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ON THE NATURE OF THINGS
New Ideas and Directions in Botany

New frontiers in the three-dimensional visualization of plant structure and function[1]

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For thousands of years, humans have acknowledged the existence of structures and organisms that exist at a scale unresolvable with the naked eye. Not until the invention of the compound microscope in the late 1500s were structures magnified sufficiently to reveal the previously unseen (Bolam, 1973). The resulting observations were revolutionary and radically altered contemporary thinking on the structure and function of living organisms.

Pioneering work by Nehemiah Grew (1682) and Marcello Malpighi (1686) identified what at the time were considered novel structures in plants. Grew clearly recognized that plant tissues needed to be conceptualized in three dimensions (3D) because they were composed of microscopic structures with distinct spatial relationships. He made some of the first attempts at reconstructing vascular elements in 3D by introducing depth and perspective to his illustrations (Figs. 1A, 2), and almost certainly dealt with the technological limitations of visualizing the inner depths of opaque, 3D tissues (Fig. 1B). Grew's illustrations provided the first indications of how complex and varied the internal organization of plants can be (Fig. 2), and in the subsequent 300 years, our understanding of the spatial organization of plant vascular systems has increased significantly.

However, major obstacles have persisted in understanding the fundamental relationships between xylem structure and function, which are directly related to the challenge of visualizing 3D structures with traditional, two-dimensional techniques. Since Grew's time, the advent of photography and advanced histological techniques have significantly improved the visualization and reproduction of xylem networks compared with manual illustrations. Yet, to reconstruct the pathway that water traverses through roots and stems, hundreds of serial cross sections are needed. Those sections must then be stacked in perfect alignment to reconstruct the xylem network manually, which is a significant and tedious task. As a consequence, the 3D internal structure of plants has remained largely unexplored.

The development of the optical shuttle technique (Zimmermann and Tomlinson, 1966) was a major step forward. Using traditional serial sectioning and light microscopy, the optical shuttle method exposes each serial section onto subsequent frames of motion picture film, thereby assigning the axial position of each serial section to a specific point in time. Playing the movie in the forward or reverse direction allows the viewer to quickly explore extraordinarily complex xylem networks. Indeed, much of what we know about xylem network connectivity and development originates from optical shuttle serial sectioning and the tireless efforts of early pioneers. For example, what appeared in two-dimensional cross sections of palm stems to be a random, scattered distribution of vascular bundles were actually an elegant and efficient arrangement of bifurcating tissues (Zimmermann et al., 1982). Even with the development of the optical shuttle method, manual reconstruction of xylem networks remains a significant obstacle. Advancements in nondestructive, 3D imaging technologies are beginning to overcome many limitations of manual serial sectioning and are providing new insight into the spatial organization of plants in both extant and fossilized material. In this essay we highlight recent advancements in nondestructive 3D imaging that have the potential to fundamentally change our knowledge of plant anatomy and physiology.

Nuclear magnetic resonance (NMR) imaging is gaining popularity for the study of plant vascular function, and plant-specific facilities have emerged to meet the growing demand (e.g., Wageningen NMR Centre). NMR imaging has the advantage of being noninvasive, allowing researchers to study intact, living plants and monitor the functional status of xylem and phloem networks without disrupting the positive and negative pressures that drive

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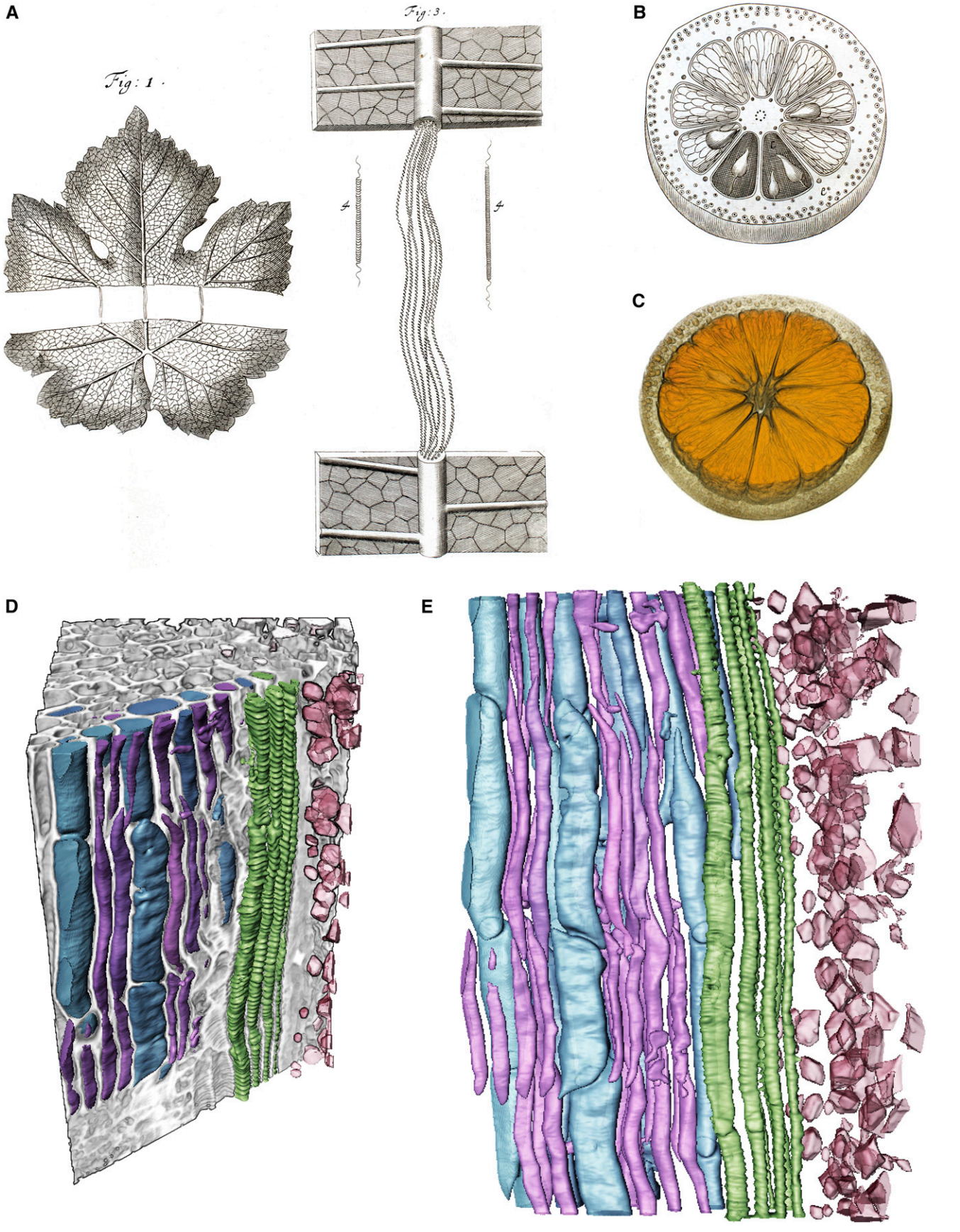
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these systems. NMR's sensitivity to hydrogen isotopes means that we can not only visualize whether xylem conduits are filled with air (i.e., embolisms) or water (Scheenen et al., 2000; Holbrook et al., 2001; Fig. 1C), but also measure sap flow velocity and direction in both the xylem *and* phloem. Such experiments have yielded some of the only estimates of phloem flow rates in living plants (Windt et al., 2006; but see Savage et al., 2013). There are, however, significant shortcomings of current NMR imaging systems that have precluded their application in many plant systems, principally image resolution, the exclusion of ferromagnetic instruments for simultaneous physiological measurements, and the logistical and dimensional constraints of current generation NMR magnets. For example, in most NMR systems the entire sample must fit inside the long (~1 m), narrow diameter (<10 cm) bore of the magnet. As a result, research has focused on potted vines, saplings, and species with large diameter vessels to overcome image resolution limitations. New NMR instruments with clamshell magnet designs, however, can accommodate a wide range of stem diameters (Windt et al., 2011; Windt and Blümler, 2015).

Some of the most diverse and promising work is emerging from implementations of X-ray computed microtomography (micro-CT). By rotating a sample positioned in an X-ray beam, hundreds of digital two-dimensional images can be reconstructed to produce 3D data sets functionally identical to Zimmermann and Tomlinson's optical shuttle method (Brodersen, 2013), but in minutes instead of hours or days. Moreover, micro-CT is nondestructive, and samples can be virtually sectioned in any plane to study the spatial relationships of internal structures (Fig. 1D, E). Though micro-CT systems were originally available only at large, synchrotron facilities, laboratory-based systems have become available that will broaden access to this method. Current micro-CT imaging systems provide a wide range of magnifications, with voxel (volumetric pixel element) resolutions from greater than 50 μm to less than 325 nm, allowing for investigations of gross anatomy down to the structure and distribution of pits in xylem conduit walls.

In addition to providing qualitative information about structure in 3D, micro-CT imaging systems facilitate quantitative analyses, such as the frequency of connections between adjacent conduits in a xylem network (Brodersen et al., 2011; Lee et al., 2013). The ability to map and analyze xylem networks in 3D will yield unprecedented information on xylem structure and function not readily available from traditional methods and, additionally, can be used to address unanswered questions in plant hydraulics. For example, one of the longstanding paradigms of xylem structure are the expected tradeoffs among hydraulic safety from embolism, conductive efficiency, and mechanical strength (Baas et al., 2004). Yet, a recent meta-analysis suggests that these tradeoffs in stems are weak at best (Gleason et al., 2015). A 3D network analysis could resolve uncertainty in the relationships of conduit properties one can measure in two dimensions using traditional methods (e.g., diameter, density, vessel grouping index) compared with 3D network properties (e.g., total pit area, degree of network connectivity).

While most work using these new methods has focused on stems, any plant structure can be imaged (e.g., Fig. 1C, D). For example, characterizing the 3D structure of leaves could better refine our understanding of the flow paths of both liquid and gaseous water, as well as the organization of air spaces inside the leaf (e.g., Verboven et al., 2015). Compared with leaves, which are often treated as planar structures, flowers and fruits are more obviously three-dimensional, fragile, and difficult to section and, thus, perfectly suited for 3D micro-CT imaging (e.g., Wang et al., 2015). At the microscopic scale, 3D imaging could be used to examine cellular shape and its influence on other traits such as optical properties of epidermal cells (Glover and Whitney, 2010). As plant biologists continue to develop a more comprehensive approach to studying whole plant structure and function, these techniques could be used to examine numerous traits in flowers, fruits, pollen, and seeds and in vegetative structures such as leaves, stems, and roots. The ability to visualize structures noninvasively is also a key advantage for studying rare specimens. Fossilized plants are often limited in abundance, and microCT imaging has been used to study morphological traits of extinct plants to infer temporal patterns in trait evolution and the phylogenetic relatedness of lineages (DeVore et al., 2006; Friis et al., 2007; see Crepet et al., 2016 in this issue).

Perhaps the most exciting opportunity afforded by these new visualization techniques is not their ability to capture static morphological and anatomical features, but rather their ability to view physiological processes in vivo. For example, time-lapse micro-CT imaging of drought-stressed grapevines has been used to track embolism spread (Brodersen et al., 2013) and repair (Brodersen et al., 2010; Knipfer et al., 2015), both of which are enigmatic phenomena that previous imaging attempts had documented but without sufficient resolution to explore specific mechanisms (Holbrook et al., 2001). Similarly, NMR imaging has been crucial in measuring rates of xylem and phloem flow to highlight water transport dynamics to developing fruits (Windt et al., 2009). Noninvasive visualization and quantification of physiological processes with these new techniques will prove to be fundamental in our understanding of the relationships between plant structure and function.

We envision two key advances in the coming years. First, instrumentation improvements will increase both the resolution of images and the size of the fields of view, and these gains will ameliorate many logistical challenges of imaging the whole macroscopic organism and all of its microscopic parts. In addition, the application of contrasting agents may help to demarcate structures in ways that X-ray absorption alone cannot, much like fluorescent dyes and markers enable confocal laser scanning microscopy to better resolve

FIGURE 1 Historic and modern visualizations of the complexity of plant anatomy. The three dimensionality of xylem (A) and other plant structures (B) were evident to Grew in the late 1600s (Grew, 1682), but the limitations of traditional light microscopy have prevented a full investigation of the spatial organization of plant tissues. New advancements in nuclear magnetic resonance imaging (C) and X-ray microcomputed tomography (microCT) (D, E) allow for nondestructive, virtual dissections of plant materials that have been otherwise impossible. New imaging techniques match the ingenuity of early botanical investigations and push the boundaries of our understanding of xylem networks. Virtual dissections of citrus petiole xylem (D, E) reveal the spatial organization of xylem formed at different stages, showing helical thickenings (green), secondary xylem (blue), fibers and axial parenchyma (purple), and calcium oxalate crystals distributed in the pith (red). The long edge of the petiole sample in (D) is 350 μm.

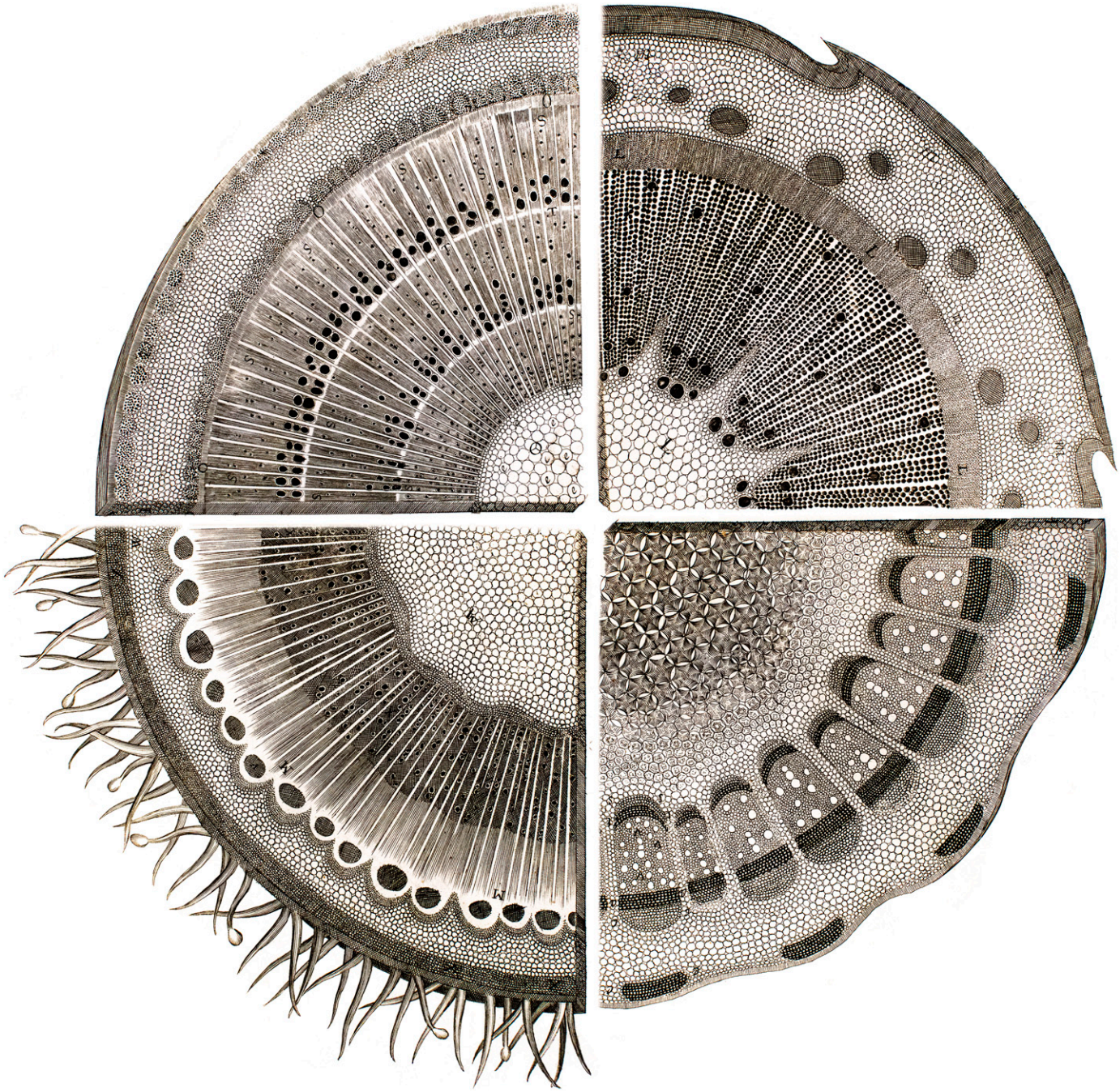

FIGURE 2 Composite image of four drawings from Nehemiah Grew's 1682 book *The Anatomy of Plants*. Grew's observations with early light microscopes acknowledged the complexity and three-dimensional properties of xylem networks that we struggle to understand today. Clockwise from upper left: plate 29, "ash branch"; plate 32, "pine branch"; plate 38, "thistle stalk"; plate 34, "sumach branch".

structures. The second type of improvement may come not from instrumentation improvement but instead from combining multiple imaging techniques to optimize the strengths of different methods (e.g., positron emission tomography, micro-CT, and X-ray fluorescence). Overall, the shift toward rapid, 3D analysis of anatomy and physiology with new imaging tools marks an important turning point in our understanding of plant structure and function and will help to resolve longstanding research bottlenecks that can be traced back to the original observations of Grew and Malpighi and the birth of botanical investigation.

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