Unravelling developmental dynamics: transient intervention and live imaging in plants

G. Venugopala Reddy, Sean P. Gordon‡ and Elliot M. Meyerowitz‡*

Abstract | Plant development is dynamic in nature. This is exemplified in developmental patterning, in which roots and shoots rapidly elongate while simultaneously giving rise to precisely positioned new organs over a time course of minutes to hours. In this Review, we emphasize the insights gained from simultaneous use of live imaging and transient perturbation technologies to capture the dynamic properties of plant processes.

Shoot apical meristem
A collection of undifferentiated cells that are located at the growing tip of a plant shoot.

Pattern formation in plants involves the generation of specific cell types in a correct positional context[1]. Understanding the mechanisms that underlie pattern formation can be addressed by the integration of dynamic imaging and transient intervention methods. During development, cells must constantly readjust their genetic programme over time and space to acquire distinct identities. The correct sequence of cell-identity transitions requires real-time observation. However, real-time observation of developmental processes may not always reveal causal mechanisms; this depends on the ability to perturb the system, and on the ability to follow the events in real time, at single-cell resolution. In addition, developing tissues are shaped and maintained by processes that control a combination of cell behaviours such as cell-division rates and orientation, cell-expansion rates, cell migration, and cell death[2]. However, cell behaviour is tightly coupled, both spatially and temporally, to cell-identity transitions. The real-time observation of transient perturbations is well-suited for investigating the genetic programmes that bridge cell behaviour and cell-fate specification. Finally, cell behaviour is influenced by structural components, such as the actin cytoskeleton, microtubule (MT) dynamics and cell-wall formation[3]. Real-time observation of transient genetic perturbations is needed to address how each cellular component affects cell behaviour.

Traditional genetic epistasis experiments have provided a basic sketch of the genetic framework that governs patterning events, and, with the recent developments in genomics and proteomics technologies, we have begun to elucidate the molecular networks that regulate these events. However, determining the spatial and temporal dynamics of molecular and cellular networks is crucial to understanding developmental patterning. Integration of real-time imaging with transient perturbation methods promises to bridge the gap between molecular networks, cell mechanics and cell behaviour.

Here, we discuss the live imaging and transient perturbation technologies and tools that are being used to study plant physiology and developmental events such as stem-cell maintenance and patterning in growing shoot and root systems. We also highlight recent studies in non-plant systems that are related to the development of new live-imaging tools, which could be applied to studying the dynamic properties of plant processes.

Temporal and spatial challenges in plants

The primary goal of live imaging is to define a least invasive method that can achieve the required sensitivity to gain adequate spatial and temporal resolution of biological events. Although the importance of live imaging for studying plant development was realized as early as the mid-twentieth century (when Ernest Ball and colleagues developed microscopic methods to record events during the development of the shoot apical meristem (SAM)), these methods lacked the required spatio-temporal resolution[4]. Several subsequent developments in light microscopy and fluorescence labelling technologies have improved the sensitivity and, therefore, the resolution at which physiological and subcellular events can be monitored (for the principles of imaging methodologies and fluorescent probes see BOXES 1,2).

The main tools for fluorescence imaging include proteins with intrinsic fluorescent properties such as green fluorescent protein (GFP), its analogous derivative chromophore-containing proteins, and luminescent proteins such as the firefly luciferase (LUC). A few of the plant-specific examples of fluorescence-based

**Department of Botany and Plant Sciences, 2150 Batchelor Hall, University of California, Riverside, California 92521, USA. ‡California Institute of Technology, Division of Biology, MC 156-29, 1200 East California Boulevard, Pasadena, California 91125, USA. Correspondence to G.V.R. e-mail: venug@ucr.edu*
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Box 1 | Principles of microscopy methods

Confocal microscopy. An optical sectioning method used to reduce the image blur caused by inclusion of light from outside the plane of focus in a cross-section of a thick sample. In confocal microscopy the path of out-of-focus light is physically blocked before detection.

Two-photon microscopy. An optical sectioning method that reduces blur from out-of-focus light. A low probability of fluorophore excitation (through simultaneous absorption of two excitation photons outside the point of focus) is used to excite the sample selectively at the focal plane, which minimizes the generation of out-of-focus light from the sample.

Wide-field microscopy with deconvolution. The blurring caused by out-of-focus light in images from systems that are not designed for optical sectioning is removed post-acquisition through image-processing algorithms.

Spinning disk confocal microscopy. Fast-scanning microscopy method in which excitation light is scanned onto the sample through multiple excitation beams; this is in contrast to the single excitation beam used for standard confocal microscopy.

Optical coherence microscopy. Deep-penetrating near-infrared light is backscattered by the sample depending on the refractive index of the illuminated substance. Backscattered light is combined with light from a reference beam to cause interference, which is then used to isolate a signal selectively from photons that have travelled a specified path length through the sample, thereby limiting image blur.

imaging studies include the use of aequorin, a calcium-sensitive luminescent protein, to monitor intracellular calcium dynamics in several cell types, and the use of fluorescently labelled probes to monitor actin and MT dynamics[5–10]. Excellent guides are available to aid the choice of appropriate fluorescent proteins, based on brightness and expression, photostability, oligomerization, toxicity, environmental sensitivity and possibilities for multiple labelling[11,12].

Box 2 | Photosensitive fluorescent proteins

Traditional fluorescent proteins are stable and, therefore, may not sufficiently report the dynamics of gene expression and protein turnover. Several new fluorescent proteins with distinct photosensitive properties have been developed, which can reveal the true molecular dynamics in plants.

Fluorescent timer protein (dsRED-E5). This protein changes its emission spectrum over time from green to red, which allows quantification of relative pools of newly synthesized versus mature protein[119]. It has been used to monitor gene-expression dynamics in developing *Xenopus laevis* embryos and in cowpea protoplasts[120].

Photo-activatable GFP (PA-GFP) variants. These increase their fluorescence yield by several times upon irradiation and can therefore mark specific protein pools. Several PA-GFPs have been generated and their spectral properties and fluorescence yields have been characterized. (For more details about PA-GFP (a derivative of WT-GFP), Kaede (a fluorescent protein from the coral *Trachyphyllia geoffroyi*)[121] and KFP1 (kindling fluorescent protein, a derivative of the asCP chromoprotein from the sea anemone *Anemonia sulcata*) see REF. 122.) The photoactivation of PA-GFP has been used in plants to follow the rates of protein movement within the endoplasmic reticulum membrane[123]. PA-GFP variants can be used to study protein turnover, lifetimes and trafficking.

DRONPA. A fluorescent protein that is engineered from a novel coral protein[124] and has unique photochromic properties: the 490-nm-induced fluorescence emission can be efficiently bleached and the bleached protein can regain fluorescence with minimal irradiation at 400 nm. This allows the protein to be reversibly highlighted and it has been used to study the real-time dynamics of shuttling extracellular regulated kinase (ERK) between the cytoplasm and the nucleus in response to epidermal growth factor (EGF) stimulation in animal cell lines. Therefore, it can be a useful tool to study stimulus-dependent gene-expression dynamics and protein localization dynamics in plants.

The choice of live-imaging methodology greatly depends on the biological question under investigation and the physical parameters of the specimen[13–16]. Live imaging is a trade-off between preserving the biological integrity of the specimen and acquiring images at a high enough signal-to-noise ratio to achieve the required spatial and temporal resolution.

Temporal resolution. Biological events occur at different rates; therefore, methods with different temporal resolution have to be developed. For example, cell-type specification in the *Arabidopsis thaliana* SAM occurs over a period of several hours and can be reliably reconstructed by imaging at intervals of hours using conventional confocal scanning microscopes[17,18] (FIG. 1a,b). By contrast, processes such as ion dynamics, cytoskeletal reorganization, cell division, cytokinesis and intracellular trafficking are completed within seconds to minutes, and therefore require shorter imaging intervals. Spinning disc confocal microscopy, with rapid acquisition speeds, was recently used to measure the rate of cellulose deposition in transgenic *A. thaliana* plants that expressed fluorescently labelled cellulose synthase[19] (FIG. 1c–e).

Spatial resolution. The three-dimensional (3D) nature of biological tissues imposes an important constraint on achieving the required spatial resolution from images that are taken at increasing depths. The optimal spatial resolution requires that images are acquired at a high signal-to-noise ratio, which can be improved in several ways. Confocal microscopes are designed to eliminate out-of-focus light and, therefore, are well-suited for imaging through thick biological specimens (BOX 1). Multi-photon imaging systems yield even better spatial resolution than confocal systems (for further information on the principles, practices and application of confocal microscopy and multi-photon microscopy in live imaging see REFS 15,16,20). Optical coherence tomography (BOX 1) has been used to image greater depths and through turbid biological samples such as plant seeds[21,22]. Wide-field microscopy (BOX 1) — coupled with computational reassignment of out-of-focus light back to its point source by deconvolution — has been shown to improve spatial resolution[23].

Objective lenses with higher numerical aperture can improve spatial resolution; for example, water-dipping or water-immersion lenses reduce refraction at the plant–medium surface and maximize light-gathering ability[12,13]. In addition, improvements in fluorescent protein quantum yield and photostability, and targeting of fluorescent labels to specific intracellular compartments have been shown to yield better cellular resolution[24–26] (FIG. 1f–i). An extensive collection of fluorescent protein tags, which target different intracellular compartments of plant cells (see the Carnegie Institution plant-cell imaging web page), and tissue-specific enhancer-trap lines (see the Haseloff laboratory homepage) has been generated and is an excellent resource for the plant community.

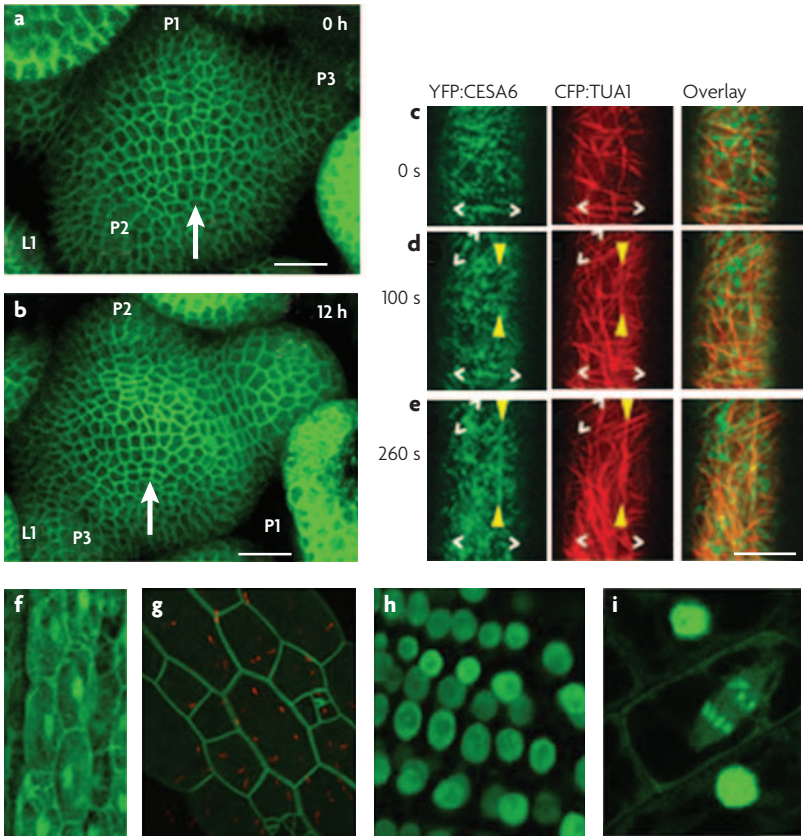

Figure 1 | **Spatial and temporal resolution in plant cells and tissues.** Panels **a**–**e** are a comparison of cellular events that occur over different timescales. Panels **a**,**b** show three-dimensional views of the L1 layer of an *Arabidopsis thaliana* shoot apical meristem (SAM), which was labelled with plasma-membrane-localized yellow fluorescent protein (YFP) and imaged on a confocal microscope. These panels are images of the same SAM taken at two time points separated by 12 hours. Time-lapse microscopy enables visual tracking of cell-division events as indicated by the deposition of new plasma membranes between cells (white arrows point to cell-division events, which occurred within the 12-hour period). P1, P2 and P3 represent flower primordia at different stages of development — P1 is the youngest and P3 is the oldest. Panels **c**–**e** show the dynamic relationship between cellulose deposition and microtubule organization, as revealed by spinning disc confocal microscopy of *A. thaliana* hypocotyls that express YFP-labelled cellulose synthase enzyme (YFP:CESA6) and cyan fluorescent protein (CFP)-labelled microtubules (CFP:TUA1). Yellow arrowheads point to newly formed microtubule bundles (CFP:TUA1 panels) and cellulose deposition (YFP:CESA6 panels). The dynamic association occurs in intervals of minutes and therefore requires faster acquisition rates to capture the event. Elapsed time is indicated. In panels **f**–**i**, targeting of green fluorescent protein (GFP) to different cellular compartments results in better spatial resolution. Panel **f** shows hypocotyl tissue that expresses untagged GFP. Panel **g** shows hypocotyl tissue that expresses plasma-membrane-localized GFP. Panel **h** shows root tissue that expresses nuclear-localized GFP. Panel **i** shows a root cell with GFP localized to chromatin during cell division. The scale bar in **a** and **b** is 20 μM and in **c**–**e** is 10 μM. Panels **a**,**b** are reproduced with permission from REF. 75 © (2004) Company of Biologists Ltd. Panels **c**–**e** are reproduced with permission from REF. 19 © (2006) American Association for the Advancement of Science. Panels **f**,**h**,**i** are reproduced with permission from REF. 24 © (2000) National Academy of Sciences, USA. Panel **g** is a kind gift from D. Ehrhardt, Carnegie Institution of Plant Biology, Stanford University, California, USA.

Dynamic imaging of nucleic acids

The organization and dynamics of the genome have been shown to influence gene expression in many organisms[27,28]. Better understanding of the influence of chromatin organization and transcriptional regulation requires dynamic analysis of chromatin behaviour. Techniques have been developed to enable the overall *in vivo* labelling of chromatin or RNAs and to label defined chromatin regions or specific RNA molecules[29,30].

Two recent studies in *A. thaliana* have used fluorescence-based methods to follow chromatin reorganization in relation to cell division and cell-fate specification. Four-dimensional (4D) live imaging of *A. thaliana* root cells that expressed GFP fused to centromere-specific histone H3 enabled the study of chromatin organization patterns and cell-division dynamics[31]. Tracking the entire set of fluorescently labelled centromeres in cells that undergo mitosis showed that the global centromere positions are not precisely transmitted from the mother cell to daughter cells in growing root meristems.

In a separate study, static 3D fluorescence *in-situ* hybridization was used on intact *A. thaliana* root epidermal tissues to follow chromatin organization around the *GLABRA2* (*GL2*) locus during cell-fate specification. In the root epidermis, cells differentiate in alternating files of hair cells and non-hair cells in a position-dependent manner. The homeodomain transcription factor GL2 is crucial for the specification of non-hair cell fate. This study has revealed that an open state of chromatin conformation around the *GL2* locus is required to control position-dependent cell-type specification[32]. In addition, it was shown that the conformational state of chromatin around the *GL2* locus is not inherited by progeny cells; the chromatin is reorganized in the G1 phase of the cell cycle. These studies provide important clues about chromatin organization and inheritance patterns among progeny cells; however, dynamic visualization of discrete chromosomal regions along with gene-expression patterns might yield further insights.

Tracking chromosomal regions as fluorescent dots. Discrete chromatin regions can be marked with the dual plasmid strategy, which is based on the bacterial *lac* operator (*lacO*) and repressor (*lacR*) system[33]. The principle of this approach is to integrate *lacO* sequences into the genome and to co-express a LacR–GFP fusion protein that is capable of binding to *lacO* sequences. This allows the tracking of discrete chromosomal regions as fluorescent dots in living cells. This method has been further modified to monitor dynamics of gene expression and associated chromatin modifications. The positioning of the *lacO* repeat sequences in front of an inducible promoter that controls the expression of a cyan fluorescent protein (CFP) has allowed the simultaneous visualization of gene-expression dynamics and related changes in chromatin structure in living cells[34]. However, understanding the influence of native chromosomal dynamics on gene-expression patterns would require the targeted insertion of *lacO* sequences into specific chromosomal regions.

Visualizing RNA localization. The dual-plasmid approach has been used to visualize RNA localization in living cells[35]. This approach uses integration of stem-loop structures into the target RNA that can bind to the bacteriophage coat protein MS2. The target RNA that carries the stem-loop structures is co-expressed with GFP–MS2 fusion proteins that can bind to the

Enhancer trap
A genetic method that allows detection of expression patterns of genes.

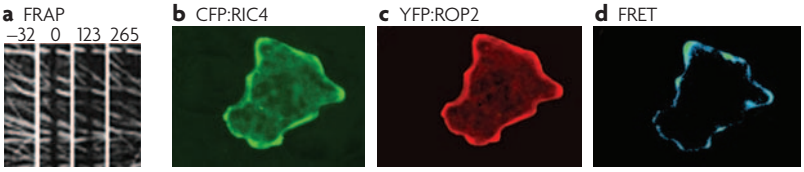

Figure 2 | **FRAP and FRET measurements in plant cells.** Panel **a** shows a time-lapse series that represents the fluorescence recovery after photobleaching (FRAP) of green fluorescent protein (GFP)-tubulin-labelled microtubules in *Arabidopsis thaliana* hypocotyl cells. The GFP-labelled tubulin has been bleached by irradiation with intense laser light and then the recovery of GFP signal has been followed over time. Total elapsed time (in seconds) before and after photobleaching is indicated above each panel in **a**. Recovery of the GFP-tubulin fluorescence signal reveals the motility of microtubules. In panels **b**–**d**, fluorescence resonance energy transfer (FRET) analysis reveals an interaction between ROP (a plant-specific member of the Rho GTPase family) and RIC4 (ROP-interactive CRIB motif-containing protein) proteins in the pavement cells of *A. thaliana* leaf epidermis — a fluorescence signal from cyan fluorescent protein (CFP):RIC4 (**b**); a fluorescence signal from yellow fluorescent protein (YFP):ROP2 (**c**); and a FRET signal due to the interaction between CFP:RIC4 and YFP:ROP2 (**d**). Panel **a** is reproduced with permission from REF. 45 © (2003) American Association for the Advancement of Science. Panels **b**–**d** are reproduced with permission from REF. 64 © (2005) Elsevier.

stem loops and thereby highlight the target RNA. This system was originally developed to visualize the mRNA of ASH1, a transcriptional repressor that is involved in mating-type switching in yeast cells. This approach has been adopted to study the transport of prolamine RNAs in rice endosperm cells[36] and it can also be applied to the study of the subcellular localization, dynamics and intracellular movement of RNA molecules.

The simultaneous visualization of chromatin behaviour, gene-expression dynamics and RNA localization patterns in real time could provide novel mechanistic insights into the process of cell-fate specification and other cellular processes.

Dynamic imaging of plant proteins

Dynamic visualization of protein localization, protein traffic and protein–protein interactions can reveal functional regulation of protein activity. Tagging native proteins with fluorescent proteins to generate functional protein chimaeras is a common method of labelling proteins *in vivo*. However, slow maturation rates of fluorescent proteins, increased stability, protein misfolding and oligomerization can impose limits on determining the true dynamics of the proteins of interest.

Venus, a variant of yellow fluorescent protein (YFP) with fast and efficient maturation rates, has been isolated and used to generate protein chimaeras in plants[18,37]. Misfolding problems that are associated with fluorescent protein chimaeras can be resolved by using mutant versions of GFP with robust folding capabilities[38] or a much smaller fluorescent tag — a tetracysteine peptide motif[39]. The tetracysteine motif can bind to exogenously supplied fluorescent-bi-arsenic ligands with high affinity, thereby highlighting the protein chimaera.

Photosensitive proteins. It can be challenging to measure protein turnover and the dynamics of spatio-temporal patterns of gene expression with GFP chimaeras[40]. In any given steady-state condition, several different species of fluorescent protein chimaeras that represent different stages of protein life can be found. Several new GFP variants could be useful in determining the dynamics of spatio-temporal patterns of gene expression. Destabilized GFPs with rapid protein turnover rates have been used in animal systems to overcome GFP stability and, therefore, can be useful for analysing the dynamics of gene expression[41]. However, destabilized GFPs can result in low fluorescence levels, which could limit their use in time-lapse imaging. An alternative approach would be to use fluorescent proteins that change their spectral properties with time (BOX 2).

Kinetic imaging methods. Protein dynamics can be studied by using kinetic imaging methods such as fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS)[42,43]. FRAP allows quantification of the average rate of protein translocation within cells[44]. FRAP has been used in plants to study MT dynamics (FIG. 2a) and protein dynamics in the endoplasmic reticulum[45].

FCS has been used in plants to determine the rate of movement and the concentration of molecular species including proteins within a living cell[46]. In addition, the cross-correlation of two distinct signals derived from spectrally resolvable, labelled species enables the determination of the molecular association of the two species. Measurement of the steady-state diffusion coefficient or rate of active transport of a molecular species may be of interest in studying the regulation of transcription factor traffic across plant cells. Coupling kinetic microscopic methods with transient genetic or chemical perturbations could reveal the regulation of organelle or molecular movements within cells.

Fluorescent hormonal sensors

Plant development involves the integration of various environmental stimuli and endogenous signals into the genetic programme. Hormones function at extremely low concentrations to influence multiple aspects of plant growth and development. Development of highly sensitive *in vivo* assays to monitor the sites of synthesis, direction of transport, and site of accumulation and action of plant hormones will help to determine their function and regulation. Mutants with altered hormonal biosynthesis and altered responses to hormones have provided mechanistic insights into the roles of various hormones in plant growth and development[47]. Recent development of sensitive fluorescence-based probes has enabled hormone signalling to be monitored in real time.

Monitoring the auxin response. Synthetic auxin-responsive promoters have become useful tools for monitoring the sites of auxin response[48]. The *DR5* promoter — a set of tandem repeats that includes an 11-bp auxin-response element from the soybean *GH3* gene fused to GFP — has been used as a biosensor to monitor the effects of auxin at cellular resolution in the study of auxin flux during *A. thaliana* embryo development, root gravitropism, cell-fate specification in the root apical meristem, and primordial patterning in the SAM[49].

Fluorescence recovery after photobleaching
Microscopic imaging technique that destroys fluorescent protein chimaeras from a well-defined region within living cells. This method is used to deduce the diffusion speed of molecules in living cells.

Fluorescence correlation spectroscopy
A correlation function derived from fluorescence intensity fluctuations. This method is applied to deduce the concentration fluctuations of fluorescent molecules in solution.

Root gravitropism
A phenomenon that describes the tendency of the plant root system to grow towards the pull of gravity.

Root apical meristem
A collection of undifferentiated cells that are located near the growing tip of roots.

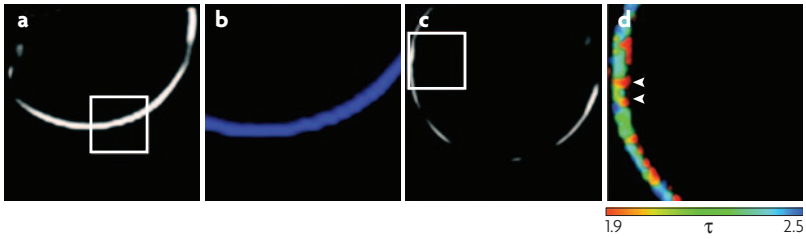

Figure 3 | **FLIM measurement of protein–protein interactions in the living plant cell.** Use of fluorescence lifetime-imaging microscopy (FLIM) analysis to determine the homodimerizing property of two membrane-localized leucine-rich repeat receptor-like kinases: SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE-3 (SERK3) and BRASSINOSTEROID INSENSITIVE-1 (BRI1). Panels show FLIM analysis on cowpea protoplasts that are transiently expressing fusions with cyan or yellow fluorescent protein (CFP or YFP, respectively). Panels **a**,**b** show *Arabidopsis thaliana* (At)SERK3–CFP/AtSERK3–YFP and panels **c**,**d** show BRI1–CFP/BRI1–YFP. Intensity images of donor CFP fluorescence are shown in panel **a** for AtSERK3–CFP and panel **c** for BRI1–CFP. Panel **b** is a pseudocolour image of the fluorescent lifetime (τ) distribution of the boxed region of the cell in panel **a** and panel **d** is a pseudocolour image of the τ distribution of the boxed region of the cell in panel **c**. Arrows in panel **d** point to areas with a significant reduction of CFP fluorescence lifetime (orange to green, τ = 1.9 to 2.0 ns) that is due to fluorescence resonance energy transfer (FRET), indicating BRI1 homodimerization, whereas the ubiquitous dark blue (τ = 2.5) in panel **b** illustrates the absence of FRET and, therefore, a lack of SERK3 homodimerization. Based on these findings, BRI1 forms homodimers, whereas SERK3 does not self-associate. Reproduced with permission from REF. 61 © (2004) American Society of Plant Physiologists.

Fluorescent protein chimaeras of PINFORMED (PIN) and AUXIN PERMEASE (AUX) proteins — which function as channels to move auxin out of and into cells and have an asymmetrical cellular localization (localized to one end of the cell) — have been used to infer the directionality of auxin transport in tissues[50,51]. Real-time imaging of *PIN1* and *DR5* reporter genes along with multiple reporter proteins of genes that are involved in floral primordial development has revealed the dynamics of auxin gradients at different stages of primordium development. Fluorescently tagged PIN protein chimaeras have also revealed the auxin-mediated regulation of PIN protein recycling and have provided mechanistic insights into feedback regulation of auxin accumulation and auxin transport[52].

The plant hormone cytokinin (CK) has been implicated in several processes from cell division to cell-fate specification[53]. CK-mediated induction of auxin-response-regulator proteins (ARRs) is involved in the regulation of phyllotactic patterning in maize and stem-cell maintenance in the *A. thaliana* shoot apex[54,55]. ARR5::GFP has been used to monitor CK responses *in planta* and revealed that CK treatment results in the upregulation of ARR expression[56]. Real-time *in vivo* monitoring of CK responses might provide important mechanistic insights into the CK-mediated control of diverse cellular and patterning events.

A non-invasive, cell-autonomous reporter system, which consists of an abscisic acid (ABA)-responsive promoter driving LUC, has also been developed to monitor the generation and distribution of physiologically active pools of ABA, a hormone that is involved in several physiological and developmental events[57]. The improved availability of sensitive fluorescence-based tools to follow the distribution of plant hormones and sites of hormone accumulation and response will further facilitate future *in vivo* real-time studies, which will lead to a better understanding of the integration of hormonal responses that regulate plant growth and development.

Fluorescence (Förster) resonance energy transfer
The excited donor fluorescent molecule transfers non-radiative energy to a second fluorescent molecule, the acceptor. The energy transfer depends on the distance between the fluorescent molecules.

Fluorescence lifetime-imaging microscopy
An imaging technique for obtaining an image that is based on the differences in the exponential decay rate of the fluorescence from a sample. It is used to deduce molecular interactions.

Studying molecular interactions

Although the resolution of conventional light microscopy is restricted to the subcellular level by the diffraction limit, statistical methodologies are extending the power of live imaging to reveal molecular interactions. Fluorescence resonance energy transfer (FRET) is a commonly used imaging method for assaying protein–protein interactions. FRET allows the determination of an association between molecular components of interest within the 1–10 nm range (FIG. 2b–d). Interactions can be assayed by direct quantification of FRET or can be derived from changes in the fluorescence lifetime of the donor, as in fluorescence lifetime-imaging microscopy (FLIM) (FIG. 3a–d). Therefore, the strength of a protein–protein interaction can be quantified through the efficiency of FRET between two protein chimaeras (aspects of FRET, FLIM and their technical variations are reviewed in REFS 58,59).

FRET has primarily been used in plants to probe the steady-state interaction of proteins that are involved in signal transduction, to follow dimerization of membrane-bound receptor kinases, and to characterize the formation of higher-order protein complexes[60–64]. The use of a bioluminescent donor for resonance energy transfer (BRET) has also been applied in plants to determine the interaction of cellular proteins[65]. BRET can reduce artefacts that are commonly observed with FRET such as auto-fluorescence and direct acceptor stimulation.

The bimolecular fluorescence complementation (BiFC) technique uses non-fluorescent fragments of intrinsically fluorescent proteins (IFPs) as tags[66,67]. These tags emit fluorescence only when the proteins of interest interact and mediate the association of IFP fragments to form a functional fluorescent complex. As in FRET, excitation of the sample is required, which can potentially lead to auto-fluorescence; however, direct stimulation of the acceptor is not an issue because the tags only fluoresce when the proteins of interest interact in a complex. In most cases, applications of FRET, BRET and BiFC in plants have been limited to probing interactions in cell culture. An important future development will be to use these techniques to study the dynamics of protein interactions in intact plants.

Structural elements and cell behaviour

Global hormonal signals interact with the local cell–cell communication machinery to influence cell behaviours such as cell-division rates and orientation as well as the rate and direction of cell expansion. In turn, cell behaviour depends on cytoskeletal elements such as actin and MTs. Therefore, simultaneous monitoring of structural elements, cell behaviour and cell-fate specification in real time might provide novel insights into developmental regulation.

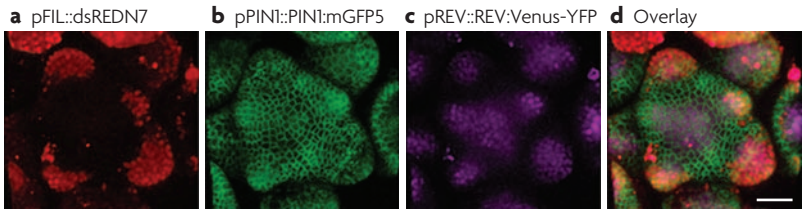

Figure 4 | **Multi-channel confocal imaging of *Arabidopsis thaliana* SAM, labelled with three spectrally variant fluorescent proteins.** Three-dimensional reconstructed top views of the *Arabidopsis thaliana* shoot apical meristem (SAM). **a** | The *FILAMENTOUS FLOWER* (a gene involved in floral primordium development) promoter drives expression of nuclear localized dsRED (a coral-derived red fluorescent protein) and marks flower primordial regions. **b** | The *PINFORMED1* (a gene involved in regulating the directionality of auxin flow) promoter drives expression of the PINFORMED1:green fluorescent protein (GFP) fusion protein and reveals the intracellular localization of PIN1 protein. **c** | The *REVOLUTA* (a gene involved in lateral organ and SAM development) promoter drives expression of the REVOLUTA:VENUS–yellow fluorescent protein (YFP) fusion protein. **d** | A combined overlay of the three channels. The images show the power of multi-channel imaging in following events that are involved in primordium development in relation to the distribution of the plant hormone auxin. The scale bar is 25 μM.

Imaging actin and MT dynamics. Several fluorescent chimeric proteins have been generated to monitor actin dynamics. The ability of the mouse talin to bind F-actin has been exploited to generate a talin:GFP chimaera to study the regulation of F-actin dynamics in developing pollen tubes and root hairs[68,69]. GFP has been fused to the second actin-binding domain (fABD2) of *A. thaliana* FIMBRIN to visualize dense actin networks[70]. Time-lapse imaging was combined with the FRAP technique to monitor GFP-labelled actin cytoskeletons, which revealed that actin filament migration and sliding of individual filaments or bundles orchestrate the dynamic reorganization of the actin scaffold.

In an effort to understand the mechanisms by which MTs undergo rapid realignment, several methods have been described to monitor and quantify MT dynamics. By injecting Rhodamine-conjugated brain tubulin into the epidermal cells of *Pisum sativum*, it was possible to follow the labelled MTs over a period of 2 hours[71]. This study demonstrated that reorientation does not occur by complete MT depolymerization in one orientation followed by polymerization of a new array in another orientation. A similar approach has been used to follow MT turnover in living stamen hair cells of *Tradescantia virginiana* that have been injected with fluorescent neurotubulin[72]. Live imaging and FRAP have been combined to measure MT turnover rates. A fluorescent chimaera that consists of a fusion between GFP and the MT-binding domain of the mammalian microtubule-associated protein-4 (MAP4) has recently been used to study MTs *in vivo*[73]. The simultaneous visualization of time-course events of labelled actin and MT cytoskeletal networks along with fluorescent reporters for cell behaviour should yield better insights into the cell mechanics that underlie cell behaviour.

Several fluorescent reporters have been developed to follow cell division, cell expansion, cytokinesis and wound-induced cell death. A HISTONE-2B:YFP chimaera has been used to monitor cell-division dynamics in root and shoot meristems, and in the syncytial endosperm of *A. thaliana*[74,75]. Fluorescent mitotic CYCLIN (CYCLIN B1;1:GFP) has been used to visualize G2 to M transition dynamics in *A. thaliana* SAM cells[75] and plasma-membrane-localized YFP (YFP 29-1) has been used to monitor cell expansion and division[75]. Specific fluorescent protein tags have been developed to follow cytokinesis in *A. thaliana* hypocotyl cells[24]. The time-lapse imaging of *A. thaliana* cells that express a GFP:NITRILASE-1 fusion protein has revealed the earliest stages of wound-induced plant cell death[76]. The use of fluorescent markers for monitoring cell behaviours along with cell-structure markers should provide important insights into the regulation of these processes during development.

Multi-spectral live imaging. With the advent of fluorescent proteins with distinct excitation and emission properties, it has been possible to achieve spatio-temporal separation of coordinated molecular and cellular events through multi-spectral imaging. Spectrally distinguishable fluorescent proteins that are expressed from native promoter elements form an important means of highlighting specific gene-expression patterns and, therefore, cell identity in plant tissues[12,17,18]. Such a strategy has been applied to follow cell identity transitions in SAMs of *A. thaliana*[18] (FIG. 4a–d).

The major challenge in multi-spectral imaging is to minimize bleedthrough of signals into inappropriate channels. Because biological reactions occur at different concentrations and the strength of promoter elements differs greatly among genes, reporters must be optimized to achieve intensities that minimize channel cross-talk. A combination of approaches can be used to solve the problem. The brightness of fluorescent proteins should be considered when assigning them to appropriate promoters; for example, fluorescent proteins with a higher quantum yield should be used in conjunction with weakly expressed promoters. Multimerized versions of fluorescent proteins that consist of 2× or 3× tandem repeats or a multimerized constitutive promoter along with a translational enhancer have been shown to provide a higher signal intensity[18,77]. Alternatively, sequestration of fluorescent proteins to specific intracellular compartments has been shown to increase their brightness[77]. Two-component transactivation systems can also be used to enhance expression from weaker promoters.

The availability of a plethora of imaging tools and methods to monitor multiple molecules, hormonal responses, molecular interactions, cytoskeletal elements and cell behaviours — all in real time — should provide unprecedented opportunities for future work aimed at integrating the multitude of responses that regulate plant processes. However, the ultimate benefits of real-time imaging in unravelling the mysteries of function and regulation of plant processes will be best realized through transient perturbations, rather than time-lapse imaging of wild-type or mutant tissues.

Transient perturbations and live imaging

The process of pattern formation involves the conversion of transient temporal signals into stable genetic circuits that involve feedforward and feedback control of gene activation[78]. Traditional genetic analyses based on terminal phenotypes can only provide clues to the network of genetic interactions, and may not reveal the real function of genes in an evolving network of interacting cells. Transient perturbations followed by real-time imaging of gene-expression patterns and cellular events could lead to better mechanistic insights. In this section, we review studies that have incorporated transient gene manipulation, chemical[79] and physical perturbations, and dynamic imaging technologies to investigate cellular processes and development.

Spatial control of gene expression. Development is regulated by the perception and interpretation of complex spatio-temporal signals. The most commonly used systems for genetic manipulation with a high degree of spatio-temporal resolution are the two-component systems, which are based on transcriptional activation of target genes (for a review of the design principles and properties of the different systems used in plants see REF. 80).

The most widely used transactivation systems in plants include the yeast-derived mGal4:VP16–UAS and the bacteria-derived pOp–LhG4 systems[81,82]. Transactivation systems consist of two components, in which a transcriptional activator binds to a consensus DNA element and activates the target gene. Both of these systems have been modified extensively to achieve optimal expression in plant cells[83]; they have been used in enhancer-trap screens and to tag characterized promoters to generate a collection of tissue-specific and cell-type-specific lines[25,26,80]. The pOp–LhG4 system has been used to achieve spatial misexpression of the transcription factors WUSCHEL (WUS) and KANADI (KAN), which are important regulators of stem-cell maintenance and lateral organ differentiation in the *A. thaliana* shoot apex[84,85]. The role of SCARECROW (SCR) in the specification of the root quiescent centre (QC) and the role of *PLETHORA* genes in root stem-cell maintenance have been studied with the mGal4:VP16–UAS system[86,87].

Temporal control of gene expression. In addition, the two-component systems have been modified further to achieve temporal control of gene manipulation. The transcription factors LhG4 and GAL4:VP16 were fused to the hormone-binding domain of the rat glucocorticoid receptor to generate chimeric transcription factors, the nuclear translocation of which can be selectively regulated by dexamethasone (DEX) treatment to activate target genes[83,88].

The DEX-inducible LhG4-GR system has been used to conditionally activate a CK biosynthesis gene and to conditionally inactivate CLAVATA3 (CLV3), a regulator of stem-cell maintenance in the *A. thaliana* shoot apex[17,83]. The effects of conditional inactivation of CLV3 on SAM organization and growth have been followed in real time by using fluorescently labelled cell-type-specific and cell-division markers[17]. This study exemplifies the power of transient genetic manipulations and real-time imaging for deciphering the function of receptor-kinase signalling in the regulation of growth and cell-fate specification. The inactivation of *CLV3* was achieved by generating fold-back double-stranded RNA against *CLV3*.

Vectors for high-throughput DEX-inducible gene silencing have been described[89,90]. The unique feature of plant microRNAs that affect only a small number of target genes (and that share high sequence complementarity with these genes) has been exploited to generate artificial miRNAs (amiRNAs) to achieve highly specific gene silencing[91]. The amiRNA system has been combined with the ethanol-inducible (Alc) system to achieve temporal control of gene silencing[92]. 17-β-oestradiol-inducible systems have also been used for temporal control of plant gene manipulation[93]. The genetically encoded, inducible two-component systems should form the basis of many experiments to manipulate gene activities in live-imaging experiments.

Genetic mosaics. Spatial and temporal alteration of gene activities can also be achieved through the creation of genetic mosaics, resulting in organisms or tissues that consist of cells with different genetic identities. Marking specific cells to follow lineages has been particularly useful in deducing several regulatory aspects of development[94]. Transposon-mediated excisions and the CRE/*loxP* and FLP/*FRT*-mediated recombination systems have been used to mark specific cells selectively as well as delete or activate genes from a specific set of cells[95]. A CRE/*loxP*-based site-specific recombination system, in which recombination events are positively marked with GFP expression, has been used to activate or delete the *SCR* gene selectively in root meristems[86]. Marking clones of cells enables them to be monitored in real time and could provide important clues about gene function and regulation during the cell divisions in development.

Genetic manipulation of protein function. Approaches have also been described that efficiently convert plant transcription factors into dominant-negative forms by fusing them to the repressor domain of the *engrailed* (*en*) gene from *Drosophila melanogaster* or to EAR motifs that are found in plant transcription factors[96,97]. Ectopic expression of such chimeric proteins has been shown to phenocopy loss-of-function mutants. The inducible activation of such chimeric transcription factors could be used to modify their activity in experiments to study dynamic responses to changes in protein function.

Chromophore-assisted light inactivation. Inactivation of specific proteins with high spatio-temporal resolution has been achieved through chromophore-assisted light inactivation (CALI). The short-lived reactive oxygen species (ROS) that are generated by the chromophore excitation event can inactivate proteins in their immediate vicinity. Originally, CALI was achieved by targeting proteins with specific antibodies that were conjugated with photosensitizer dyes such as malachite green or

Ethanol-inducible (Alc) system; 17-β-oestradiol-inducible system
Genetically encoded transactivation systems, which can be activated by exposing the plant tissues to ethanol or oestrogen, respectively.

CRE/*loxP* and FLP/*FRT*-mediated recombination systems
Commonly used genetically encoded systems for sequence-specific recombination that induce recombination either between two different chromosomes or between different segments of a chromosome. The systems are used to mark cells by inducing or deleting genes.

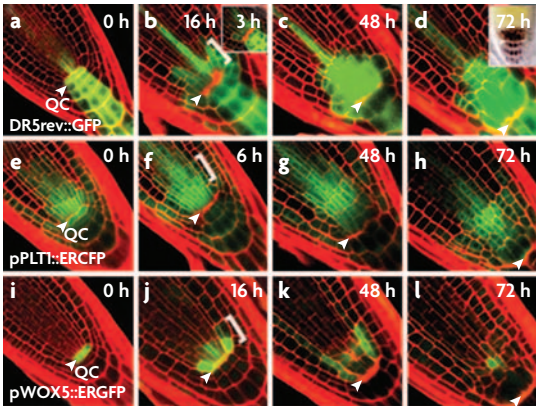

Figure 5 | **Cell ablations, transient intervention and live imaging.** Temporal sequence of effects of quiescent centre (QC) ablation on the distribution of the plant hormone auxin and cell-fate identities in the *Arabidopsis thaliana* root meristem. Effects of QC ablation on redistribution of the auxin response as measured by the auxin-response gene *DR5rev* promoter activity (**a**–**d**), *PLETHORA1* (PLT1, which is involved in stem-cell maintenance) expression (**e**–**h**) and a QC-specific marker, *WOX5* (**i**–**l**). Disruption of auxin flow by QC ablation results in rapid upregulation of the auxin response followed by cell-fate changes. Arrowheads indicate the QC (**a**,**e**,**i**) and the position of the ablated QC in the other panels. Fluorescent reporter expression is shown in green and propidium iodide staining is shown in red. Reproduced with permission from REF. 104 © (2006) American Association for the Advancement of Science.

fluorescein[98]. However, the generation and delivery of antibodies into cells can be limiting factors. Therefore, genetically encoded CALI, in which the target protein is tagged with small tetracysteine motifs that specifically bind to membrane-permeant photosensitizer dyes has been developed[99]. Subsequent developments have resulted in a fully genetically encoded CALI in which the target proteins are tagged with fluorescent proteins that function as photosensitizers.

In a recent study, multiphoton excitation was used for CALI of an enhanced GFP (EGFP)–protein chimaera to inactivate protein function without nonspecific photodamage in living mammalian cells[100]. With the identification of a highly efficient photosensitizer such as KillerRed (a GFP homologue derived from the hydrozoan chromoprotein anm2CP), CALI has several potential applications in plants[101]. The expression of KillerRed protein chimaeras can be controlled by specific promoters to achieve a high degree of spatio-temporal manipulation of protein activities or cell ablations to investigate cell-fate decisions in real time.

Physical perturbations. Multicellular tissues represent an interconnected network of distinct cell types. It has been proposed that, in plants, positional signals influence growth and patterning events. Physical perturbations such as laser-mediated ablations have been used to investigate the importance of positional information from neighbouring cells in cell-fate determination, and to deduce the source and directionality of signals involved in stem-cell maintenance and patterning in the *A. thaliana* root meristem[102,103].

Several studies using the *A. thaliana* root meristem have combined transient gene manipulation, cell ablations and time-lapse imaging to explore the regulatory interactions involved in stem-cell maintenance and wound-induced regeneration. A recent study used laser-induced ablations along with fluorescent cell-type-specific markers to monitor auxin flux during cell-type respecification during regeneration of root tissue[104] (FIG. 5a–l). An elegant time-lapse experiment combined transient manipulation of the levels of the RETINOBLASTOMA-RELATED protein (RBR) and ablation of specific root QC cells to obtain mechanistic insights into the interactions between cell-fate determinants and cell-cycle regulators[105]. Combining advanced multi-modal imaging methods with transient manipulation of gene activities, protein activity or direct manipulation of cells will be critical for developing an understanding of developmental interactions and the causal interactions between molecules, intracellular compartments and cells.

Conclusions and future perspectives

We have reviewed recent progress in the use of multi-modal live-imaging technologies and transient perturbation methodologies in plants. Impressive strides have been made in improving the spatial and temporal resolution of biological imaging. It is now possible to resolve several spectral variants of fluorophores through multi-spectral imaging. The technological breakthroughs in multi-spectral imaging along with experimental approaches to optimize fluorescence signals should facilitate simultaneous visualization of molecular, organelle and multicellular interactions in real time. Transient perturbation methodologies involve genetic and physical methods, and the use of chemical modulators. Equally impressive progress has been made with genomic technologies to decipher gene functions and gene networks that regulate developmental processes in diverse organisms[78]. Inducible gene activation and genome-wide expression profiling in plants have yielded primary insights into the genetic circuits that operate in the patterning of *A. thaliana* roots, floral induction pathways and pattern formation in flowers[106–108].

An important challenge is to transform molecular maps into networks of functional interactions. Progress in the area of proteomics promises to establish maps of protein interactions[109]. However, gene expression maps and protein interaction maps do not take into account the cellular context of signalling pathways and cell–cell interactions. Therefore, the transformation of genomic and proteomic maps into maps that show the networks of functional interactions of cells will require quantitative and dynamic understanding of the spatio-temporal organization of tissues, and of the regulatory interactions of their cells. This can be accomplished through quantitative real time imaging of molecular interactions within living cells, and of cellular activities within living tissues.

Quantitative imaging will require the development of new fluorescent probes that reflect the true dynamics of the proteins to which they are attached, and they must be combined with kinetic imaging methods to assay dynamic molecular, organelle and cellular events. Quantitative imaging also requires the development of computational methods to navigate, visualize and track image data over time[110,111]. With the quantitative understanding of discrete molecular and cellular events, it should be possible to develop realistic computational models of developmental processes. Data from live imaging have been exploited to construct computational models that explain individual developmental events[112–115]. However, genome-scale determination of expression patterns of genes and gene functions in regulating developmental processes will be crucial for generating integrated whole-organism models of development. A genome-scale, cell-type-specific gene-expression map has been described for *A. thaliana* root meristem cells[116].

A genome-wide RNA interference screen has been combined with high-throughput time-lapse microscopy for phenotypic profiling of all genes that are required for the first two rounds of cell division in the *Caenorhabditis elegans* embryo[117]. The data obtained have been integrated with data from protein interactions, along with similarities in expression profiles and protein function, to decipher modules that regulate cytokinesis in *C. elegans* embryos[118]. The application of similar methods to plants is now possible. Together with the spatial and temporal control of gene and protein activity in real time — and the ability to monitor cellular behaviour, protein localization and gene activity in living tissue — the application of these methods will lead to an entirely new view of the mechanisms of plant growth, development and environmental response.

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Competing interests statement
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