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Advanced imaging techniques for the study of plant growth and development

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A variety of imaging methodologies are being used to collect data for quantitative studies of plant growth and development from living plants. Multi-level data, from macroscopic to molecular, and from weeks to seconds, can be acquired. Furthermore, advances in parallelized and automated image acquisition enable the throughput to capture images from large populations of plants under specific growth conditions. Image-processing capabilities allow for 3D or 4D reconstruction of image data and automated quantification of biological features. These advances facilitate the integration of imaging data with genome-wide molecular data to enable systems-level modeling.

Combining imaging and modeling

Even the simplest organisms are highly complex systems in which countless dynamic biochemical processes occur simultaneously. To reach a comprehensive and quantitative understanding of such a complex molecular machine, the ability to accurately characterize dynamic processes at different scales is essential. Traditional molecular, genetic, and biochemical studies have successfully identified regulators of plant growth and development; however, these approaches often fail to address the timing of molecular events. To capture the dynamic behavior of biological systems, molecular activities need to be analyzed with regard to their spatial and temporal properties. To generate a comprehensive model of developmental processes, gene expression patterns have to be recorded with high-spatial resolution and combined with morphological, genetic, and functional data. Such approaches have been used to comprehend the role of genes during cell fate

decisions [1] or in response to environmental perturbations [2,3]. Recent developments in the field of imaging have provided the tools to study processes during growth and development with high spatial and temporal resolution and with a high throughput (Figure 1). For the first time, this allows combining a comprehensive set of genome-wide data with imaging techniques and computational modeling, enabling the generation of quantitative models of plant development. Here we highlight imaging techniques that have led to novel biological insights with regard to plant growth and development, and furthermore hold the promise to generate data that will advance our understanding of the molecular systems that govern plant growth and development.

Imaging to capture the time domain

To achieve a comprehensive characterization of the dynamic cell behavior responsible for organ growth and development, it is necessary to measure gene expression, cell division, and cell expansion, as well as their rates and

Glossary

Confocal microscopy: laser microscopy that allows image acquisition of fluorescent molecules producing images with high horizontal resolution and depth selectivity.

Light sheet illumination: technique in which the sample is illuminated perpendicular to the direction of observation.

Micro-computed tomography: 3D image computed from multiple 2D projections from different angles obtained by interaction of matter and X-rays.

Microfluidics device: apparatus in which small volumes of liquid can be controlled.

Multi-angle image acquisition three-dimensional reconstruction and cell segmentation-automated lineage tracking (MARS-ALT): integrated and automated approach to track cell lineages over time, in which images of organs are acquired from multiple angles, computationally merged and segmented.

Optical projection tomography: 3D image computed from multiple 2D projections from different angles obtained by optical microscopy.

Quantitative trait loci (QTL): genome regions that underlie the quantitative variation of a trait.

Recombinant inbred lines (RILs): collection of lines, each containing chromosomes which constitute a genetic mosaic of two parental lines.

Selective plane illumination microscopy (SPIM): light sheet illumination-based microscopy allowing for image acquisition with high-spatial and temporal resolution.

Super resolution microscopy: microscopy technique that can yield images with a resolution higher than the diffraction limit would allow.

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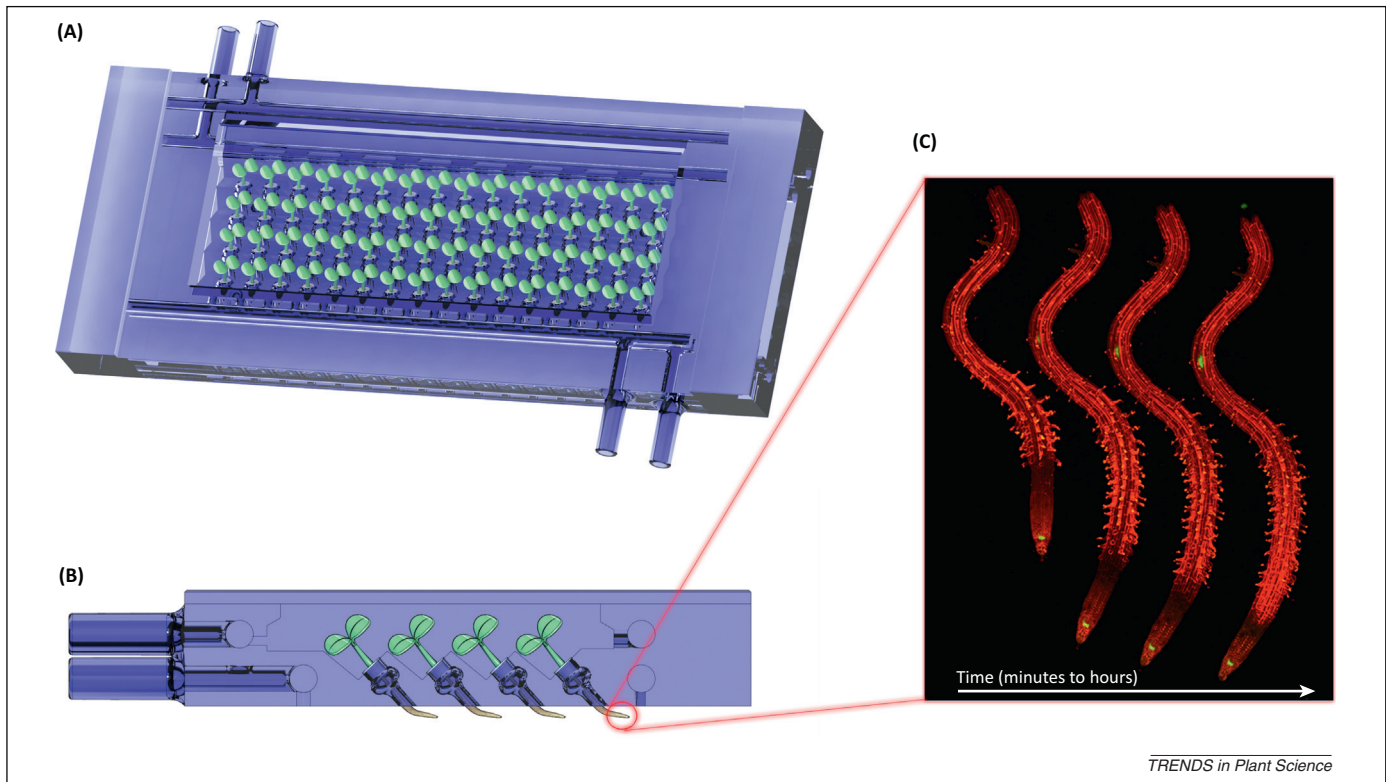


Figure 1. Schematic representation of the RootArray microfluidics device [71]. **(A)** Front view of 64 *Arabidopsis thaliana* seedlings grown over several days without manual intervention owing to gaseous and liquid exchanges through the tubes on the top left and bottom right. **(B)** Side view of the microfluidics device. Red circle highlights roots imaged by confocal microscopy. **(C)** Root stained with propidium iodide expressing a transgenic reporter line and tracked over time.

spatial distribution. Early work showed how image-based experimentation could define cell lineage and division patterns in shoot [4,5] and root meristems [6]. Subsequently, there was a need to develop imaging tools to visualize cellular dynamics in the living organism. In an effort to correlate observable molecular-level processes with plant development, several groups are now combining experimental approaches with computer modeling to analyze data and to make testable predictions [7–9]. Results from biological experiments (e.g., z-stacks of confocal microscope images) can be used to construct predictive mathematical and graphical models that account for the behavior of the system (reviewed in [10,11]). These models are then used to make non-intuitive predictions that provide further insight into the processes involved and can be tested experimentally [12]. An example of morphodynamics, which combine modeling and cell tracking of mutants with altered division patterns, was used to explain the variability in cell size among sepal epidermal cells [13]. In addition to showing that the timing of cell division is irregular, this work highlights the importance of quantitative measurements of plant features for developing and testing morphodynamic models. Similarly, a study using a powerful combination of existing data with live imaging studies and modeling proposed a model in which polarity switching and asymmetric divisions are key to the precise sequence of patterning events that lead to the formation of guard cells in *Arabidopsis* (*Arabidopsis thaliana*) leaves [14]. Furthermore, live imaging and computational modeling have been combined to predict the position of new organs in response to mechanical signaling [15,16]. Overall, these studies have shown that

a combination of time-resolved, high-resolution morphological and gene expression data is a powerful way to generate models that predict developmental processes.

Although gene expression is an important parameter for models of growth and development, the abundance of bioactive molecules in cells or organs is critically important information. To measure temporal dynamics of such agents *in vivo*, readouts from highly specific molecular sensors are required. Tools based on changes in probe intensity, color, localization, and energy transfer [17,18], together with a wide array of sensors used to assay a variety of key molecules such as sugars, amino acids, ions such as ammonium and hormones, are now available [18–21]. Among these, a new sensor has been engineered that enables dynamic changes in endogenous auxin levels to be detected. The DII-VENUS sensor enables quantitative measurement of auxin signaling activity at high spatio-temporal resolution during growth and development [20]. Additionally, microfluidic devices probing sensor responses under controlled conditions with high time resolution can be used. For instance, a microfluidics-based device, the RootChip, was used to quantify changing metabolite levels in multiple roots in real time [22].

Microscale resolution

Every image acquisition method has specific limits that are defined by attributes such as enhanced resolution, sensitivity, phototoxicity, penetration depth, and the properties of available dyes and fluorophores. In the past 5–10 years, significant progress has been made to develop new methods to push these limits. One of the most notable developments has been the development of super resolution

microscopy (see [Glossary](#)). This technology enables the user to resolve features smaller than the limit of diffraction, thus significantly enhancing the ability to capture biological processes at a molecular scale. Different technologies achieving this goal have been developed: stimulated emission depletion microscopy (STED), structured illumination microscopy (SIM), photoactivated localization microscopy (PALM), and stochastic optical reconstruction microscopy (STORM) (for a comprehensive review on super resolution microscopy, see [\[23,24\]](#)). In plants, this enhanced resolution was used to explore plant membrane organization [\[25\]](#), to resolve and quantify lateral diffusion of PIN proteins in membranes [\[26\]](#), and characterize viral protein movements through plasmodesmata [\[27\]](#). Nonetheless, although the physical resolution has increased, other severe constraints exist that limit the potential temporal resolution and the applicability to *in vivo* imaging for many super resolution systems.

Unwanted effects caused by refracted and scattered light, such as phototoxicity and photobleaching, have long been one of the major bottlenecks for *in vivo* confocal microscopy limiting both temporal and spatial resolution. Due to its fast image acquisition capability and high dynamic range, selective plane illumination microscopy (SPIM) is becoming the preferred method for imaging live fluorescent samples in 3D. This technique makes use of a thin (1–10 μm) light sheet across successively deep sections of the sample and in subsecond time frames, which minimizes phototoxicity and photobleaching [\[28,29\]](#). Notably, the thickness of the light sheet is adjusted depending on the sample to ensure homogeneous imaging. SPIM-based live images of emerging lateral roots (LRs) have been acquired to understand how LRs form in 3D and 4D. *In vivo* imaging of LR development showed that rather than following a strict pattern of cell division, it is the intrinsic mechanical constraint of the surrounding tissues that plays a role in the formation of LRs [\[30,31\]](#). Its technical advantages and current initiatives to make this technology more accessible to the research community by developing more cost-effective and easier to assemble and operate light sheet microscopes [\[32,33\]](#) are likely to make SPIM the tool of choice and thereby the source of the many 3D/4D image datasets needed for systems-level understanding of growth and development.

In addition to the development of new microscope technologies, sophisticated experimental setups and image processing and analyses algorithms can extend the ability to capture 3D or 4D phenotypes using conventional laser scanning confocal microscopy. For instance, real-time, automated 4D imaging during plant development has been achieved using multi-angle image acquisition three-dimensional reconstruction and cell segmentation-automated lineage tracking (MARS-ALT) [\[34\]](#). Furthermore, there are techniques such as optical projection tomography (OPT) that have tremendous power for 3D/4D analyses. OPT has been used to quantify the size and shape of clonal sectors in Snapdragon (*Antirrhinum majus*) flowers, which led to new insights into the role of tissue polarity organizers in the development and evolution of organ shape [\[35\]](#).

With this battery of new tools in hand, we are beginning to have the ability to quantify the mechanistic basis of cellular and molecular dynamics in living organisms, which is an important step in shifting from descriptive to more quantitative biology.

Macroscale resolution

Image analysis algorithms are the primary drivers in advancing imaging-based studies that require the quantification of macroscopic structures such as roots, stems, leaves, seeds, and flowers. A useful website (www.plant-image-analysis.org) describes 92 different image analysis software tools for studying plant biology [\[36\]](#). Some of the tools require user inputs such as manual point selection, whereas others are automated or semiautomated, which is important when the task is to analyze dynamic processes captured in multiframe time sequences ([Figure 2](#)). Roots have received considerable attention, because they can be grown within transparent gel media or on vertical gelled surfaces for the purposes of collecting images. Some tools have been designed to track growth rate, tip angles, and local curvature minute-by-minute to quantify mutant phenotypes [\[37–40\]](#), whereas other tools are designed to capture the overall architecture of branched root systems [\[41,42\]](#). Series of 2D images taken at different angles of rotation, rather than at different times, have been used to generate macroscopic 3D renditions of root system architecture [\[43,44\]](#). A third class of tools analyzes the time series of macroscopic images to characterize growth-related movement of material along the root with tracking algorithms to generate a kinematic description with microscopic spatial resolution [\[45–49\]](#). Micro-computed tomography, which uses X-rays instead of visible light, can be used to generate high-resolution 3D images of roots grown in soil [\[50\]](#).

Measurements of hypocotyl length in *Arabidopsis* seedlings have long provided important information about the mechanisms of light and hormone action. Formerly researchers would manually measure end-point lengths, but now computerized image analysis methods are being used. Different tools have been developed to measure responses to ethylene, light, and gravity, pinpointing when key events occur or mutant phenotypes begin to emerge [\[38,51–55\]](#).

The *Arabidopsis* rosette has also received a great deal of attention from the perspective of measuring growth from images. The relatively planar structure of the *Arabidopsis* rosette is effectively captured by overhead images that can be analyzed to determine features such as leaf area and compactness. Sequences of images can be automatically processed to determine rates of change in these descriptors [\[56–59\]](#). A laser-scanning technique has been shown to produce 3D renditions of *Arabidopsis* rosette morphology that can also capture hourly changes [\[60\]](#). This technology has also been used to study root system architecture [\[61\]](#) and will undoubtedly be used in other plant growth monitoring applications in the future.

The seeds from which all the above structures ultimately originate are also good examples of macroscopic structures that can be quantified with high resolution by image analysis [\[62–64\]](#).

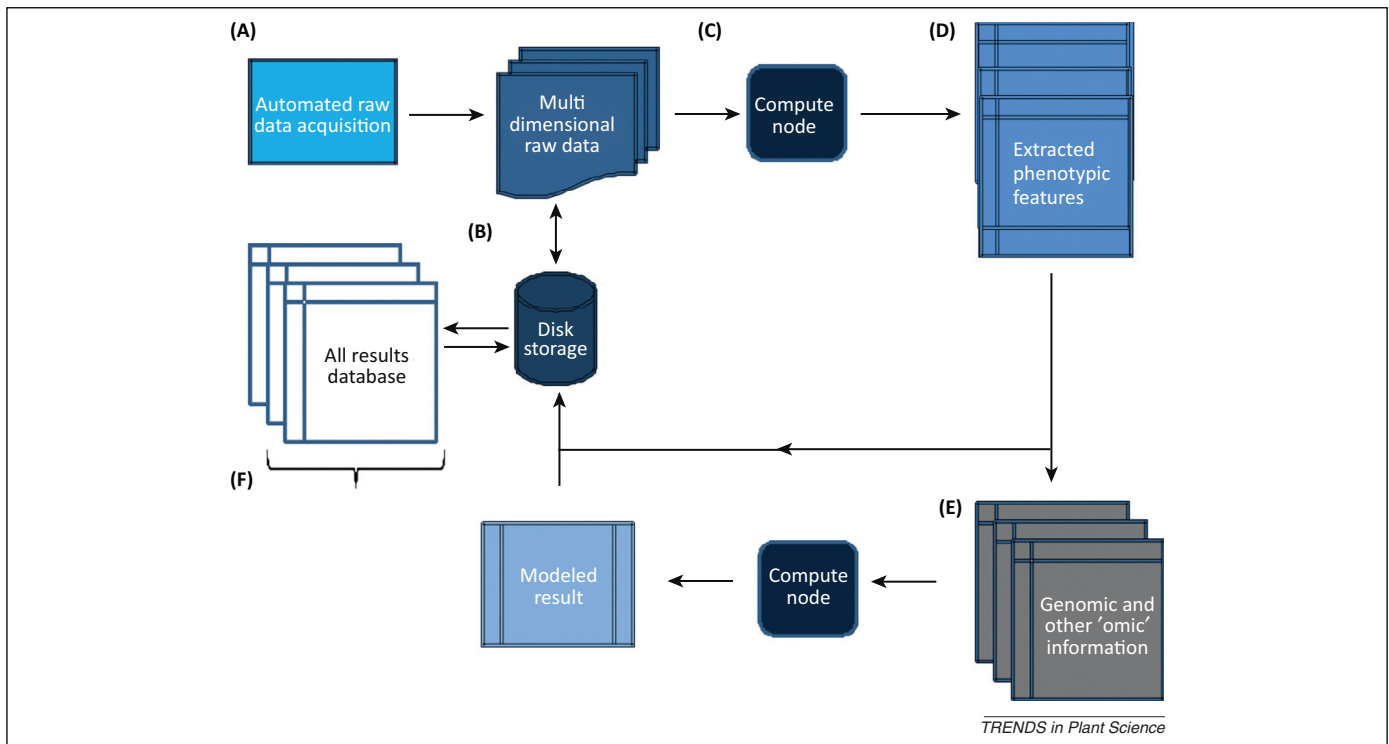


Figure 2. A typical workflow for the acquisition and processing of images. (A) An imaging device can produce multidimensional raw data such as z-stacks or time series of images that need to be saved to a disk. (B) Extracting information from those images requires a suitable algorithm and appropriate computing resources. (C) The features quantified from the images must be organized in some data structure such as a database in a manner that facilitates integration with other data types that will be combined to capture key features of the system under study (D). (E) These integrative analyses, collectively called modeling, can be computationally demanding. (F) Databases that can present the raw data as well as the modeled results to the broad scientific community, including image scientists, statisticians, mathematicians, bioinformaticists, as well as plant scientists, would be very enabling for modeling biological processes.

An important area of development in all the approaches discussed above is increasing automation so that large-scale population studies are feasible.

Throughput to capture populations and systems

Applying image-based trait measurement methodologies to studies of large populations, such as panels of natural accessions, collections of recombinant inbred lines (RILs), or populations of mutants will advance our understanding of biological systems because of the quantitative rigor inherent in the approach. Despite the remarkable advances in quantitative phenotyping capabilities effective over a range of scales, their systematic application to the study of large numbers of plants is still a tremendous challenge. A key issue for increasing the scope of experimentation to include populations of plants is maintaining a strictly controlled and reproducible growth environment over the course of multiple experiments. There has been substantial interest in phenotyping soil-grown plants to determine shoot growth, biomass, and responses to environmental stresses. Platforms designed for this purpose employ either a robotically movable camera or move plants to a stationary camera. In setups designed for use with *Arabidopsis*, a top view image of the rosette is acquired and the rosette area is measured using automated image analyses [59,65,66]. Image-based phenotyping for monocots, such as rice, is complicated by their morphology. For these, a simple top view is often not sufficient and thus side view images are required. Due to the large sizes of shoot phenotyping devices, the heterogeneous nature of soil and time

frames that typically span several weeks, a major challenge is to provide uniform growth conditions for each plant. This is complicated by micrometeorological conditions that exist within glass houses or growth chambers. Platforms such as GlyPh (a low-cost platform for phenotyping plant growth and water use), WIWAM [65], and PHENOPSIS [67] attempt to address these issues by weighing pots and adjusting for evaporated water. However, such systems are still subject to position-dependent environmental effects. The Phenoscope pipeline [66] not only adjusts for water loss but also automatically, and at regular intervals, rotates the position of all pots.

Phenotyping of root-related traits usually requires the plants to be grown in transparent, non-soil media. Although this limits the applications to more reductionist experimental designs, it also alleviates many of the challenges associated with micro-environmental noise. Furthermore, the simple structure of the root and its optical properties facilitate the development of a broad range of applications at different spatial and time scales. Aided by the small size of *Arabidopsis* seedling roots, various platforms have been developed that can harbor many individuals in parallel and thus provide a larger throughput. The observation of gravity stimulus-induced growth responses at relatively small time scales and with a high throughput was initially achieved by multiplexing automated image acquisition. Parallel data acquisition was performed using 11 identical setups in which plants grown on the agar surface of plates and illuminated from behind were imaged using charge-coupled device CCD

cameras [68]. More than 10^6 images were captured and subsequently used to map quantitative trait loci (QTLs) underlying the natural variation of gravitropic growth responses thereby providing a first glimpse into the time-resolved genetic architecture of a dynamic trait. By substituting the multiplexing of cameras with a robotic platform, further improvement of throughput for similar assays was achieved. Backlit plates are arranged in a vertical grid and two cameras, each providing a different resolution, are mounted on a three-axis robot whose position is controlled based on real-time detection of roots [69]. Not only physiological responses at the organ level can be assessed at a large scale but also processes at the cellular level. To achieve this, the microscopy needs to be automated. A simple agar-filled growth chamber in conjunction with automated confocal microscopy has been used for morphometric measurements on hundreds of natural accessions of *Arabidopsis*, allowing genome-wide association mapping of cellular traits [70]. A further level of automation is required to capture responses at the cellular level, such as spatiotemporal changes in gene expression upon changing growth conditions. This was achieved with a microfluidics device called the RootArray (Figure 1). In this platform, up to 64 plants at a time can be subjected to growth condition changes. An integrated software solution controls a confocal microscope to acquire high-resolution images of root tips. Automated image processing is then utilized to extract expression changes of fluorescent marker proteins in different root tissues at different time points [71].

Other challenges are encountered for phenotyping older roots and for understanding the 3D architecture of root systems. A critical issue is to preserve the topology of root architecture during image acquisition and to deal analytically with the complex 3D structures of root systems. A relatively simple but efficient high-throughput approach has been developed for rice (*Oryza sativa*) roots: plants are grown in a hydroponic environment and subsequently scanned and computationally analyzed [72]. Although this approach is very high-throughput, information about 3D root system architecture (RSA) cannot be captured. An alternative method provides 3D information on RSA with some reduction of throughput, using a transparent gellan gum medium. Plants are grown in transparent cylinders and imaged on a computer-controlled turntable with a stationary camera to acquire images from different angles. From these images, a 3D representation of the root system can be generated and traits derived. This provides a powerful tool for studying the genetic bases of a complex 3D developmental process [44].

Although methods providing the throughput to quantitatively phenotype whole populations are still in their infancy, these examples indicate the potential of such approaches. The benefits from using such procedures are the ability to: (i) sample a greater genotypic or treatment area; (ii) compare different experimental series; (iii) assess and reduce the environmental noise that confounds measurements; (iv) assess the significance and the magnitude of the effect compared with an empirically acquired distribution derived from all plants in the assay; and (v) free observations from human bias. This leads to a

more powerful method for detecting phenotypic changes in an unbiased and quantitatively robust manner [71], of revealing the genetic bases of complex genetic traits that govern plant form and growth [44,66,72], mapping genes and alleles underlying such traits [70], and of re-evaluating classical assays, models, and assumptions [65].

Concluding remarks

Recent technical developments for imaging plant growth and development pave the way for obtaining data with unprecedented spatial and temporal resolution and throughput. Whereas higher resolution directly determines the level of detail of biological processes that can be observed and analyzed, higher throughput can provide a solid statistical basis for quantitative observations. The integration of image-based data with data from genome-wide approaches provides the capability of modeling biological processes over time and at different scales. The quantitative system-level investigation of development with novel imaging and image processing technologies has only just begun. The future success of such studies will critically depend on combining technological and conceptual advances in different disciplines.

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