Video Article

Using High Resolution Computed Tomography to Visualize the Three Dimensional Structure and Function of Plant Vasculature

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Abstract

High resolution x-ray computed tomography (HRCT) is a non-destructive diagnostic imaging technique with sub-micron resolution capability that is now being used to evaluate the structure and function of plant xylem network in three dimensions (3D) (*e.g.* Brodersen *et al.* 2010; 2011; 2012a,b). HRCT imaging is based on the same principles as medical CT systems, but a high intensity synchrotron x-ray source results in higher spatial resolution and decreased image acquisition time. Here, we demonstrate in detail how synchrotron-based HRCT (performed at the Advanced Light Source-LBNL Berkeley, CA, USA) in combination with Avizo software (VSG Inc., Burlington, MA, USA) is being used to explore plant xylem in excised tissue and living plants. This new imaging tool allows users to move beyond traditional static, 2D light or electron micrographs and study samples using virtual serial sections in any plane. An infinite number of slices in any orientation can be made on the same sample, a feature that is physically impossible using traditional microscopy methods.

Results demonstrate that HRCT can be applied to both herbaceous and woody plant species, and a range of plant organs (*i.e.* leaves, petioles, stems, trunks, roots). Figures presented here help demonstrate both a range of representative plant vascular anatomy and the type of detail extracted from HRCT datasets, including scans for coast redwood (*Sequoia sempervirens*), walnut (*Juglans* spp.), oak (*Quercus* spp.), and maple (*Acer* spp.) tree saplings to sunflowers (*Helianthus annuus*), grapevines (*Vitis* spp.), and ferns (*Pteridium aquilinum* and *Woodwardia fimbriata*). Excised and dried samples from woody species are easiest to scan and typically yield the best images. However, recent improvements (*i.e.* more rapid scans and sample stabilization) have made it possible to use this visualization technique on green tissues (*e.g.* petioles) and in living plants. On occasion some shrinkage of hydrated green plant tissues will cause images to blur and methods to avoid these issues are described. These recent advances with HRCT provide promising new insights into plant vascular function.

Video Link

The video component of this article can be found at http://www.jove.com/video/50162/

Introduction

Water is transported from plant roots to the leaves in a vascular tissue called xylem - a network of interconnected conduits, fibers, and living, metabolically active cells. Transport function of plant xylem must be maintained to supply nutrients and water to leaves for photosynthesis, growth, and ultimately survival. Water transport in xylem conduits can be disrupted when the xylem network is compromised by pathogenic organisms. In response to such infections plants often produce gels, gums, and tyloses as a means to isolate pathogen spread (*e.g.* McElrone et al 2008; 2010). Drought stress can also limit water transport in xylem. As plants lose water during prolonged drought, tension builds in the xylem sap. Water under tension is metastable (*i.e.* at a certain threshold the tension becomes great enough to cavitate water columns contained in xylem conduits). After cavitation occurs, a gas bubble (embolism) can form and fill the conduit, effectively blocking water movement (Tyree and Sperry 1989), a phenomenon analogous to decompression sickness (*i.e.* "the bends") in deep sea divers.

Despite the importance of xylem water transport for optimal plant function as demonstrated by a vast body of historical and contemporary literature on this topic (Tyree & Zimmermann, 2002; Holbrook *et al.*, 2005), there are still aspects of xylem networks that remain elusive. Several research groups have recently begun utilizing High resolution x-ray computed micro-tomography (HRCT) to evaluate finer details of wood anatomy and vascular tissue (*e.g.* Mayo et al; 2010, 2008; Mannes *et al.* 2010; Brodersen *et al.* 2010, 2011, 2012a,b; Maeda and Miyake, 2009; Steppe *et al.* 2004). HRCT is a nondestructive technique used to visualize features in the interior of solid objects and to obtain digital information

on their 3-D structural properties. HRCT differs from conventional medical CAT-scanning in its ability to resolve details as small as a micron in size, even for high density objects. Recent advances in synchrotron HRCT technology have improved image resolution and signal to noise ratio sufficiently so that plant vessel networks and intervessel connections can be visualized, assigned 3D coordinates, and exported for hydraulic model simulations. Brodersen *et al.* (2011) recently advanced this technique by combining 3D reconstructions generated by synchrotron HRCT with a Fortran model that automatically extracts data from the xylem network at much higher resolution than was ever possible with traditional anatomical methods (*i.e.* serial sectioning with a microtome and image capture with light microscopy, *e.g.* Zimmermann 1971). This work has also been used to optimize hydraulic models of the xylem system and identified unique characteristics of transport (*i.e.* reverse flow in some vessels during periods of peak transpiration) (Lee *et al.*, in review).

Synchrotron HRCT can now be used to visualize xylem functionality, susceptibility to cavitation, and a plants' ability to repair embolized conduits. Failure to re-establish flow in embolized conduits reduces hydraulic capacity, limits photosynthesis, and results in plant death in extreme cases (McDowell *et al.* 2008). Plants can cope with emboli by diverting water around blockages via pits connecting adjacent functional conduits, and by growing new xylem to replace lost hydraulic capacity. Some plants possess the ability to repair breaks in the water columns, but the details of this process in xylem under tension have remained unclear for decades. Brodersen *et al.* (2010) recently visualized and quantified the refilling process in live grapevines using HRCT. Successful vessel refilling was dependent on water influx from living cells surrounding the xylem conduits, where individual water droplets expanded over time, filled vessels, and forced the dissolution of entrapped gas. The capacity of different plants to repair compromised xylem vessels and the mechanisms controlling these repairs are currently being investigated.

Description of the ALS facility Beamline 8.3.2

Our work to date has been conducted on the Hard X-ray Micro-Tomography Beamline 8.3.2 at the Advanced Light Source in Lawrence Berkeley National Lab (Berkeley CA USA). Plant samples are placed in a lead-lined hutch located 20 m from the x-ray source, generated by a 6 Tesla superconducting bend magnet dipole within the Advanced Light Source electron storage ring operating at a critical energy of 11.5 KeV. A schematic of the end station is shown in **Figure 1**. The x-rays enter the hutch with a beam size of 40x ~4.6 mm and pass through the sample that is mounted on a motorized rotating stage. The transmitted x-rays impinge on a crystal scintillator (two materials commonly used are LuAG or $CdWO_4$) which convert x-rays to visible light that is relayed via lenses onto a ccd for image collection. The camera, scintillator and optics are contained in a light tight box that is on rails that allows the sample-to-scintillator distance to be optimized for phase contrast imaging.

All samples are mounted on the 10 cm diameter rotary stage which in turn is mounted on horizontal and vertical translation stages for sample positioning. A living plant sample, with the root system mounted in a custom built plant pot holder and the foliage contained in an acrylic tube, can be seen in **Figure 2**. Typical exposure times can range from 0.1- 1 sec using 10-18 KeV, and scan durations will range from 5-40 min depending on the settings optimized for a particular sample. For tall samples (typical of plant xylem networks), data scans can be tiled by repeating the measurement with the sample at different heights, which is controlled automatically, allowing seamless serial sections along a maximum sample height of ~ 10 cm. Maximum sample width when imaging at 4.5 µm resolution is ~1 cm for samples that are nearly perfect in vertical orientation. Data generation and processing is completed using the protocol listed below. Because of the difference in x-ray attenuation between air and water, excellent image contrast can be obtained in plants without the use of contrast solutions typical of medical CT systems. The air-filled vessel lumen is easily distinguishable from the surrounding water-filled tissue in hydrated plants.

Protocol

Protocol details described below were written specifically for work at the Advanced Light Source 8.3.2 beamline. Adaptations may be required for work at other synchrotron facilities. Proper safety and radiation training is required for use of these facilities.

1. Sample Preparation for Live Plants

1. Grow plants in ~10 cm diameter pots, and ensure that the main stem (or portion of the plant to be scanned) is as centered as possible and oriented vertically in the pot. The physical dimensions of the HRCT instrument hutch at the Advanced Light Source limits live plants to ~1 m in height. As a consequence, imaging of live plants is best performed on seedlings/saplings grown in small pots. Depending on the experiment, different soil types can be used to control soil moisture content (*e.g.* in drought experiments), and for some plants with flexible shoots (*e.g.* vines) longer shoots can be carefully tucked into the acrylic tube described below (see **Figures 1** and **2**).
2. Mount the live potted plants in a custom-made rigid aluminum pot holder. The top plate height can be adjusted to accommodate a range of pot heights. The top of the plate is designed to align with the top of the soil surface, and the plant protrudes from the center of the two-part plate. The purpose of the pot holder is to ensure the plant stem is held firmly in place to minimize vibration or sample motion. Minimizing sample motion during a scan is essential.
3. Once mounted in the holder, measure the stem water potential or leaf transpiration using a Scholander style pressure chamber or a clip-on leaf porometer, respectively, to determine the physiological status of the plant prior to scanning.
4. Place a thin walled acrylic cylinder over the plant and on top of the aluminum plant holder and secure it in place with clay putty to stabilize the sample (**Figure 2**). Any vibration or movement of the upper foliage will be transmitted down the stem and cause the plant tissue within the scanned area to move, ultimately leading to image distortion. The cylinder is used to contain plant foliage and prevent plant leaves from rubbing against other pieces of equipment in the hutch that would result in vibrations during a scan. Additional plastic wrap, paper towels, and tape should be used to further minimize vibration and movement of plant parts (see problems associated with sample movement in **Figure 4**). To reduce its x-ray absorption (which can decrease the image quality at a given exposure time), the containing cylinder should have as thin walls as possible while maintaining sufficient rigidity to perform its function.
5. Attach the custom pot holder to the air bearing stage and lock it (screw) into place between the x-ray source and the imaging sensor and camera equipment. Position the stem as vertical as possible and center on magnetic chuck base to ensure the sample stays in the field of view during rotation.

2. Sample Preparation for Fresh, Excised Plant Tissue

1. Fresh plant material, typically stems or petioles, can be scanned after immediate removal from a live plant. If the intent of the experiment is to visualize the entirety of the xylem network, water within the vessels must be evacuated and replaced with air. To do this, mount the sample in a Scholander style pressure chamber and push compressed air or nitrogen through the sample at low pressure (< 0.05 MPa) for approximately 5 min. Species will differ in the time required to evacuate the vessel network. If the intent is to evaluate the extent of embolism formation in the fresh plant tissue, then excise samples from the plant using a fresh razor blade and make the cuts under water.
2. Wrap the sample in a layer of Parafilm to prevent desiccation during the scan.
3. Mount the sample in a drill-chuck fixed to a metal plate that is screwed into the air bearing stage. Center and orient the sample vertically as described above to ensure the sample remains in the field of view.

3. Sample Preparation for Dried Woody Tissues

1. For optimal tissue sample visualization and image contrast, it is necessary to slowly dehydrate the entire woody tissue sample. Cut samples to approximately 6 cm in length. Select samples that are as straight as possible in the targeted scan region and have a diameter of ≤1 cm.
2. Place the woody tissue sample into a drying oven at low temperature to slowly dry the sample without causing any cracking or splitting of the tissue. This process is likely to differ between species and tissues. For woody stems, 12 hr in a 40 °C oven is typically sufficient to provide excellent contrast without causing significant changes in the physical structure of the stem (see problems with rapid drying demonstrated in **Figure 3**).
3. In some situations it is desirable to have a fiduciary marker within the sample such that subsequent dissection and visualization with scanning electron microscopy can be oriented to specific points in the HRCT image. To do this, affix a metal or glass bead or wire to the outside of the stem using Parafilm. Another method is to use a silicone resin (*e.g.* RTV-141, Bluestar Silicones, East Brunswick, NJ) that can be injected into a single xylem conduit (see examples in Brodersen *et al* 2010). Once hardened, the silicone resin is clearly visible in the sample and easily distinguished from the other air-filled vessels. Use this marker to precisely locate specific regions of the sample.
4. Mount the sample in the drill chuck and center as described above.

4. Sample Preparation for Leaf Tissue for Two Dimensional (2D) Radiograms

1. To visualize vessel contents in leaves in near-real-time, leaves can be scanned to produce a 2D radiogram, similar to a dental x-ray. Mount the leaf between two sheets of thin acrylic plastic, and secure the edges with clips. Then attach the mounted sample to a post-holder system and position the optical breadboard next to the imaging system and x-ray source.

5. Scanning the Sample in the 8.3.2 Hutch

1. Decide the magnification that will work best for your application. ALS Beamline 8.3.2 has the capability to scan with lenses with magnifications of 2x, 5x, and 10x. These result in image pixel sizes of 4.5, 2.25, and 0.9 µm, respectively. Depending on the magnification, the sample must be of appropriate size, as the field of view decreases with increasing magnification. See details for choice of camera and lens and the resultant image parameters in **Table 1**.

	PCO.4000 (4008x2672)		**PCO.Edge (2560x2160) (Optique Peter)**	
Lens	**pixel (µm)**	**field of view (mm)**	**pixel (µm)**	**field of view (mm)**
10x	0.9	3.6	0.65 (0.69)	1.7 (1.7)
5x (4x)	1.8	7.2	1.3 (1.72)	3.3 (4.4)
2x	4.5	18	3.25 (3.44)	8.3 (8.8)
1x	9	36	6.5 (-)	16.6 (-)

Table 1. Details regarding available cameras and lenses at ALS 8.3.2.

2. Set the x-ray energy to 15 keV. This has been shown to provide excellent image contrast for most plant applications (see Brodersen *et al.* 2010, 2011, 2012a,b). Exposure times are generally dependent on the thickness and density of the sample (and thus the magnification used) range between 100 and 1,000 msec. Longer exposure times (as long as detector pixels are not saturated) will generally lead to higher signal to noise ratio, but at the cost of increased scan times.
3. Choose an angular increment that is appropriate for your application. Samples are rotated 180° during a scan, and the number of images taken during the rotation can have a significant impact on size of the dataset, length of the scan interval, and final image quality, but there are generally diminishing returns in quality. Typical scans are performed at 0.25° increments, yielding 721 images per scan. Decreasing the increment to 0.125° results in better images for visualizing fine details, but yields 1,440 images and thus a much larger dataset (for a typical region of interest, this means ~10-30 GB of data vs. 5 GB). However, the signal to noise ratio is often improved and worth both the increased scan time and data size. Dry stems that are unlikely to deform/shrink during a scan can be subjected to longer intervals (smaller angular increment) without detriment. When imaging live plants, where biological processes (*e.g.* embolism repair) take place on short time scales, opting for the shorter scan intervals is preferable to limit potential damaging effects of x-ray radiation on this tissue- although this comes at a potential loss of image quality. Shorter scan intervals can be achieved using the Continuous Tomography setting during which the sample continuously rotates while the images are captured.

4. For each scan, "bright field" and "dark field" images must be corrected. Bright field images are images without the sample in the beam. These are often collected before and after the scan of the sample by horizontally translating the sample. Dark fields are collected by closing the x-ray shutter—this measured the amount of signal the camera shows with no x-rays.

6. Data Processing

1. Transfer the "raw" 2D .TIF images, which were exported from the acquisition computer to a file server, to a data processing computer. If the computer has sufficient RAM, the data can be copied to a so-called "RAM Drive" (a portion of the RAM appears as a hard drive on the computer). In this way the software does not have to access a spinning hard drive, which is comparatively slow compared to a solid state drive or flash memory. This step significantly reduces the amount of time required to process datasets.
2. The images must be converted to a percent transmission scale. Beamline 8.3.2 has a custom background normalization plug-in that can be downloaded and used with the freely available software packages ImageJ or Fiji (http://fiji.sc/). It subtracts the dark counts from the images and normalizes the sample images by the bright fields to yield images that show percent transmission. Load normalized images into the Octopus software package (http://www.inct.be/en/software/octopus) and "reconstruct" the 3D dataset from the 2D raw .TIF files using the designated processing steps (Normalize images, Ring removal, Sinogram creation, Parallel beam reconstruction). This process then yields a series of .TIF transverse (cross sectional) images composed of "voxels" (volumetric pixel elements), each with an x, y, z coordinate and intensity values representing the x-ray linear absorption coefficient.

7. Visualization

1. Visualize the stack of images in one of a variety of software packages. Freeware (*e.g.* Drishti, http://anusf.anu.edu.au/Vizlab/drishti/index.shtml) can be used to visualize volumes or individual or stacks of images (*e.g.* ImageJ or FIJI). Other software packages can be used for 3D visualization. Our research group uses the Avizo software package (http://www.vsg3d.com/avizo/overview), but others such as Amira (http://www.amira.com/) and VGStudioMax (http://www.volumegraphics.com/) are also commonly used.
2. Load datasets into system memory and display the sample in virtual transverse, longitudinal, or radial slice orientations. Because of the 3D attributes of the dataset, virtual slices through the sample can be rotated in any plane to align with the regions of interest, a significant improvement over traditional serial light microscopy (see Movies 1-3 for detailed examples).
3. To visualize the sample as needed in 3D, "segment" the sample using the variety of semi-automated and manual routines in Avizo to separate vessel lumens or other structures from the surrounding tissue. Segmentation refers to defining boundaries between objects of interest, thus separating or segmenting them into separate regions. Rendering volumes in 3D is performed by the visualization software. One method to do this is direct volume rendering, where each point in a volume is assumed to emit and absorb light; the amount and color of emission and absorption can be defined using a "colormap", and the resulting projection in a given direction is displayed on the screen. Alternatively, a wireframe or 3D mesh surface representing the segmented boundaries is constructed to show a 3D model of the structure of interest. The 3D mesh is composed of polygonal elements, and the total number of elements will affect both the fidelity of structure reproduction and the size of the associated data file (*i.e.* more elements leads to higher fidelity but larger file size). A variety of image processing modules are available within the visualization software to control the volume rendering outputs, as well as control for image brightness, contrast, transparency, noise reduction, *etc.*

8. Quantification

1. Once segmentation has been accomplished, it is possible to quantify the target plant structures or functional changes in volume, length, width, presence or absence of water, air, *etc.* For example, Brodersen *et al.* (2010) used Avizo software to quantify the volume change of water droplets inside grapevine refilling vessels. Plants were scanned every 30 min over four to eight hours creating a time-lapse sequence of vessel refilling. Each scan was reconstructed and loaded into Avizo, where individual droplets were measured over time as their volume increased.

Representative Results

Synchotron HRCT scans have been successfully implemented on a wide variety of plant tissues and species using beamline 8.3.2 (**Figure 5**), and have provided new insights into the structure and function of plant xylem at unprecedented resolution in 3D. The visualization and exploration capabilities provided by the 3D reconstructions (as illustrated in **Figures 6-8**; and Movies 1-3) allow for precise determination of location and orientation of structures with the xylem networks on both excised samples and in living plants.

In some situations, sample movement or unintended vibrations have caused distortions in the final images, rendering the scans unusable (*e.g.* **Figure 4**), but the improvements to decrease scan time (with continuous tomography) have minimized the detrimental effects of such data losses because many more scans can now be completed in the limited beamtime allocated to each user. These shorter scan times also enable repeated measures of a single replicate over time to capture the dynamics of processes like embolism spread and repair.

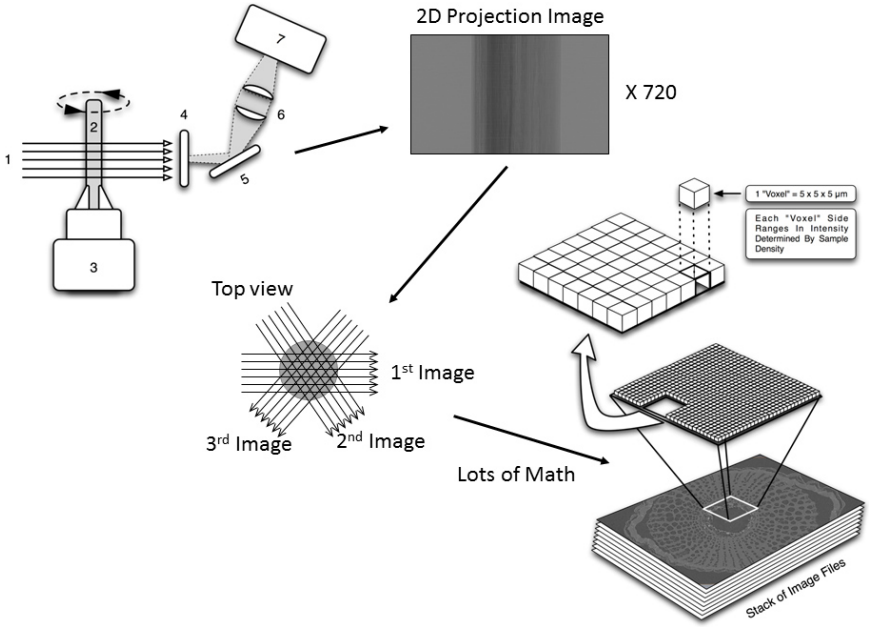

Figure 1. Schematic of sample scanning procedure and setup inside the hutch at ALS beamline 8.3.2. Upper left: The x-ray source beam (1) is projected through the sample (2) that is attached to the air table with a drill chuck that rotates during scanning. The x-rays that pass through the sample impinge on a crystal scintillator (4) which fluoresces visible light that is redirected by a mirror (5) through lenses (6) to a ccd camera (7) that captures a digital image. The "raw" 2D x-ray images (upper right image- example is a plant stem sample rotated 180 ° during a full scan at an increment of 0.25 ° resulting in 720 2D images) are transformed and result in a stack of transverse images (bottom right) that are used for the 3D reconstructions. Click here to view larger figure.

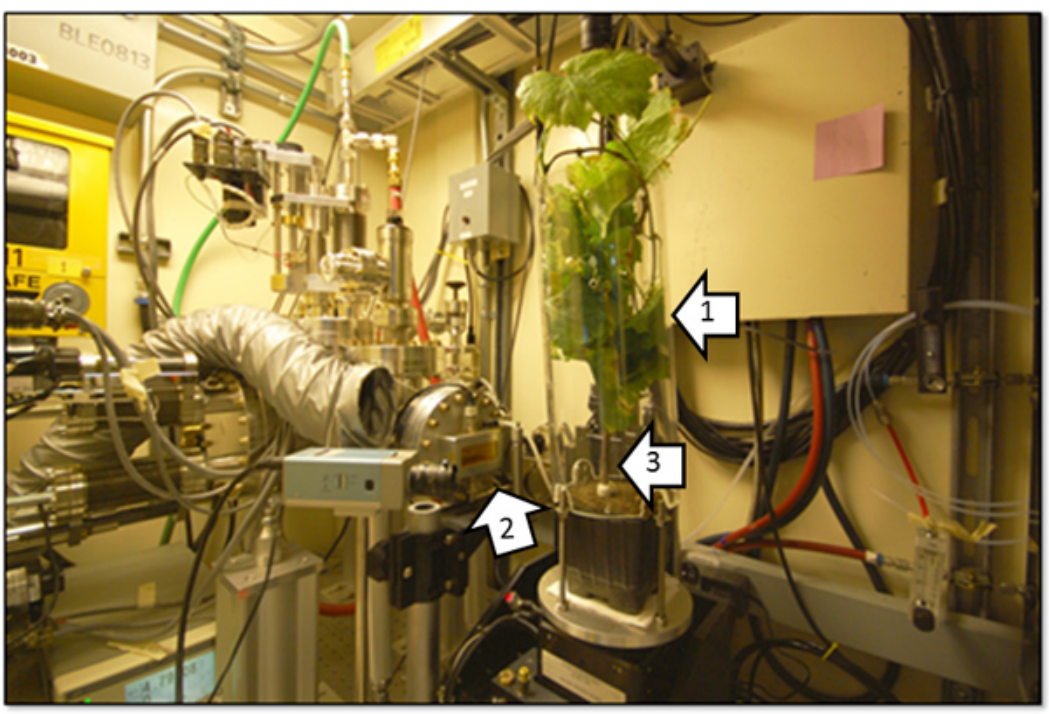

Figure 2. Image taken inside the hutch of the ALS beamline 8.3.2 showing a live, potted grapevine prepared for scanning. The vine is contained in an acrylic tube (1). The x-ray beam enters the hutch to the left (2), then passes through the sample (*e.g.* the grapevine stem) (3) and then enters a light tight box containing the camera, scintillator and optics (box not shown in this image).

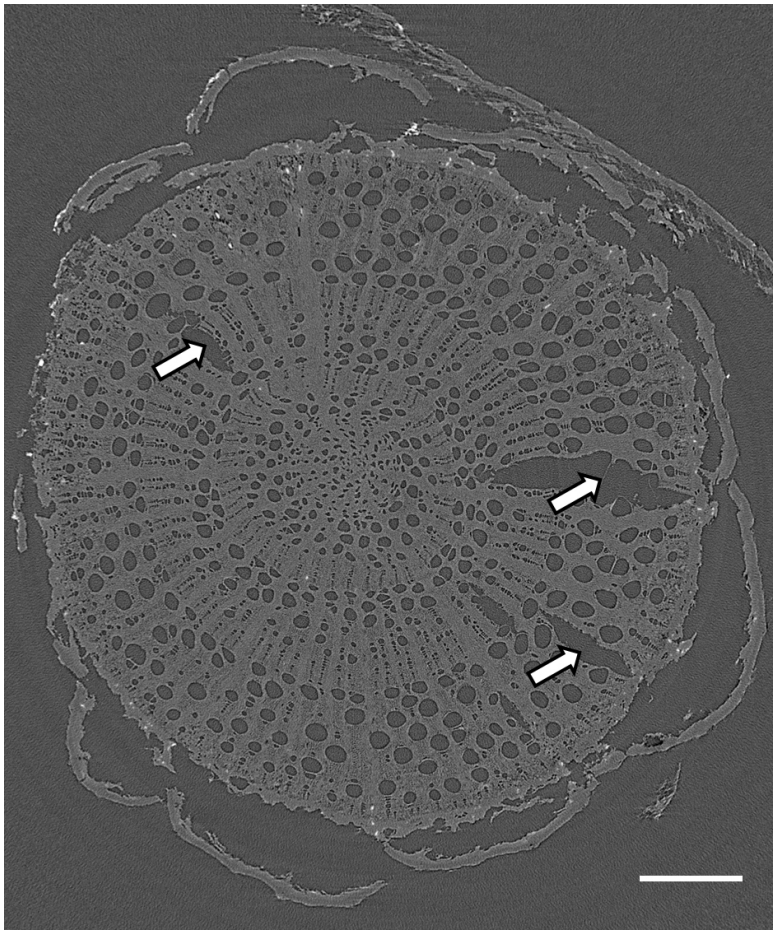

Figure 3. Example of sample cracking (denoted with the white arrows) when a woody root (seen here) was subjected to drying for too long and/or at too high a temperature. To avoid this damage and to maintain structural integrity and faithfulness to tissue structure *in vivo* dehydration requires some testing ahead of time. Scale bar = 1 mm.

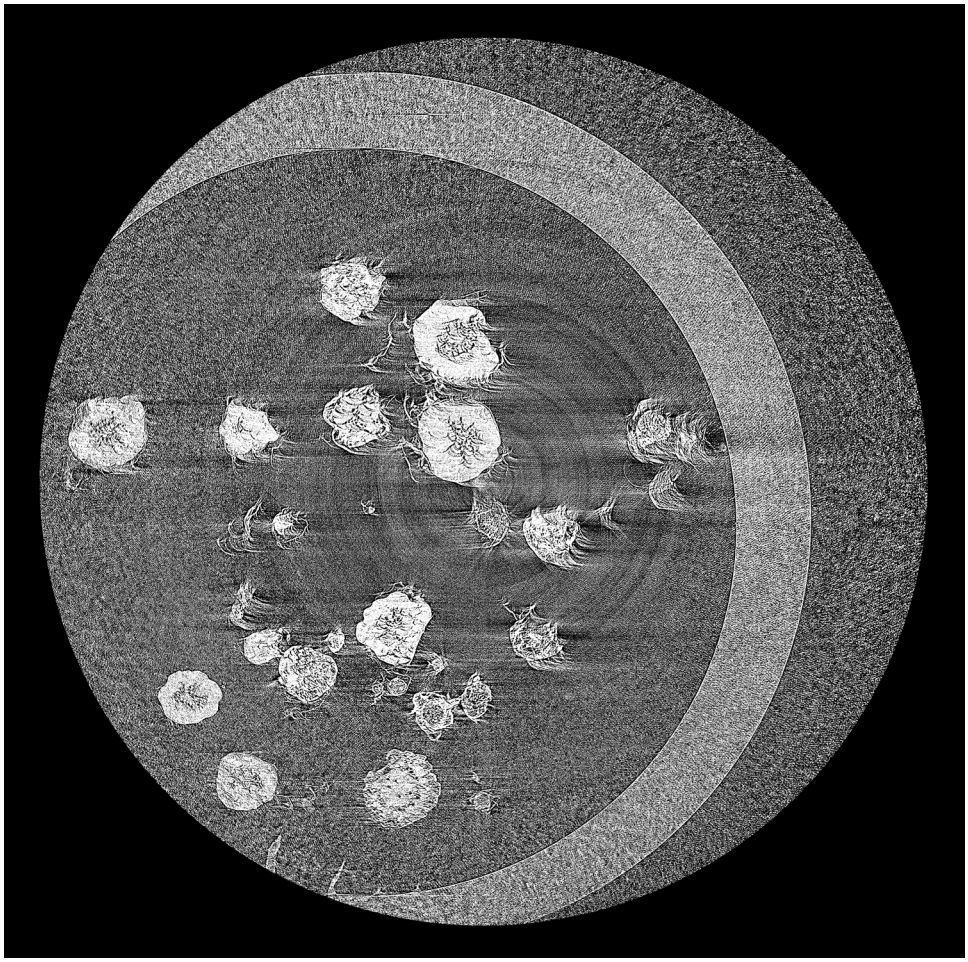

Figure 4. Image distortions, as seen here for numerous small woody roots, result from movement of the sample during the scan period. In this example a column of small woody roots (each bright white spot is a single root) still attached to a living plant were scanned and apparently moved during the scan and resulted in the distorted image. To overcome this issue samples need to be securely stabilized with additional padding inside the acrylic tube surrounding the plant.

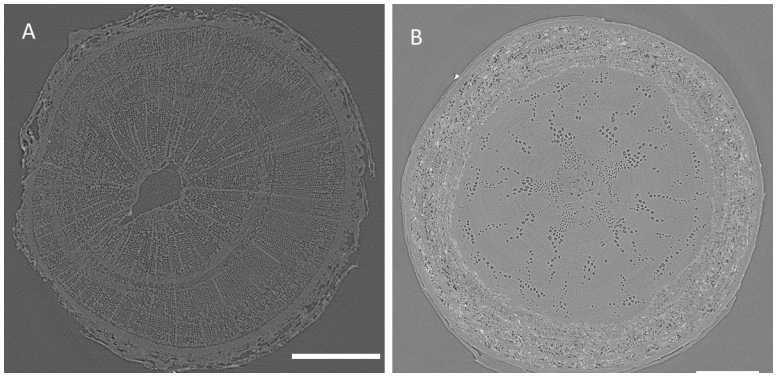

Figure 5. Examples of transverse images of woody stems scanned for (A) Coastal Redwood and (B) Valley Oak. White scale bars are 1.0 mm in both images. Click here to view larger figure.

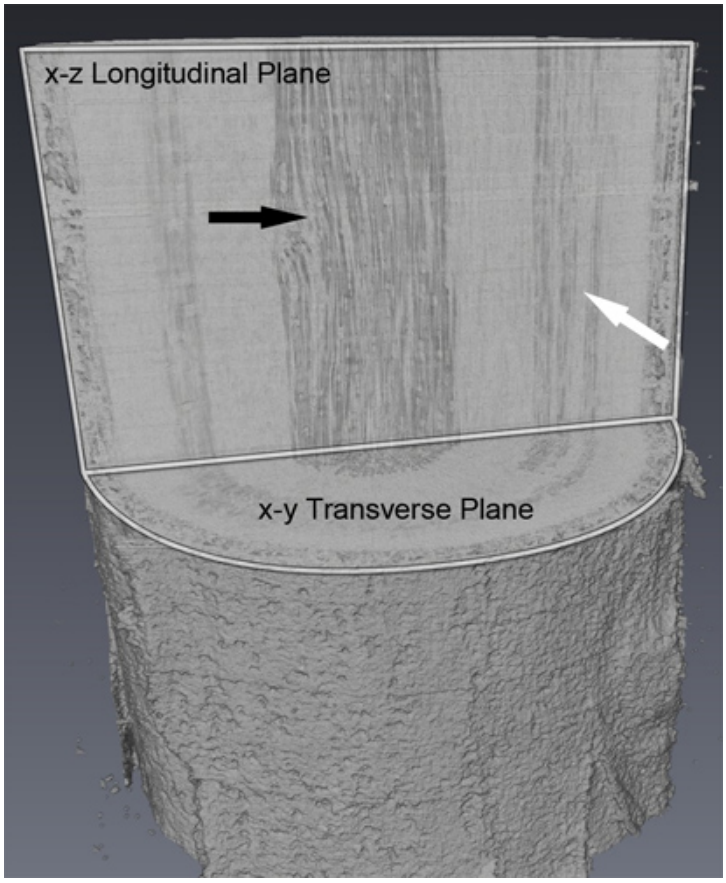

Figure 6. 3D reconstruction of a stem generated from a HRCT scan of a living coastal redwood sapling shown with a longitudinal and transverse plane exposed. Most of the xylem seen in this image is water-filled, while there are air filled conduits at the center of the stem (black arrow) that resulted from cavitation during a drought experiment. This scan also captured conduits in the act of cavitating- see the intermediate gray scale conduits forming a ring about halfway between the center and stem exterior (white arrow).

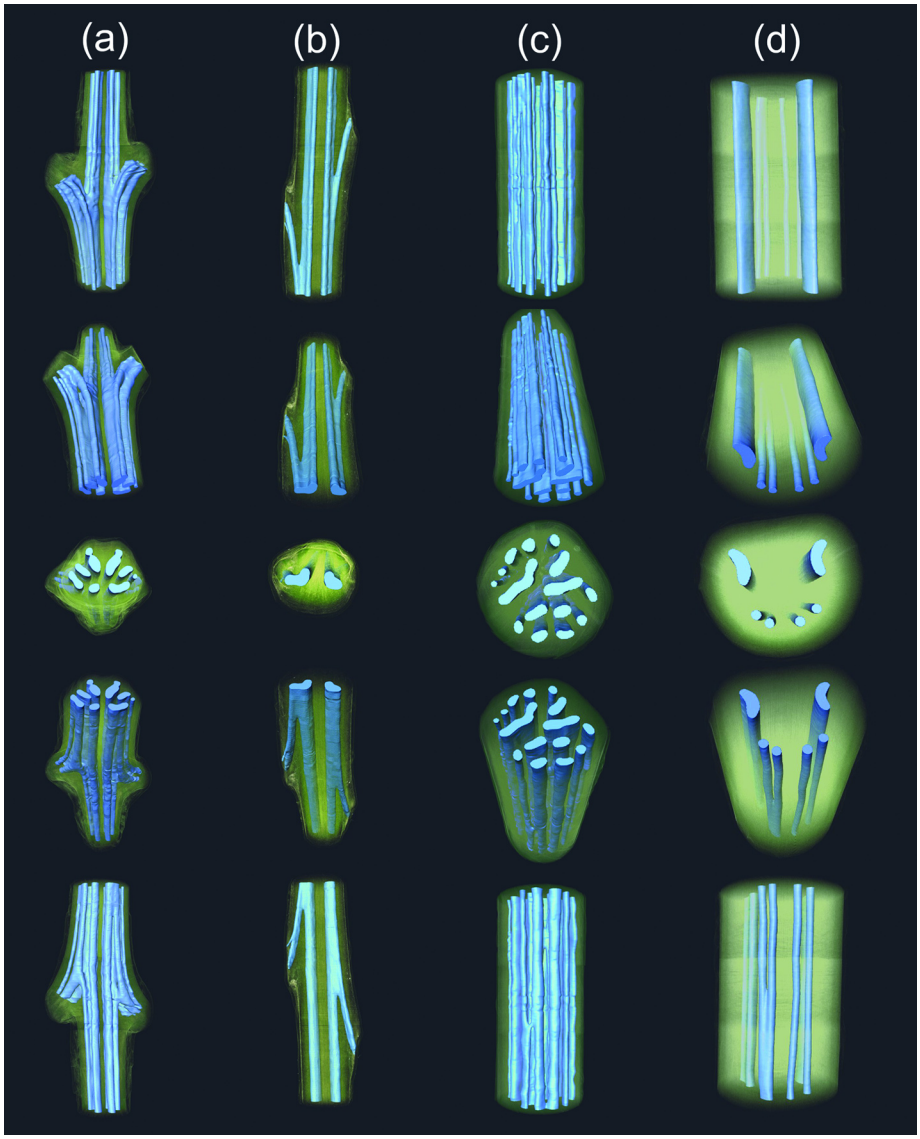

Figure 7. Image from Brodersen *et al* 2012- *Plant, Cell & Environment* demonstrating the 3D reconstruction of xylem vascular arrangement in two fern species scanned at two different points on the frond. Vascular bundles are visible in blue while the surrounding tissue is in green. In *Pteridium aquilinum*, the vascular bundles are optimized for high conductivity with many connections in both the frond tip (a) and base (c). In contrast, Woodwardia fimbriata has a much more conservative vascular arrangement with few connections between bundles in the frond tip (b) and base (d). The resulting vascular arrangements lead to high photosynthetic rates in *P. aquilinum* but at the expense of low tolerance to drought, while *W. fimbriata* is optimized for frond longevity with lower photosynthetic rates but higher drought tolerance. Frond tip and base sections are approximately 4 mm and 9 mm in diameter, respectively.

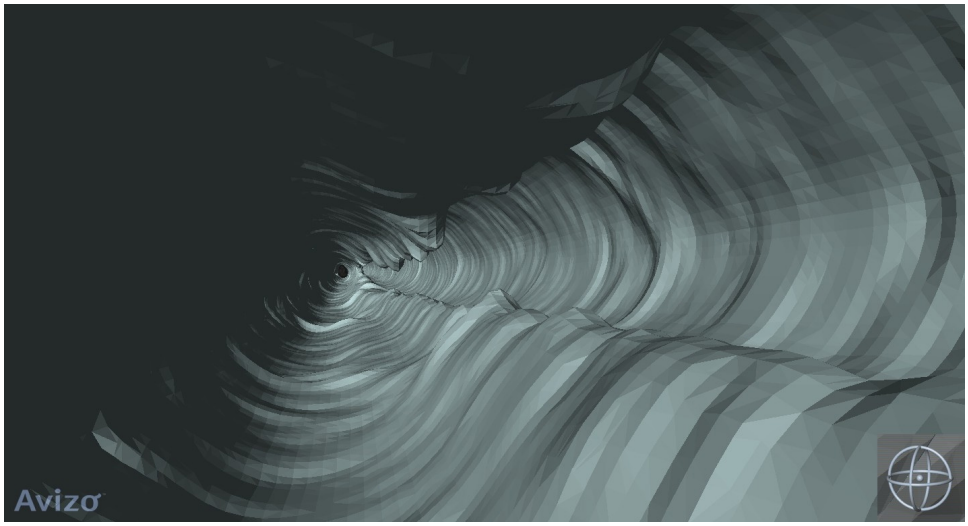

Figure 8. 3D reconstruction generated from a HRCT scan of walnut stem xylem. This image helps to demonstrate the capacity for exploring the tissue in incredible resolution as these are two adjacent xylem conduits that share an interconnected wall for much of their length. Here, the image processing and smoothing have removed the thin shared vessel wall in the volume rendering. Exact location and thickness of this vessel wall is retained in the raw image data and can be used to study connectivity. Each of the connected vessels in this image have ~ 40 µm diameter.

Movie 1. Click here to view movie.

Movie 2. Click here to view movie.

Movie 3. Click here to view movie.

Discussion

Synchotron HRCT provides plant biologists with a powerful, non-destructive tool to explore the inner workings of plant vasculature in incredible detail. This technology has been used recently to identify previously undescribed anatomical structures in grapevine xylem that differentially alter xylem network connectivity in various grapevine species (Brodersen *et al.* 2012b, in press)- this connectivity can drastically alter the ability of vascular pathogens and emboli to spread destructively throughout xylem networks. The first successful scans of living plants have also revealed fine scale detail of dynamic processes like embolism spread and repair (Brodersen *et al* 2010; McElrone *et al* 2012 New Phytologist 196(3):661-665), and helped to implicate the role of a specific living cell type in repairing embolism- the spatial resolution provided by HRCT at ALS 8.3.2 made this possible. Specifics about these processes and other aspects of xylem networks still remain elusive- HRCT will likely play a key role in continued discovery particularly when paired with other high resolution techniques (*e.g.* Laser Capture Microdissection), and could be paired with other recently developed advanced visualization techniques for use in plant biology (*e.g.* Lee *et al*, 2006; Truernit *et al*, 2008; Jahnke *et al.* 2009; Iyer-Pascuzzi *et al* 2010).

Disclosures

We have nothing to disclose.

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