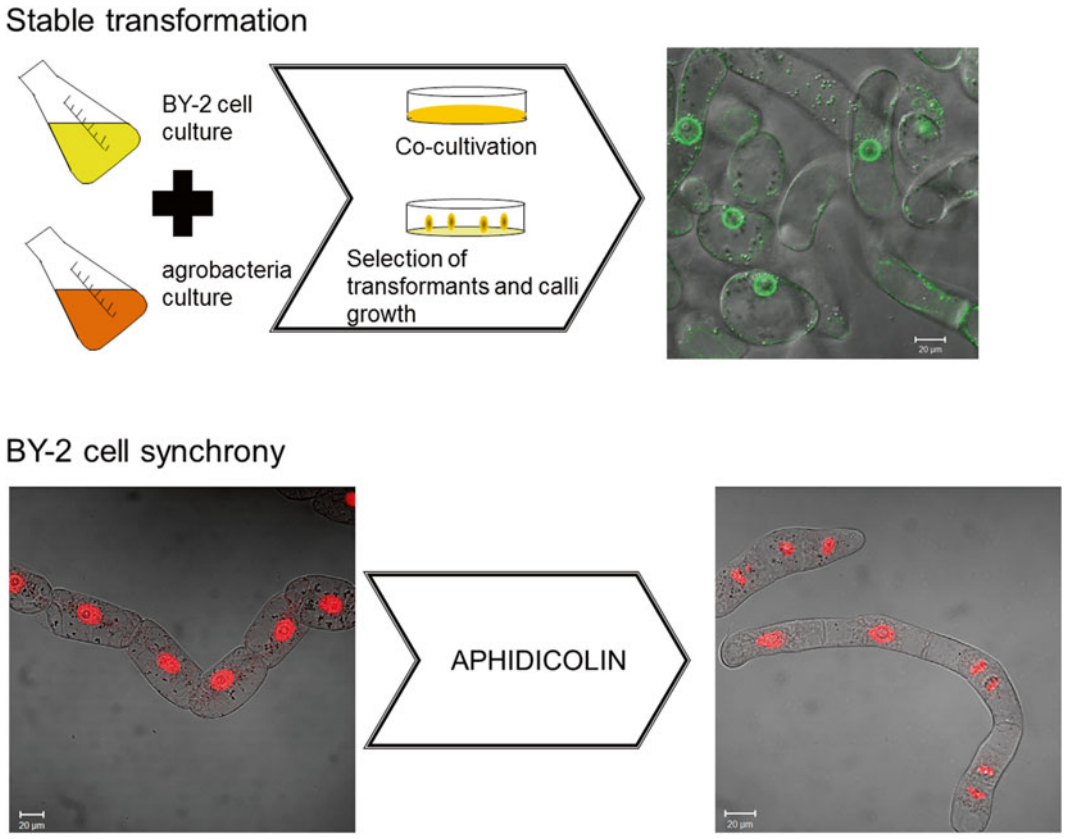

Fig. 1 Methodology overview: Stable transformation of BY-2 cells is achieved by co-cultivation with *Agrobacterium tumefaciens* carrying a binary expression vector. In this image, AtSUN2-YFP (*green*) can be seen in the NE. Synchronization of BY-2 cells with aphidicolin significantly increases the amount of mitotic cells for observation and downstream analysis. Nuclei stained with Draq5 (*red*); confocal image size bars = 20 μm

confocal microscopy) provides alternatives, though in both cases cannot be considered "live cell" imaging due to the use of Triton. Dual labeling (e.g., with histone H2B fused to cyan fluorescent protein [CFP] and a nuclear envelope protein fused to yellow fluorescent protein [YFP]) allows studies of behavior of nuclear envelope proteins relative to chromatin. Other multiple fusions permit exploration of co-localization.

Stable transformed, synchronized BY-2 cell cultures can be used for various microscopic and biochemical analysis of mitotic processes, from Western blots and kinase assays [7] to live cell and ultrastructural imaging [5, 8, 9], Particularly, live cell imaging may not only be used for localization studies, but also to investigate protein interactions in vivo: for instance, by observing protein mobility using fluorescence recovery after photobleaching (FRAP), or more directly by fluorescence resonance energy transfer (FRET) and bimolecular fluorescence complementation (BiFC) assays ([7]; Fig. 2b).

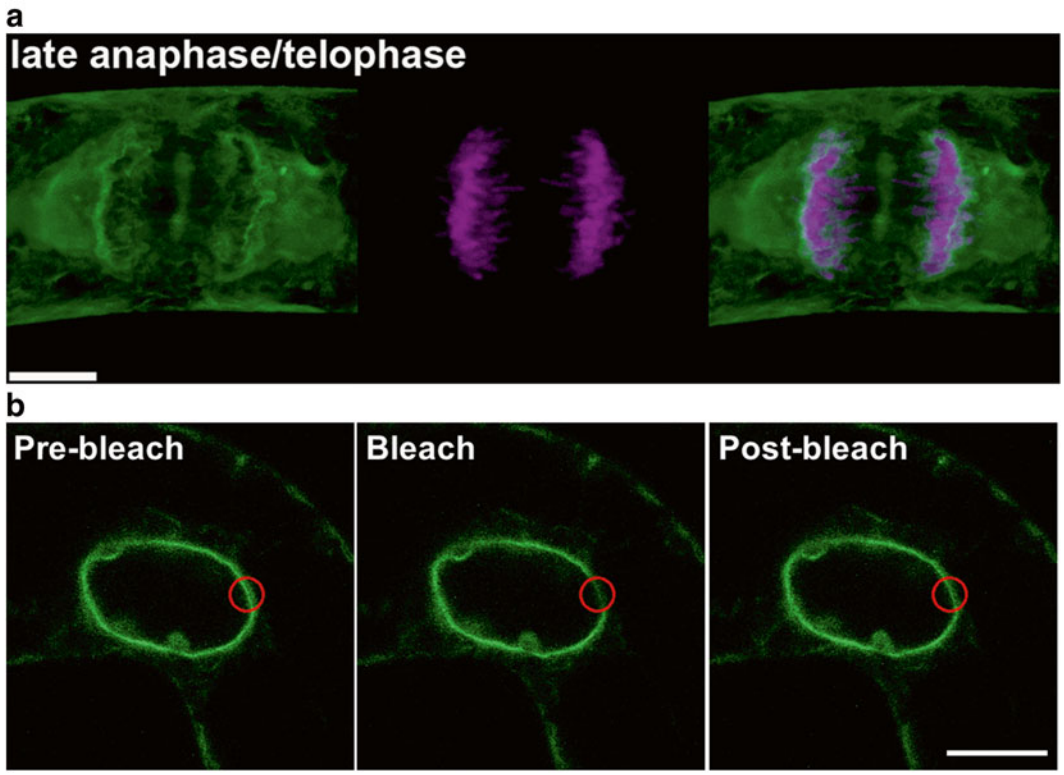

Fig. 2 (**a**) Projection of late anaphase/telophase cell stably expressing NE marker AtSUN1-YFP (*green*) and chromatin marker H2B-CFP (magenta). (**b**) FRAP in an interphase BY-2 cell at the NE labeled with YFP-AtSUN1, fluorescence measured in *red circled region of interest* before and after the bleach. Size bars = 20 μm

2 Materials

2.1 Production of BY-2 Suspension Cultures

2.1.1 Hormone Stock Solution

2,4-Dichlorophenoxyacetic acid (2,4-D), 1 mg/ml in ethanol

1. Dissolve 1 mg 2,4-D in 1 ml 70 % (v/v) ethanol.
2. Store for up to 6–8 weeks at 4 °C.

2.1.2 Antibiotics

Antibiotics should be prepared according to manufacturer's instructions and sterilized as follows

1. Aspirate the solution into a sterile syringe without a needle.
2. Attach a 0.2 μm syringe filter and expel the solution through the filter into a sterile tube.

2.1.3 Medium for Suspension Cultures of BY-2 Cells

1. Place 750 ml water in a 1 l beaker and add 30 g AnalaR-grade sucrose. Stir until dissolved.
2. Add 4.3 g Murashige and Skoog medium (without sucrose, indole acetic acid, kinetin, agar).

3. Add 200 ml 1 mg/ml 2,4-D.
4. Adjust pH to 5.8 with 0.1 M KOH.
5. Make up to 1000 ml with water, mix well, and autoclave. Allow to cool and store for up to 2 weeks at 4–8 °C.

2.1.4 Medium for Culturing BY-2 Callus (See **Note 1***)*

1. Add agar (plant cell specific; Bacto Agar) to liquid BY-2 medium (see above) to a final concentration of 1 % (w/v).
2. Autoclave and allow to cool until flask can be held for pouring.
3. Store medium until required. Pour fresh plates just before use. For selection of stable transformants, add timentin, carbenicillin, and appropriate selection antibiotics.

2.1.5 Materials for Generating BY-2 Cell Suspension Cultures

1. Petri dish containing callus of wild-type or transformed BY-2 cells grown on solid BY-2 medium in a petri dish (obtained from a lab working with BY-2 cell lines; this is the best way to store and transport the cell lines as they require constant agitation while in liquid culture).
2. Conical flasks (50 ml) containing 20 ml liquid BY-2 medium covered with aluminum foil caps and autoclaved.
3. Conical flask (100 ml) containing 50 ml liquid BY-2 medium (alternatively, 250 ml conical flask containing 100 ml liquid BY-2 medium).
4. Filter-sterilized antibiotics as required for selection of transformed BY-2 cells.
5. Sterile single-edged razor blades.
6. Sterile packet of aluminum foil squares for covering flasks.
7. Orbital shaking incubator at 130 rpm, 25 °C, no light.
8. Sterile wide-bore pipette.
9. Laminar flow hood.

2.2 Stable Transformation of Tobacco BY-2 Cells

1. LB medium with and without appropriate filter-sterilized bacterial selection antibiotic (*see* **Note 2**).
2. Acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone) as 100 mM stock in ethanol.
3. Sterile antibiotic stock solutions
   Timentin (ticarcillin disodium/potassium clavulanate) and carbenicillin should be used to kill agrobacteria after co-cultivation. The working concentration of these antibiotics is as follows: 20 mg/l timentin, and 100 mg/l carbenicillin. Antibiotics are also required for selection of transformants. The choice of the antibiotics is dependent on the vector used but can include kanamycin and hygromycin B. Working concentrations for these two are as follows: 100 mg/l kanamycin, and 40 mg/l hygromycin B.

Table 1
Nuclear and nuclear envelope markers used for BY-2 transformation

Marker	Plasmid	BY-2 selection	Description	Citation
LBR-GFP	pVKH18En6	Hygromycin B	Nonfunctional NE marker; derived from human LBR	[4]
YFP-SUN1 YFP-SUN2	pCambia1300	Hygromycin B	NE marker; derived from Arabidopsis	[10]
H2B-CFP CFP-H2B	pK7CWG2[a] pK7WGC2[a]	Kanamycin	Chromatin marker; derived from Arabidopsis	[5]
WEE1-YFPC	pKanII-SPYCE	Kanamycin	Nuclear marker; derived from Arabidopsis; for BiFC	[7, 11]
SKIP-YFP^N	pSPYNE	Kanamycin	Nuclear marker; derived from Arabidopsis; for BiFC	[7, 12]

[a]Obtainable from Universiteit Gent Plant Systems Biology http://gateway.psb.ugent.be/search

4. *Agrobacterium tumefaciens* strains GV3101 or EHA105 or LBA4404 transformed with vector (Table 1) containing appropriate construct should be used.
5. BY-2 suspension culture (3 days old; prepared as described above).
6. Sterile liquid BY-2 medium (approximately 70 ml per construct).
7. Sterile solid BY-2 medium plates without antibiotics (one plate per construct) and plates with selectable antibiotic, 100 mg/l carbenicillin and 20 mg/l timentin (ten plates per construct).
8. Stationary incubator at 25 °C, no light.
9. Shaking incubator, 25 °C set to 130 rpm, no light.
10. Sterile pipette tips and pipettes Sterile 1.5 ml microcentrifuge tubes (one per construct) and 15 ml sterile centrifuge tube (two per construct).
11. Forceps, sterile.
12. Laminar flow hood.
13. Tin foil.
14. Parafilm.
15. Benchtop microcentrifuge.
16. Cold room or refrigerator.

2.3 Synchronizing BY-2 Suspension Culture Cell Division with Aphidicolin

*CAUTION:*Aphidicolin is very toxic and should be handled with great care. It should be handled wearing gloves and skin contact should be avoided; wear lab coat and eye protection.

Inhalation of dust should be avoided. Disposal of aphidicolin should be according to local regulations.

1. Two 100 ml conical flasks with 50 ml liquid BY-2 medium, sterile (for larger cultures 100 ml in 250 ml flasks).
2. Liquid BY-2 medium for washing (for 50 ml culture use 500 ml media for washing).
3. Sterile dH_2O (50 ml).
4. Aphidicolin (5 mg/ml, in dimethyl sulfoxide, observe the manufacturer's instructions). Add suitable filter-sterilized antibiotics, for transformed BY-2 cells only.
5. Wild-type or transformed stationary-phase BY-2 cells (7-day-old cultures) grown in suspension.
6. Retort stand.
7. Autoclaved sintered glass funnel (G3 [porosity 3], borosilicate glass; either 80 ml or 250 ml).
8. Shaking incubator at 130 rpm, 25 °C.
9. Liquid waste container.
10. Laminar flow hood.
11. Rubber tubing and Hoffman clamp if required.
12. Sterile pipette and pipette tips.

2.4 Determining Mitotic Index

1. 20 % Triton X-100 (1 μl Triton X-100 in 5 μl dH_2O).
2. DRAQ5 used according to the manufacturer's instructions.
3. Hoechst stain (100 μl stock): 1 μl 10 mg/ml 2 μl Triton X-100 and 97 μl of distilled water stored at 4 °C.
4. Other nuclear stains such as DAPI or ethidium bromide can also be used (*see* **Note 3**).
5. 1.5 ml microcentrifuge tube.
6. Sterile pipette tips.
7. Microscope slides.
8. Cover slips.
9. Lens oil (if required).
10. Confocal or fluorescence microscope.

2.5 Confocal Imaging of Live Cells

1. Confocal microscope.
2. Microscope slides.
3. Cover slips.
4. Solid BY-2 medium (0.7 % w/v agarose).
5. Sterile pipette tips.
6. Laminar flow hood.

3 Methods

3.1 Production of BY-2 Cell Suspension Culture

1. Work in sterile conditions in a laminar flow cabinet.
2. Cut a 2–3 mm diameter piece of wild-type or transformed BY-2 callus with a sterile razor blade without removing it from the petri dish on which it is growing. If single, isolated calli on the plate, use 1 callus. Using 1 callus will make the culture more homogenous.
3. Holding it facing into the sterile airflow, open a sterile 50 ml conical flask containing 20 ml liquid BY-2 medium. Add filter-sterilized antibiotics as required.
4. Open a sterile packet of aluminum foil squares.
5. Transfer the cut callus to the 50 ml flask with medium.
6. Gently pipette the culturing medium up and down to break up callus. Alternatively, use sterile forceps or a sterile pipette tip to squash the callus against the glass side of the flask.
7. Seal with aluminum foil and place in an orbital incubator (130 rpm and 25 °C, no light).
8. After 1–2 weeks the culture should be quite thick; passage by pipetting into fresh medium, using a wide-bore pipette tip. Depending on growth rate of culture transfer 0.5–2 ml of old culture to make the new culture. Passage cells into a larger culture—50 ml medium in 100 ml conical flask. Repeat **step 8** every 7 days until the culture is growing stably.
9. Suspension cultures can be further bulked up by using 100 ml medium in 250 ml conical flasks. Depending on growth rate use up to 3 ml of old culture to transfer into 100 ml new culture medium.
10. Established cultures can be used after four passages for downstream applications.

3.2 Stable Transformation of Tobacco BY-2 Cells

3.2.1 Growing Agrobacteria

1. Inoculate 5 ml LB medium (containing appropriate filter-sterilized bacterial selection antibiotic) with a single colony of *Agrobacterium tumefaciens* GV3101 or EHA105 or LBA4404 strain transformed with a binary vector containing the appropriate construct. Alternatively, use swab of glycerol stock for inoculation. Incubate for 16–20 h at 180 rpm and 28 °C.

3.2.2 Stable Transformation of BY-2 Cells

Use a 3-day-old BY-2 suspension culture (either wild type for single transformation or transformed line for co-expression).

Prepare agrobacteria as follows:

1. Centrifuge 1 ml liquid overnight agrobacteria cultures at 5000 × *g* for 5 min at RT. In a laminar flow hood or by the flame, remove supernatant and resuspend cells in 1 ml LB

medium containing acetosyringone (add 20 µl acetosyringone stock to 10 ml medium). Repeat centrifugation to finish first wash step. Repeat wash step two more times. After third wash, resuspend cells in 1 ml LB medium containing acetosyringone and incubate for 1 h at 4 °C.

2. In a laminar flow cabinet, transfer 7 ml of the 3-day BY-2 culture to a 15 ml sterile tube, add 1.2 µl acetosyringone and 100 µl of prepared agrobacteria. Gently invert tube several times to mix cultures and then pour onto plate containing solid BY-2 medium with no antibiotics.
3. Seal plate with micropore tape, either wrap in aluminum foil or place in a blacked out box, and incubate for 3 days at 25 °C in the dark without shaking.
4. After incubation, in laminar flow hood, transfer BY-2 cells from plate for washing by gently tapping the petri dish to loosen cells and then rinsing cells off the plate with 5–10 ml liquid BY-2 medium; transfer to sterile 15 ml tube.
5. Wash the BY-2 cells three times with 15 ml liquid BY-2 medium containing 100 mg/L carbenicillin and 20 mg/L timentin; for each wash step, centrifuge the cells at 500 - 1000 xg (ca 3000 rpm) for 5 min (set breaks and acceleration to 0), remove supernatant, and resuspend in approximately 10 ml medium (final volume 15 ml). Alternatively to centrifugation, cells can be left for 10 min to settle naturally before removing supernatant. However, centrifugation causes fewer cells to be lost.
6. After the final wash step resuspend cells to a total of 10 ml. Transfer 1 ml of resuspended cells onto a plate containing solid BY-2 medium, timentin, carbenicillin, and appropriate selection antibiotic.
7. Gently rotate plate to spread cells over the surface of the solid medium.
8. Seal plate with Parafilm and incubate in the dark at 25 °C without shaking for 4–6 weeks until calli appear.
9. If constructs are fluorescent, use fluorescence stereomicroscope to screen the calli. Transfer calli to suspension as detailed in method for producing BY-2 suspension culture.
10. Subculture cells for at least 4 weeks before other downstream applications.
11. For double-transformation, BY-2 cells are first transformed with one construct and, once stably expressing suspension cells are established, these may be used for a second transformation with the other construct.

3.2.3 Synchronizing BY-2 Suspension Culture Cell Division with Aphidicolin

1. Use a 7-day-old BY-2 suspension culture (either wild type or stable transformed).
2. In a laminar flow hood, transfer 7 ml of the 7-day-old culture into 50 ml fresh medium (in 100 ml conical flask, sterile).
3. Add 50 µl of 5 mg/ml aphidicolin and incubate culture for 24 h in a shaking incubator at 130 rpm, 25 °C with no light.
4. After the incubation, remove aphidicolin by washing ten times with 50 ml sterile BY-2 medium, in a laminar flow hood (*see* **Note 4**). The wash steps should be carried out as follows.
5. In the laminar flow hood, fasten an autoclaved sintered glass funnel (for 50 ml culture use 80 ml funnel) in a retort stand and place a beaker for collecting waste.
6. Rinse the funnel with 50 ml autoclaved dH_2O.
7. Once the water has completely drained, transfer the BY-2 cell suspension culture into the funnel and wait for the cells to settle at the bottom and most of the medium to drain off. It is important that the cells do not dry out but a small meniscus of medium remains to cover the cells.
8. Add 50 ml of fresh BY-2 medium and allow it to drain through the funnel. Repeat this wash step nine more times. During the wash, cells can be gently resuspended with a wide-bore pipette.
9. If the flow rate of the washing medium is faster than 50 ml/min, attach rubber tubing to the base of the funnel and a Hoffman clamp to the tubing. Set the clamp so that the flow through is lowered to less than 50 ml/min.
10. After the last wash, resuspend the cells in 50 ml of fresh BY-2 medium and transfer the culture to a new, sterile 100 ml conical flask.
11. Return culture to incubator and remove 1 ml samples every hour to check mitotic index. Depending on BY-2 cell line, maximum mitotic index can be observed approximately 8 h after aphidicolin wash out.

3.3 Determining Mitotic Index

3.3.1 DRAQ5: For Use with Fluorescence or Confocal Microscope

1. To 500 µl cells add 1 µl 20 % Triton X-100 (1 µl Triton X-100 in 5 µl dH_2O) and 0.2 µl of 5 mM DRAQ5 for a working concentration of 10 µM.
2. Incubate for 1–2 min before imaging.
3. Mount 60 µl on microscope slide, and add cover slip and, if required, lens oil (or water).
4. Excitation with any laser from 488 nm upwards; capture excitation in LP650.

3.3.2 Hoechst Staining for Use with a Fluorescence Microscope

1. Work with a Hoechst stock containing Triton X-100 to enhance uptake of the stain. 2. To make 100 µl stock, use 1 µl Hoechst, 2 µl Triton X-100, and 97 µl dH_2O; stock can be stored at 4 °C.
2. Take 100 µl of cells from the culture flask with a wide-bore sterile pipette.
   (a) Place the cells in a 1.5 ml Eppendorf tube and add 5 µl of Hoechst stock.
   (b) Place 20 µl of cells on a slide, add cover slip, and view with a fluorescence microscope (excitation 350 nm, emission 460 nm). Score for mitotic index by counting mitotic cells as a percentage of total number of cells.

3.4 Confocal Imaging of Live Cells

For time-lapse imaging (keeping cells for more than 25 min on slide and imaging) cover the microscope slide first with solid BY-2 medium. This is useful to keep cells alive for longer time lapses such as imaging one whole mitotic division [5]. To keep medium sterile, prepare these slides in flow hood.

1. Prepare solid BY-2 medium as above but use 0.7 % w/v agarose.
2. Cover microscope slide evenly with approximately 1 ml of warm, liquefied medium and allow setting. Prepare slides fresh before use.
3. Mount cells (approximately 60–100 µl), and add cover slip and, if required, lens oil or water.

For confocal imaging, use settings appropriate for fluorescent probe. For longer time-lapse imaging, keep laser output and transmission as low as possible to avoid bleaching. Time-lapse imaging has previously been used to visualize the dynamics and relationships of nuclear and mitotic membranes as well as chromatin (*see* **Note 2**) throughout an entire mitotic division as well as specific mitotic phases [4, 5, 8].

In addition to tracking the localization of a protein throughout the cell cycle, confocal imaging can also be used to analyze protein mobility and protein interactions. Cook et al. [7] have used BiFC to report protein interactions of nuclear kinase WEE1 with SKIP1 of the 26S proteasome system. Analysis of protein mobility by FRAP and protein interactions by apFRET requires a more elaborate setup but similar to FRAP or FRET using other plant tissue [8, 10, 13, 14]. In addition to interphase structures, however, FRAP and apFRET can also be carried out on mitotic structures such as the mitotic spindle membranes, phragmoplast, and cell plate. Graumann and Evans [5] used FRAP to observe the mobility of NE proteins AtSUN1 and AtSUN2 during NE breakdown and reformation, in mitotic spindle membranes and at the

cell plate. Similarly, mobility and turnover of tubulin and microtubules at the phragmoplast and cell plate have been recorded [15].

3.5 Conclusion

Stable transformation and synchrony of BY-2 suspension cells offer a great platform to analyze mitotic processes in plants. Specifically, the use of fluorescent tags and dyes coupled with confocal microscopy make it possible to analyze living, dividing cells to study mitotic processes in real time. In addition to nuclear and nuclear envelope markers, multiple cellular proteins and components can be tagged to study the localization, dynamics, interactions, and functions of these throughout the cell cycle.

4 Notes

1. Achieving high levels of synchrony in the cultures requires careful attention to all stages of the experiment. It is important to optimize culture growth to commence with cultures that are growing well and the cells are in good health. Once aphidicolin has been added, care must be taken to wash it out carefully; maintain a steady flow of wash buffer by adjusting the level of inflow and outflow as described in the protocol. Never let the cells dry out during the process and use an appropriate size of funnel. If 100 ml cultures are used, instead of 50 ml, the funnel size should be increased to 250 ml. A larger funnel should also be used if the culture is very dense.
2. The stains suggested to observe chromatin are very useful for imaging, but all will have an affect on viability and cell cycle. In fact, most stains require mild permeabilization of the cells. If chromatin dynamics are to be observed, it is best to use fluorescent protein markers for chromatin or chromatin stains that do not require permeabilization. The stage of the confocal microscope is a relatively hostile environment for prolonged time courses. Stress on cells can be reduced by using minimum laser power to achieve results and by keeping the room and stage temperature as constant as possible.
3. Plates for BY-2 callus growth containing antibiotics should be prepared freshly as storage affects the potency of the antibiotic. Stored plates also become more easily infected due to condensation.
4. LB medium is used to grow most *Agrobacterium tumefaciens* strains such as GV3101 but other strains may require different medium such as YEB. Therefore, stick to lab-based protocols when growing agrobacteria.

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Chapter 10

Immunolabeling of Nuclei/Chromosomes in *Arabidopsis thaliana*

Michael Sandmann, Joerg Fuchs, and Inna Lermontova

Abstract

The cell cycle is a complex sequence of events by which cells grow and divide mitotically or meiotically. Mitosis results in the generation of two identical daughter cells, while meiosis generates gametes as a prerequisite for sexual reproduction. To study the localization and dynamics of proteins involved in the regulation and proceeding of the cell cycle, life cell imaging of proteins fused to fluorescent tags can be performed. However, in some cases this approach cannot be applied, e.g., due to low fluorescence intensity, fast bleaching or degradation of recombinant proteins by the proteasome pathway. Instead, immunolabeling with protein-specific antibodies represents a useful approach for the analysis of intact cells. Alternatively, immunolabeling can also be applied to isolated and/or flow-sorted nuclei of particular cell cycle stages (G1, S, and G2) or of different endopolyploidy levels. This chapter details indirect immunolabeling protocols to analyze the subcellular localization and distribution of cell cycle-specific proteins in *Arabidopsis thaliana*.

Key words *A. thaliana*, Immunolabeling, Chromosomes, Mitosis, Meiosis

1 Introduction

To understand the functional importance of proteins involved in the control of cell cycle dependent processes, studies of their subcellular localization are required. In some cases these analyses are done by life cell imaging of proteins fused to fluorescent tags (e.g., GFP or YFP). However, in many cases the expression level of cell cycle dependent proteins is very low and, consequently, the expression of the fusion constructs under control of an endogenous promoter not strong enough to generate detectable fluorescence signals and the expression under control of a constitutive promoter might result in unspecific localization patterns. In these cases the immunolabeling approach might be a useful alternative and/or in the latter case could be used to confirm results obtained by

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expression of GFP fusion constructs under control of a constitutive promoter.

Immunolabeling is a biochemical process allowing the spatial and temporal localization of antigens (usually proteins) within nuclei, cells, tissues or organs. Immunolabeling can be applied either directly or indirectly. In the direct variant the protein-specific primary antibody is directly linked to a tag (most frequently fluorescent compounds). Although this method guaranties a minimum of cross-reaction, it is less frequently used compared to the indirect method. The indirect method employs a two-step protocol in which the unlabeled protein-specific primary antibody is detected by a tagged secondary antibody in a second step (Fig. 1). This allows the universal use of commercially available secondary antibodies of any available color. Usually the indirect method results in stronger signals per antigen.

The application of immunolabeling allows the analyses of high numbers of cells at different mitotic (Fig. 2a) and meiotic stages as a prerequisite for statistical analysis. Furthermore, quantification of immunosignal intensities can be used to study the dynamics of proteins during mitotic [1, 2] and meiotic [3] cell cycles. Double-immunostaining experiments (Fig. 2b) can be applied to visualize and localize two proteins simultaneously [4], provided that they are raised in different animals.

Here we describe detailed protocols on the indirect immunolabeling of cell cycle-specific proteins on nuclei of *A. thaliana*.

2 Materials

2.1 Preparation of Slides

1. Three-day-old seedlings or inflorescences of 4–6-week-old plants of *A. thaliana*.
2. Microtubule-stabilizing buffer (MTSB): 50 mM PIPES, 5 mM $MgSO_4$, 5 mM EGTA, pH 6.9.
3. Fixation buffer I: 4 % (w/v) paraformaldehyde in MTSB buffer.
4. Enzyme mixture: 2.5 % (w/v) pectinase (1 U/mg), 2.5 % (w/v) cellulase (10 U/mg), 2.5 % (w/v) pectolyase (0.3 U/mg, Sigma Aldrich, St. Louis, USA) in MTSB buffer.
5. Embryo dishes.
6. Stereomicroscope.
7. Dissection needles and a fine forceps.
8. Polysine-coated slides.
9. Cover slips 24 × 24.
10. Coplin jar.
11. Platform shaker.

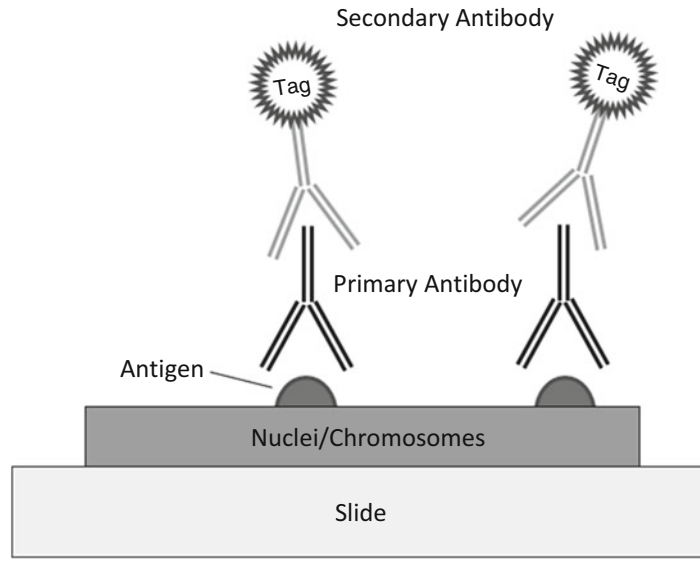

Fig. 1 Schematic view of indirect immunolabeling procedure

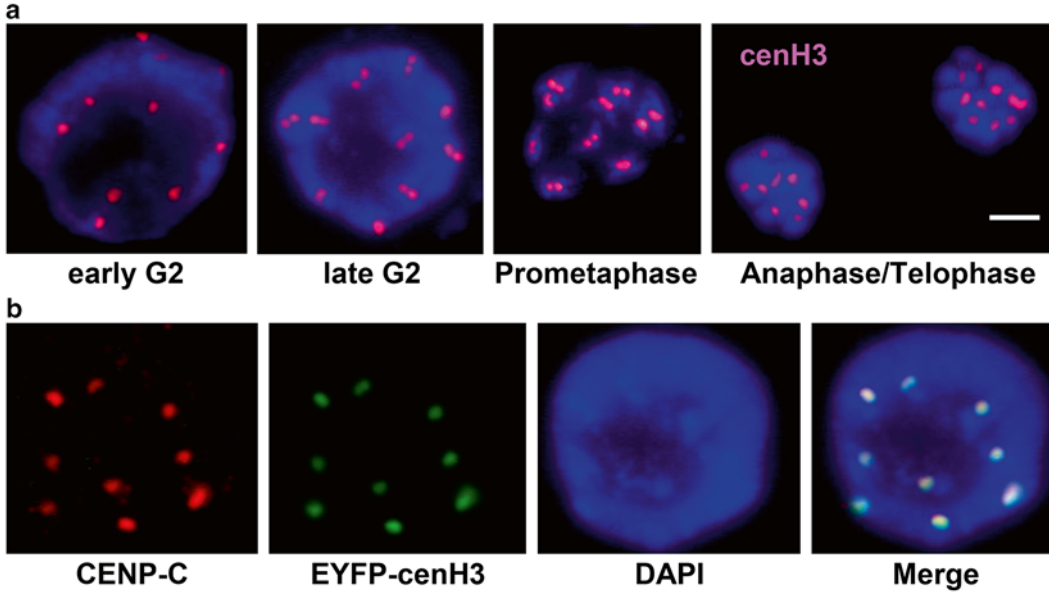

Fig. 2 (**a**) Immunolabeling of centromeric histone H3 (cenH3) at centromeres of *Arabidopsis thaliana* with anti-cenH3 antibody through the mitotic cell cycle. (**b**) Double immunolabeling of centromeric proteins CENP-C (*red*) and EYFP-cenH3 (*green*) at chromocenters of a meristematic nucleus of EYFP-cenH3 transformant with anti-CENP-C and anti-GFP antibodies, respectively. DNA is counterstained with DAPI (*blue*). Bar = 2 μm

2.2 Flow Sorting of Nuclei

1. Leaves of 4–6-week-old *A. thaliana* plants.
2. Tris buffer: 10 mM Tris–HCl, 10 mM Na_2EDTA, 100 mm NaCl, 0.1 % Triton X100, pH 7.5.
3. Fixation buffer II: 4 % (w/v) formaldehyde in Tris buffer.
4. Nuclei isolation buffer: 15 mM Tris–HCl, 2 mM Na_2EDTA, 0.5 mM Spermin, 80 mM KCl, 20 mM NaCl, 15 mM mercaptoethanol, 0.1 % Triton X100, pH 7.5.
5. Sucrose buffer: 100 mM Tris–HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.05 % Tween 20, and 5 % sucrose, pH 7.9.
6. 0.5–2 μg/ml 4′, 6-diamidino-2-phenylindole (DAPI).
7. Exsiccator.
8. Petri dishes.
9. Standard microscope slides.
10. Flow sorter.

2.3 Immunolabeling

1. Blocking solution: 8 % BSA, 0.1 % Triton X100 in MTSB buffer.
2. Antibody dilution buffer: 1 % BSA in MTSB buffer.
3. Primary and secondary antibodies.
4. 0.5–2 μg/ml DAPI.
5. Coplin jars.
6. Humidity chamber: A hermetic container (e.g., plastic box for microscope glass slides) with wet paper on the bottom.
7. Fluorescence microscope equipped with a high-resolution CDD camera.

2.4 Sources of Primary and Secondary Antibodies for Immunolabeling Experiments

2.4.1 Commercially Available Antibodies

Antibodies against various antigens, including plant-specific antigens, are produced by many different companies in the world. The following link compiles a brought collection of primary antibodies that can be purchased from different companies: http://www.antibodies-online.com/search.php. The antibodies are available as polyclonal and/or monoclonal once. While a polyclonal antibody represents a mixture of antibodies from different B cells that are able to recognize multiple epitopes on the same antigen, a monoclonal antibody is an antibody from a single B cell and binds only to one unique epitope. Polyclonal antibodies are faster to generate and less expensive but also less specific with a higher potential for cross-reactivity. Monoclonal antibodies in contrast are highly specific with a reduced probability for cross-reactivity but significantly more expensive and more time consuming to generate. While monoclonal antibodies are usually generated in mice a broad range of animals (e.g., rabbit, rat, goat, sheep, and guinea pig) is available for the production of polyclonal antibodies.

Before selecting commercial antibodies for immunolabeling experiments it has to be checked whether these antibodies are suitable for this application, usually this information is given in the data sheet of the product. It is easily possible that antibodies which give specific signals on Western blots are not suitable for the immunolabeling experiments or vice versa.

2.4.2 Custom-Made Antibodies

Although the list of commercially available antibodies against plant antigens is constantly increasing it is still mainly restricted to antibodies against commonly used marker proteins. Antibodies against specific proteins of interest have to be generated either from isolated and purified proteins or from synthetic peptides. Using full-length recombinant proteins as antigens for raising antibodies enables the production of a conformational epitope which might be useful for the recognition of folded proteins. However, the purification of recombinant proteins is time consuming and expensive. The use of synthetic peptides requires less effort and is cheaper but bears the risk of a lower antigenicity.

2.4.3 Antibodies Against Synthetic Peptides

Antibodies against synthetic peptides can be raised using programs offered by different companies. These programs include the synthesis of peptides, their conjugation to carriers and the immunisation of animals of your choice (http://www.eurogentec.com, http://www.genscript.com, http://www.abcam.com). Alternatively, peptides synthesized by companies can be used for immunisation using facilities of your home institution if available.

2.4.4 Antibodies Against Recombinant Proteins

To generate antibodies against recombinant proteins either the whole protein or only certain protein domains can be used as antigens. It is preferable to generate and use for the immunization soluble recombinant proteins. To achieve this task, several references can be used as guidelines [5, 6]. Depending on the protein of interest an optimization of the protocol might be required. This includes optimizations on the genetic/vector level (gene/codon usage engineering, choice of vector and solubility promoting tags like MBP [Maltose binding protein]) as well as on the host/induction level (choice of host [*Escherichia coli*, yeast, etc.] including choice of strains and expression conditions [temperature, concentration of inducer, etc.]).

Furthermore, if certain domains of the protein increase the insolubility, then it might be possible to express only the soluble part. Smaller protein antigens often deliver more specific antiserum than larger proteins.

3 Methods

3.1 Preparation of Slides

1. For immunostaining of mitotic chromosomes and meristematic nuclei germinate seeds (100–200) of *A. thaliana* by placing them in a row on wet filter paper in Petri dishes. Incubate Petri dishes vertically for 3 days at 21 °C (16-h light/8-h dark) in a growth chamber. For the analysis of meiosis (and mitosis) in flower buds use 4–6-week-old *A. thaliana* plants grown in soil.
2. Collect seedlings or inflorescences into a embryo dish with MTSB buffer.
3. Remove MTSB and add 2 ml of *freshly prepared* fixation buffer I, apply vacuum (using a Speed Vac) for 1–2 min in case of root tips or 20 min (*see* **Note 1**) for flower buds and incubate the samples afterwards for 20 min on ice with constant gentle shaking on a platform shaker.
4. Remove the fixation solution, briefly rinse, and afterwards wash material 2 × 20 min in MTSB on ice with constant shaking.
5. Remove MTSB and add ~150 µl of enzyme mixture and incubate seedlings for 5 min at 37 °C and flower buds for 25 min (*see* **Note 2**).
6. Stop the reaction by adding 1–2 ml of MTSB (plant material has to be covered with buffer). Remove the solution using a pipet and add 1–2 ml of fresh MTSB buffer.
7. Cut root tips (5–6 per slide) (*see* **Note 3**) or isolate anthers and ovaries (from 5 to 6 flower buds of appropriate stage (0.5–1 mm)) under a stereomicroscope on polysine-coated slides (*see* **Note 4**) in a drop of MTSB buffer, cover the material with a cover slip, and remove excess of liquid by putting the slides between two sheets of filter paper. Squash the samples (with thumb and/or preparation needle) and put the slides to liquid nitrogen. Remove the cover slips using a razorblade, put the slides into a Coplin jar with MTSB and use them for immunolabeling as described below.

3.2 Flow Sorting of Nuclei

1. For isolation of leaf nuclei grow plants in soil in a cultivation room for 4–6 weeks.
2. Fix 3–4 young leaves for 20 min in 10 ml freshly prepared fixation buffer II on ice under vacuum in an exsiccator.
3. Wash leaves 2 × 10 min in Tris buffer on ice.
4. To isolate the nuclei chop leaves using a sharp razorblade in 1 ml nuclei isolation buffer in a Petri dish.

5. Filter the nuclei suspension using, e.g., 5 ml Polystyrene Round-Bottom Tube with Cell-Strainer cap 35 μm (BD Biosciences) to remove cell debris.
6. Stain nuclei with 0.5–2 μg/ml DAPI and sort them according to their different fluorescence intensities corresponding to the different ploidy levels into separate collection tubes using a flow sorting facility (7). For flow sorting each flow cytometer with a sorting device and appropriate light source and filter equipment for DAPI excitation and emission can be used.
7. Pipette 10–15 μl of sucrose buffer onto microscopic slides and add equivalent amounts of sorted nuclei, gently mix it and air-dry the slides overnight.
8. Use slides for immunolabeling or transfer them for longer storage to −20 °C.

3.3 Immunolabeling

1. Add ~100 μl of blocking solution per slide (Subheading 3.1, **step 7**), cover it with parafilm, and incubate it for 1 h at room temperature in a humidity chamber. Before applying the blocking solution to slides with flow-sorted nuclei (*see* **Note 5**) (Subheading 3.2, **step 9**) wash them for 5 min in MTSB in a Coplin jar, fix them for 10 min in fixation buffer I on ice, and wash them as described above (Subheading 3.1, **step 4**). Perform washing and fixation steps on a platform shaker with constant gentle shaking.
2. Dilute primary antibodies in dilution buffer (*see* **Notes 6** and **7**), remove the blocking solution, add ~40 μl of primary antibodies per slide, cover slides with parafilm and incubate them overnight at 10 °C or 1 h at room temperature in a humidity chamber (*see* **Note 8**).
3. Wash the slides 3 × 5 min in MTSB in a Coplin jar with constant shaking.
4. Add ~40 μl diluted secondary antibodies (*see* **Notes 9** and **10**) to each slide, cover the slides with parafilm, and incubate them 1 h at room temperature in a humidity chamber.
5. Wash the slides 3 × 5 min in MTSB in a Coplin jar with constant shaking.
6. Add a drop (8 μl) of antifade (Vectashield) containing 0.5–2 μg/ml DAPI as a DNA counterstain to each slide and cover it with a cover slip (24 × 24).
7. Perform microscopic analysis.

4 Notes

1. Apply vacuum in 5-min intervals to allow a better penetration of the fixation solution into the plant material.
2. Incubation time has to be optimized each time for newly prepared enzyme mixtures. For seedlings it can vary from 5 to 8 min and for flower buds from 25 to 30 min.
3. After incubation in the enzyme mixture root tips very often separate from seedling and can be collected by pipet with small volume pipet tip (2–10 μl) under a binocular.
4. It is recommended to use polysine-coated slides which allow better adhesion of plant material.
5. Slides with flow-sorted nuclei stored at −20 °C should be equilibrated to room temperature for ~30 min and then dried for 30–60 min at 60 °C.
6. The optimal dilution of antibodies has to be determined empirically. When a new antibody has to be tested, it is advisable to try different dilutions, starting with 1:100, 1:500, and 1:1000 and afterwards using smaller dilution steps if required.
7. For double-immunolabeling experiment dilute the two selected primary antibodies to the optimal concentration in dilution buffer (*see* **Note 6**). It is important that these antibodies have been raised in different animals. Alternatively, double immunostaining experiment can be performed using a sequential approach: incubation with first primary and the corresponding secondary antibody (*see* Subheading 3.3, **steps 2–5**), fixation for 10 min in fixation buffer I following by washing in MTSB, and then incubation with second primary and the corresponding secondary antibody (*see* Subheading 3.3, **steps 2–6**).
8. Incubation time and temperature have a severe impact on the quality of staining. These conditions need to be optimized for each antibody and sample to achieve a staining of high specificity with low cross reactivity. Usually the specificity of the immunolabeling is higher by incubating the primary antibody overnight at low temperatures (i.e. 10 °C).
9. For the dilution of secondary antibodies follow the manufacturer's instruction. For optimal results an adjustment of the antibody dilution might be required.
10. For the double-immunostaining experiments combine secondary antibodies conjugated to different fluorescent dyes: for instance, green (Alexa 488; FITC) and red (Rhodamine, Alexa 594).

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Chapter 11

In Vivo Imaging of Microtubule Organization in Dividing Giant Cell

Marie-Cécile Caillaud and Bruno Favery

Abstract

Mitosis which is a major step during plant development can also be observed in physiopathological conditions. During the compatible interaction between the root-knot nematode *Meloidogyne incognita* and its host *Arabidopsis*, the pathogen induce through repeated divisions without complete cytokinesis the formation of hypertrophied and multinucleate feeding cells, named giant cells. Due to the presence of hypertrophied plant cell material surrounding the giant cells, classical live cell imaging gave therefore very poor resolution. Here, we describe a protocol which allows the in vivo observation of the mitotic apparatus in developing giant cells using confocal imaging of vibrosliced tissues. This approach can also be used to visualize in vivo other cellular processes occurring in different steps of giant cells.

Key words Microtubule, Mitosis, Giant cells, Nematode, Arabidopsis

1 Introduction

The nematode infection provides an original model to explore key processes such as plant cell mitosis. Nematodes are obligate biotrophic pathogens which evolve the ability to induce "giant cells," permanent feeding cells that constitute their unique source of nutrients [1]. Giant cells result from synchronous repeated karyokinesis without complete cell division [2]. Fully differentiated giant cells reach a final size about 400 times that of root vascular cells and contain more than a 100 polyploid nuclei, which have also undergone extensive endoreduplication [3]. Giant cell development is accompanied by division and hypertrophy of surrounding cells, leading to a typical root gall formation, the primary visible symptom of infection.

The distribution of the plant cell cytoskeleton in developing giant cells have been first determined using fixed tissues by immunostaining of α, β-, and γ-tubulins in butyl-methylmethacrylate embedded galls [4, 5]. New approach of live cell imaging was used to study the dynamic of cytoskeleton organization during

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giant cells ontogenesis using vibroslices through galls [6]. This technique allows the visualization in vivo of the mitotic apparatus in giant cells. Fluorescent markers were used——the MAP4 microtubule-binding domain (MBD)-GFP and H2B-YFP [7] as well as the microtubule-associated protein MAP65-3 [6]——to characterize accurately dynamic changes in the microtubules organization in mitotic giant cells. Imaging of these fluorescent-coupled proteins led to the confirmation of the presence of multiple microtubule spindles during the early steps of giant cell formation (Fig. 1a, b). Time-lapse in vivo also revealed the presence of early synchronous phragmoplast arrays with a restricted out-growth which lead to the formation of a novel cell plate structure—the giant cell mini cell plate—that does not extend across adjacent faces of the cell [6]. Here, we describe the protocol which allowed the observation in vivo of the mitotic apparatus in dividing giant cells using vibrosliced galls. This protocol would be compatible with study of any protein involved in nematode feeding site ontogenesis or physiology, and it make an ideal tool to study protein dynamics in this living tissue.

2 Material

2.1 Equipment

1. Plastic and glass Petri dishes (245 × 245 × 20 mm).
2. Laminar-flow hood.
3. 100, 10 and 0.5 μm sieve.
4. 10 mL syringe.
5. 50 ml cornings.

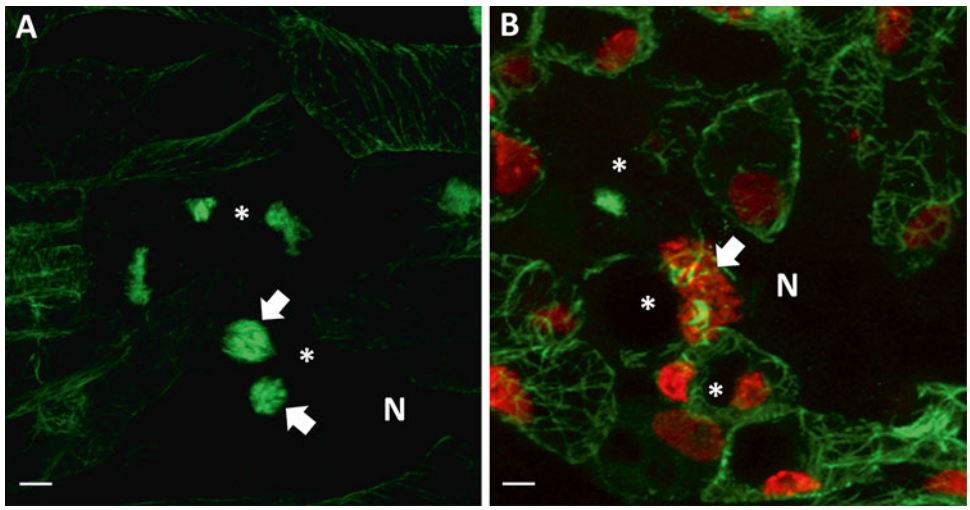

Fig. 1 Microtubule organization in giant cells of Arabidopsis plant expressing (**a**) MBD:GFP (*green*) or (**b**) MBD:GFP (*green*) and H2B:YFP (*red*) using confocal microscope. *Asterisks* giant cells, *N* nematode; *arrows* mitotic structures in giant cells. Scale bar: 2.5 μm

6. Syringe-driven filter unit and 3 μm membrane.
7. Gelrite (phytagel, Sigma-Aldrich, St. Louis, United States of America).
8. Fluorescent stereomicroscope.
9. Tweezers, scalpel, scissors.
10. 1 ml tube.
11. 5 ml pipette tips and plastic Pasteur pipettes.
12. HM650V vibrotome Microm (Walldorf, Germany).
13. Glass slides and cover slips.
14. Inverted confocal microscope (model LSM510 META; Zeiss, Germany).

2.2 Culture Media and Buffers

1. Plant in vitro medium: *Murashige* and *Skoog* (MS) ½ medium, containing 1 % sucrose, 0.7 % plant cell culture-tested agar. Adjust pH (with potassium hydroxide, KOH) to 6.5 and autoclave at 121 °C for 15–20 min.
2. Arabidopsis seed sterilization:

   2.63 % sodium hypochlorite (NaOCl) in water.

   Ethanol 70 %.
3. Nematode ultracleaning solutions:

   0.5 % NaOCl in water.

   0.01 % mercuric chloride ($HgCl_2$) in water (*see* **Note 1**).

   0.7 % Streptomycin sulfate in water (*see* **Note 2**).

   1 L of sterile water.
4. Embedding medium: 7 % agar (or agarose for molecular biology) dissolved in boiling distilled water.

2.3 Plant Material

1. *Arabidopsis* line expressing the N-terminal domain of the microtubule-binding domain of MAP4 fused to the GFP (*Pro35S:MBD:GFP*).
2. *Arabidopsis* line expressing Histone H2B fused to YFP (*Pro35S:H2B:YFP*).
3. Plant expressing *Pro35S:MBD:GFP* were crossed with plant expressing *Pro35S:H2B:YFP*. Progeny expressing both constructions were selected for fluorescence using fluorescent stereo microscope. Homozygous progeny was used for microscopy analysis.
4. *Arabidopsis* seed were surface sterilized by soaking for 5 min in ethanol 70 % and then for 5 min in 2.63 % sodium hypochlorite in a laminar flow hood. Seeds were extensively washed with ethanol 70 %, dried, and dispensed onto Petri dishes containing in vitro media (*see* Subheading 2.2). Plants were grown at 20 °C under a cycle of 8 h of light and 16 h of darkness for 5 days.

2.4 Pathogen Material

1. *M. incognita* was grown on greenhouse-grown tomato (*Solanum lycopersicum* 'St. Pierre'). The infected roots were collected, washed with tap water, and 5 cm-long root pieces grinded with 0.5% NaOCl in a blender.
2. Eggs were collected by filtration through a strainer and two sieves (100 µm and 10 µm) and washes with tap water to get rid of all the bleach. The eggs were transferred on a 5 µm sieve and the sieve were placed in a tray containing sterile tap water for eclosion.
3. J2 larvae were hatched in the water of the tray and were collected 7 days after the hatching on a 0.5 µm sieve. The number of larvae/mL was evaluated using cell counting chamber. Larvae attaching to the plastic, maximize the use of glass materials.

3 Methods

3.1 Ultra-Cleaning of M. incognita

1. In a laminar-flow hood, put the freshly hatched larvae on a 3 µm membrane (maximum 50,000 larvae per membrane) placed inside a syringe-driven filter unit, using a 10 mL syringe (*see* **Notes 3** and **4**).
2. Using 10 mL syringe, inject slowly the 0.01 % $HgCl_2$ solution (*see* **Note 1**) through the syringe-driven filter unit containing the nematodes (*see* **Note 5**). The nematode larvae need to be in contact with the solution for 9–10 min.
3. Using the 10 mL syringe, inject slowly the 0.7 % streptomycin sulfate solution (*see* **Note 2**) through the syringe-driven filter unit containing the nematode larvae. Wash three times by injecting through the syringe-driven filter unit with sterile water.
4. Resuspend the larvae in 0.5 % gelrite in order to obtain 20 larvae per µL. To do so, pull out the membrane using sterile tweezers and place it in a glass Petri dish with the nematode larvae facing up. Take off the larvae from the membrane by gently pipeting up and down the gelrite solution on top of the membrane, using a cut 20 µl tip (*see* **Note 6**).

3.2 In Vitro Inoculation of Arabidopsis Seedlings with M. incognita

1. Five days after sowing, screen *Arabidopsis* transgenic line expressing *Pro35S:MBD:GFP* and/or *Pro35S:H2B:YFP* for good level of fluorescent protein on the root meristem using fluorescent stereo microscope.
2. In a laminar flow hood, prick out ten plants expressing a good level of fluorescent proteins into fresh MS (*see* Culture Media and Buffers) square Petri dishes.

3. Let grow the seedlings for 10 days at 20 °C under a cycle of 8 h of light and 16 h of darkness. Petri dishes must be inclined at an angle of 60° to allow the roots to grow along the surface.
4. In a laminar flow hood, add 100 surface-sterilized *M. incognita* J2s on each seedling by deposing droplet of 10 µl close to the root apex (*see* **Note 7**).
5. Seal the Petri dishes with parafilm to prevent evaporation and keep them on the bench at RT for 5–6 days.

3.3 Preparation of the Infected Material Prior to Observation

1. 5–6 days after inoculation of the seedlings with the nematode larvae, extract from the Petri dish root systems infected with the nematodes using tweezers.
2. Wash carefully the root system of each plant with distilled water to remove all the pieces of plant medium and place them in an empty Petri dish (*see* **Note 8**).
3. Excise galls under the stereo microscope using tweezers and scalpel and place them in a 1 ml tube filed with distilled water (*see* **Note 9**).
4. To prepare the mould, cut the last 1 cm of a 5 ml pipette tip using scissors and place it in an open small Petri dish (Fig. 2a).
5. Using tweezers place two to three galls in the bottom part of the mould and pour on top cooled embedding medium (*see* Culture Media and Buffers) using plastic Pasteur pipette (Fig. 2b).
6. After solidification, extract the preparation from the plastic mould using pencil back.
7. With razor blade, cut the top of the preparation to flatten it and fix the preparation with glue on the vibratome receptacle putting the galls in the top part of the block (Fig. 2c).
8. Vibroslice the block into sections of about 100 µm thick using a vibratome (Fig. 2d).
9. Dispose the sections in a glass slide and add distilled water with a plastic Pasteur pipette before covering them with a cover slip (*see* **Note 10**, Fig. 2e).
10. Prepare a humid box by placing a soaked-paper roll on the bottom of a square Petri dish and display the slides on it before closing the lid.
11. Let the preparation in the humid box until observation with confocal microscope.

3.4 Imaging of the Mitosis in Giant Cells

1. For imaging of the dividing giant cells, observe the vibroslice with a ×63 water immersion apochromat objective using confocal microscope.
2. In order to image both dividing nucleus and microtubule mitotic structures in giant cells of *Arabidopsis* transgenic line

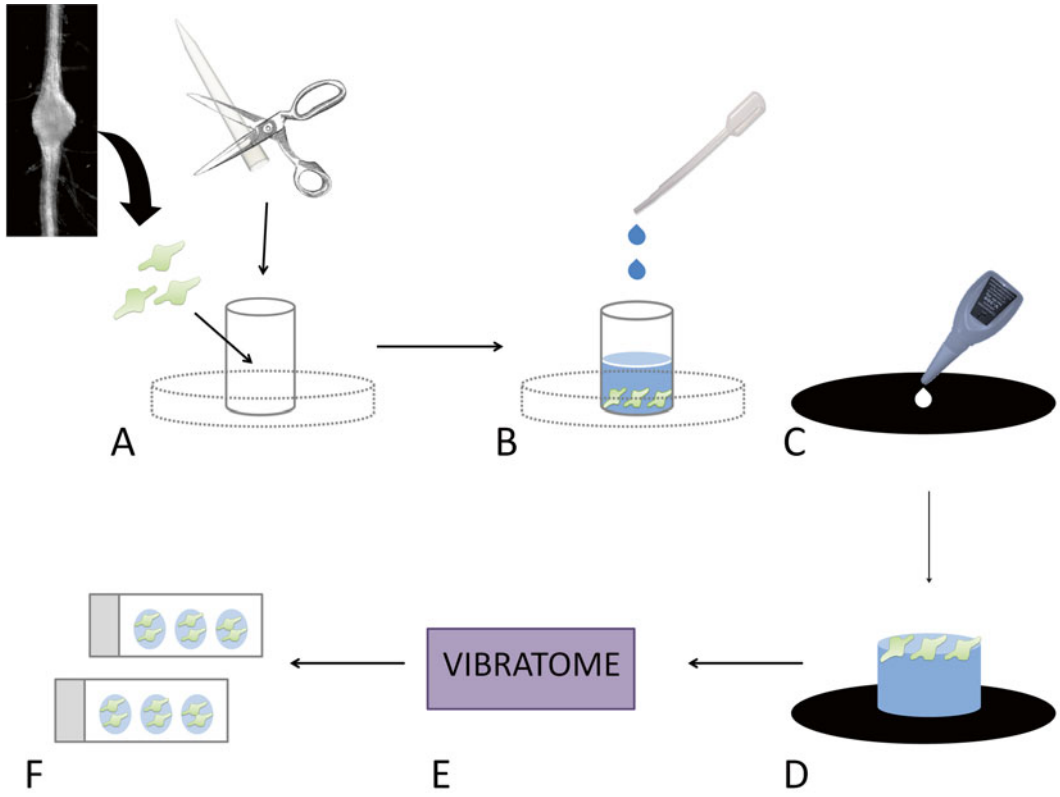

Fig. 2 Scheme of the preparation of the infected material prior to observation. (**a**) Cut the last 1 cm of a 5 ml pipette tip using scissors and place it in an open small Petri dish. Using tweezers place on it two to three galls. (**b**) Pour on top cooled embedding medium using plastic Pasteur pipette. (**c**) After solidification, extract the preparation from the plastic mould using pencil back. (**d**) With razor blade, cut the top of the preparation to flatten it and fix the preparation with glue on the vibratome receptacle putting the galls in the top part. (**e**) Vibroslice the block into sections of about 100 μm thick, using a vibratome. (**f**) Dispose the sections in a glass slide and add distilled water with a plastic Pasteur pipette before covering them with a cover slip until observation with confocal microscope

expressing *Pro35S:MBD:GFP* and *Pro35S:H2B:YFP*, use the Lambda acquisition mode (*see* **Note 11**) with a 499–550 nm beam path (488 nm excitation line).

3. Select two region of interest (ROI) which correspond to the YFP signal of H2B:YFP in the dividing nucleus and the GFP signal of MBD:GFP in the microtubule mitotic structure.
4. In the graph plotting the pixel intensity versus the mean wavelength of each band, verify that the fluorescence observed matches the desired fluorophore or represents nonspecific emission.
5. Choose two different colors to distinguish YFP signal of H2B:YFP in the dividing nucleus and the GFP signal of MBD:GFP in the microtubule mitotic structure.

6. In order to reconstruct in 3D the structures observed in giant cells, use Z-stack mode.

4 Notes

1. Mercuric chloride is Very toxic (T+), Corrosive (C), Dangerous for the environment (N). Wear two pairs of gloves and discard it in the appropriate chemical bin (ICSC 0979; R-phase: R28, R34, R48/24/25, R50/53; S-phrases: (S1/2), S36/37/39, S45, S60, S61.
2. The streptomycin sulfate is used as standard antibiotic to prevent bacterial infection in the culture. Wear gloves.
3. Autoclave the syringe-driven filter unit with the membrane in place.
4. To prevent contamination, the number of larvae/membrane must be inferior at 50,000.
5. Do not push too strongly and verify using stereomicroscope that there is no leak: you shouldn't see nematode in the solution placed in a Petri dish.
6. The homogenized solution must become more and more opaque, due to the presence of the nematodes.
7. Before inoculating the plant, observe with the stereomicroscope the larvae to be sure that they are numerous and still alive.
8. Be careful to let the root system infected with nematodes always immerged in water to not let it dry.
9. When you excise galls under the stereo microscope using tweezers and scalpel, let 1 mm on each side of the gall: this will allow you to manipulate them with tweezers without damaging the Giant cells.
10. When you start to cut through the block containing the giant cells, observe some of the section with the stereomicroscope to see if you are close to the giant cells. When you will manage to remove enough tissue around them, increase the size of the sections in order to not cut through the giant cells and then empty them. A young giant cell is about 100–150 μm in diameter.
11. The lambda acquisition mode collects the fluorescence in a stack that sorts the emission output into a series of wavelength bands.

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Part IV

Cell Division and Morphogenesis

Chapter 12

Real-Time Lineage Analysis to Study Cell Division Orientation in the Arabidopsis Shoot Meristem

Cory J. Tobin and Elliot M. Meyerowitz

Abstract

Cells in the Arabidopsis shoot apical meristem are small and divide frequently throughout the life-time of the organism making them good candidates for studying the mechanisms of cell division in plants. But tracking these cell divisions requires multiple images to be taken of the same specimen over time which means the specimen must stay alive throughout the process. This chapter provides details on how to prepare plants for live imaging, keep them alive and growing through multiple time points, and how to process the data to extract cell boundary coordinates from three-dimensional images.

Key words Live imaging, Image processing, Cell division, Segmentation, Arabidopsis

1 Introduction

The Arabidopsis shoot apical meristem is a dome-shaped structure at the apical end of the shoot that is responsible for generating most of the above-ground tissue [1]. The cells in the are organized into three distinct layers, the L1 (the epidermal layer), the L2 (sub-epidermal), and what is often termed the L3, which is more a region than a layer, as it consists of all of the cells below the L2. Very few cells move between layers [2, 3]. The cells in the top two layers expand such that they remain the same thickness but push on their neighbors within the same layer towards the periphery of the meristem. These cells divide anticlinally which preserves the thickness of the layer, limiting it to be one cell deep. Additionally, since plant cells are bound to each other by common walls and cannot move past each other, the position of the walls created during cytokinesis is critical to patterning and morphogenesis at the tissue and organ level.

These properties make the shoot meristem a useful tissue for studying cell division in plants. For example, the fact that these cells rarely divide periclinally means that the division geometry only needs to be tracked in two dimensions (along the surface of a

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three dimensional structure). This also means that most of the interesting geometry is visible in the *xy* plane (where the *z*-axis runs the direction of the shoot) and confocal microscopes usually have better resolution on the *x*- and *y*-axes compared to the *z*-axis. Additionally, the cells are small, so many cell divisions can be captured in a single, high-magnification image.

In this chapter we cover two different methods for keeping Arabidopsis plants alive while imaging them during a time series. One method involves plants grown in soil while the other involves plants grown in an agar medium. There are advantages and disadvantages to both methods (*see* **Note 1**).

1.1 Plant Culture

Plants grown in soil must be grown in very small dishes so that they can fit underneath the microscope objective. Unless the microscope has a stage that can be positioned 10–20 cm below the front of the objective then plants grown in planting pots will not fit. But growing in small dishes presents some unique problems. The soil will dry out rapidly requiring frequent watering. More importantly, assuming that a water-dipping objective will be used (which is desirable for the larger numerical aperture) getting water to stay in between the meristem and objective, as seen in Fig. 1a is difficult and requires practice. Growing the plants in agar means that imaging will be simpler. As shown in Fig. 1b, the plant and agar medium will be inside a high-walled box which can be filled with water during imaging. Unfortunately, for plants grown in agar sterilization becomes an issue and many plants will succumb to mold growth if measures are not taken to prevent contamination. Additionally, if the plants are left submerged in water for an extended period of time, many will die. This can be diminished to some extent by using water with 1/2× Murashige and Skoog salts but this may not be optimal for the objective. Please consult the objective manufacturer before attempting this.

1.2 Microscopy

Since the meristem is not flat and if we wish to observe the cells on the surface of the meristem, we must use three dimensional microscopy techniques. If we were working with a flat tissue with relatively thick cells such as a rosette leaf, bright-field microscopy might be sufficient. The most suitable methods in this particular case are laser scanning confocal and spinning disc microscopy. Both of these methods will capture three-dimensional images (z-stacks) which will allow us to extract just the first layer of cells using image processing techniques.

Choosing the fluorophore to use will have a great effect on the ease of data collection. Bright and photostable fluorophores will allow use of less light during imaging allowing the plants to live longer without photo-damage. Fluorescent dyes are not desirable in this application as over time the dye will build up in internal organelles making image processing difficult. A plasma

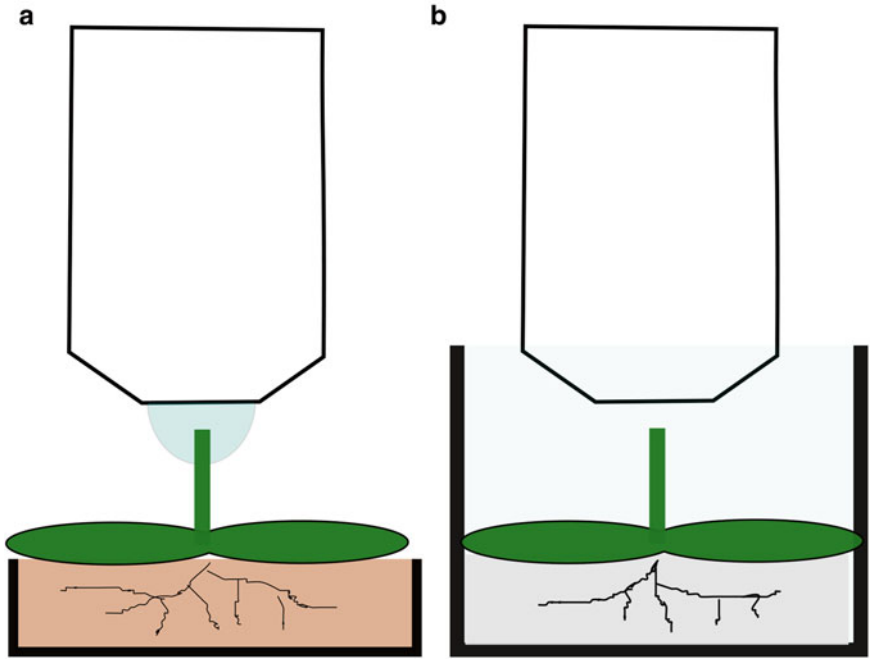

Fig. 1 Schematic of two different imaging configurations. (**a**) The plant is grown in soil in a petri dish. The shoot apical meristem pierces a water drop hanging from the bottom of a water immersion objective. (**b**) The plant is grown in a sterile box with nutrient agar. The box is filled with water during imaging and a water immersion objective is dipped into the box

membrane-localized fluorescent protein driven by an ubiquitin promoter will be best. 2xYPF is a good choice as it is bright and very photostable.

1.3 Overview of Software Workflow

A large part of tracking and quantifying plant cell division involves using software to analyze images. The software used in this chapter is MorphoGraphX developed by Richard Smith [4]. This software is particularly well suited for analyzing cell division geometry on the surface of a curved tissue like the shoot meristem. But other software can be used as well. MARS-ALT will work although it is specifically developed for three-dimensional segmentation which is not necessary for tracking cells on the surface of the shoot meristem [5]. Both of these software packages are free. Commercial software exists that will work sufficiently. In particular, Imaris by the company Bitplane has features for segmenting cell boundaries in two and three dimensions.

The overall goal during image processing is to extract the boundary positions of each cell from the images at each time point. These data are then fed into your own analysis.

The first step when using MorphoGraphX is to convert the image data into a solid structure void of cell boundary data. This allows us to create a mesh of vertices representing the surface of

the meristem. The image data is then mapped on to the mesh. We decide how deep into the meristem we want to collect data from (the first layer of cells is about 4 μm deep). All of the data from the surface reaching down to that particular depth is projected on to the mesh. A watershed segmentation algorithm is then used to label the boundaries of cells on the mesh. These labels are converted into a series of coordinates which can be exported and further used in your own custom analysis of cell growth, geometry and lineage.

2 Materials

2.1 Equipment

Upright fluorescent microscope.

- Inverted microscopes will not work.
- Preferably laser scanning confocal or spinning disc.
- Water immersion objective.
- Solid, flat-stage (instead of a slide holder) dissecting microscope.
- Preferably binocular.
- Minimum 10× total (objective × eyepiece) magnification.

Sharpened forceps.

Growth chamber or growth lights.

200 μL adjustable pipette.

2.2 Materials for Growing Plant in Agar

Murashige and Skoog Basal Medium.

Murashige and Skoog Vitamin Solution.

Sucrose.

Phytoagar.

Carbenicillin.

Deionized water.

Western incubation box 2.875″ × 2.00″ × 1.25″.

Filter tape, nonwoven, pressure sensitive.

1 M Potassium hydroxide.

Aluminum foil.

25 ml Serological pipet.

pH meter.

Magnetic stir plate.

Autoclave.

Two flasks.

Two stir bars.

Analytical balance.

Electronic serological pipettor.

2.3 Materials for Growing Plants in Soil

High-wall petri dishes.

Sunshine mix.

SuperSoil Potting Soil.

Vermiculite #2 Coarse.

Perlite.

Marathon Insecticide 1 %.

Spray or squirt bottle.

2.4 Computer Hardware

At least 16 GB of RAM.

Nvidia graphics card, 2 GB of RAM or more recommended.

2.5 Software

Linux operating system (*see* **Note 2**).

MorphoGraphX [4] (Lab of Richard Smith, Max Planck Institute for Plant Breeding Research) (*see* **Note 3**).

ImageJ [6] (*see* **Note 4**).

3 Methods

3.1 Method to Grow Plant in Soil

1. Before planting, *see* **Note 5**.
2. Mix enough soil for the quantity of plants you will be using, about 200 mL of soil per plant.

2 parts Sunshine Mix, 2 parts SuperSoil Potting Soil, 1 parts Vermiculite, 1 parts Perlite, 0.02 parts Marathon insecticide

3. Fill each high-walled petri dish with soil and pack it firm.
4. Add water to each dish using a squirt or spray bottle until the soil is soaked.
5. If you are using plants selected on agar medium, transplant them to the soil and make sure that the roots are covered in soil.
6. If you are starting from seed, place two or three seeds in the center of the soil.
7. Place lids on dishes.
8. Move dishes to a 4 °C area for 3 days to stratify.
9. Remove dishes from cold and place under grow lights.
10. When seeds germinate, use forceps to remove excess plants.
11. Water plants with a squirt bottle or a water bottle every day.

12. Wait for the shoot to produce flower primordia and grow to a height of about 1 cm.
13. Using sharp forceps and a dissecting microscope, dissect away any flowers and flower primordia close to the meristem so the meristem is visible (*see* **Note 6**).
14. Fill a small container (such as a petri dish) with deionized water.
15. Dip the microscope objective in the water dish such that a droplet of water sticks to the end of the objective covering up the front lens.
16. Place the dish with the plant under the objective and raise the stage (or lower the objective) until the tip of the shoot pierces the surface tension of the hanging water drop.
17. Use a 200 μL pipette to add more water to the drop.
18. Move the stage up and down to make sure that the plant's shoot can move freely in the water drop (*see* **Note 7**).
19. Use the microscope and acquire a z-stack of meristem (see the section below on imaging).
20. After acquiring images, remove the plant from underneath the microscope.
21. Put the plant back in the growth room until the next time point.
22. Before imaging at the next time point, return to **step 13** and check to make sure no flowers have grown over the top of the meristem. Dissect away any excess material.

3.2 Plants in Agar

1. Make enough growth medium for the quantity of plants you have chosen to use.
2. For 1 L of growth medium:
   (a) To a 2 L flask, add the following:
      - 1 L of deionized water
      - 2.2 g Murashige and Skoog Basal Medium
      - 10 g sucrose
      - Stir bar

   (b) Use a magnetic stir plate to mix the ingredients.
   (c) Use the pH meter, pipette, and 1 M KOH to adjust the pH to 5.7.
   (d) Add 7 g of phytoagar.
   (e) Cover the flask with aluminum foil.
   (f) Autoclave for 30 min on liquid cycle.
   (g) After removing the flask from the autoclave, continue to stir using the magnetic stir plate.

(h) When the flask is cool enough to touch add 100 mg of carbenicillin.

3. Place the 1.25 in. western incubation boxes in a sterile hood.
4. Bring the flask of growth medium into the hood.
5. Using sterile technique, open each box and pour in enough medium to fill the box at least 1.5 cm.
6. Leave the lids open to prevent condensation on the inside of the boxes.
7. For 250 mL of top medium: To a 1 L flask, add the following 250 mL of deionized water 1.75 g of phytoagar stir bar
   (a) Use a magnetic stir plate to mix the ingredients.
   (b) Cover the flask with aluminum foil.
   (c) Autoclave for 30 min on liquid cycle.
   (d) After removing the flask from the autoclave, continue to stir using the magnetic stir plate.
   (e) When the flask is cool enough to touch add 25 mg of carbenicillin.
8. After the growth medium in the boxes has solidified bring your flask of top medium into the sterile hood.
9. Pour a thin layer of top medium into each box.
10. Wait for the top medium to cool.
11. Once the medium has cooled and solidified, seedlings can now be transferred to the boxes.
12. In each box, use a sterile 200 μL pipette tip to gouge a small hole in the center of the agar (*see* **Note 8**).
13. In the sterile hood, use forceps to pick a single seedling from the selection plate and transfer it to a box on top of the hole in the agar.
14. Use a pipette tip to push the seedling's root into the hole in the agar so the seedling is upright.
15. Close the box and seal it with filter tape.
16. Place the box in a growth chamber or under growth light.
17. When the plant's shoot has grown to about 1 cm, move the box to a work area that has a dissecting microscope (*see* **Note 9**).
18. Using sharp forceps and a dissecting microscope, dissect away any flowers and flower primordia close to the meristem so the meristem is visible (*see* **Note 10**).
19. Move the box to the stage of the microscope that will be used for imaging.
20. Gently fill the box with sterile deionized water using a 25 mL serological pipette so that there is at least 1 cm of water over the top of the meristem (*see* **Note 11**).

21. Position the box under the objective and raise the stage (or lower the objective) until the front of the objective is near the specimen (*see* **Note 12**).
22. Proceed to capture a z-stack by following the suggestions in the Imaging section.
23. After imaging, remove and dispose of the water using a 25 mL serological pipette.
24. Close the box and place back under the growth lights until the next time point.
25. Before imaging at the next time point, return to **step 19** and check to make sure that no flowers have grown over the top of the meristem. Dissect away any excess material.

3.3 Imaging

Specific microscopy instructions cannot be given since they will differ depending on the particular brand, model, and configuration of microscope used. But the following are suggestions that should be considered before imaging.

1. Period between time points
   (a) The period should be chosen to expose the meristem to as little laser light as possible.
   (b) A 2-h period is a good starting point
   (c) If the tissue is not changing much in 2 h, increase to 4 h.
   (d) If the tissue is rapidly growing consider doing 1 h.
   Six hours and above should not be used as a small percentage of cells will divide twice within one period.
2. Resolution
   (a) 512×512 will probably be good enough to quantify cell geometry.
   (b) All else being equal, a higher resolution will not provide substantial benefit to the downstream segmentation algorithms and will expose the specimen to more light.
3. Light intensity
   (a) Experiment with light intensity and detector gain.
   (b) Less light is better for keeping the specimen growing.
   (c) If you use too little light you will need to increase the detector gain which can lead to increased background noise.
   (d) If the background looks grainy to the eye, increase the light intensity and drop the gain until the background looks smooth.
   (e) If the background is grainy the downstream segmentation algorithm will be unable to accurately detect the cell boundaries.

4. Bit depth
   (a) Ideally your images should be 16 bit.
   (b) By default Zeiss LSM microscopes record in 12 bit.
   (c) MorphoGraphX will not read 12-bit images properly.
   (d) Many microscope systems default to 8 bit, so double-check this before starting.

3.4 Image Processing

The first step for importing your data into MorphoGraphX is to convert your z-stack into TIFF format. The easiest tool for this job is ImageJ or Fiji, which is a particular distribution of ImageJ with many useful plug-ins already installed. If you are using ImageJ proper you will need to install a plug-in which allows you to read your particular z-stack image format (LSMToolbox for Zeiss LSM files, Loci_tools for Leica LIF files, Bioformats for Nikon ND2 or Zeiss CZI files).

One thing to note is that you need the first image in the series to be the top of the meristem. If the order is reversed you will need to export the data as a series of individual TIFF files rather than a single multi-TIFF, so the images can be reversed.

Before beginning retrieve the *x*,*y*,*z* dimensions of the voxels from the image file metadata.

3.4.1 Multiple TIFF Images

To export the data as a series of TIFF images:

1. Open ImageJ or Fiji.
2. Click on File, then Save As, and then Image Sequence.
3. Select the TIFF format and start the numbering at 0.

   Click OK (*see* **Note 13**).

The following Bash script (OSX, Linux, or Cygwin environments only) can be used to reverse the numerical order of the TIF file names if needed. This assumes your files are named like "image3.tif". Modify the second line of the script if your files are named differently. Copy this script into the same directory as your multiple TIFF files and run the script:

```
#!/bin/bash
image=(image*.tif)
MAX=${#image[*]}
for i in ${image[*]}
do
  num=${i:5:3} # grab the digits
  compliment=$(printf'%03d' $(echo $MAX-$num |
  bc))
  ln $i copy_of_image$compliment.tif
done
```

To import these images into MorphoGraphX:

1. Start the MorphGraphX program.
2. In the top menu bar select Stack then Stack1 then Main then Image Sequence. Click Add Files.
3. Select all of the image files in the series.
4. Click OK.
5. Input the X, Y, and Z dimensions of the voxels in micrometers (*see* **Note 14**).
6. Click Start and your stack should appear in the main window in a semi-transparent state as seen in Fig. 2a.

3.4.2 Single TIFF Image

To export the data as a single multi-TIFF image:

1. Click on File, then Save As, and then Tiff.
2. Select the TIFF format and start the numbering at 0.
3. Open the MorphoGraphX program.
4. Drag the TIFF file into the main window (*see* **Note 15**).
5. Click Start and your stack should appear in the main window in a semitransparent state as seen in Fig. 2a.

3.4.3 Building the Mesh

To convert the image data into a mesh representing the surface of the meristem:

1. On the right side, select the Process tab, and then the Stack tab.
2. Expand the Morphology section.
3. Click on Closing.
4. Change the X Radius, Y Radius, and Z Radius parameters to 15.
5. Click Go.
6. Confirm that the cell boundaries are no longer visible and the meristem looks like a solid mass (*see* **Note 16**).
7. Click on Edge Detect.
8. Modify the parameters that appear towards the bottom (*see* **Note 17**).
9. Make sure the surface looks relatively smooth as in Fig. 2b (*see* **Note 18**).
10. Erase any structures that you do not want included in the mesh like flower primordia
    (a) At the top of the window select the Pixel Edit tool.
    (b) To erase, hold Ctrl and Alt and click regions with the left mouse button.

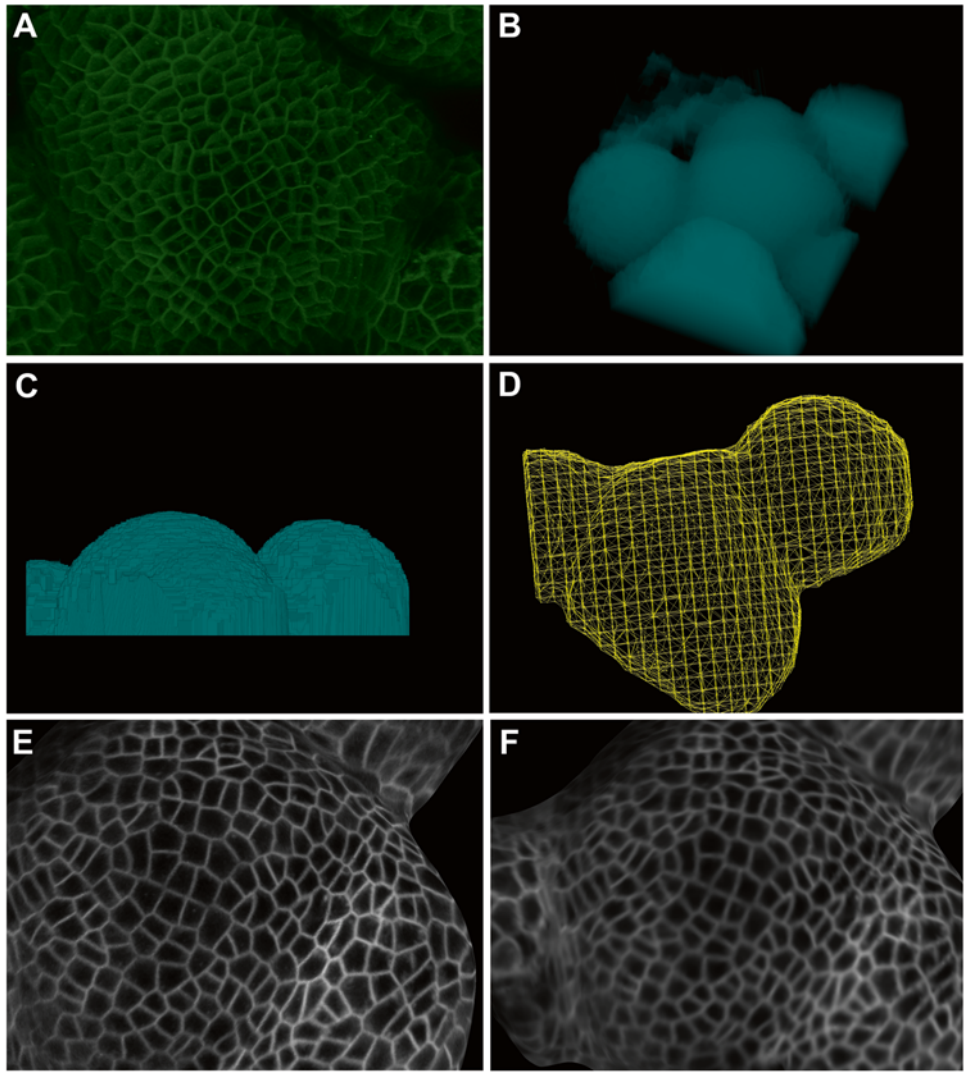

Fig. 2 Example of an image processed in MorphoGraphX. (**a**) Z-stack loaded. (**b**) After surface detection. (**c**) Side view after editing of surface to remove extraneous parts. (**d**) Creation of coarse mesh. (**e**) Image data mapped onto mesh surface. (**f**) Smoothing of the data on mesh surface

(c) When not holding Ctrl and Alt use the mouse to move and rotate the image.

(d) Use the mouse scroll wheel to zoom in and out.

(e) Look for small extraneous bits around the meristem and erase those, too.

(f) With the opacity turned up, it should look like Fig. 2c.

11. In the Process tab, click the Mesh tab and expand the Creation section.
12. Select Marching Cubes Surface.

13. Try the default parameters and click Go.
14. To view the mesh:
    (a) Go to the Main tab, and then the Stack 1 tab.
    (b) Disable Main and Work
    (c) Enable Mesh
    (d) Choose ALL from the View dropdown menu.
    (e) It should look similar to Fig. 2d.
15. Go to the Process tab, then the Mesh tab, in the Structure section select Smooth Mesh and click Go.
16. Select Subdivide Mesh and click Go.
17. Repeat **steps 15** and **16** until there are at least one million vertices (*see* **Note 19**).
18. Remove the bottom of the mesh.
    (a) Position the meristem so you are facing it from the side. Double-clicking will snap it into place.
    (b) On the right side of the window, choose the Select Points in Mesh button.
    (c) Hold Ctrl and Alt and use the mouse to select the entire bottom of the mesh.
    (d) Use the Del key to remove the selected vertices.
19. Map the image data onto the mesh.
    (a) Select the Main tab.
    (b) Make sure that the Main stack is selected rather than the Working stack (*see* **Note 20**).
    (c) Select the Process tab.
    (d) In the Mesh tab expand the Signal section.
    (e) Select Map Signal.
    (f) Choose 1 μm for the Min Dist parameter and 4 μm for the Max Dist parameter.
    (g) Click Go.
    (h) Go to the Main tab. Unselect Work and select Mesh. This should display the image data mapped onto the mesh.
    (i) It should look similar to Fig. 2e.
20. Confirm that the image data mapped on to the mesh looks good.
21. If deeper cell layers are appearing on the surface, experiment with the parameters in **step 19f**.
22. Select Smooth Mesh Signal from the Signal section.

23. Click Go. This will smooth out the image data to help the segmentation algorithm produce straighter boundaries. It should look similar to Fig. 2f.

Now the data are mapped onto the mesh. You can see it better by turning off the stacks. If the image looks jagged or overly pixelated, add more vertices to the mesh using **steps 15** and **16** and then re-map the data onto the mesh using **step 19**. The image is now ready to be segmented.

The previous steps can be automated using MorphGraphX's Python scripting capabilities. The following code will perform the steps automatically but you won't be able to pause it and view intermediate steps. Comment out parts of the code if you want to see intermediate states.

To run Python code in MorphoGraphX, first save the code to a text file that has the .py file extension. In MorphoGraphX click on the System tab, and then select Python. Choose the file that contains the Python code, and then click Go:

```
Stack.Closing(15,15,15)
Stack.Edge_Detect(100000,2,0.3,30000)
Mesh.Marching_Cubes_Surface(5,5000)
Mesh.Smooth_Mesh(1)
Mesh.Subdivide()
Mesh.Smooth_Mesh(3)
Mesh.Subdivide()
Mesh.Smooth_Mesh(1)
Mesh.Subdivide()
Mesh.Subdivide()
Mesh.Project_Signal("No",1,3,0,50000)
Mesh.Smooth_Mesh_Signal()
```

If you're running an older version of MorphoGraphX the stack may require more processing before the edge detection step. In that case use the following code:

```
Stack.Resize_Canvas("Yes","Yes",0,0,40)
Stack.Shift_Stack(0,0,20)
Stack.Average(1,1,1,1)
Stack.Closing(15,15,15)
Stack.Edge_Detect(100000,2,0.3,30000)
Mesh.Marching_Cubes_Surface(5,5000)
Mesh.Smooth_Mesh(1)
Mesh.Subdivide()
Mesh.Smooth_Mesh(3)
Mesh.Subdivide()
Mesh.Smooth_Mesh(1)
Mesh.Subdivide()
Mesh.Subdivide()
Mesh.Project_Signal("No",1,3,0,50000)
```

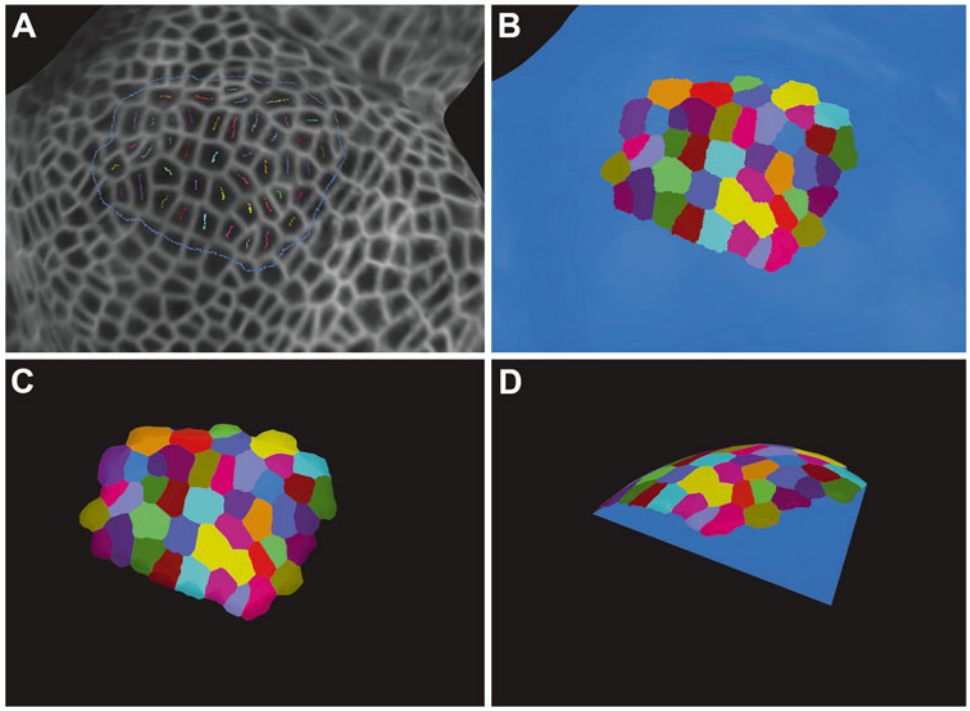

Fig. 3 Segmentation of cells in MorphoGraphX. (**a**) Seeds drawn on the mesh. One seed is drawn in the center of each cell. This helps the watershed segmentation algorithm correctly differentiate between different cells. (**b**) After watershed segmentation. (**c**) Creation of cells using 1 μm line segments. (**d**) Side view of the cells

3.4.4 Segmenting

To detect the edges of the cells in the mesh:

1. At the left side of the window, click the Add New Seed tool.
2. Hold Ctrl and Alt and use the mouse to click the interior of a cell.
3. Do this for each cell as seen in Fig. 3a.
   (a) Each cell should turn a different color.
   (b) You can drag the mouse to color a larger portion of the cell interior.
   (c) This can help if the background noise is high.
   (d) Each time you click you are drawing with a new cell seed.
   (e) If you have already picked your mouse up but want to keep drawing with the same seed, select the Add Current Seed tool and draw with that.
   (f) To revert back to making new seeds, select the Add New Seed tool again.
   (g) To erase a seed:
   - Click on the Label Color (the colored box at the top of the window). The color should disappear.

- Choose the paint bucket tool.
- Click on the seed that you want to erase.

(h) With a new seed, draw a circle around the area you want segmented as seen in Fig. 3a.

4. In the Process tab, expand the Segmentation section.
5. Click on Watershed Segmentation.
6. Use the default parameters.
7. Click Go.
8. Wait for the segmentation to complete.
9. The mesh should look similar to Fig. 3b.
10. If you are satisfied with the segmentation, proceed to **step 13**.
11. If there are corners between the cells that are not assigned a color:
    (a) In the Process, Mesh, Cell Mesh section select Fix Corners.
    (b) Click Go.
    (c) In the Segmentation section, choose Watershed Segmentation.
    (d) Click Go.
    (e) The corners should now be fixed.
12. If you are not satisfied with the smoothness of the boundaries:
    (a) In the Process, Mesh, Structure section select Subdivide Adaptive Near Borders.
    (b) Map the signal data onto the mesh again using the procedure described previously at **step 19** in Subheading 3.4.3.
    (c) In the Process, Segmentation section select Watershed Segmentation.
    (d) Use the default parameters.
    (e) Click Go.
13. Expand the Cell Mesh section and select Convert to Cells.
14. Choose a value of 1 μm for the Minimum Distance parameter (*see* **Note 21**).
15. Save the cell data.
    (a) From the top menus, select the Mesh dropdown menu.
    (b) Select Mesh 1, and then Export.
    (c) Choose Cells for the file type (*see* **Note 22**).
    (d) Click OK.
16. Save the mesh.
    (a) From the top menus, select the Mesh drop-down menu.

(b) Select Mesh 1, and then Save (*see* **Note 23**).

(c) Click OK.

If you receive errors while trying to create cells (**step 13**), there may be a problem with the structure of the mesh. Use the following Python code to try to fix the problem. After running this code, go to **step 13** and try to build the cells again:

```
Mesh.Segment_Mesh(20000)
count = 0
while count < 2:
  try:
    Mesh.Make_Cells(1)
    break
  except:
    Mesh.Fix_Corners()
    Mesh.Smooth_Mesh(1)
    Mesh.Segment_Mesh(20000)
  count += 1
```

3.4.5 Lineage Tracking

During segmentation, each cell is assigned a unique number as an identifier. When two separate images from a time series are segmented, the cell ID numbers (labels) from one image do not correspond to the same cells in the other image. In order to compare cells from one time point to the next we must match up cells in each image. MorphoGraphX has tools for comparing two meshes at once and reassigning labels to match up cells between time points. If you have more than two time points you will first compare mesh 1 with mesh 2, readjusting the labels in mesh 2. Then compare the adjusted mesh 2 with mesh 3, adjusting the labels in mesh 3 to match those of mesh 2 (and therefore mesh 1 as well). At the end, all of the cells with the same lineage will have the same label. If a cell has divided, both daughter cells will have the same label.

1. Load the first mesh by clicking on Mesh in the top menu bar, then selecting Mesh 1.
2. Choose the file of the saved mesh.
3. Click OK.
4. Repeat **steps 1** through **3** but in **step 1** choose Mesh 2 instead.
5. In the Main tab, click the Stack 1 tab.
6. Turn Stack off.
7. Turn Surface off with Labels active.
8. Turn Mesh on.
9. Select Cells from the View drop-down menu.
10. Change the color of this mesh using the color palette.
11. Click the Stack 2 tab.

12. Turn Stack off.
13. Turn Surface off with Parents active.
14. Turn Mesh on.
15. Select Cells from the View drop-down menu.
16. Make sure that this mesh has a different color than the mesh from step 10.
17. In the Main tab set the Control-Key-Interaction to Stack 1.
18. Hold down the Ctrl key and move the cell wireframe using the left mouse button.
19. Move the wireframe so that mesh 1 is above mesh 2.
20. Rotate the view to look at the meristems from the top.
21. Rotate the mesh so that the cells from both meshes line up as best as possible.
22. The meshes can be scaled if needed to aid in aligning cells.
23. Transfer labels from mesh 1 to mesh 2.
    (a) In the Stack 2 tab check Surface and Parents boxes.
    (b) Click on the Grab Label from Other Surface tool from the left side of the window.
    (c) Hold the Ctrl and Alt keys.
    (d) Click on a cell in mesh 1.
    (e) The label will be transferred to the cell in mesh 2 that you "clicked through."
    (f) Click all the cells that you want labels transferred for.
24. Save the second mesh with new labels.
    (a) Make sure that the Stack 2 tab is active.
    (b) Click the Mesh drop-down menu from the top of the window.
    (c) Click Save.
    (d) Choose a file location.
    (e) Click OK.
25. Save the cells from the second mesh.
    (a) Make sure that the Stack 2 tab is active.
    (b) Click the Mesh drop-down menu from the top of the window.
    (c) Click Export.
    (d) Select Cells for the file type (*see* **Note 24**).
    (e) Click OK.

Now you should have a series of cell files (as created in **step 25**), one for each time point. These files contain the coordinates of all

the cell boundaries and the label numbers should be consistent throughout the series of files. These are the data you should use for any custom geometric or lineage analysis (*see* **Note 25**).

4 Notes

1. Comparison of growing plants in soil vs. in agar.

   In soil:

   Advantages:

   - Don't have to worry about sterility when planting.
   - The plant will never be submerged in water.
   - Higher percent of plants survive extended imaging.

   Disadvantages:

   - Plants require frequent, individual watering.
   - Placing immersion fluid (water) in between the specimen and objective is difficult and requires practice.

   In agar:

   Advantages:

   - Plants don't need individual watering.
   - Getting immersion fluid (water) in between the specimen and objective is simple.

   Disadvantages:

   - Seeds must be sterilized before planting.
   - Planting must occur in sterile environment.
   - Large percent of plants will succumb to contamination.
   - Plants must be fully submerged in water during imaging.
   - Some plants will not survive past the first observation time point.
2. Ubuntu or Kubuntu will be simplest if you are not an experienced Linux user. The distribution must be able to run proprietary NVIDIA graphics drivers.
3. Other software that can be used in place of MorphoGraphX.
   - MARS-ALT [5]
   - Bitplane Imaris
4. Fiji [7] is preferable over ImageJ.
5. Before Planting
   - Find a suitable line with a bright enough fluorescent protein localized to the plasma membrane.
   - Decide if you will be growing in soil or agar
   - Decide how many plants you will need

6. No flowers should protrude above the top of the meristem as these will block light during imaging. If the plant has been well watered you should be able to remove the older flowers by pinching the base of the pedicel with sharp forceps. Younger primordia can be removed by placing one arm of the forceps between the meristem and flower and pushing down to shear off the flower. Be careful not to touch the meristem with the forceps otherwise epidermal cells will die.
7. The goal is to allow the stage and plant to move freely so a z-stack can be acquired. Too little water and the meristem will not move freely through the drop. Too much water and the drop will fall from the objective. Experiment to get the optimal drop size.
8. The hole should pass through the top agar and go into the growth agar.
9. If the shoot has grown too much, the long stem will sway during imaging. If the shoot is still too short, the objective will touch the rosette leaves during imaging causing the plant to move. The optimal height will depend on the size of your objective and its working distance.
10. No flowers should protrude above the top of the meristem, this will block light during imaging. If the plant has been well watered you should be able to remove the older flowers by pinching the base of the peduncle with sharp forceps. Younger primordia can be removed by placing one arm of the forceps between the meristem and flower and pushing down to shear off the flower. Be careful not to touch the meristem with the forceps otherwise epidermal cells will die.
11. If there is too much water it will spill out due to the displacement created by the objective. If there is not enough water the objective can leave the water during imaging causing aberrations.
12. If your microscope has bright field mercury illumination, use the UV/DAPI configuration during positioning of the specimen. This will allow for easy visualization of the light cone which can be seen through the side of the box when the room is dark. Once you have the specimen positioned at the focal point of the light cone, use the binoculars and fine stage controls to precisely position the specimen for imaging.
13. This will create multiple image files. One file for each slice in the z-stack.
14. These data should be available in the metadata associated with the original image file.
15. This will create multiple image files, one for each slice in the z-stack. This file can now be imported into MorphoGraphX.

16. Your original data is still unmodified in Main, Stack1, Work. If you can still see cells, increase the Radius parameters.
17. The values for these parameters will depend on the brightness and the amount of background noise in your data. Start with the default values but change Threshold to 100,000.
18. There should be no large spikes protruding from the surface. Experiment with different parameters.
19. This number can be found at the bottom of the window. More vertices will improve accuracy at the expense of memory usage. At some point more vertices is no longer helpful.
20. This determines which data will be mapped onto the mesh.
21. This determines the smallest wall segment. Smaller values increase the number of points in the final dataset. Larger values decrease the number of points but may miss small details in the cell boundaries. The mesh should now look similar to 3C and 3D.
22. This creates a text file will coordinates of cell boundaries only. The entire mesh data is not included.
23. This creates a .mgxm file. All of the mesh data is included. This file can be used to reload the mesh later.
24. This creates a text file with coordinates of cell boundaries only. The entire mesh data is not included.
25. If you are only interested in making heatmaps you should instead load the .mgxm files as created in **step 24**. MorphoGraphX has features for making heat maps of cell growth magnitudes and principal growth directions.

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Chapter 13

Cell Proliferation Analysis Using EdU Labeling in Whole Plant and Histological Samples of Arabidopsis

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Abstract

The ability to analyze cell division in both spatial and temporal dimensions within an organism is a key requirement in developmental biology. Specialized cell types within individual organs, such as those within shoot and root apical meristems, have often been identified by differences in their rates of proliferation prior to the characterization of distinguishing molecular markers. Replication-dependent labeling of DNA is a widely used method for assaying cell proliferation. The earliest approaches used radioactive labeling with tritiated thymidine, which were later followed by immunodetection of bromodeoxyuridine (BrdU). A major advance in DNA labeling came with the use of 5-ethynyl-2′deoxyuridine (EdU) which has proven to have multiple advantages over BrdU. Here we describe the methodology for analyzing EdU labeling and retention in whole plants and histological sections of Arabidopsis.

Key words DNA replication, DNA labeling, EdU, 5-Ethynyl-2′deoxyuridine, Cell division, Cell proliferation, Histology

1 Introduction

In vivo labeling of replicating DNA has been a useful tool for biologists for at least 60 years. Early experiments in plants used the uptake and incorporation of tritiated thymidine during DNA replication to demonstrate the quiescent nature of the QC in roots [1, 2]. This was later followed by analysis of replication in the shoot meristem [3]. The radioactive nature of tritiated thymidine and the difficulty with detection of labeled samples led to the development of fluorescent immunostaining of DNA that had incorporated bromodeoxyuridine (BrdU) in the 1970s and 1980s [4, 5]. A major disadvantage of BrdU is that the immunodetection requires denaturation of the DNA duplex for efficient antibody binding, which leads to changes in overall tissue structure. Additionally, in plants, antibody delivery is inhibited by the cell wall such that detection requires cell wall digestion. While BrdU has been successfully used

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for cell cycle studies for 30 years, it is rapidly being replaced by detection of incorporated 5-ethynly-2′deoxyuridine (EdU).

EdU is a thymidine analog, replacing the 5′methyl in the pyrimidine ring with an alkyne. To visualize incorporation, EdU is reacted with a fluorescent azide in a CU(I) catalyzed reaction [6]. Major advantages of EdU labeling are that there is no need to denature DNA, and the small molecules required for the chemical reaction penetrate plant tissues efficiently. EdU was rapidly adopted for use in plants [7] and has been successfully used for multiple studies of root development [8, 9], analysis of plant DNA damage response [10], chromatin modifications [11, 12], and plant growth and development [13–17]. However, none of these reports have analyzed cell proliferation in shoot meristems, or on histological sections.

A concern with all metabolic labeling experiments is the toxicity of the labeling molecule. EdU is no exception, and has been shown to have toxic effects at concentrations required for efficient labeling. This led to the creation of F-ara-EdU, which retains labeling efficiency with much lower toxicity [18]. F-ara-EdU has recently been used for long-pulse experiments to examine cell division rates in living roots [19].

Here we describe protocols we have developed for pulse-chase labeling of Arabidopsis meristems using EdU. These methods should be widely applicable to analysis in most plant systems, and can be substituted with F-ara-EdU for longer labeling pulses. In this protocol, germinating seedlings are pulse labeled with EdU. Following this pulse, plants can be grown for varying lengths of time in media lacking EdU as a "chase." Following the chase period, plants are fixed and reacted with fluorescent azides in order to visualize labeled DNA. For short chase periods, these experiments allow one to visualize which cells were replicating during the pulsed period. For longer chase periods, one can visualize cell populations with slower division rates.

2 Materials

2.1 EdU Labeling

1. Laminar flow hood or equivalent for sterile plant culture work.
2. Suitable growth space for plant tissues.
3. 4-, 6-, and 24-well tissue culture plates (*see* **Note 1**).
4. Parafilm or culture tape.
5. 40–100 μm nylon mesh holders for transferring seeds between liquids, e.g., Cell Strainer (Semadeni) (*see* **Note 2**).
6. Ethanol.
7. 50 % bleach.

8. Suitable liquid growth medium: In our experiments with Arabidopsis we have used ½ MS medium plus vitamins supplemented with 1 % sucrose.
9. Suitable solid growth medium: Depending on the length of the pulse and chase times, this can either be soil or culture media. In our experiments with Arabidopsis we have used ½ MS medium plus vitamins supplemented with 1 % sucrose, and 3 % phytagel. For root experiments, square plates that can be grown vertically facilitate removal of the roots from the media.
10. EdU or F-ara-EdU: EdU is available as a reagent in Click-iT® EdU Imaging Kits from Invitrogen or separately from Sigma. F-ara-EdU is available from Sigma. Both compounds are prepared as a 10 mM stock in DMSO.
11. Light microscope, e.g., Leica DM 1000.

2.2 EdU Detection General

1. Fixative: We have used both 3:1 ethanol:acetic acid and 3.7 % formaldehyde (*see* **Note 3**).
2. PBS
3. Wash buffer, 3 % BSA in PBS.
4. Permeabilization buffer, 0.5 % Triton X-100 in PBS.
5. EdU detection reagents, e.g., Click-iT® EdU Imaging Kit (Invitrogen), which will be detailed in this protocol (*see* **Note 4**).
6. Filter paper.
7. DAPI for DNA counter-staining (stock solution 2 mg/mL), we have also used Hoechst with good results.
8. Microscope slides and appropriate cover slips.
9. 50 % glycerol.
10. Plastic cover slips cut very slightly larger than the slides being used (*see* **Note 5**).
11. Coplin jars (*see* **Note 6**).
12. Humidity chamber: We use short rectangular metal tins lined on the bottom with paper towels and then covered with glass transfer pipettes to form a solid surface for slides to rest on.
13. Vectashield.
14. Rubber cement to seal slides.

2.3 EdU Detection Roots/Whole Tissues

1. Confocal microscope equipped with appropriate fluorescent filters, e.g., Karel Zeiss LSM 780.
2. Image acquisition software, e.g., ZEN2011.
3. Deconvolution software, e.g., Huygens (SVI, The Netherlands).

2.4 Paraffin Embedding and Sectioning for Shoot Meristems

1. Tissue cassettes.
2. Eosin.
3. Tissue processor, e.g., LOGOS for dehydrating and clearing fixed tissue for paraffin embedding.
4. Paraffin.
5. Embedding molds.
6. Forceps
7. Microtome and blades, e.g., Microm HM325.
8. Clearing reagent, e.g., Histo-Clean (Fisher Scientific)
9. Fluorescent microscope with appropriate filters: Confocal imaging is not required due to the thin sections produced

3 Methods

3.1 EdU Labeling

1. Surface sterilize dry seeds by briefly (less than 30 s) washing them with 70 % EtOH in an Eppendorf tube. Following this wash, seeds are incubated with 50 % bleach for 7 min (*see* **Note 7**).
2. Remove the 50 % bleach solution in a sterile environment (laminar flow hood or equivalent) and wash twice with sterile water to remove any remaining bleach.
3. Place sterile seeds into a nylon mesh holder, and, using sterile forceps, place this into a tissue culture plate containing enough culture media to cover the seeds. Seal the tissue culture plate with parafilm or culture tape.
4. Seeds are germinated under standard conditions, 22 °C and 24-h daylight (*see* **Note 8**).
5. Following 3 days of germination, emerging radicles should be visible with a dissecting microscope from the majority (>70 %) of seedlings. Adjust germination time until radicle emergence is visible.
6. Prepare a working solution of EdU in ½ MS liquid media with sucrose. We have obtained good results with a concentration of 5 μM (*see* **Note 9**).
7. Under sterile conditions, transfer the nylon mesh containing germinating seedlings to the working EdU solution in a 4-well tissue culture plate. The solution should fully cover the seedlings.
8. Label seedlings with EdU for 1 h (*see* **Note 9**).
9. Transfer the nylon mesh holder to a 6-well tissue culture plate containing fresh liquid culture medium. Wash the seedlings by

transferring them to fresh medium at least twice to remove any remaining EdU solution.

10. Depending on the experimental plan, transfer seedlings either to fresh liquid media (for short chase experiments up to 48 h), to vertical solid culture media (for longer chases to examine roots), or to soil (for longer chases to examine shoots or other aerial tissues).

3.2 EdU Detection in Seedlings

1. After the desired chase period (*see* **Note 9**) in liquid media lacking EdU, seedling must be fixed prior to EdU labeling.
2. Transfer the seedlings to be labeled to 2 mL Eppendorf tubes and fix with a fixative. The fixative volume should be in gross excess to the volume of tissue (10:1) (*see* **Note 10**).
3. Incubate seedling in fixative for 1 h at room temperature. Replace with fresh fixative and repeat for a total of 2–3 times on the same day.
4. Incubate seedlings in fixative overnight at 4 °C. Replace with fresh fixative in the morning (*see* **Note 11**).
5. Return seedlings to nylon mesh filters to facilitate the next washing steps [6–12].
6. Wash seedlings with two changes of wash buffer in 6-well tissue culture plants. Following each wash, quickly dry the mesh on filter paper to remove remaining liquid.
7. Remove the wash solution and replace with permeabilization buffer. Incubate at RT for 20 min.
8. During this incubation, prepare the EdU reaction cocktail according to Table 1 in the order listed. The reaction buffer should be used immediately.
9. Quickly dry the mesh filters on filter paper and wash twice with 1 mL of wash buffer, drying the mesh on filter paper between washes.
10. Transfer the mesh to 4 well tissue culture plates. Add 250 μL of Click-iT reaction cocktail to each well. Shake the plate briefly to ensure that the reaction buffer is equally distributed over the seedlings.
11. Incubate for 30 min, RT, in the dark.
12. Remove reaction buffer, dry, and wash once with washing buffer.
13. Wash once with PBS.
14. During the washing step, prepare a working solution of DAPI by diluting the stock solution 1:1000.
15. Transfer seedlings to 500 μL of working DAPI solution.
16. Incubate for 30 min, RT, in the dark.

Table 1
Click-iT reaction buffers for whole tissues

	# of samples				
	1	**2**	**4**	**8**	**10**
Distilled water	193.5 µL	387 µL	774 µL	1.620 mL	1.98 mL
10× Click-iT stock	21.5 µL	43 µL	86 µL	180 µL	220 µL
$CuSO_4$	10 µL	20 µL	40 µL	80 µL	100 µL
Fluorescent-azide	0.6 µL	1.2 µL	2.5 µL	5 µL	6 µL
Reaction buffer additive	25 µL	50 µL	100 µL	200 µL	250 µL
Total	250 µL	500 µL	1 mL	2 mL	2.5 mL

Prepare the reaction cocktail in the order listed and use immediately. The volumes given here are appropriate for tissue culture plates with wells of 17 mm diameter. Newer Click-iT Plus kits use a modified copper reagent resulting in slight changes to the volumes listed here

17. Remove the DAPI and wash twice with PBS.
18. On a fresh microscope slide, drop 30 µL of 50 % glycerol.
19. Transfer seedlings individually from the mesh holders to the slide.
20. Cover carefully with cover slips.
21. Dry slides at 50 °C to distribute the glycerol under the cover slip.
22. Seal slides with rubber cement.
23. Slides are ready to use, and can be stored at 4 °C in the dark.
24. Visualize EdU labeling with a fluorescent microscope and appropriate filters. Oil immersion 40× objectives give sufficient magnification to clearly visualize individual cells in Arabidopsis seedlings. To confirm labeling of chromosomes, the EdU signal should clearly overlap the signal from DAPI.

3.3 EdU Detection Roots

1. After the desired chase period, plants must be fixed prior to labeling. Carefully remove plants from solid, vertically grown media and place them singly into wells of a 24-well tissue culture plate (*see* **Note 12**).
2. Incubate plants in fixative for 1 h at room temperature. Replace with fresh fixative and repeat for a total of 2–3 times on the same day (*see* **Note 10**).
3. Incubate plants in fixative overnight at 4 °C. Replace with fresh fixative in the morning (*see* **Note 11**).

4. Wash plants with two changes of wash buffer. In each case, carefully remove the previous solution without damaging the roots.
5. Remove the wash solution and replace with permeabilization buffer. Incubate at RT for 20 min.
6. During this incubation, prepare the EdU reaction cocktail according to Table 1. The reaction buffer should be used immediately.
7. Wash plants twice with 1 mL of wash buffer.
8. Add 250 μL of Click-iT reaction cocktail to each well. Ensure that the reaction buffer is equally distributed over the plant and that the plants are completely covered.
9. Incubate for 30 min, RT, in the dark.
10. Remove reaction buffer and wash once with washing buffer.
11. Wash once with PBS.
12. Create a working solution of DAPI by diluting the DAPI stock 1:1000 in PBS.
13. Remove PBS and cover plants with DAPI working solution.
14. Incubate for 30 min, RT, in the dark.
15. Remove the DAPI and wash twice with PBS.
16. On a fresh microscope slide, drop 30 μL of 50 % glycerol.
17. Transfer plants from tissue culture wells to the slide (*see* **Note 13**).
18. Cover carefully with cover slips.
19. Dry slides at 50 °C to distribute the glycerol under the cover slip.
20. Seal slides with rubber cement.
21. Slides are ready to use, and can be stored at 4 °C in the dark.
22. Visualize EdU labeling with a confocal fluorescent microscope and appropriate filters. The large tissue depth in intact roots necessitates confocal imaging.

3.4 EdU Detection of Sectioned Material

1. In contrast to seedlings and roots, visualization of interior structures of larger shoot apical meristems is difficult due to the depth of the tissue, which causes problems both in penetration of the labeling cocktail and in microscopy. In this protocol, shoot meristems are first embedded in wax, sectioned, and then reacted with fluorescent molecules for visualization.
2. Harvest the desired aerial portions of soil grown plants. To facilitate further processing, remove leaves from the meristem by cutting the petiole as close to the stem as possible (*see* **Note 14**).

3. Incubate plants in fixative for 1 h at room temperature. Replace with fresh fixative and repeat for a total of 2–3 times on the same day.
4. Incubate plants in fixative overnight at 4 °C.
5. Replace fixative with 70 % ethanol/5 % eosin on the next day. Stain plants for approximately 30 min (*see* **Note 15**).
6. Transfer stained plants to appropriate tissue cassettes and store in 70 % ethanol.
7. Dehydrate tissues for paraffin embedding using a tissue preparation machine (*see* **Note 16**).
8. Using pre-warmed molds and forceps, embed the dehydrated tissue in paraffin at 60 °C. To facilitate cutting at later steps, position the tissue flat at the bottom of the mold (*see* **Note 17**).
9. Place the bottom of the tissue cassette onto the top of the mold and add paraffin so that it covers the base of the cassette. This allows the cassette to later be clamped to the microtome.
10. Allow the paraffin-embedded samples to harden either on a cooling plate or in a fridge or a freezer. Once hardened, the embedded tissues can be stored at RT.
11. Trim as much paraffin as possible surrounding the tissue using a razor blade or scalpel (*see* **Note 18**).
12. Set the microtome for appropriate-sized sections. We have routinely cut sections at 10 μm (*see* **Note 19**).
13. Prepare a microscope slide by covering the surface with a thin layer of water.
14. Section the tissue. Use forceps to gently pull the wax sections away from the microtome. Individual sections should remain attached together in a strip (*see* **Note 20**). Continue cutting until the length of the strip is slightly shorter than the length of the slide
15. Carefully lay the wax strip onto the water-coated microscope slide and gently position it at the top of the slide.
16. Continue sectioning strips until the slide is covered (*see* **Note 21**). Maintain the orientation of the strips such that the first section of the new strip is always located under the first section of the previous strip.
17. Gently remove remaining water, either with a pipette or by air-drying. This can be facilitated using a slide heater and heating the slides to 42 °C.
18. Visualize the slide with a low magnification light microscope. The shoot apical meristem should be clearly distinguishable in the sections by the appearance and disappearance of a dome in the center of the stem.

19. Continue producing slides from one stem until the appearance and disappearance of the meristem is confirmed by light microscopy (*see* **Note 22**).
20. Allow slides to air-dry overnight.
21. Transfer slides to Coplin jars (*see* **Note 6**).
22. Incubate in clearing reagent at RT, twice for 10 min, or according to the manufacturer's instructions.
23. Rehydrate the slides in an ethanol series.

    Absolute ethanol 1 min

    95 % ethanol 1 min

    85 % ethanol 1 min

    50 % ethanol 1 min

    30 % ethanol 1 min

    Distilled water, twice for 1 min
24. Wash the slides twice with washing buffer by flooding with 1 mL of solution holding the slide at an angle over a beaker, allowing the washing solution to run off.
25. Incubate in permeabilization buffer in Coplin jars for 20 min at RT.
26. Prepare Click-iT reaction buffer according to Table 2.
27. Wash the slide twice with washing buffer (*see* **Note 23**).
28. Add 50 μL of Click-iT reaction buffer to each slide, cover with a plastic cover slip, ensuring that the buffer is evenly distributed over the slide. Place in a closed humidity chamber and incubate at RT for 30 min.
29. Remove the plastic cover slip and wash each slide twice with PBS.
30. Prepare DAPI working solution by diluting stock solution 1:1000 in PBS.
31. Add 50 μL of DAPI working solution to each slide and cover with a plastic cover slip, ensuring that the buffer is evenly distributed over the slide. Place in a closed humidity chamber and incubate at RT for 30 min.
32. Remove the plastic cover slip and wash the slide once with PBS. Add 25 μL of Vectashield supplemented with 2 μg/mL DAPI and carefully cover with a glass cover slip.
33. The slides are ready to use and can be stored at 4 °C in the dark for periods of at least 2 weeks.
34. Visualize slides with a suitable fluorescent microscope. Due to the thin sections, confocal microscopy is not necessary.

Table 2
Click-iT reaction buffer for sectioned tissues on slides

	# of slides		
	2–6	**6–12**	**12–24**
Distilled water	193.5 µL	387 µL	774 µL
10× Click-iT stock	21.5 µL	43 µL	86 µL
$CuSO_4$	10 µL	20 µL	40 µL
Fluorescent-azide	0.6 µL	1.2 µL	2.5 µL
Reaction buffer additive	25 µL	50 µL	100 µL
Total	250 µL	500 µL	1 mL

Due to the time required for processing, we generally limited the number of slides processed at one time to 24 or less. Prepare the reaction cocktail in the order listed and use immediately. Newer Click-iT Plus kits use a modified copper reagent resulting in slight changes to the volumes listed here

4 Notes

1. EdU pulse chasing and subsequent labeling requires multiple washing steps in different buffers. As it is generally desirable to pulse many seeds at once for short time periods, we place sterile seeds into mesh holders to facilitate transfer from EdU containing media to wash media. The washing steps are carried out efficiently in tissue culture plates. As EdU detection reagents are costly, costs can be reduced by using tissue culture plates with the smallest size wells practical. We have used plates with a well diameter of 17 mm for these experiments. Washing buffers are less expensive and washing is more effective with larger volumes; thus we routinely perform washing steps in 6-well tissue culture plates (36 mm diameter).
2. We produced nylon mesh by removing the tops of 1, 5, or 10 mL pipette tips with a razor, depending on the diameter of the wells of the culture plate being used. The bottom of the cut tip is then melted briefly with a Bunsen burner and immediately placed onto the nylon mesh, fusing the two. We used 60 µM Millipore Nylon net filters (Millipore, NY6004700), though mesh sizes from 40 to 100 µM are acceptable.
3. This procedure has also been used successfully with fixation in 3.7 % formaldehyde in PBS, with and without vacuum infiltration. We prefer ethanol:acetic acid fixative due to its reduced toxicity and stability of the fixed tissue for long term storage; it is, however, not compatible with the use of GFP-tagged proteins. For experiments involving the use of a co-localizing

marker, formaldehyde or paraformaldehyde should be used. In either case, the volume of fixative should be in much greater excess to the tissue volume (10:1). The fixative times we listed in the protocol are specifically for ethanol:acetic acid fixation and are extended to ensure that the fixed material is stable for long periods of time. We have also had success with fixing tissue for as little as 1 h, although further processing of the tissue should be performed immediately.

4. Due to the nature of the chemical reaction used for EdU labeling, different fluorophores can be used for labeling DNA. At the time of this writing, Invitrogen offers Alexa 488, 555, 594, and 647. We routinely use Alexa 488, and have also used Alexa 594, both with good results in plants.
5. Plastic cover slips are less expensive than glass for the multiple incubations performed on slides, are faster to use, easier to handle, and are less damaging to the tissue on the slide. We cut them out of plastic, although parafilm can also be used. The slips should be cut to the size of the slide, with little extra material. In our experience larger slips are often dislodged or moved during subsequent handling, damaging the tissue.
6. When dealing with large numbers of slides, Coplin jars with slide racks that allow easy transfer of all slides in one step to a jar with another solution, save significant amounts of time. We have used the EasyDip slide staining system from VitroVivo.
7. Longer time periods in bleach lead to a dramatic reduction in germination efficiency. Seven minutes should be considered the maximum time for sterilization.
8. Synchronization of germination is important. We have had good luck with synchronous germination from seed stocks that are at least several months old. Younger seed stocks may require stratification at 4 °C for up to 5 days to increase synchrony.
9. We recommend empirical determination of EdU concentrations as well as pulse times for all experiments. In our hands, EdU labeling was readily visible with a 1-h pulse of 1 μM EdU. We observed an increase in labeling up to 5 μM, with little or no apparent increase for higher concentrations. EdU is cytotoxic, although we have observed little or no growth defects for pulses up to 24 h with 5 μM EdU. Longer pulses will allow more cells, and especially more slowly dividing cells, to incorporate the EdU label. For longer pulses, F-ara-EdU, which is much less toxic, can be substituted, and seedlings can be germinated on plates with the label [19]. Importantly, we have observed that even after removal of the EdU for long periods of time in the "chase" phase, cells which did not divide during the pulse period became labeled at later time points.

We believe this is due to cells retaining the EdU within the nucleotide pool for long periods of time, and incorporating it at the next division.

10. Seedlings are fixed in 3.7 % Formaldehyde in PBS for at least an hour. Vacuum infiltration can be applied for 15 min, though in our experience this has made no difference to final results.
11. Once fixed, plants can be stored in either fixative or 70 % ethanol at –20 °C. We have used fixed tissue for EdU labeling up to 1 month after fixation with no effect on quality; our experience with other cytological techniques suggests that tissue fixed in ethanol:acetic acid is stable at –20 °C for up to 1 year. Formaldehyde fixed tissue is much less stable, and should be processed within a few days, maximally 1 week.
12. Although fewer reagents may be used by washing multiple samples together, untangling roots, especially for plants several weeks old, is painstaking.
13. It facilitates microscopy when the aerial portion of the plant is placed at one end of the slide, and the root is stretched to the other. For longer roots, stretch it to one end of the slide and then loop back to the other. Repeat as necessary. For especially long roots, the root can be cut and placed on multiple slides.
14. Sectioning can be a painstaking procedure, and is made slightly less so by strictly limiting the amount of tissue embedded in paraffin. In our experiments, we removed as much leaf tissue as possible and embedded only the central stem of the plant containing the shoot apical meristem. This helps primarily by allowing the tissue to be placed in the paraffin at the desired angle for sectioning, and maximizing the number of sections that can be placed on a single slide. Sectioning is a skill that requires some practice and is best performed on tissue that is not experimentally valuable.
15. Eosin stains the tissue pink, facilitating the visualization of the otherwise small, white tissue samples within the larger white paraffin block. This also helps to determine the section containing the sample during sectioning.
16. In case tissue preparation devices are not available, samples can be dehydrated by hand. A sample protocol is available in [20].
17. Significant amounts of time can be saved by embedding tissues in the orientation in which they will be cut. For longitudinal sections of the shoot apical meristem, we have placed stems near a corner and at the very bottom of the mold. This facilitates removal of excess paraffin, and reduces the number of sections that must be cut before the sample is sectioned.
18. Cut the paraffin at an angle away from the sample, and do not cut blocks so that the area bound to the cassette is smaller than

0.5 cm^2 as smaller blocks occasionally break off from the tissue holder.

19. Although thinner sections are technically possible, we found 10 μm to be a good balance between ease of sectioning and tissue depth. 10 μm sections generally had one to two cell layers per section.
20. If individual sections do not remain together, but curl up, it helps to adjust the angle of the cutting blade or the position of the blade with respect to the sample. Pushing the blade too hard against the sample can also cause the sections to curl.
21. We routinely fit 3–5 strips per slide. Higher strip density limits reagent costs and facilitates later screening.
22. To reduce time on fluorescent microscopes, mark the slide with which rows contain the meristem. However, slides must be visualized shortly after sectioning, as once the sections have dried it is extremely difficult to distinguish the tissue from the surrounding wax.
23. From this step on, the slides should not be allowed to dry. When processing multiple slides, parallel processing occasionally results in the earliest processed slides drying out. For this reason, we suggest processing the slides in a sequential manner, meaning wash one slide, add the Click-iT or DAPI solution to that slide, cover it with a plastic cover slip and place it in the humidity chamber before beginning with the next slide.

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Chapter 14

Studying Cell Division Plane Positioning in Early-Stage Embryos

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Abstract

Unraveling the mechanisms that govern division plane orientation is a major challenge to understand plant development. In this respect, the *Arabidopsis* early embryo is a model system of choice since embryogenesis is relatively simple and cell division planes orientation is highly predictable. Here, we present an integrated set of protocols to study 3D cell division patterns in early-stage *Arabidopsis* embryos that combine both cellular and sub-cellular localization of selected protein markers with spatial organization of cells, cytoskeleton, and nuclei.

Key words Embryo, Whole-mount, Immunolocalization, Cell wall, Microtubules, Fluorescent marker, *Arabidopsis*

1 Introduction

Cell division, migration, growth, and differentiation determine the hierarchical organization of tissues and organs in multicellular organisms. Symmetric, proliferative divisions give rise to identical daughter cells, whereas asymmetric divisions are mainly associated with initiation of new cell types and developmental patterns. In asymmetric divisions, the position of the cleavage plane is often governed by cell polarity, a classical paradigm being that symmetry breaking relates to asymmetric distribution of polarity determinants within the cell [1, 2]. As a consequence of differential partitioning, daughter cells are committed toward distinct developmental fates. Alternatively, daughter cells may initially share the same distribution of cell fate determinants, but are then subjected to different positional cues inducing distinct developmental fates. Because of the absence of cellular mobility during plant development, the orientation of cell division determines the relative positions of cells within the tissue, and is thus essential for coordinated organogenesis and growth [3]. Hence, unraveling mechanisms governing the

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orientation of both symmetric and asymmetric cell division is crucial to understand plant development.

Early embryogenesis in *Arabidopsis thaliana*, starting from a single zygotic cell, involves a well-characterized pattern of symmetric and asymmetric cell divisions. The topological orientation of these divisions is remarkably invariant, making the *Arabidopsis* embryo a fruitful experimental system to study developmental processes at play during early embryonic development of angiosperms [4]. To explore and quantify the role of intrinsic/extrinsic factors involved in cleavage plane selection such as cell geometry, nuclear positioning or cytoskeleton arrays, efficient imaging methods are required. Ideally, such protocols should allow to simultaneously visualize the cell envelope (either cell walls or membranes, thus enabling 3D reconstruction of the embryo), the cytoskeletal organization, the nucleus, and should also allow to follow the subcellular localization of protein markers related to embryo development.

In seed plants, embryogenesis takes place within seeds, and embryos are surrounded by the endosperm and maternal tissues. Early embryos are even less accessible due to their small size, making them even more challenging to study. Because of that, live-imaging techniques are not easily applicable and up to now, most experiments are performed on fixed embryos (for examples *see* [5–10]). Ideally, protocols designed to image early-stage embryo development should be rapid, reliable, easy to perform with no specific technical skills required. They should use intact whole-mount embryos in order to allow 3D reconstruction of whole embryos. To get reliable statistical figures, they should also permit acquisition of numerous samples with limited time and effort.

Here, we present an integrated set of protocols to study 3D cell division patterns and protein markers in early-stage *Arabidopsis* embryos. The first protocol based on propidium iodide staining provide high-resolution 3D images of cell walls, within intact ovules and is suitable for the study of both embryo [9, 10] and maternal tissue development [11]. A second alternative method on isolated embryos combines cell wall and nucleus staining in order to get information on the spatial positioning of nuclei within cells. A third method relies on whole-mount immunolocalization coupled to DAPI staining, and is compatible with additional detection of fluorescently tagged proteins such as GFP markers. Altogether, these methods provide an integrated set of 3D data, combining the cellular and sub-cellular localization of selected protein markers with spatial organization of cells, cytoskeleton, and nuclei within *Arabidopsis* embryos.

2 Materials

2.1 Equipment

1 cm diameter incubation baskets 100 μm (12.440) (Intavis, Koeln, Germany).

24-well plates.

Microscopy slides.

22 × 22 mm glass coverslips (for the immunolocalization and cell wall/nuclei staining protocols).

50 × 22 mm glass coverslips (for the cell wall imaging protocol).

Tweezer with super fine tips (Dumont Tweezer Style 5) (EMS, Hatfield, USA).

Paracentesis needle (HL1105) (Collin, Bagneux, France).

Microsurgery knife (72047-15) (EMS, Hatfield, USA).

Double-sided adhesive tape.

Agitator.

Heat chamber (37 °C).

Binocular microscope with a diascopic base.

Laser scanning confocal.

2.2 Solutions

2.2.1 Solutions for Immunolocalization and Cell Wall/Nuclei Staining

1. Microtubule Stabilizing Buffer (MTSB ½): Stir 7.56 g PIPES (25 mM), 0.61 g $MgSO_4 \cdot 7H_2O$ (2.5 mM) and 5 mL of 0.5 M EGTA (2.5 mM, *see* **Note 1**) in 990 mL of distilled water. Adjust the pH to 6.9 with 1 N KOH and add additional distilled water to obtain a total volume of 1 L.
2. Paraformaldehyde fixation buffer: 4 % paraformaldehyde in MTSB ½ (*see* **Note 2**), 0.1 % Triton X100.
3. Agarose solution: Low gelling temperature agarose 1 % in water. Weigh 1 g of agarose and add 100 mL of distilled water. Heat the solution until complete dissolution of the agarose. Cool down the agarose solution before use.
4. Digestion buffer: stir 2.44 g MES (25 mM) in 430 mL of distilled water and adjust pH to 5.5 with NaOH 10 N. Add 0.44 g $CaCl_2$ (8 mM), 54.65 g mannitol (600 mM), 0.1 g pectolyase y23 (0.02 %) (MP biomedicals, Illkirch Graffenstaden, France), 0.5 g macerozyme r-10 (0.1 %) (Yakult Honsha, Tokyo, Japan) and adjust the volume to 500 mL with distilled water. The digestion buffer may be aliquoted and stored at −20 °C (*see* **Note 3**).
5. Washing buffer: To make a 1 M stock solution, dissolve 0.75 g of glycine in 10 mL of 1× Phosphate Buffered Saline (PBS) solution made from a dilution 1:10 in distilled water of a ready-to-use 10× PBS solution. This stock solution may be aliquoted

and stored at –20 °C. To obtain the washing buffer (50 mM Glycine), make a 1:20 dilution of the stock solution using 1× PBS.

6. Staining buffer: The Pontamine Fast Scarlet 4B (S479896) (Sigma-Aldrich, St. Louis, USA) stock solution is a 25 mg/mL solution in distilled water. It can be aliquoted and stored at –20 °C. The working dilution is 125 μg/mL.
7. Mounting medium: Add 700 μL of Citifluor AF1 (Agar Scientific, Stansted, United Kingdom) to 200 μL of a 0.1 mg/mL DAPI solution (*see* **Note 4**).

2.2.2 Solutions for Cell Wall Staining

1. Fixation solution (*see* **Note 5**): Mix 5 mL of methanol with 1 mL of acetic acid and 4 mL distilled water.
2. NaOH/SDS solution: Mix 200 μL of 10 N NaOH and 500 μL of 20 % SDS in 9.3 mL of distilled water. This solution (0.2 N NaOH, 1 % SDS) must be freshly prepared.
3. Digestion buffer: To obtain the stock solution, dissolve 100 mg of α-amylase (A4551) (Sigma-Aldrich, St. Louis, USA) in 10 mL of distilled water. This stock solution is stored at –20 °C as 20 μL aliquots (*see* **Notes 3** and **6**). Add 980 μL of distilled water to a 20 μL aliquot to obtain the digestion buffer (0.2 mg/mL α-amylase).
4. Bleach solution: Make a 1:8 dilution of a bleach commercial solution (9.6 % NaOCl) with distilled water.
5. Oxidation solution (1 % Periodic acid): Add 20 μL of periodic acid 50 % (w/v in distilled water) to 980 μL of distilled water. The periodic acid 50 % (w/v) solution may be aliquoted and stored several weeks at room temperature. The oxidation solution must be freshly prepared.
6. Staining solutions (diluted MPS and PI solutions) (*see* **Note 5**): Prepare a diluted modified pseudo-Schiff (MPS) solution by adding 1.9 g of sodium metabisulfite to 3 mL of 5 M HCl and distilled water up to 100 mL. The propidium iodide (PI) stock solution is a 2 mg/mL solution in distilled water (P4170) (Sigma-Aldrich, St. Louis, USA). This PI stock solution may be aliquoted and stored at –20 °C.
7. Clearing solution (*see* **Note 5**): Add 25 mL of glycerol and 50 mL of distilled water directly in the bottle containing 100 g of chloral hydrate (C8383) (Sigma-Aldrich, St. Louis, USA). The clearing solution may be stored for several months in a chemical fume hood.
8. Mounting medium (Hoyer's solution) (*see* **Note 5**): Stir 200 g of chloral hydrate and 20 g of glycerol in 50 mL of distilled water. Then add 30 g of gum arabic (G9752) (Sigma-Aldrich, St. Louis, USA).

2.3 Plant Material

Arabidopsis thaliana (Col0 ecotype) plants were grown in the greenhouse under 14 h of light, a diurnal temperature of 19 °C, and a nocturnal temperature of 16 °C.

Embryo stages from the undivided zygote to embryos at the globular, transition or heart stages, can roughly be deduced from the position of the silique along the inflorescence stem. Each silique from the inflorescence stem is numbered from the youngest to the oldest one. Silique #1 corresponds to the youngest pollinated flower displaying an elongated silique. Silique #1 harbors seeds with either a zygote or a one-celled embryo proper where the zygote has underdone a transverse cell division, producing an apical cell (the embryo proper) and a basal one that will develop after several rounds of division in the suspensor. Siliques #2 to #4 harbor seeds containing 2- to 8-celled embryos, siliques #5 and #6 correspond to embryos at the globular stage, and siliques #7 to #9 to embryos at the transition and early heart stages.

An *Arabidopsis* line expressing the pWOX5::GFP reporter [12, 13] was used: in this line, GFP starts to accumulate at the early-globular stage in the hypophysis, the uppermost cell of the suspensor that will differentiate to form part of the root cap.

2.4 Antibodies

The monoclonal anti-α-tubulin antibody produced in mouse (clone B-1-5-2, Sigma-Aldrich, St. Louis, USA) was used to label microtubule arrays.

The polyclonal anti-KNOLLE produced in rabbit [6] was a gift of Gerd Jürgens (University of Tübingen, Germany). The anti-KNOLLE antibody allows to identify cells in early mitosis, as this cytokinesis-specific syntaxin starts to accumulate at the trans-Golgi network during the G2/M transition. KNOLLE is expressed all along mitosis and during cytokinesis where it accumulates at the expanding cell plate.

Secondary antibodies were purchased from Life Technologies (Carlsbad, USA): Alexa Fluor® 488 Goat Anti-Mouse (A-11017), Alexa Fluor® 555 Goat Anti-Rabbit (A-21430), Alexa Fluor® 647 Goat Anti-Mouse (A-21235).

3 Methods

3.1 Whole Mount Immunolocalization (Fig. 1, *See* Note 7)

3.1.1 Dissection and Fixation

A dissecting microscope is used all along the procedure from the dissection of siliques to the attachment of embryos to coverslips. The dissection step consists in the removal of the valves of each silique to perform fixation on exposed seeds. Hold the silique pedicel with a tweezer, the replum facing up. Then slide a paracentesis needle on each valve margin from the base of the silique toward the tip to open it, and remove each valve using the needle. Insert a plastic bucket (*see* **Note 8**) into a well of a 24-well plate filled with 1 mL of fixation buffer. The seeds attached to the septum are

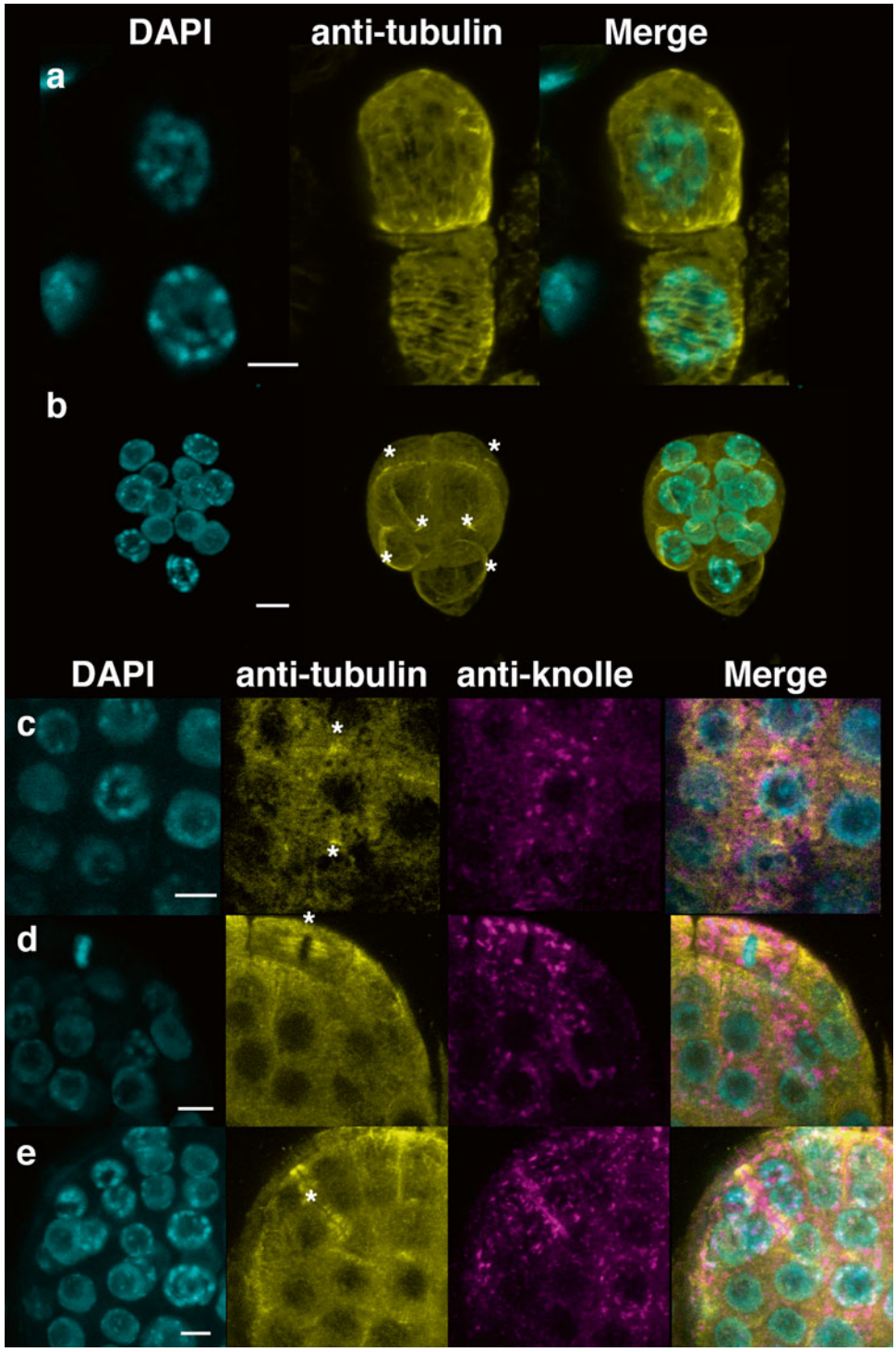

Fig. 1 Combination of immunolocalization and DAPI staining in interphasic and dividing embryonic cells at different stages of development. (**a**) Maximum projection of z-stack images of a 1c-stage embryo. The suspensor cell (*bottom cell*) displays transverse microtubule cortical arrays while the *upper cell* corresponding to the embryo proper has longitudinal cortical microtubules. (**b**) 3D volume rendering of a 14c-stage embryo. *Asterisks* indicate four cells with a prominent preprophase band. (**c**) 2D image of a globular embryo cell at the preprophase stage. Condensed chromatin is hardly visible with DAPI while the anti-tubulin antibody (*yellow*) revealed the preprophase band (*asterisks*). KNOLLE starts to accumulate at this stage.

transferred into the basket. Place the 24-well plate in a vacuum desiccator and apply vacuum using an oil pump. Pull the vacuum until bubbles form in the fixation buffer then break it and apply the vacuum again for 1 h. Rinse two times for 10 min in 1 mL of MTSB ½. The fixation step is done under a chemical fume hood because of formaldehyde toxicity.

3.1.2 Embryos Attachment to Coverslips

Remove three septa from the same developmental stage from the MTSB ½ buffer and put them on a coverslip placed on a microscopy slide (*see* **Note 9**). In a drop of MTSB ½, using a paracentesis needle, remove the seeds from each septum, then discard the septum. Place a second coverslip on the first one and apply a mild pressure with a paracentesis needle to gently push the embryos out of the seeds one by one (*see* **Note 10**). Do not use a tweezer to exert the pressure or you may break the coverslip. Separate the two coverslips using a tweezer, and place each one on a slide. Let the buffer evaporate for a few minutes without drying out the embryos. Spread 20 μL of 1 % low gelling temperature agarose on the coverslip to embed all embryos (*see* **Note 11**). Once the agarose is hardened (but not dried out in order to keep the embryos wet), put the slides in a wet chamber and cover the agarose with around 250 μL of MTSB ½.

3.1.3 Permeabilization, Rehydratation, and Digestion

All the remaining steps are done in a wet chamber (i.e. a Petri dish containing wet filter papers) to avoid desiccation of the samples. Remove the MTSB ½ and add 250 μL of 100 % methanol to permeabilize the tissue. Incubate 10 min at room temperature in a chemical fume hood. Remove methanol and rehydrate during 10 min with 250 μL of MTSB ½. Rinse another time with 250 μL of MTSB ½. For cell wall digestion of embryo cells, cover the sample with 250 μL of digestion buffer and incubate for 1 h at 37 °C.

3.1.4 Hybridization

Rinse two times with 250 μL 1× PBS for 10 min at room temperature. Remove the PBS solution and incubate with 250 μL of the primary antibodies at least overnight at room temperature (*see* **Note 12**). The anti-tubulin antibody is used at a dilution of 1:1000 in 1× PBS and the anti-KNOLLE antibody at a dilution of 1:2000 in 1× PBS.

Fig. 1 (continued) (**d**) A typical acentrosomal spindle (*asterisk*) is visible in this maximum projection of z-stack images of a globular embryo cell at metaphase. The metaphase plate is revealed using DAPI staining. (**e**) Maximum projection of z-stack images of globular embryo cells at telophase. Two adjacent cells display a phragmoplast, as revealed with the anti-α-tubulin antibody (*asterisks*) while the anti-KNOLLE antibody highlights the growing cell plate. The *cyan* color corresponds to DAPI staining, *yellow* to the anti-α-tubulin antibody, *purple* to the anti-KNOLLE antibody, and the last column to merged colors. Scale bar = 5 μm (in **a**, **c**, **d**, and **e**) and 10 μm (in **b**)

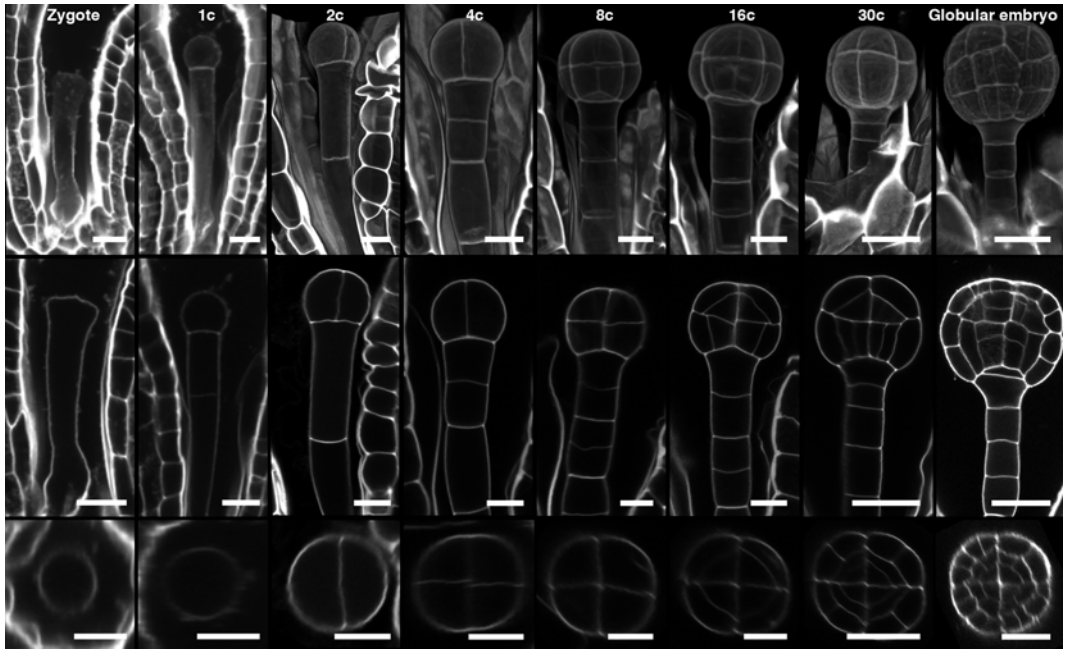

Fig. 2 Series of *Arabidopsis* embryo development stages from the zygote to the globular stage. 3D volume rendering (*upper row*), longitudinal section (*middle row*) and transverse section of the central domain (*bottom row*) of a zygote, and 1c-stage, 2c-stage, 4c-stage, 16c-stage, 30c-stage and globular stage embryos. Scale bar = 10 μm (zygote and 1c- to 16c-stage embryos) and 20 μm (30c-stage and globular embryos)

Rinse two times with 250 μL of washing buffer for 10 min. Incubate with 250 μL of the secondary antibody solution (1:1000 dilution in 1× PBS) for at least 4 h at room temperature (*see* **Note 12**).

3.1.5 Mounting

Rinse the sample two times with 250 μL of washing buffer for 10 min at room temperature. Add a drop of mounting medium on each coverslip, and turn it upside down on a slide. Seal the coverslip to the slide with transparent nail polish.

3.2 Cell Wall Staining (Fig. 2, See Note 13*)*

3.2.1 Dissection and Fixation

Proceed exactly as for the dissection step used during the immunolocalization experiment except that the seeds attached to the replum are placed in bucket inserted in a 24-well plate filled with 1 mL of cell wall fixation buffer. Seeds are incubated at least overnight although better results are obtained after a 3-day incubation. Samples can be stored for several weeks at this stage. The fixation step is done under a chemical fume hood because of methanol toxicity.

3.2.2 Rehydratation, Permeabilization and Digestion

Remove the fixation solution and rehydrate the samples using a graded ethanol series (10 min per bath): 50 %, 30 %, 15 % and distilled water twice. Incubate 3 h in a NaOH/SDS solution at 37 °C and rinse two times in distilled water. Incubate 1 h at 37 °C in the amylase digestion buffer. Rinse two times with distilled water.

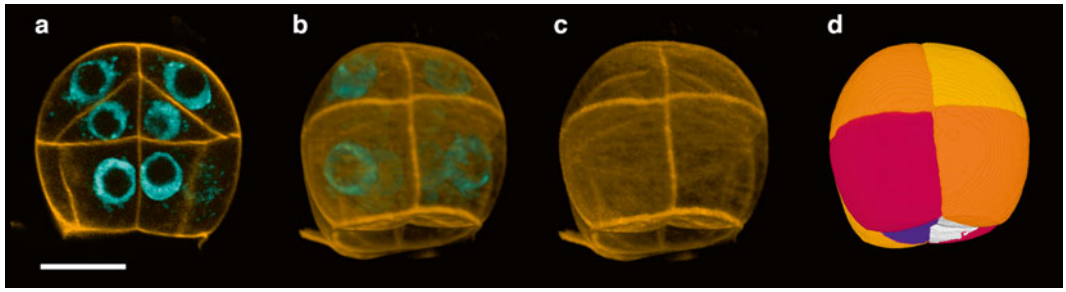

Fig. 3 Pontamine Scarlett and DAPI staining of a 16-cell embryo. (**a**) Median optical section of a 16c-embryo, stained with Pontamine Scarlett (*yellow*) and DAPI (*cyan*), and showing the typical cellular organization at this stage. (**b**, **c**) 3D volume rendering image of the same embryo. (**d**) 3D segmentation image obtained using the morphological segmentation plugin (*see* **Note 13**). Scale bar = 10 μm

3.2.3 Discoloration, Staining and Clearing

While under the dissecting microscope, transfer the samples to the bleach solution and incubate the seeds until most of them turn white: for very early-stage embryos (siliques #1 to #7), 10–20 s are usually enough. Later stages (silique #7 and older ones) may require up to 60 s. Rinse three times in distilled water. Transfer the samples to 950 μL of MPS for 30 s, then add 50 μL of the PI stock solution. Incubate overnight. Rinse two times in distilled water and transfer in the clearing solution and incubate for at least 2 h. At this stage, samples can be left in the clearing solution overnight. The clearing step is done under a chemical fume hood because of chloral hydrate toxicity.

3.2.4 Mounting

We use two layers of adhesive double-sided tape as spacers to avoid sample flattening. Put the spacers at about 3 cm from each other in the middle of the slide, return the basket, and push down the grid to empty it. Detach the seeds from the septa and scatter them between the spacers. Add few drops of Hoyer's and put a coverslip in the middle of the slide. Be sure to add enough mounting medium to fill the space between the slide and the cover-slip. The mounting step is done under a chemical fume hood because of chloral hydrate toxicity.

3.3 Combination of Cell Wall and Nuclei Staining (Fig. 3, See Notes 13 *and* 14*)*

3.3.1 Fixation

3.3.2 Staining

Proceed exactly as the fixation step for immunolocalization.

Take three septa from the same developmental stage and put them on a slide. Then using a microsurgery knife, cut the seed coat at the seed tip (i.e. at the pole opposite to the micropyle) while keeping the seeds attached to the septum. This step will enable the dye to enter the seed and stain the embryo. Samples are incubated at room temperature for 60 min in the Pontamine Fast Scarlet 4B solution and rinsed two times with 1 mL of PBS for 10 min at room temperature.

3.3.3 Embryos Extraction

In a drop of PBS, using a paracentesis needle, remove the seeds from each septum, then discard the septum. Place a 22 × 22 mm coverslip on the slide and apply a mild pressure with a paracentesis needle to gently push the embryos out of the seeds one by one. Do not use a tweezer to exert the pressure or you may break the coverslip.

3.3.4 Mounting

Separate the coverslip from the slide using a tweezer, add a drop of mounting medium and put the coverslip back on the slide. Seal the coverslip to the slide with transparent nail polish.

3.4 Observation

Fluorescence was recorded after a 405 nm (DAPI), 488 nm (GFP and Alexa 488), 488–561 nm (propidium iodide), 561 nm (Alexa 555 and Pontamine Fast Scarlet 4B) and 633 nm (Alexa 647) excitation and a selective emission of 410–485 nm for DAPI, 495–533 nm for GFP and Alexa 488, 565–600 nm for Alexa 555 and Pontamine Fast Scarlet 4B, 565–720 nm for propidium iodide and 637–713 nm for Alexa 647. The acquisitions are made sequentially because of the overlap between DAPI and GFP/A488, between GFP/A488 and A555, and between Pontamine Fast Scarlet 4B and A647. To image GFP fluorescence combined with immunolocalization staining, start to image it before acquiring DAPI/A555/Pontamine Fast Scarlet 4B/A647 to avoid photobleaching of the signal. The acquisitions are made with an inverted Zeiss Observer Z1 spectral confocal laser microscope LSM 710 and with a 40× objective (506179, NA: 1.3, WD: 210 μm).

4 Notes

1. To prepare a 0.5 M EGTA (ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) stock solution, stir 3.8 g of EGTA into 15 mL of distilled water using a magnetic stirrer. To dissolve EGTA, add solid KOH to adjust the pH to 8.0, the EGTA will slowly go into solution as the pH nears 8.0. Add KOH slowly and check the pH regularly so that the pH does not go beyond 8.0. Dilute the solution to 20 mL with distilled water.
2. Weigh-in of PFA and all further steps of the PFA solution preparation (Paraformaldehyde, 158127, Sigma-Aldrich, St. Louis, USA) must be done in a chemical fume hood to avoid hazardous exposures to PFA. To prepare 200 mL of a 4 % PFA solution, weigh 8 g of PFA directly into a bottle and add 150 mL of MTSB1 ½. Adjust the pH to 9.0 adding 8 N NaOH then heat the solution to 80 °C to depolymerize the PFA. When the solution is translucent, cool it at room temperature and adjust the pH to 7.0 using sulfuric acid (H_2SO_4, 95 %). Dilute

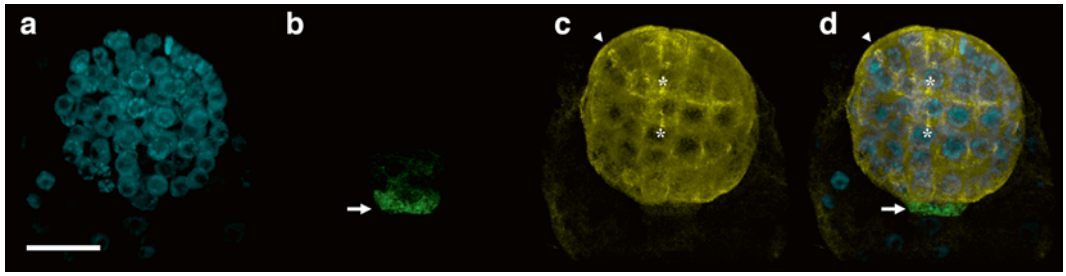

Fig. 4 Combination of immunolocalization and fluorescence imaging from a GFP reporter gene. Here is an example of whole-mount immunolocalization of tubulin in a globular embryo expressing the pWOX5:GFP reporter. (**a**) Chromatin staining with DAPI. (**b**) GFP fluorescence from the pWOX5:GFP reporter specifically expressed in the hypophysis cell (*arrow*). (**c**) Immunodetection of tubulin. *Asterisks* indicate the position of a preprophase band, and *arrowheads* point to two phragmoplasts in two adjacent cells. (**d**) Overlay. Scale bar = 20 μm

the PFA solution to 200 mL with MTSB1 ½. The PFA solution may be aliquoted and stored at −20 °C.

3. You may have to adjust the enzyme concentration depending on the enzyme lots since no enzyme-specific activity is indicated by suppliers. It is therefore recommended to adjust the amount of enzymes added during the digestion step when a newly prepared enzyme solution has been done.
4. DAPI (4′,6-diamidino-2-phenylindole) solution stock: Dissolve 10 mg of DAPI powder (Roche Diagnostics, Bâle, Switzerland) in 1 mL of distilled water and store this 10 mg/mL stock solution in aliquots at −20 °C. The DAPI working dilution is a 0.1 mg/mL solution. Take 10 μL of the DAPI stock solution and add 990 μL of distilled water. This DAPI working dilution can be stored at 4 °C up to 2 weeks.
5. The preparation of the fixation, the MPS, the staining, the clearing, and the mounting solutions must all be done in a chemical fume hood to avoid exposures to hazardous compounds.
6. Since PI stains most polysaccharides (such as starch, callose, cellulose, etc.), and to get specific cell wall signal, an amylase digestion is performed on tissues that are enriched in starch granules.
7. This protocol for immunolocalization preserves the fluorescence from GFP or any other fluorescent proteins. It is thus possible to combine the visualization of fluorescence from Arabidopsis marker lines expressing fluorescently tagged proteins with the antibody-based fluorescence after immunodetection (see for example Fig. 4).
8. Use plastic baskets to transfer samples from well to well during the fixation step. This prevents sample damage and loss during the procedure. To empty the baskets, turn it upside down on

the coverslip, and push carefully on the removable grid to let the samples fall on the glass surface.

9. Putting the coverslip on a microscopy slide at this stage enables to more easily manipulate and move the coverslip.
10. Using square 22 × 22 mm coverslips for this step ensures to keep all embryos gathered within a small surface area which saves time during the imaging step since the tiny and difficult to spot early embryos are not scattered on the entire slide surface. Furthermore, attaching embryos to coverslips (and not to slides) enables to have embryos at the closest distance from the objectives during the imaging steps and thus ensures to get an optimal image quality.
11. To spread the low gelling agarose solution on the embryos, take a new coverslip and use it like a paintbrush.
12. Both primary antibodies and both secondary antibodies are hybridized at the same time: Since the primary anti-tubulin and anti-KNOLLE antibodies were raised in different host species, the secondary species-specific antibodies cannot cross-react with each of the primary antibodies.
13. Both PI and Pontamine Fast Scarlet 4B are convenient to stain cell wall in fixed cells [9, 10, 14, 15], although PI is less specific (*see* **Note 4**). Note that MPS-PI staining is not compatible with DAPI staining, immunolocalization nor GFP detection whereas the Pontamine Fast Scarlet 4B staining is. Both staining provide confocal images of cell wall with high resolution and low background. Stacks of image are used to segment cell envelopes in 3D using appropriate software (http://fiji.sc/Morphological_Segmentation or http://imagejdocu.tudor.lu/doku.php?id=plugin:segmentation:morphological_segmentation:start) and to access to quantitative data (surface, volume, shape, etc.). See for example Fig. 3 and [10].
14. Cell wall and DAPI staining can be combined with immunolocalization and fluorescence imaging from fluorescently tagged proteins expressed in marker lines.

Acknowledgements

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Part V

Cytokinesis

Chapter 15

Characterization of Cytokinetic Mutants Using Small Fluorescent Probes

Andrei Smertenko, Panagiotis Moschou, Laining Zhang, Deirdre Fahy, and Peter Bozhkov

Abstract

Cytokinesis is a powerful paradigm for addressing fundamental questions of plant biology including molecular mechanisms of development, cell division, cell signaling, membrane trafficking, cell wall synthesis, and cytoskeletal dynamics. Genetics was instrumental in identification of proteins regulating cytokinesis. Characterization of mutant lines generated using forward or reverse genetics includes microscopic analysis for defects in cell division. Typically, failure of cytokinesis results in appearance of multinucleate cells, formation of cell wall stubs, and isotropic cell expansion in the root elongation zone. Small fluorescent probes served as a very effective tool for the detection of cytokinetic defects. Such probes stain living or formaldehyde-fixed specimens avoiding complex preparatory steps. Although resolution of the fluorescence probes is inferior to electron microscopy, the procedure is fast, easy, and does not require expensive materials or equipment. This chapter describes techniques for staining DNA with the probes DAPI and SYTO82, for staining membranes with FM4-64, and for staining cell wall with propidium iodide.

Key words Fluorescent probes, Cell plate, Cytokinesis, Mutants, DNA, Cell wall, Lipids

1 Introduction

Plant cells are surrounded by rigid oligosaccharide walls and, unlike their animal counterparts, are immobile. Usually, once generated plant cells occupy a given space in the plant body throughout its entire lifespan. Consequently, correct tissue patterning must be established simultaneously with the production of new cells through precise positioning of partitions (cell plates) between daughter cells. A plethora of functionally divergent proteins are required for efficient cell plate assembly [1]. Cell plates are constructed by a specialized structure called the phragmoplast. The morphology of the phragmoplast is maintained by microtubules [2]. Microtubules in addition serve as tracks for delivery of cell plate materials. Apart from microtubules, the phragmoplast con-

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sists of many organelles including endoplasmic reticulum, Golgi, mitochondria, membrane vesicles, and actin filaments [1]. Therefore, the phragmoplast appears to be a complex and self-sufficient molecular machine. One of the main functions of the phragmoplast is synthesis of the cell plate primer that is made of membrane materials, proteins, and oligosaccharides. The phragmoplast disassembles after primer synthesis is complete and the cell plate undergoes maturation [2]. During the maturation step, the composition of the plasmalemma and cell wall in the cell plate undergoes changes to become similar to that of the mother cell.

Considering the complexity of biochemical and vesicle trafficking pathways required for cell plate synthesis, cytokinesis may be considered a valuable model for understanding a wide range of processes in plants. Mutants with defects in cytokinesis and aberrant cell plate formation have been instrumental in advancing our understanding of plant biology, in particular molecular mechanisms of signaling [3–5], microtubule organization [6–10], membrane trafficking [11–14], cell wall synthesis [15], and orientation of cell division plane during tissue patterning [14, 16–18]. Despite significant progress in identification and functional characterization of proteins that are essential for cytokinesis, the molecular mechanisms of cytokinesis in plants remain poorly understood mainly owing to the complex nature of cytokinesis. Therefore, identification and characterization of new mutants with cytokinetic defects will continue to be an important tool for analysis of molecular mechanisms of plant cell division.

Genes that regulate cytokinesis also play an essential role in plant development and therefore corresponding mutants can exhibit phenotypes associated with developmental abnormalities and infertility [3, 5, 6, 9, 11–13]. The cytokinetic phenotype of the promising mutant lines identified in forward or reverse genetic screens has to be confirmed by microscopic analysis. Specific markers of cytokinetic defects are appearance of multinucleate cells and cell wall stubs. Electron microscopy provides comprehensive evidence for both features (Fig. 1) [10, 11, 15]; however, this procedure is time-consuming and relatively expensive. Small fluorescent probes provide a useful alternative for identification of specific defects in mutants suspected of cytokinetic failure. Preparation of the specimens is easy as some probes work in living cells and do not require specific pre-treatments, while others work with simple formaldehyde fixation. The staining is very quick and specimens can be imaged using compound fluorescence microscope. Typically dyes that label DNA, membranes, or cell walls are used for the analysis. In this chapter, we describe procedures for application of several commonly used probes such as DAPI, SYTO82, FM4-64, and propidium iodide (Fig. 2).

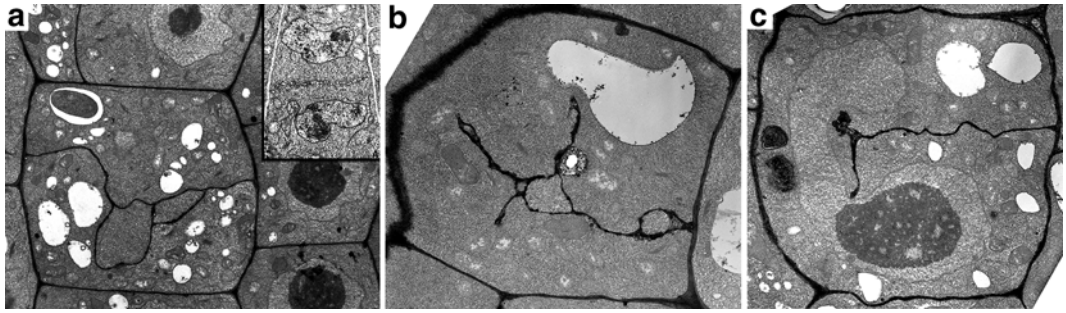

Fig. 1 Electron micrographs of cytokinetic defects in *mor1-1* allele [10]. Roots of *mor1-1* plants were incubated at non-permissive temperature before fixation and preparation of the samples. (**a**) The partition between daughter cells is wavy with several loops; magnification 2000. The *inset* shows cytokinesis in a control cell. (**b**) Branched cell plate that does not connect to the opposite side of the mother cell wall; magnification 3250. (**c**) An example of cell wall stub that does not reach the opposite side of mother cell, magnification 2300

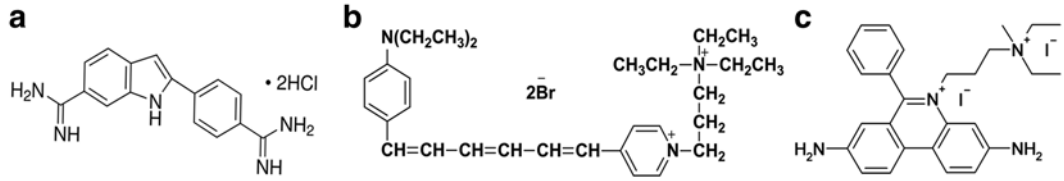

Fig. 2 Chemical structures of DAPI (**a**), FM4-64 (**b**), and Propidium iodide (**c**)

2 Materials

All solutions are prepared either in double-distilled water (ddH_2O) or analytical grade DMSO as detailed in Table 1. The stock solutions are stored at −20 °C or at −80 °C with the exception of propidium iodide which must be stored at +4 °C.

2.1 Plant Material

This protocol is designed to be compatible with wide range of plant material including roots, leaves, hand-cut or microtome-cut sections, and tissue-culture cells. A suitable material can be derived from plants grown in soil, hydroponically or in sterile tissue culture conditions. *Arabidopsis* plants used here were grown on solid 1/2MS medium, at 21 °C, 14 h/8 h day/night settings.

2.2 1/2MS Medium for Growing Arabidopsis Plants

0.22 % (w/v) Murashige and Skoog Basal Salt Mixture.

1 % (w/v) Sucrose.

pH 5.7.

Table 1
General information about the probes

Probe	MW	Solvent	Stock concentration (mM)	Final concentration	Fixation	Excitation laser line (nm)	Emission (nm)
DAPI	350.25	DMSO	1	10–100 nM	Aldehyde	405	420–600
SYTO 82	~350	DMSO	5	2 μM	Live cells	561	570–650
FM4-64	607.51	DMSO	5	2 μM	Live cells	488	500–650
Propidium iodide	668.39	ddH_2O	15	15 μM	Live cells	561	530–650

2.3 Fixative

For solid medium add 0.7 % (w/v) of plant agar.

4 % (w/v) Formaldehyde.

50 mM HEPES, pH 7.0.

0.4 % Triton X-100.

2.4 Phosphate Buffered Saline (PBS)

2 mM KH_2PO_4.

10 mM Na_2HPO_4.

150 mM NaCl.

pH 7.2.

2.5 Stock Solutions of Fluorochromes

Composition and concentration of stock solutions for all fluorochromes are listed in Table 1.

DAPI can be sourced from many chemical suppliers (e.g. # 14285, Cayman Chemical).

SYTO 82 is supplied by Life Technologies as 5 mM stock solution in DMSO.

FM4-64 (Invitrogen) can be sourced under the name NeurotransRed C_2 by Setareh Biotech.

Propidium (PI) iodide is supplied by Sigma (Sigma, #P4170).

Materials for Mounting Specimens

Standard microscope slides and coverslips (thickness No 1, 22 × 22 mm).

2.6 Special Equipment

Fluorescence or laser scanning confocal microscopes equipped with excitation filters and appropriate light sources for excitation at 405, 488 and 561 nm, and emission filters for blue, green and red part of the spectrum.

3 Methods

3.1 DAPI (4′,6-Diamidino-2-Phenylindole)

Overview. DAPI binds to the minor grove of DNA double-helix and therefore is a specific stain for DNA. DAPI is a very popular probe due to several advantages: (1) stable binding to DNA; (2) the fluorescence intensity increases upon binding, providing good signal to background ratio during imaging even if DAPI is not washed off extensively; (3) high stability; solutions containing bacteriostatic agents can be stored at +4 °C for many months. DAPI remains active in formaldehyde solutions. However, for most types of living plant cells, DAPI cannot penetrate the cell wall and membrane, and requires fixation prior to staining. In addition, DAPI emission spectrum spans the wavelengths from 390 to 600 nm and for this reason becomes unsuitable for simultaneous image acquisition with many commonly used probes. In such cases, the best results are obtained with sequential image acquisition mode using laser scanning confocal microscope.

3.1.1 Procedure

1. Fix roots, leaves, or any other appropriate material in Fixative solution for 30 min at room temperature.
2. Rinse in PBS once for 3 min, once for 5 min, and once for 10 min.
3. Incubate material in DAPI solution in PBS for 10 min (*see* **Note 1**).

Alternatively, DAPI can be added directly to the fixative solution. In this case the plant material is fixed for 20–30 min and then can be imaged after three washes of 3 min each in PBS. Use compound fluorescence or laser scanning confocal microscope equipped with the filters combination compatible with DAPI fluorescence characteristics as indicated in Table 1.

3.1.2 Outcome

DAPI will stain DNA in mitochondria, plastids, and in the nucleus. Nucleoli are not stained making nuclei appear as rings (Fig. 3a, b). Cytokinetic failure manifests by: (1) cells containing several nuclei (Fig. 3b); (2) irregular-shaped big cells with multiple nuclei (Fig. 3a). Some nuclei could have different shape and size and are illustrated for SYTO 82 below. The cell outlines are usually identified on the corresponding bright-field images collected on the same microscope as fluorescence images.

3.2 SYTO 82

Overview. SYTO 82 stains both DNA and RNA, and may produce a high background signal that in some cases compromises staining of chromosomes or nuclear DNA. SYTO 82 is a vital stain and does not require fixation. In samples fixed with formaldehyde

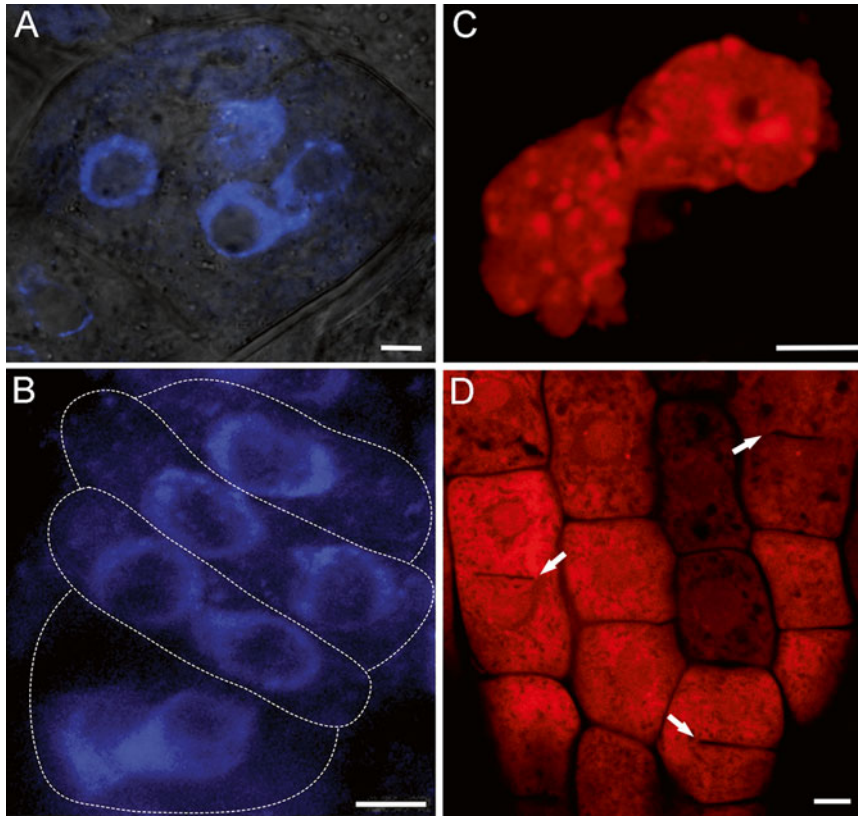

Fig. 3 Abnormalities of nuclei in root tip cells of *ple6* [7] detected using DAPI and SYTO 82. (**a**) Representative big irregular-shaped cell with multiple nuclei clustered in the cell center, DAPI staining. (**b**) Three bi-nucleate cells stained with DAPI. The cell borders are indicated with the *dashed white line*. (**c**) SYTO 82 stains irregularly shaped nuclei in a bi-nucleate cell. (**d**) SYTO 82 stains cytoplasm in formaldehyde-fixed cells shadowing the cell wall stubs (*arrows*). Scale bars 5 μm

SYTO 82 stains cytoplasm. SYTO 82 works well with root tips and tissue culture cells.

3.2.1 Procedure

1. Immerse material in the solution of SYTO 82 diluted in water or appropriate medium and incubate for 30 min at room temperature.
2. Wash samples in water or medium for 15 min. Image fluorescence signal of SYTO 82 within 2–3 h using combination of excitation and emission characteristics specified in Table 1.

3.2.2 Outcome

Similarly with DAPI, SYTO 82 stains multiple irregularly shaped nuclei in cells with cytokinetic failures (Fig. 3c). In fixed cells, SYTO 82 stains cytoplasm creating a negative staining effect of cell wall stubs (Fig. 3d).

3.3 FM4-64 or NeurotransRed C_2 (N-(3-Triethylammonium-Propyl)-4-(6-(4-(Diethyl-Amino) Phenyl)Hexatrienyl) Pyridinium Dibromide)

Overview. FM4-64 is a lipophilic probe with low fluorescence intensity in water. However, upon binding to membranes, the intensity of fluorescence increases. FM4-64 first binds to the plasma membrane, then is internalized and stains endosomes and the cell plate. This makes it a useful stain for live cell imaging of dividing cells and for visualization of cells in which cytokinesis has failed. FM4-64 works only with living cells; however, versions that can be fixed after incorporation into the membrane are now commercially available (FM4-64Fx, Invitrogen). FM4-64 (Invitrogen) is sold under the name NeurotransRed C_2 by Setareh Biotech.

3.3.1 Procedure

1. Incubate seedlings or any part of the plant (Note D) with FM4-64 diluted in water or liquid tissue culture medium (1/2MS in case of *Arabidopsis*, medial for individual plant systems can vary) for 5–30 min (*see* **Note 2**).
2. Transfer seedlings or other material to water or medium without FM4-64 for 5 min and image fluorescence signal within 3 h (*see* **Note 3**) using combination of excitation and emission characteristics as specified in Table 1.

3.3.2 Outcome

The homogeneous labeling along the plasma membrane (*see* **Note 4**) outlines the cells (Fig. 4). Cytokinetic defects appear as cell-wall "stubs," incomplete cell plates that attach only to one side of the mother cell wall. The stubs can be very short (Fig. 4b) or very long with quite elaborate shape (Fig. 4a).

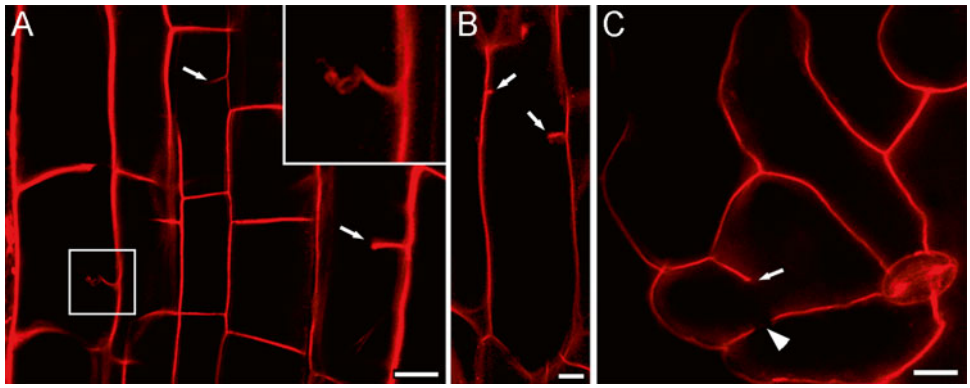

Fig. 4 Staining of cell wall stubs in *ple6* with FM4-64. (**a**) Staining of cell wall stubs (indicated by *arrows*) in root tip cells. The *inset* shows higher magnification of a cell wall stub with a twisted end. (**b**) Enlarged root epidermis cell with several wall stubs (*arrows*). The lack of clearly defined apical and basal cell walls suggests multiple cytokinetic failures. (**c**) Cell wall stub (*arrow*) and a gap in the cell wall (*arrowhead*) in the leaf epidermis. Scale bars, 10 μm

3.4 Propidium Iodide (3,8-Diamino-5-{3-[Diethyl(Methyl) Ammonio]Propyl}-6-Phenyl Phenanthridinium Diiodide)

Overview. Propidium iodide is a water-soluble probe that binds to nucleic acids by intercalating between the bases, with little or no sequence preference, and to plant cell walls. Upon binding to the nucleic acids fluorescence excitation maximum is 535 nm and the emission maximum is 617 nm. PI shows an enhancement of 20- to 30-fold when bound to DNA, while SYTO dyes show a 500-fold increase. PI is unable to permeate cell wall and membrane of living cells, and therefore requires formaldehyde fixation. PI can also be used to stain nuclei in unfixed dead cells with compromised plasma membrane integrity.

3.4.1 Procedure

1. Incubate seedlings or root cuttings (*see* **Note 5**) with fresh PI working solution in water or culture medium for approximately 1 min. Incubation time can vary between 30 s and 3 h. However, long incubation times lead to strong background signal.
2. Wash samples three times 2 min each in dH_2O or culture medium to minimize toxic effect of PI (*see* **Note 6**).
3. Transfer seedlings or root cuttings under a coverslip, either in staining solution or water (PI is incompatible with glycerol-based mounting medium). Image specimens immediately (*see* **Note 7**).

3.4.2 Outcome

The homogeneous labeling along the cell walls outlines the cells (Fig. 5). Cytokinetic defects appear as cell-wall "stubs," incomplete cell plates that attach only to one side of the mother cell wall (Fig. 5b–d).

4 Notes

1. DAPI penetrates slowly through tissue layers. Stain samples overnight at +4 °C if short incubation does not give the desired results.
2. Leaves for this assay work as well as roots; however, cytokinetic defects are commonly identified in root tips.
3. FM4-64 staining is reversible and if samples are left in a wash solution for too long, the signal gradually decreases.
4. If too many cytoplasmic vesicles are stained, incubate the specimens on ice.
5. PI can penetrate through the membrane punctures of dead cells and stain nuclei. For this reason, this procedure works better with fresh specimens.
6. The effect of PI on human body is not known, but it is considered as a potential hazard. Handle in fume cupboard.

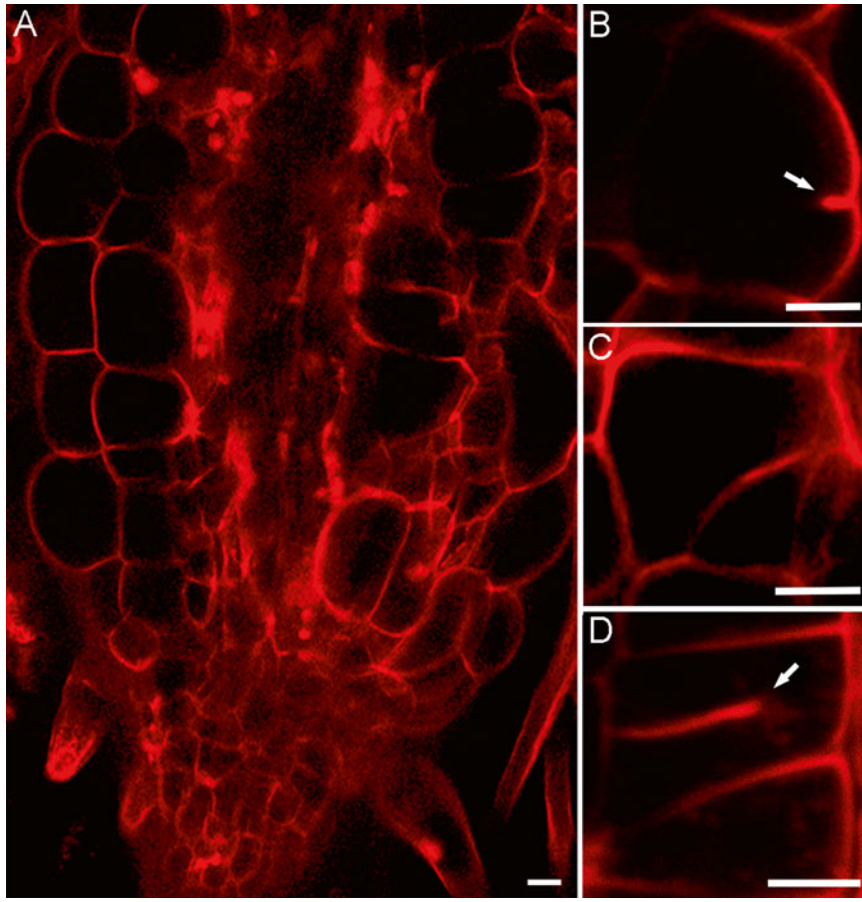

Fig. 5 Visualization of cytokinetic defects in *rsw4* with propidium iodide. The seedlings were incubated at the restrictive temperature. (**a**) Overall root-tip morphology. (**b**) A short cell-wall stub. (**c**) A cell with multiple cell plates. (**d**) Cell wall stub. Arrows indicate the cell wall stubs. Scale bars, 10 μm

7. Ensure careful handling of samples throughout the imaging procedure, since rough handling, such as applying excessive pressure on the coverslips increases number of dead cells and gives higher background signal.

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Chapter 16

Using Live-Cell Markers in Maize to Analyze Cell Division Orientation and Timing

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Abstract

Recently developed live-cell markers provide an opportunity to explore the dynamics and localization of proteins in maize, an important crop and model for monocot development. A step-by-step method is outlined for observing and analyzing the process of division in maize cells. The steps include plant growth conditions, sample preparation, time-lapse setup, and calculation of division rates.

Key words Maize, Mitosis, Plant, Live-cell imaging, Microtubules, Cell division, Fluorescent proteins, Confocal, Microscopy

1 Introduction

Live-cell imaging in plant cells is used to study many dynamic processes including meristem growth [1–3], development [4], root hair growth [5], organelle movement [6], and microtubule and actin dynamics [7–11]. Live-cell imaging has also provided insight into the mechanisms of cell division in plants [12–16]. The recent development of stably transformed maize lines expressing fluorescently tagged proteins makes it possible to answer questions regarding the dynamics of cell division and other processes [17, 18]. Maize is an ideal model system for this type of study because its relatively large cells divide within the framework of other cells while growing in a precisely defined developmental gradient.

Live plant cell imaging performed to understand the process of division has primarily used *Arabidopsis thaliana*, *Tradescantia virginiana*, and tobacco-cultured cells. Reliable, stable transformation of *A. thaliana* [19] has resulted in the fusion of many proteins of interest to fluorescent proteins with subsequent examination of protein dynamics. A more challenging technique that has also yielded dynamic information is microinjection of fluorescently labeled proteins and subsequent live-cell imaging in the spiderwort

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DOI 10.1007/978-1-4939-3142-2_16,

T. virginiana [20–22]. Another powerful model used to answer questions about cell division is the cultured tobacco cell line bright yellow 2 (BY-2). BY-2 cells are easy to transform [23], synchronize [24], and observe in vitro on the microscope [25–29]. *Physcomitrella patens* recently emerged as a model for live cell imaging [8, 30, 31] which will likely soon be expanded to explore the dynamics of division.

Maize has lagged as a model system for live plant cell imaging because there were very few available maize lines expressing proteins fused to fluorescent proteins. A notable exception to the lack of live cell imaging is the analysis of chromosome movement during meiosis, which does not require fluorescent proteins [32]. Although bombardment of maize leaf cells has been used to transiently express fluorescent proteins, bombardment has the disadvantage of transforming a small number of cells. Moreover, it is a damaging process that requires lengthy in vitro culture of isolated tissues, which may alter protein dynamics or localization [33]. Stable transgenic lines expressing a variety of proteins fused to fluorescent proteins circumvent some of the problems with transient maize transformation, allowing live-cell imaging of dynamic processes.

Maize is an excellent model system for development and cell biology in monocots because its leaves develop progressively and reproducibly. This reproducible developmental gradient in the leaves is referred to as the "base to tip gradient" [34, 35]. Near the base of the leaf, cells divide symmetrically. Further from the base, some cells divide asymmetrically to differentiate and to establish specialized cell types [36]. Finally, towards the tip of the leaf, cells expand rapidly. This reproducibility allows direct comparisons to be made regarding distinct developmental stages, including a recent explosion in large-scale "-omics" analysis [37–43]. The recent influx of genomics resources together with the developmental gradient and live-cell imaging tools will synergize to improve our understanding of monocot biology, potentially impacting next-generation crop production.

To explore the subcellular localization and dynamics of many processes, including cell division, stable transformed maize lines expressing live-cell markers have been created [17, 18]. Like many other land plant cells, maize cells form typical dividing structures in symmetric and asymmetric divisions [44–47]. A preprophase band is formed before mitosis and is thought to predict the future site of the new cell wall [48]. The preprophase band is a ring of microtubules, microfilaments and a large number of other proteins that usually forms at the cell cortex around the nucleus [12]. The preprophase band disassembles during metaphase while the spindle forms [49]. The spindle is an organized antiparallel array of microtubules that moves chromosomes during anaphase. During telophase, the plant-specific structure called the phragmoplast

forms. The phragmoplast is composed of microfilaments and an antiparallel array of microtubules that serve as tracks for the transport of cell-wall components to the newly forming cell plate. The cell plate is the nascent cell wall, and eventually it expands outwards to the cortex to complete cytokinesis [50, 51].

The method described here provides a protocol for live-cell imaging of actively dividing maize cells using YFP-TUBULIN as an example. In it, plant growth conditions are outlined, as well as sample preparation for microscopy, setting up the time lapse, and finally analyzing the results. Recognition of common problems, such as sample damage and movement, is discussed and solutions presented.

2 Materials

2.1 Greenhouse Materials

1. Maize seeds that produce plants expressing one or more live-cell markers such as YFP-TUBULIN [18]. Full list available at (http://maize.jcvi.org/cellgenomics/index.php).
2. Soil.
3. Slow release fertilizer.
4. Calcium-magnesium supplementary fertilizer (e.g., Peters Excel Cal-Mag Special 15-5-15 water-soluble fertilizer).
5. 10 cm square pots in 15 pot flats.
6. 4 g/L glufosinate-ammonium in 0.1 % Tween 20.
7. Large 7.5 L ("2 gallon") pots.
8. Cotton applicators.
9. LED lights or other supplemental lighting. Light intensity should be ~230 $\mu E/m^2/s$ at a height of 1 m. A detailed protocol for greenhouse growing conditions can be found at agron-www.agron.iastate.edu/ptf/protocol/Greenhouse%20Protocol.pdf.

2.2 Microscopy Materials

1. Confocal microscope system. Either a spinning disk or a point scanning confocal system can be used together with an inverted microscope, an EM-CCD camera, and appropriate lasers and filters for imaging various fluorescently labeled proteins. Various microscopes have benefits and drawbacks (*see* **Note 1**).
2. Airstream incubator or thermostat.
3. Mid-range infrared thermometer.
4. Glass cover slips.
5. Rose chamber for holding the sample still during long time lapse. Rose chambers or alternatives can be used to stabilize the sample within the field of view (*see* **Note 2**).

6. Vacuum grease loaded into a 10 cm^3 needle-less syringe.
7. Water.
8. 200 μL pipette and tips.
9. Digital calipers.
10. Straight scalpel blade.
11. Forceps.
12. Software for running the microscope (e.g., Micromanager 1.4 https://www.micro-manager.org/).

2.3 Data Analysis Materials

1. ImageJ or FIJI (a regularly updated and modified version of ImageJ available at http://fiji.sc/Fiji).
2. Statistical package to import and analyze data.

3 Methods

3.1 Plant Growth

Temperature and lighting conditions will affect the growth rate and cell division rate of maize. Consistent growth conditions, consistent plant age, and tissue type are required for any comparative quantitative analyses.

The following steps list a reproducible method of growing plants.

1. Plant seeds ~2 cm deep in pre-wetted, pre-fertilized soil in 10 cm square pots loaded into a 15-pot flat for easy transport.
2. Germinate seedlings in standard long-day greenhouse conditions. The conditions are 16 h light at 25 °C, 8 h dark at 21 °C with supplemental lighting provided by LED or high-pressure sodium and metal halide lamps.
3. Water plants when the soil is dry, likely three times a week.
4. Fertilize with the Cal-Mag fertilizer once a week.
5. After 1 week of growth, use a permanent marker to mark the second or third leaf, and then apply 4 g/L glufosinate-ammonium in 0.1 % Tween to the marked location using a cotton-tipped applicator.
6. Two to three days after herbicide application, score resistant and sensitive plants (*see* **Note 3**).
7. Transplant resistant plants into 2-gallon pots.
8. Grow for a total of 3–5 weeks after planting. Plants should have at least seven visible leaves at this stage of growth. Do not use diseased or poorly growing plants. Figure 1a shows acceptable growth for a 4-week-old plant and describes the parts of the plants discussed in this paper (*see* **Note 4**).

9. Figure 1b indicates the part of the plant dissected to observe many dividing cells (described in more detail below in sample preparation for microscopy and *see* **Note 4**).

3.2 Sample Preparation for Microscopy

1. Set the thermostat. Alternatively, turn on the airstream incubator and point it onto the stage for at least 10 min prior to placing the sample (Fig. 2a). Determine what setting will provide the correct temperature before use. I used 21 °C (*see* **Note 5**).
2. Use a mid-range-infrared thermometer to measure the temperature using the laser to guide the thermometer to stage right next to the sample (Fig. 2b).
3. Place a clean glass cover slip on the lower half of the Rose chamber (Fig. 3a).
4. Prepare a glass cover slip for the sample by applying a thin film of vacuum grease in a circle with ~1 cm diameter (*see* **Note 6**).
5. Add a ~100 mL drop of water inside the vacuum grease circle with a pipette (*see* **Note 7**).

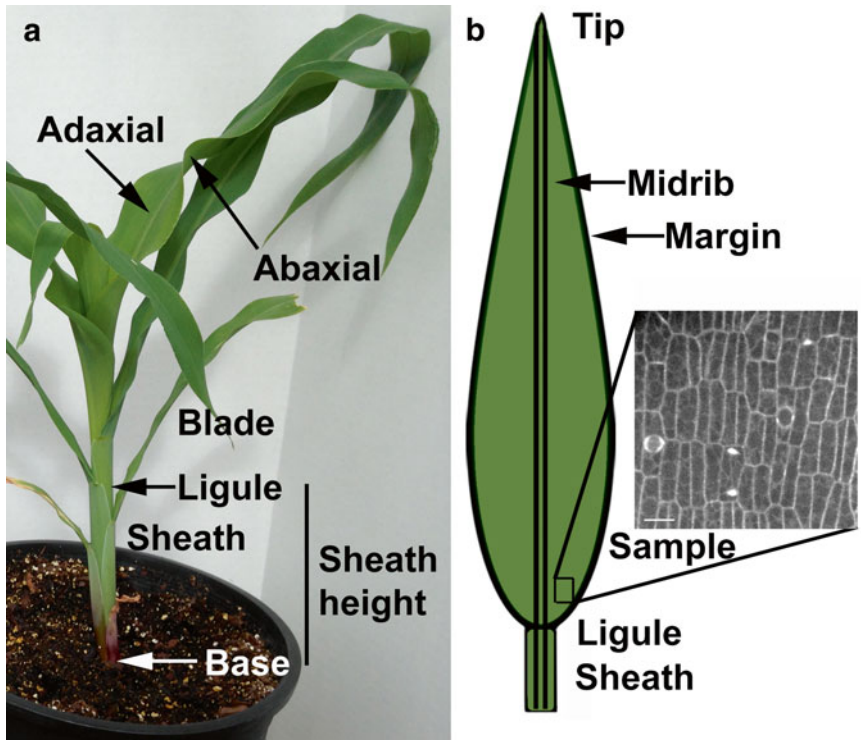

Fig. 1 Representative example of a maize plant and descriptions of the parts of the leaf used in this method. (**a**) A 28-day-old plant grown in standard greenhouse conditions with relevant parts and descriptors of the plant indicated. (**b**) Schematic of a plant leaf, with more descriptors, as well as a micrograph of a young maize leaf expressing YFP-TUBULIN. Bar is 50 μm

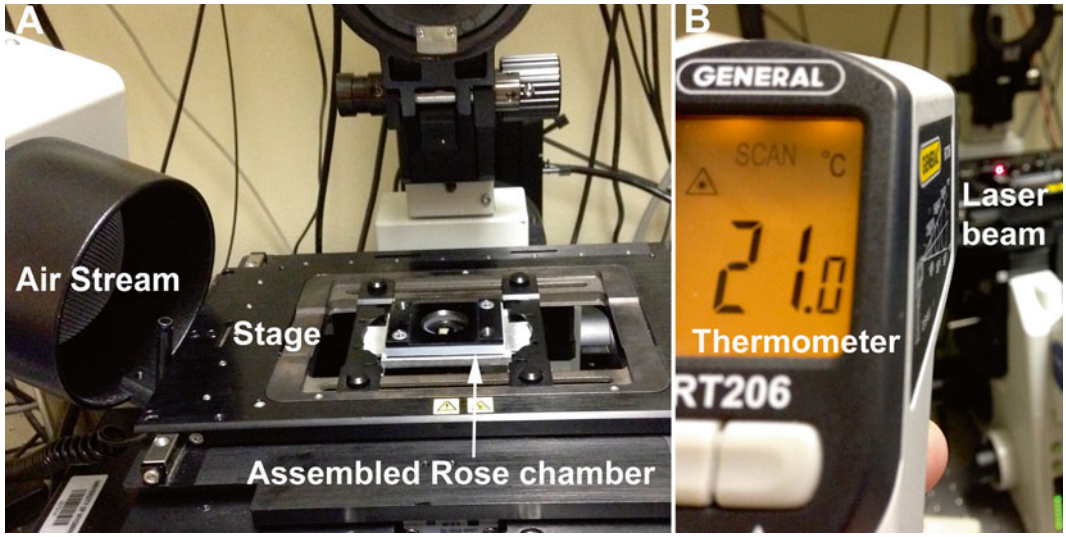

Fig. 2 Temperature control. (**a**) Photograph of the inverted stage with the airstream pointing toward the sample. The Rose chamber is assembled on the microscope. (**b**) The infrared thermometer is shown with the laser readout pointed toward the sample on the microscope

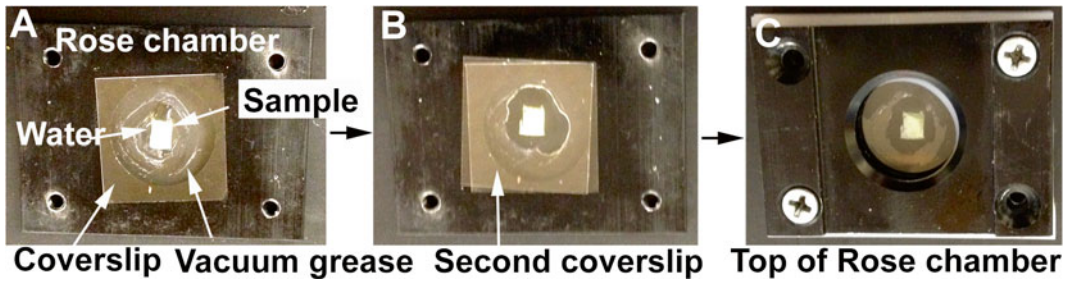

Fig. 3 The Rose chamber holds the sample steady during time-lapse imaging. This figure shows the steps needed to assemble it. (**a**) The bottom part of the rose chamber is shown with the cover slip, vacuum grease, water and sample mounted. (**b**) The second cover slip is carefully mounted on top of the sample, spreading the water evenly. (**c**) The top of the Rose chamber is screwed into place, and the sample is ready to be loaded onto the holder and the stage

6. After 3–5 weeks of growth, harvest plants for microscopy. Refer to Subheading 3.1 for growth conditions and Subheading 3.1, **step 8** for acceptable plants to harvest.
7. Cut off the above-ground portion of the maize plant, at the base of the sheath, leaving behind the roots (Fig. 1b).
8. Sequentially peel away the outer leaves until the sheath height is less than 3 mm. Sheath height can be measured with digital calipers or a ruler.
9. Excise a ~0.2–1.0 cm^2 leaf piece just above the ligule and between the midrib and the margin (Fig. 1b) using a straight scalpel blade.

10. Carefully peel the excised portion from the rest of the leaf, holding the sample by the edge with forceps. Avoid touching the tissue directly.
11. Mount the adaxial side down in the water droplet towards the objective when the rose chamber is fully assembled (*see* Figs. 1a and 4 and **Note 4**).
12. If there are air bubbles (*see* Fig. 5d), carefully remove your sample from the water and set it down again (*see* **Note 8**).
13. Gently place another cover slip on top of the sample and bottom cover slip (Fig. 3b).
14. Adjust the sample so that it is in the middle of the Rose chamber (*see* **Note 9**).
15. Place the top half of the Rose chamber, including the silicone sandwich, onto the top cover slip, and carefully screw it down (Fig. 3c).
16. Load the rose chamber into the holder and mount it on the microscope stage after adding the correct immersion liquid for the objective (*see* **Notes 10** and **11**).
17. Figure 2a shows a Rose chamber loaded onto the stage and Fig. 4 shows the schematics of the Rose chamber and its final orientation relative to the objective.

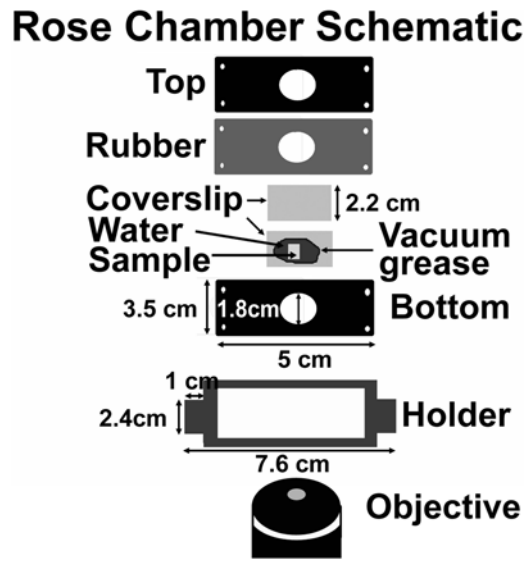

Fig. 4 Schematic of the Rose chamber with parts and measurements labeled. All pieces but the metal holder will eventually be screwed together to gently sandwich the sample and stabilize it during time-lapse imaging. The cover slip at the bottom of the Rose chamber will come into contact with the immersion liquid and the objective

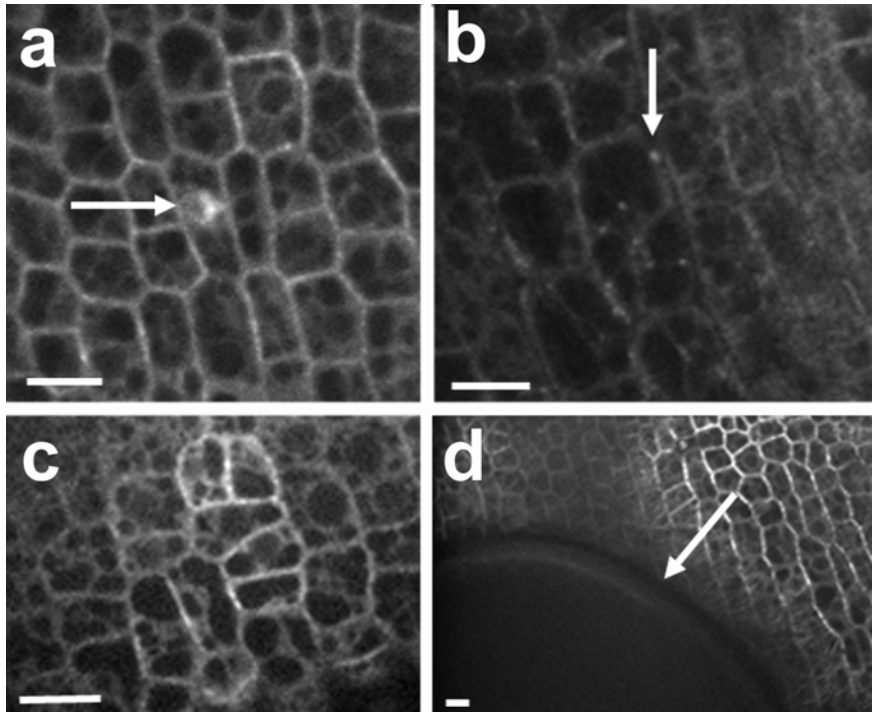

Fig. 5 Examples of cells expressing YFP-TUBULIN that are damaged and an air bubble. (**a**) Aberrant division structure indicated by an *arrow*. (**b**) Abnormal microtubule structure that looks like a dot indicated by an *arrow*. (**c**) Example of cells with a lot of small vacuoles. (**d**) Air bubble indicated by an *arrow*. Bar is 20 μm

3.3 Microscopy

1. Start the computer, microscope, camera, focus and filter controller, laser launch, etc. Consult a microscope manual or knowledgeable person for details on its operation.
2. Find the epidermal cell layer using bright field. Avoid taking images near the cut sites.
3. Switch to the camera view to start observing the fluorescent proteins in the cells. If cells are damaged or there is an air bubble, prepare a new sample. Examples of damaged cells are shown in Fig. 5 (*see* **Note 8**).
4. Adjust the exposure time, EM gain, and laser attenuation (if necessary) so that the illuminated sample has high dynamic range (*see* **Note 12**).
5. If the goal of the time lapse is to capture the entire process of division, prioritize capturing cells in late prophase at the start of the time lapse. Figure 6, time 0, shows an example of a cell in late prophase with a disassembling preprophase band. More examples of cells in late prophase are shown in Fig. 7 (*see* **Note 13**).
6. Use the multi-acquisition tool to set up your time lapse. Specify Z stack positions, exposure time, EM gain, and amount of

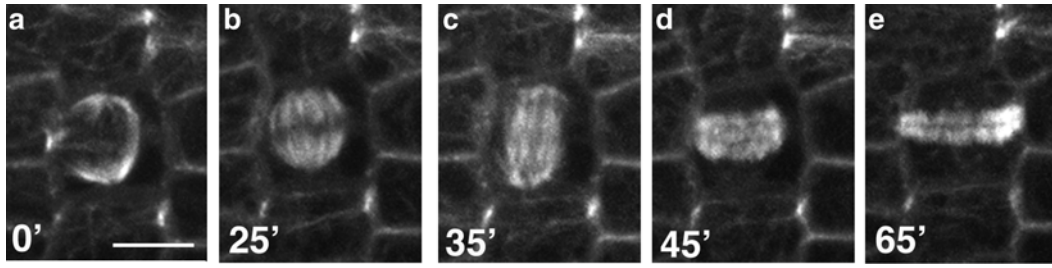

Fig. 6 Time lapse of a cell progressing from late prophase to the end of telophase and examples of cells in late prophase. Microtubules are labeled with YFP-TUBULIN. Time is indicated in the *bottom left-hand side* in minutes. (**a**) Late prophase. Note that the preprophase band has almost fully disassembled. (**b**) The bipolar spindle is formed in metaphase. (**c**) The anaphase spindle elongates to separate the chromosomes. This is the shortest stage in the cell cycle and generally takes 10–15 min. (**d**) A phragmoplast, an antiparallel set of microtubule arrays, forms in telophase. The cell wall materials are transported along the microtubule tracks towards the phragmoplast midline. (**e**) The phragmoplast expands outwards towards the cortex to complete cytokinesis. Bar is 10 μm

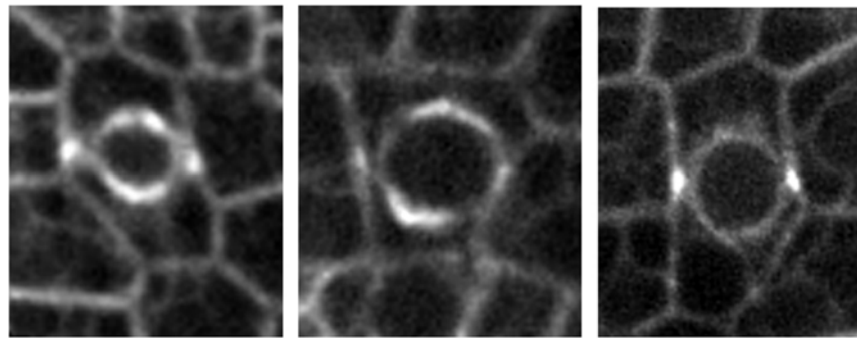

Fig. 7 Three examples of cells transitioning between prophase and metaphase. Note the prominent microtubule accumulations around the nuclear envelope

time between image collection. For observing mitosis in maize, 5-min time lapse is a good compromise between photobleaching and loss of temporal resolution (*see* **Note 14**).

7. Choose the "save as function" to save time-lapse images as they are produced into a single folder so that images are not lost in case of power outages (*see* **Note 15**).
8. Start the time lapse.
9. Adjust for sample movement, especially in the first 20 min. The most frequent trouble is slow drift in one direction. If there is a lot of drift, the sample is incorrectly positioned on the cover slip and a new sample should be loaded.
10. Check the time lapse for sample movement every hour. Compare the start of the time lapse with the new positions, and readjust your sample if the *x*, *y*, or *z* planes shift during the time-lapse.

11. Stop the time lapse after the cells are finished dividing or 5 h, whichever occurs first. Empirically, 5 h was the maximum amount of time that division progressed with this sample type at this temperature.

3.4 Data Analysis

1. Open stacks in the image analysis program Fiji or ImageJ.
2. Make maximum projections of the Z stacks: Process: Batch:Macro. Then specify input (the folder with the Z stacks) and output files (a new folder to put the max projections) with output format [Tiff]. Use this macro: run ("Z Project…", "start = 1 stop = 10 projection = [Max intensity]") (*see* **Note 16**).
3. Open the maximum projections as an image sequence (File:Import:Image sequence) and scroll through the file.
4. If the sample drifts a lot during the time-lapse, perform a correction by loading Plugin:Registration:StackReg or Plugin:Registration:Register Virtual Stack Slices and choose "Translation" (*see* **Note 17**).
5. Use the timestamps on original Z stacks to calculate the timing of division stages. Figure 5 illustrates the microtubule structures to look for at various stages of the cell cycle, and illustrates one potential way of indicating time.
6. Measure the amount of time needed to progress from one stage to another. These transitions are very clear when time-lapse images are viewed as a movie. The time can be analyzed in by calculating (the time between frames) × (number of frames cells are in a particular stage) = total time cells are in that state. An alternative method, if the stacks are not generated in 5-min intervals, is to compare the time-stamps on the images as they were originally saved.
7. Scroll through the image sequence focused on one mitotic structure. The spindle microtubules will start accumulating before the preprophase band disassembles. Preprophase band disassembly is observed as a loss of fluorescence at the cortex.
8. Next, the spindle, a football-shaped structure, coalesces perpendicular to the final division site and then expands during anaphase.
9. A sharp transition occurs from the orientation of the spindle to the orientation of the phragmoplast. The spindle is generally perpendicular to the final division site, while the phragmoplast is generally aligned with the final division site. This visually striking morphological change is usually obvious from one frame to the next.
10. The phragmoplast expands towards the cortex, sometimes contacting one side before the other [52]. Viewed from the side, it looks like two microtubule-containing disks with the

midline containing very few microtubules. As the phragmoplast expands, the interior microtubules may disassemble. Viewed from the top the phragmoplast looks like an expanding torus.

11. Finally, the phragmoplast reaches the cortex and starts to disassemble. Once it is completely disassembled, stop the time lapse and measure the complete time of all the steps.
12. Import the time values into Microsoft Excel or another spreadsheet program. Usually, the long and unpredictable amount of time that cells spend in preprophase/prophase means that gathering this type of data is more difficult. If cells do not progress, they may be damaged (Fig. 5 and *see* **Note 8**).
13. Other measurements can also be made such as the rate of phragmoplast expansion or the rotation and movement of the spindle. These are not discussed further in this method.

4 Notes

1. *Microscope selection*

   A confocal microscope must be used to avoid out of plane fluorescence with intact plant tissues. Either a laser scanning microscope or a spinning disk microscope can be used. The benefits of using a spinning disk microscope are speed and reduced photo-damage to the specimen. For fluorescent proteins that photo-bleach rapidly, a spinning disk microscope with a sensitive camera is essential. If the fluorescently tagged protein is abundant and does not photo-bleach easily, such as YFP-TUBULIN, it is possible to use a point-scanning microscope for time-lapse imaging. If necessary, the images can also be binned to reduce both photo-damage and the time required to gather each individual image.

2. *The Rose chamber*

   The Rose chamber as it is used here prevents movement of the sample by applying enough pressure to flatten a curved sample, but not too much to damage the cells. There is no commercially available option for purchase of a Rose chamber, but it is straightforward for a machine shop to make one. It consists of a metal holder, two pieces of metal and one piece of silicone or rubber with a width of 5 cm × 3.5 cm with a hole cut in the middle of both 1.8 cm diameter, four aligned screw holes, illustrated with dimensions shown in Fig. 4. An inexpensive alternative to the Rose Chamber is a glass slide sealed with VALAP (This is a 1:1:1 mixture by weight of paraffin, lanolin and Vaseline, doi:10.1101/pdb.rec12380*Cold Spring Harb Protoc 2010.*). VALAP, a solid at room temperature, is

gently heated on a hot plate to liquefy. VALAP provides a strong and biologically inert attachment between the slide and the cover slip. Do not use Vaseline or vacuum grease unless the sample is thin because the cover slip may slip during imaging.

3. *PCR to distinguish segregating transgenes*

   When multiple transgenes are segregating, it is helpful to confirm the genotype using a PCR based method, particularly when the plants express fluorescent proteins only in dividing tissue. A general method for maize DNA extraction and PCR can be found at http://rasmussenlab.weebly.com/protocols.html.

4. *Age and type ofmaizematerial suitable for imaging cell division by time lapse.*

   Overall, it is important to observe similar samples from one experiment to the next so that the data can be combined in the quantitative analyses. This is also why temperature control (*see* **Note 5**) and growth conditions are vital to the success of the experiment. Several parts of the plant are actively dividing including the base of the leaf, the meristem, the young sheath material and the root tips. Maize primary roots are thick, and cutting them appropriately for stable time-lapse imaging can be difficult. Young blade or sheath tissue is preferred because it is flat, has mostly undeveloped chloroplasts (and therefore little to no autofluorescence), and has a high proportion of dividing cells. If asymmetric divisions are preferred, older leaf tissue should be used. Either the adaxial or abaxial side of the leaf can be used. If juvenile leaves are preferred, either because the fluorescent protein is better expressed or the developmental stage is more ideal for the experiment, plants ~2 weeks old can be harvested.

5. *Temperature control*

   The temperature must be the same between experiments if comparisons are going to be made between samples. The main reason to keep the temperature then same is that microtubule dynamics (and other protein dynamics) are different at different temperatures [53]. Other methods that can be used to control temperature are a thermostat-regulated room or a heated chamber for microscopy.

6. *Vacuum grease*

   Vacuum grease is recommended to form the small well between the two cover slips because it has the correct viscosity and is chemically inert. It is important that a thin, consistent layer is applied for optimal time-lapse microscopy. If the layer is too thin or if it is spread unevenly, the sample will slip during time lapse (*see* **Note 8** on sample slipping). Other materials

that can be used are Vaseline and VALAP. Vaseline will work in a pinch, but it may not adequately protect the sample during mounting because it is much softer than vacuum grease. VALAP is tricky to apply in a thin layer because it solidifies very quickly.

7. *Water*

   Use water to mount maize samples. Other materials, such as mineral oil or phosphate buffered saline, will damage the cells and prevent timely progression of mitosis. Other materials, such as perfluorocarbons [54], may be better for imaging, but I have not had an opportunity to use them.

8. *Avoid sample damage and air bubbles*

   Damage and air bubbles both cause significant trouble for time-lapse imaging. Avoid smashing, squashing, or otherwise disturbing the sample. If the sample is damaged, take a new slice from the plant, which should be wrapped in moist paper towels to preserve it for a few hours if necessary. The most obvious signs that cells are damaged are a lack of organized cortical interphase microtubule array in nondividing cells, cells with many small vacuoles, cells that fail to progress in the cell cycle or cells with abnormal mitotic arrays (*see* Fig. 5). Air bubbles will cause your sample to slip out of focus and may alter the rate of division. If the sample has an air bubble, carefully take the sample out, and set up a fresh cover slip, vacuum grease, and water to place the sample.

9. *Sample placement*

   Placing the sample in the middle of the Rose chamber is very important because if it is not correctly centered, the objective may run into the side of the chamber. This has the potential to scratch the objective, disturb correct Z stack imaging, and make the sample slip.

10. *Objective selection*

    For semi-high-throughput imaging, the lowest objective that still allows unambiguous identification of mitotic structures should be used. I used a 20× objective for this purpose. For producing a time-lapse figure, a higher objective (such as 40 or 60×) should be used to more clearly illustrate the mitotic figures. An alternative, should it be feasible with your microscope setup, is to use *x*,*y* motorization to move between multiple different samples during time-lapse.

11. *Immersion liquid*

    The correct immersion liquid must be used on your objective, but what type of objective is best for time-lapse imaging? Some objectives do not require any immersion liquid but use air instead. Unfortunately, the refractive index of air is very different than plant cells. The benefit of using an oil objective is

that the immersion oil will not evaporate during imaging. However, it reduces the quality of imaging because the refractive index of immersion oil is not very similar to plant cells. Water has a closer refractive index to your sample, and will produce a more beautiful micrograph. However, water objectives are not ideal for long time lapse because the water can evaporate within 1 h of time-lapse imaging. Glycerin or silicone oil may provide the best compromise between a good refractive index and slower evaporation but I do not have experience with either of these materials. Perfluorocarbon immersion liquids can also be used.

12. *Laser power*

    Keep laser power low to minimize photo-damage to the cells. Instead of increasing laser power, consider increasing EM gain or exposure times. Empirical testing may be done to confirm that the settings do not cause photo-damage before long time lapses are performed. Use a sample to test for photo-damage by collecting the total number of images the time lapse will acquire during one session. This can be done in micromanager using the "burst" function. After imaging, measure fluorescence loss and observe whether damage occurs.

13. *Cells in lateprophase*

    Since the amount of time a cell spends in preprophase can be quite long (>3 h), it is best to focus the sample such that a cell in late prophase is in the middle of the field of view. These cells are characterized by an obvious accumulation of microtubules at both poles of the imminent spindle, but still have a preprophase band (Fig. 7).

14. *Selecting appropriate image collection intervals*

    One goal of time-lapse imaging is good temporal resolution but another more important goal is prevention of photo-damage. Short time intervals (e.g., 2 min) can increase the risk of photo-damage to the cells, which impedes division. Long time intervals (e.g., 15 min) can result in data loss. For example, anaphase, the shortest stage of the cell cycle (~10 min) will not be observed within 15-min intervals. A decent time compromise is 5-min intervals for dividing cells, but this will need to be optimized depending on the type of sample and temperature (*see* **Note 5**). An alternative way to minimize photo-damage is to capture a single image or a short Z stack, such as 4–1 μm intervals at each time point instead of a full Z stack (covering the top through the bottom of the cells usually at 1 or 1.5 μm stacks for 10–20 separate Z stacks).

15. *Automatic saves*

    The "save as" function saves images as they are produced, showing an accurate timestamp on each Z stack. The save as

function also protects against loss of already acquired images during power failures or other malfunctions that can occur during time-lapse imaging.

16. *Set the Z stack so that the maximum projection will provide a clear image*

    Maximum projection, a post-acquisition method of visualizing the Z stacks compressed into one image, can be used to more clearly distinguish structure types and changes in mitotic structures. Sometimes the maximum projection to produce the clearest image will not be the entire Z stack, particularly if the sample is thick.

17. *Automatic corrections inImageJor FIJI*

    Two plug-in programs in ImageJ/FIJI can be used to adjust for sample movement during imaging: Stack Reg and Registration:Register Virtual Stack Slices. Both can automatically correct the slow drift caused by plant growth or movement in one direction. However, if large manual adjustments are performed during time-lapse imaging, these programs may not be able to correct the image. Manually correct large shifts in ImageJ, and then run StackReg or Register Virtual Stack Slices.

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Chapter 17

Ratiometric Fluorescence Live Imaging Analysis of Membrane Lipid Order in Arabidopsis Mitotic Cells Using a Lipid Order-Sensitive Probe

Patricia Gerbeau-Pissot, Christophe Der, Markus Grebe, and Thomas Stanislas

Abstract

Eukaryotic cells contain membranes exhibiting different levels of lipid order mostly related to their relative amount of sterol-rich domains, thought to mediate temporal and spatial organization of cellular processes. We previously provided evidence in *Arabidopsis thaliana* that sterols are crucial for execution of cytokinesis, the last stage of cell division. Recently, we used di-4-ANEPPDHQ, a fluorescent probe sensitive to order of lipid phases, to quantify the level of membrane order of the cell plate, the membrane structure separating daughter cells during somatic cytokinesis of higher plant cells. By employing quantitative, ratiometric fluorescence microscopy for mapping localized lipid order levels, we revealed that the Arabidopsis cell plate represents a high-lipid-order domain of the plasma membrane. Here, we describe step-by-step protocols and troubleshooting for ratiometric live imaging procedures employing the di-4-ANEPPDHQ fluorescent probe for quantification of membrane lipid order during plant cell division in suspension cell cultures and roots of *Arabidopsis thaliana*.

Key words Di-4-ANEPPDHQ, Membrane order, Cell plate, Mitosis protocols, Arabidopsis roots, Arabidopsis suspension cells

1 Introduction

Eukaryotic membrane is formed of various lipids predominantly belonging to the three main classes of sterols, sphingolipids, and phospholipids [1]. For several decades, membrane researches were dominated by the idea that proteins were the key factors with regulatory functions in membranes and that lipids rather served structural roles, such that membrane lipids were regarded as a solvent in which membrane proteins would freely diffuse [2]. However, a more recent concept of "membrane rafts" proposed that the lipid bilayer is not a sole homogeneous solvent, based on the observation of a preferential association of sphingolipids, sterols, and some proteins, conferring potential lateral segregation of the membrane

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into microdomains [3]. Indeed, such sterol-rich domains have been defined at microscale by observing accumulation of fluorescent marker recognizing sterol-enriched domains, such as filipin, e.g., at the tip of either the growing pollen tube [4] or the elongation tube of filamentous fungus [5], and in Arabidopsis root [6]. With the establishment of super-resolution imaging methods, the observation of lateral membrane organization and its lipid dependence could be further refined to nanoscale [7, 8]. Biophysical studies have accordingly shown partitioning of domains by identifying delimited areas in connection with their specific components and the interactions between them. Upon mixing certain types of lipids, particularly cholesterol and phospholipids, distinct phases can be observed to coexist within the membrane [9], an ordered- (*lo* phase) with a high conformational order in the lipid acyl chain due to strong interactions between lipid molecules, a disordered- (*ld* phase), and a crystal- (*lc* phase) liquid phases [10]. Förster resonance energy transfer (FRET) experiments conducted on artificial membranes with two matched fluorescent lipid donors having different affinities for *lo* versus *ld* phases, revealed an inhomogeneous mixing of lipids into regions of the bilayer and especially the formation of *lo*-like domains with a diameter of 10–40 nm at the physiological temperature of 37 °C [11]. These experiments provided an improvement of the "membrane raft" theory postulating the existence of small (20–200 nm) sterol- and sphingolipid-enriched liquid-ordered assemblies within the membrane [12].

Subsequently, experimental approaches suitable for in situ characterization of such heterogeneity in live cells were developed. Initial approaches focused on the characterization of spatial protein distribution and studies on proteins attached to the plasma membrane (PM) by a glycophosphatidylinositol (GPI) anchor suggested that these can occur in clusters with a diameter of less than 70 nm [13]. FRET experiments combined with theoretical modeling confirmed that GPI-anchored proteins are present at the cell surface within nanoscale (<5 nm) cholesterol-sensitive cluster [14] and immuno-electron microscopy experiments showed a lateral segregation of proteins into islands, at the same scale [15]. These data established that the intrinsic heterogeneity of the PM can be observed at the nanometer scale and, thus, is unfortunately below the limit of observation by optical microscopy imposed by the diffraction of light [12]. Such obstacles can be circumvented by using "single-particle tracking" (SPT), which tracks the trajectory of individual proteins in microscopy with antibodies coupled to a gold particle or predominantly fluorescent latex beads. This approach was used to reveal the existence of a dynamic association into specialized domains and based on this it was proposed that "membrane rafts" may be enriched by several specific proteins [16]. Another technique using total internal reflection fluorescence (TIRF) to monitor single quantum dots was also used to

analyze the cholesterol dependent diffusion of GPI-anchored proteins. This revealed that these proteins dynamically distributed both outside and within nanodomains containing the ganglioside GM1, which is a typical lipid enriched in "membrane rafts" [17]. All these investigations on the spatial organization and dynamics of membrane proteins and lipids provided a better definition on the membrane property, defining as "Membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes." [18].

However, technical limitations to observe microdomains in vivo, essentially due to the limitation of available tools, induced doubts about the biological existence of these specialized areas [19]. New technologies were thus needed for imaging ordered domains at nanoscale. Development of super-resolution imaging methods such as stimulated emission depletion (STED) microscopy provided a tool for observing lateral membrane organization and for addressing its lipid dependence at nanoscale [7, 8]. In addition, the characterization of the spatial organization of ordered- and disordered- domains has been addressed at the surface of living cells by using various microscopy techniques including two-photon microscopy [20], imaging of partitioning of fluorescent lipids and proteins [21] or environmentally sensitive probes [22, 23]. A potentially powerful strategy is the use of lipid order-sensitive probes coupled with fluorescence spectroscopy. This approach takes advantage of the fact that the degree of lipid packing alters the physical properties of the environment around the dye inserted into the bilayer and subsequently modifies the spectral properties of the fluorophore [24]. A palate of fluorophores for the quantitative imaging of lipid order in model membranes [23, 25] and in cell membranes [26, 27] are now available. The mainly used lipid order-sensitive probes are Laurdan (6-lauryl-2-dimethylamino)-naphthalene, [28] and the related probe C-Laurdan, which shows higher solubility in aqueous media than Laurdan and is more photostable. Both versions require an UV or multiphoton excitation [29] and display a 50 nm blue-shift in their emission maxima when inserted into ordered phases compared to disordered phases of the membrane [30]. A new series of fluorescent probes for imaging membrane order was recently characterized in artificial bilayers as well as in intact live zebrafish embryos, the fluorescent dyes PY3304, PY3174, and PY3184 [27], but they are not yet marketable.

Similarly, 1-[2-hydroxy-3-(*N*,*N*-dimethyl-*N*-hydroxyethyl) ammoniopropyl]-4-[β-[2-(di-*n*-butylamino)-6-napthyl]vinyl] pyridinium dibromide (di-4-ANEPPDHQ) is a commercially available dye previously employed as a probe for membrane order in artificial membranes as well as in cells [23, 31]. As for Laurdan, lipid order affects the fluorescence emission spectrum of di-4-ANEPPDHQ,

where the spectral region between 500 and 580 nm reflects the ordered phase and between 620 and 750 nm the disordered phase of the membrane [26]. The degree of membrane lipid order can be quantified by ratiometric imaging and subsequent calculation of the generalized polarization value (the GP value, 26). Di-4-ANEPPDHQ can be excited and detected with most conventional confocal laser scanning microscopes, as it can be excited at a wavelength of 488 nm. The probe has been successfully used in live animal cells, such as at the immunological synapse periphery in T cells [20] and in HeLa cells [26]. Di-4-ANEPPDHQ has been applied for the detection of membrane order in cells or organs of living plants. This includes studies on membrane order during pollen tube tip growth in *Picea meyeri* [4], the imaging of PM domains at micrometer scale in *Nicotiana tabacum* during plant defense signaling [32], lipid-order imaging during cytokinetic cell plate formation [33] as well as PM-order imaging in cells of the root meristem of *Arabidopsis thaliana* [34].

During cytokinesis of somatic cells in higher plants the cytoplasm of a cell is divided to form two daughter cells by the inside-out formation of a transitory membrane compartment, the cell plate [35–37]. The cell plate grows centrifugally by localized deposition of membrane and cell wall material in the plane of cell division [35, 38]. Using ratiometric live imaging and di-4-ANEPPDHQ (Fig. 1), we demonstrated that the cell plate represents a dynamic, high-lipid-order membrane domain in *Arabidopsis thaliana* cells from root [33] and suspension cultures (Fig. 2). By continuous recruitment of vesicles, the cell plate expands and fuses with the lateral PM to form two new individual PMs and to complete cell division [35, 38]. Such membrane traffic seems thus to preferentially implicate proteins and/or lipids enriched in ordered domains, which is in agreement with the recent measurement of the high lipid order level of endosome [34].

Taken together, di-4-ANEPPDHQ is a lipid order-sensitive probe that can be easily used to quantify the degree of membrane packing and to establish the spatial and dynamic distribution of ordered domains in vivo. Here, we provide a detailed protocol for membrane order imaging employing di-4-ANEPPDHQ that requires standard CLSM equipment, is easy to perform especially when using the image processing macro provided by Owen and colleagues [26], and generates reproducible results for roots and cell suspension cultures of *Arabidopsis thaliana*.

2 Materials

2.1 Plant Materials

The ecotype of *Arabidopsis thaliana* employed in this study was Columbia (*Col-0*).

1. Seedlings were grown vertically for 5 days on Murashige and Skoog (MS)-agar plates prepared as square Petri dishes

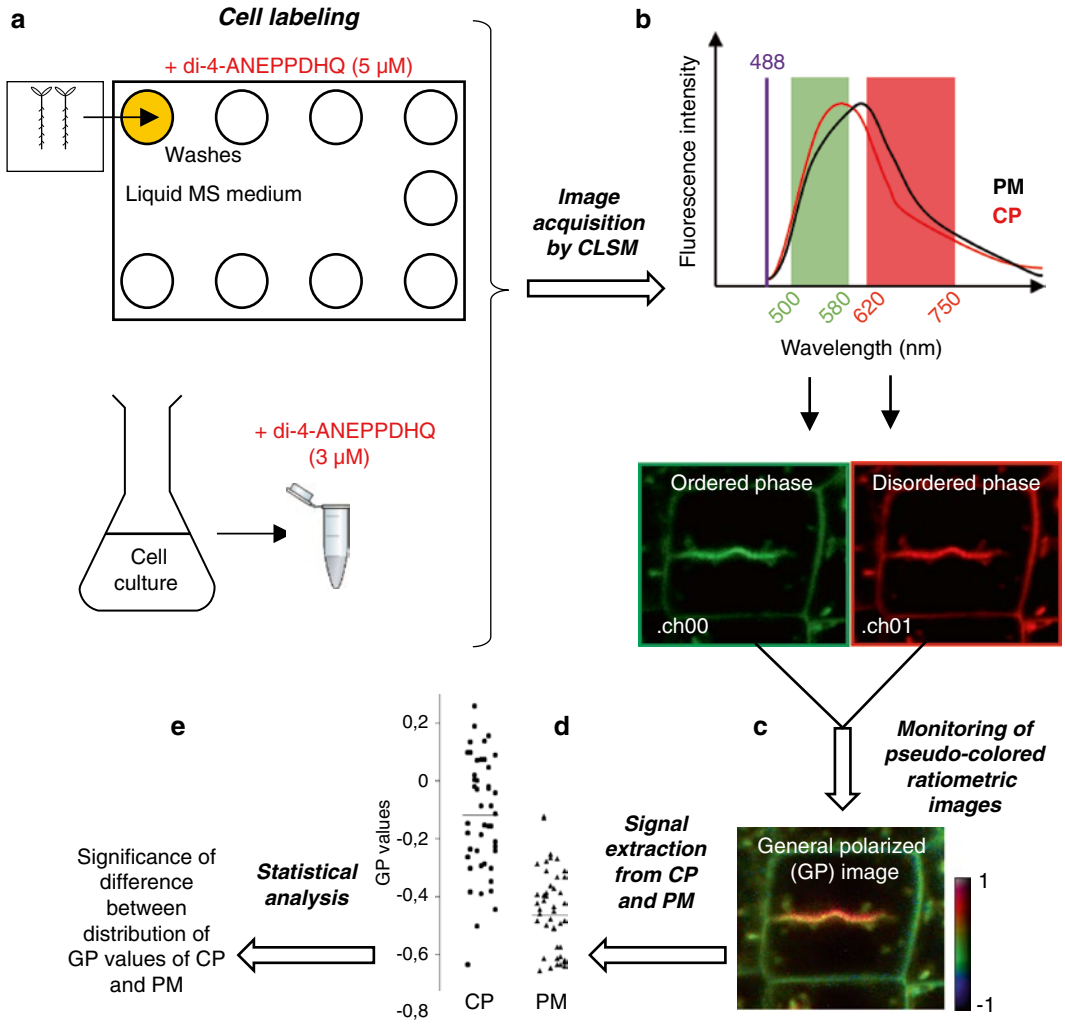

Fig. 1 Work flow from di-4-ANEPPDHQ labeling of live Arabidopsis roots or cell suspension cultures, fluorescence image acquisition, ratiometric processing, GP image generation, GP value extraction graphic display, and statistical analysis. (**a**) Five-day-old seedlings or 3-day-old suspension cells were submerged in liquid MS medium containing di-4-ANEPPDHQ. (**b**) After mounting, dividing cells were imaged upon excitation of di-4-ANEPPDHQ at 488 nm and fluorescence emission was acquired in two spectral regions corresponding to the ordered- (*green*) and disordered- (*red*) phases, indicated by *green* and red *boxes*. (**c**) The pixel intensity value data from these images was used to generate a ratiometric General polarized (GP) image as described in the text. (**d**) Extracted mean GP values from CP and PM were determined and the results from a larger number of cells plotted in a graph and (**e**) statistical analysis was used to determine as to whether the two distributions were statistically different or not

(12 cm × 12 cm). The photoperiod was set up to 16 h light/8 h dark at 22 °C.

2. Suspension cells were grown in the following supplemented MS medium at pH 5.6, containing MS salt supplemented with 0.01 mg/l kinetin, 0.5 mg/l 1-Naphthaleneacetic acid and

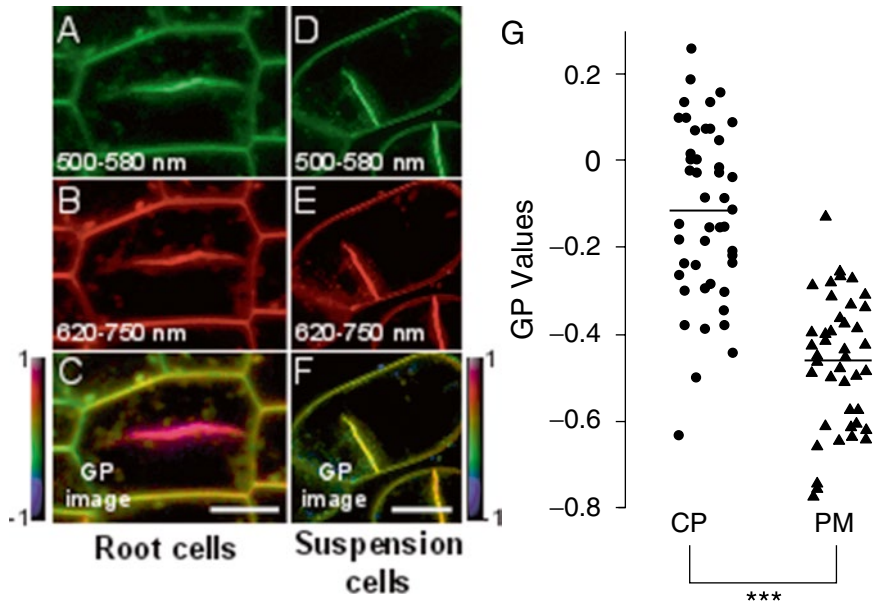

Fig. 2 di-4-ANEPPDHQ fluorescence labeling of (**a**–**c**) dividing root cells and (**d**–**f**) dividing suspension cells recorded between (**a**, **d**) 500–580 nm, representing high lipid order and (**b**, **e**) 620–750 nm, representing low lipid order levels. (**c**, **f**) Ratiometric pseudo-color-coded GP images obtained after processing images recorded at 500–580 and 620–750 nm. According to the accompanying color scales, *red* corresponds to highest lipid order and *black* to lowest lipid order membrane regions. Scale bars represent 5 μm. (**g**) Quantitative analysis of mean GP value distributions from CPs and PMs of 47 cells. The horizontal lines indicate the means of the non-normal distributions. The *P* value obtained using the non-parametric, two-tailed Mann–Whitney test indicates that the difference between the distributions is very highly significant (***$P = 3.2 \times 10^{-12}$)

30 g/l sucrose. For subculturing of cell suspensions, 20 ml of a 2-week-old Arabidopsis cell suspension culture were transferred into new 250 ml flask containing 100 ml of fresh supplemented MS medium. Cells were maintained under continuous light conditions (200 μE/m²/s) on a rotary shaker (140 rpm).

2.2 Buffers and Solutions

1. *Murashige and Skoog* (*MS*)*-agar plates*: 1× MS, 1 % sucrose, 0.8 % plant agar, and 2.5 mM MES, buffered to pH 5.8 with KOH.
2. *Liquid MS medium*: MS-agar-plate medium as above but without plant agar and buffered to pH 7 with KOH.
3. Di-4-ANEPPDHQ (1 mg, Molecular Probes) was dissolved in 300 μl or 1 ml of DMSO to a stock solution of 5 mM or 1.5 mM for labeling of Arabidopsis roots or suspension cells, respectively. Add the DMSO directly to the glass vial and ensure that the probe is dissolved completely. Dispend aliquots of 10 μl of stock solution in 0.1 ml reaction tubes and stored them sealed in an airtight, lightproof vial at room temperature (21 °C) for up to 6 months.

2.3 Materials

1. 12-Well multiwell plates.
2. Slides 76 × 26 mm.
3. Cover slip 24 × 24 mm.
4. Cover slip 24 × 50 mm.
5. Cover slip 18 × 18 mm.
6. Double-sided tape.
7. Forceps.
8. A rotary shaker (140 rpm).
9. Any confocal laser scanning microscope (CLSM) setup with a 488 nm excitation laser line and the possibility for two-channel detection at 500–580 and 620–750 nm.

   (a) In this study, we used a Zeiss LSM 780 Axio Observer Z1 inverted microscope for observation of Arabidopsis seedling roots and

   (b) A Leica TCS SP2 upright microscope for Arabidopsis cell suspension observation.
10. Objectives used:

    (a) A C-Apochromat 40/1.2 DIC water-corrected objective (M27, Zeiss).

    (b) An HC Plan Apochromat 63/1.40 oil immersion objective (Leica).
11. ImageJ (http://rsbweb.nih.gov/ij/) with di-4-ANEPPDHQ analysis macro installed [26].

3 Methods

3.1 Di-4-ANEPPDHQ Labeling of Arabidopsis Roots and Analysis of Cytokinetic Cells

1. Place 2 ml of liquid MS medium into a 12-well plate (Fig. 1a).
2. Add 2 μl of di-4-ANEPPDHQ stock solution to obtain a working concentration of 5 μM. Ensure that the medium is well cooled prior to transfer of seedling.
3. Submerge 12 five-day-old seedlings in liquid MS medium containing di-4-ANEPPDHQ. Seedlings should be completely submerged in MS liquid medium. If not, pipet the liquid medium on top of the seedlings.
4. Incubate for at least 30 min at room temperature, or longer if visualization of additional endomembrane compartments is desired (*see* **Note 1**).
5. Wash the seedlings three times in MS medium without di-4-ANEPPDHQ. To this end, replace the di-4-ANEPPDHQ solution with three changes of liquid MS medium in 90-s intervals, pipetting the MS medium up and down on the seedlings and let it incubate for 90 s each time.

6. Mount the seedlings in liquid MS medium between a 24×50 mm cover slip and a 24×24 mm cover slip "sandwich" separated by double-sided tape placed at the edges of the 24×50 mm cover slip ([39], *see* **Note 2**). Approximately ten roots can be placed in the middle of this arrangement.
7. Proceed with image acquisition by CLSM immediately after mounting (*see* **Note 3**).

3.2 Di-4-ANEPPDHQ Labeling of Arabidopsis Suspension Cells

Prior to observation cells were maintained under agitation with a rotary shaker (140 rpm).

1. Place 500 µl of cell culture in a 2 ml reaction vial.
2. Add 1 µl from the 1.5 mM di-4-ANEPPDHQ stock solution to obtain a final concentration of 3 µM.
3. Gently shake for a few seconds.
4. Mount approximately 15 µl between a microscope slide and a 18×18 mm cover slip when viewing with an upright microscope. Approximately 3–5 suspension cells can be visualized in the observation field when employing the 63× objective.
5. Proceed with image acquisition by CLSM immediately after mounting.
6. The time between mounting and observation must not exceed 15 min to minimize cell stress.

3.3 Image Acquisition at CLSM

1. Activate the 488 nm laser line set a low laser power (*see* **Note 4**).
2. Set up the two spectral acquisition windows for di-4-ANEPPDHQ fluorescence at 500–580 nm and at 620–750 nm (Fig. 1b). Set the pinhole size to 1 Airy, the scan speed to 400 Hz, and the line average to 4.
3. Turn on the transmitted light detector and select continuous acquisition mode focusing on a dividing cell (*see* **Note 5**). Cell plates (CP) are easily identified, since they display slightly higher di-4-ANEPPDHQ fluorescence signal compared to the signal observed at the PM.
4. In order to obtain an equivalent signal in both acquisition channels, select the Q-Lut mode and modify laser power and/or PMT settings to obtain a signal in both channels that does not display pixel saturation (*see* **Note 6**).
5. Save the setup (*see* **Note 7**).
6. Acquire images and save as 8-bit grayscale .tiff format files named "XX".ch00 for the green channel (ordered phase, Fig. 2a, d) and "XX".ch01 for the red channel (disordered phase, Fig. 2b, e) (*see* **Note 8**).

3.4 Calibration Factor

To compensate for differences in the efficiency of collection in the two channels, correct GP values using the G factor. In order to obtain the G factor, the same microscope set-up employed for imaging root samples is used to image the fluorescence of a drop of undiluted di-4-ANEPPDHQ stock solution.

1. Place 2 μl of di-4-ANEPPDHQ stock solution on an empty slide on the microscope stage.
2. Load the same set-up used during image acquisition from roots.
3. Image at three different laser power settings, 0.3 %, 0.5 %, and 1 % for the Zeiss LSM780, and 10 %, 20 %, and 30 % for the Leica SP2 setup, respectively.
4. Extract the mean pixel intensities for the channels .ch00 (ordered) and .ch01 (disordered) in ImageJ.
5. Calculate the corresponding GP_{mes} values according to the equation

$$G = \frac{GP_{ref} + GP_{ref}GP_{mes} - GP_{mes} - 1}{GP_{mes} + GP_{ref}GP_{mes} - GP_{ref} - 1}$$

GP_{ref} is a reference value for di-4-ANEPPDHQ in DMSO, here fixed at −0.85 [23]. Define GP_{mes} by extracting the mean pixel intensities of the channels .ch00 (ordered) and .ch01 (disordered) in ImageJ and by using the equation

$$GP_{mes} = \frac{I_{500-580} - I_{620-750}}{I_{500-580} + I_{620-750}}$$

3.5 Image Analysis

Calculate the GP values and generate pseudo-colored ratiometric images in ImageJ according to the equation

$$GP = \frac{I_{500-580} - GI_{620-750}}{I_{500-580} + GI_{620-750}}$$

To this end, employ the custom-written macro for ImageJ available in (26, Fig. 1c)

1. Download the macro provided in [26].
2. Open ImageJ and run the macro (Plugins, Macros, run …). A dialogue box will open.
3. Select the folder in which you store the .ch00 (order) and .ch01 (disorder) images.
4. Set the threshold value for the analysis to “15,” set the color scale for the output GP images to “grays,” and select no immunofluorescence mask. Set the factor according to the value defined in Subheading 3.4 and select “Yes” to accept to use G factor for GP image calculation. Press “OK” and a dialogue box will open.

5. Select a representative image from the list, keep the order channel in the brightness folder and the 16_colors.lut and click "OK."
6. On the new open box, press "auto" to define the value of the minimum and maximum colors and press "OK." A new folder is generated in the folder containing the raw data.

3.6 Data Analysis

1. Open a representative GP image in ImageJ (Fig. 2c, f).
2. Select an ROI at the cell plate employing the polygons tool and extract the mean intensity value. Do the same for the closest plasma membrane. Copy and paste these two values (comprise between 0 and 255) in a table file.
3. Normalize extracted values by using the formula

$$\mathrm{GP} = \frac{\text{extracted values}}{127.5} - 1 \ \left(\text{Value comprise between } -1 \text{ and } 1\right).$$

4. Plot the final values in a graph displaying the GP values at the *y*-axis and displaying PM or CP at the *X*-axis (Figs. 1d and 2g, *see* **Note 9**).
5. Use a nonparametric test such as two-tailed Mann–Whitney U rank-order-sum test (http://elegans.som.vcu.edu/~leon/stats/utest.html) and/or Kolmogorov-Smirnoff test (http://www.physics.csbsju.edu/stats/KS-test.html) to test for significance of difference between distribution of GP values from the CP and from the PM (*see* **Note 10**).

4 Notes

1. The PM and the CP will be labeled by di-4-ANEPPDHQ in 30 min which will equilibrate between these membranes. However, increasing incubation times will allow for the labeling of additional endomembranes.
2. Two thin strips of double-sided tape (1×30 mm) are placed longitudinally at the edges of the 24×50 mm cover slip. Seedling roots are then aligned longitudinally in between the strips of tape in a drop of MS liquid medium and a 24×24 mm cover slip is placed top of the medium and the double-sided tape. Cotyledons are left outside of the cover slip in a small drop of medium, so that it is possible to feed the seedlings with liquid MS medium during the experiment and to avoid drying of the seedlings.
3. After mounting, the seedlings can be observed for 40 min or longer but small drops of MS medium may need to be resupplied to the cotyledons. To this end, the seedlings should

be visually inspected during image acquisition and medium resupplied if necessary. At the same time, start a new incubation round to optimally use the time spent at the CLSM.

4. Di-4-ANEPPDHQ displays high fluorescence intensity therefore on our Zeiss LSM780 a 488 nm laser intensity of 0.5–1 % was sufficient while laser power at the Leica TCS SP2 needed to be adjusted to 20 %.
5. When imaging Arabidopsis roots, focus on the area close to the root tips, where cell actively divide. For suspension cells, it is more difficult to identify cell plates. Dividing cells may be located anywhere within the cell population on the slide which needs to be scanned through.
6. Any saturated pixels will result in a GP calculation giving erroneous results. Therefore, it is essential to ensure for each image that the signal does not reach saturation in any pixel and signal intensities at the cell plate may vary from one cell to another.
7. In order to compare your different GP images, it is critical that you keep the same setup during the whole acquisition process and on comparing for example wild type and mutants or chemically treated or non-treated cells.
8. Images acquired with a Leica SP2 CLSM software will automatically have the file labeling .ch00, .ch01. Zeiss CLSM software does not have this specific nomenclature and adjustments will need to be made by either employing a macro or by manual modification of the image file names.
9. In Fig. 2, we monitored two regions of the emission spectrum of di-4-ANEPPDHQ, reflecting the ordered- (Fig. 2a, d) and disordered- phases (Fig. 2b, e), using two different type of Arabidopsis cells. Generating ratiometric pseudo-colored GP images (Fig. 2c, f), we observed that the cell plates are represented by colors corresponding to higher GP values when compared to plasma membrane colors, suggesting a higher lipid order level at the CP. This was reproducible irrespective of whether Arabidopsis seedling tissues or whether suspension cells were examined. Quantitative analysis of 47 individual cells of an Arabidopsis suspension culture confirmed that GP values extracted from cell plates consistently displayed a higher GP value than the GP values extracted from plasma membrane (Fig. 2g).
10. Since distributions of the measured populations of cells did not follow a normal distribution, a nonparametric test should be employed. The two populations need to comprise a same number of values when employing the Mann–Whitney test but may vary when employing the Kolmogorov-Smirnoff test.

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Index

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