

Journal of Visualized Experiments

Utilization of high resolution computed tomography to visualize the three dimensional structure and function of plant vasculature --Manuscript Draft--

Manuscript Number:	JoVE50162R2
Article Type:	Invited Methods Article - JoVE Produced Video
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Date: June 14, 2012

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Subject: Submission of Invited manuscript to the Journal of Visualized Experiments

Dear Dr. Singh,

We have uploaded our manuscript file and figures to the JoVE submission website. We appreciated the extra time (June 15th submission deadline) that you permitted for this submission. If you have any questions, please contact myself or the corresponding author (Dr. Brodersen).

Regards,

Andrew J. McElrone

Title: Using high resolution computed tomography to visualize the three dimensional structure and function of plant vasculature

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Keywords: CT scans, advanced visualization techniques, xylem networks, plant vascular function, synchrotron, x-ray micro-tomography, ALS 8.3.2

Short Abstract (50 word max)

High resolution x-ray computed tomography (HRCT) is a non-destructive diagnostic imaging technique that can be used to study the structure and function of plant vasculature in 3D. We demonstrate how HRCT facilitates exploration of xylem networks across a wide range of plant tissues and species.

Long Abstract (150-300 word max)

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.

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 20m 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 ~4.6mm 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 CdWO₄) 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 4" 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 second 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 ~ 10cm. Maximum sample width when imaging at 4.5 μ m 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 (2-3 pages, written in imperative tense, in step by step format)

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

1.1 Grow plants in ~10cm 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 ~1m 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 & 2).

1.2. 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.

1.3. 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.

1.4. 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.

1.5. 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

2.1. 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 minutes. 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.

2.2. Wrap the sample in a layer of Parafilm to prevent dessication during the scan.

2.3. 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

3.1. 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.

3.2. 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 hours 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).

3.3. 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.

3.4. Mount the sample in the drill chuck and Center as described above.

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

4.1. 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

5.1. 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.

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

	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 (-)

5.2. 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 1000 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.

5.3. 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 ~10-30GB of data vs. 5GB). 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.

5.4 For each scan, “bright field” and “dark field” images must be collected. 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

6.1 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.

6.2 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

7.1. 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.

7.2. 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 1-4 for detailed examples).

7.3. 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 polygonalelements, 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

8.1. 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 minutes 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

Synchrotron HRCT scans have been successfully implemented on a wide variety of plant tissues and species using beamline 8.3.2 (Fig. 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 Figs. 6-9; and Movies 1-4) 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. Fig. 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.

Figures and Tables

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.

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 1.0mm in both images.

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 9mm 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 ~ 40µm diameter

Figure 9: Water droplets (blue) forming on the inside of a xylem vessel (green) as captured in a live grapevine recovering (after rewatering) from drought stress. The vessel is approximately 150 µm in diameter. See Brodersen et al 2010 for details.

Links to Movies:

Movie 1:

<https://www.dropbox.com/s/wrei90kiu6kjxer/Quercus.mov>

Movie 2:

http://onlinelibrary.wiley.com/store/10.1111/j.1365-3040.2012.02524.x/asset/supinfo/PCE_2524_sm_MovieS1.mov?v=1&s=f4e4086ebc6c05743e6ae70d2bb2096c8cf08ac0

Movie 3:

http://onlinelibrary.wiley.com/store/10.1111/j.1365-3040.2012.02524.x/asset/supinfo/PCE_2524_sm_MovieS2.mov?v=1&s=0f7e030bb72d2057d5a891215e375093e27e1102

Movie 4:

<http://www.plantphysiol.org/content/suppl/2010/09/14/pp.110.162396.DC1/162396BrodersenMovie.mov>

Discussion

Synchrotron 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 in press), 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).

Acknowledgements:

The authors would like to thank S Castorani, AJ Eustis, GA Gambetta, CM Manuck, Z Nasafi, and A Zedan. This work was funded by: the U.S. Department of Agriculture- Agricultural Research Service Current Research Information System funding (research project no. 5306–21220–004–00; The Advanced Light Source is supported by the Director, Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.); and NIFA Specialty crops research initiative grant to AJM..

Disclosures (conflict of interest statement): We have nothing to disclose

Table of Specific reagents and equipment

Name of the Reagent	Company	Catalogue Number	Comments (optional)
NA			

Material Name/Equipment	Company	Catalogue Number	Comments (optional)
See specifics listed above regarding equipment at the Advanced Light Source beamline 8.3.2			

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Title: Using high resolution computed tomography to visualize the three dimensional structure and function of plant vasculature

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Keywords: CT scans, advanced visualization techniques, xylem networks, plant vascular function, synchrotron, x-ray micro-tomography, ALS 8.3.2

Short Abstract (50 word max)

High resolution x-ray computed tomography (HRCT) is a non-destructive diagnostic imaging technique that can be used to study the structure and function of plant vasculature in 3D. We demonstrate how HRCT facilitates exploration of xylem networks across a wide range of plant tissues and species.

Long Abstract (150-300 word max)

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.

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 20m 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~4.6mm 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 CdWO₄) 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 10cm 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 second 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 ~ 10cm. Maximum sample width when imaging at 4.5 μ m 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 (2-3 pages, written in imperative tense, in step by step format)

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

1.1 Grow plants in ~10cm 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 ~1m 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 & 2).

1.2. 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.

1.3. 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.

1.4. 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.

1.5. 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

2.1. 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 minutes. 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.

2.2. Wrap the sample in a layer of Parafilm to prevent desiccation during the scan.

2.3. 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

3.1. 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.

3.2. 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 hours 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).

3.3. 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.

3.4. Mount the sample in the drill chuck and Center as described above.

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

4.1. 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

5.1. 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.

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

	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 (-)

5.2. 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 1000 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.

5.3. 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 ~10-30GB of data vs. 5GB). 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.

5.4 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

6.1 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.

6.2 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

7.1. 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.

7.2. 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 1-4 for detailed examples).

7.3. 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 polygonalelements, 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

8.1. 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 minutes 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

Synchrotron HRCT scans have been successfully implemented on a wide variety of plant tissues and species using beamline 8.3.2 (Fig. 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 Figs. 6-9; and Movies 1-4) 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. Fig. 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.

Figures and Tables

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.

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 = 1mm.

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 1.0mm in both images.

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 9mm 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 ~ 40µm diameter

Figure 9: Water droplets (blue) forming on the inside of a xylem vessel (green) as captured in a live grapevine recovering (after rewetting) from drought stress. The vessel is approximately 150 µm in diameter. See Brodersen et al 2010 for details.

Links to Movies:

Movie 1:

<https://www.dropbox.com/s/wrei90kiu6kjxer/Quercus.mov>

Movie 2:

http://onlinelibrary.wiley.com/store/10.1111/j.1365-3040.2012.02524.x/asset/supinfo/PCE_2524_sm_MovieS1.mov?v=1&s=f4e4086ebc6c05743e6ae70d2bb2096c8cf08ac0

Movie 3:

http://onlinelibrary.wiley.com/store/10.1111/j.1365-3040.2012.02524.x/asset/supinfo/PCE_2524_sm_MovieS2.mov?v=1&s=0f7e030bb72d2057d5a891215e375093e27e1102

Movie 4:

<http://www.plantphysiol.org/content/suppl/2010/09/14/pp.110.162396.DC1/162396BrodersenMovie.mov>

Discussion

Synchrotron 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 in

press), 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).

Acknowledgements:

The authors would like to thank S Castorani, AJ Eustis, GA Gambetta, CM Manuck, Z Nasafi, and A Zedan. This work was funded by: the U.S. Department of Agriculture- Agricultural Research Service Current Research Information System funding (research project no. 5306-21220-004-00; The Advanced Light Source is supported by the Director, Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.); and NIFA Specialty crops research initiative grant to AJM..

Disclosures (conflict of interest statement): We have nothing to disclose

Table of Specific reagents and equipment

Name of the Reagent	Company	Catalogue Number	Comments (optional)
NA			

Material Name/Equipment	Company	Catalogue Number	Comments (optional)
See specifics listed above regarding equipment at the Advanced Light Source beamline 8.3.2			

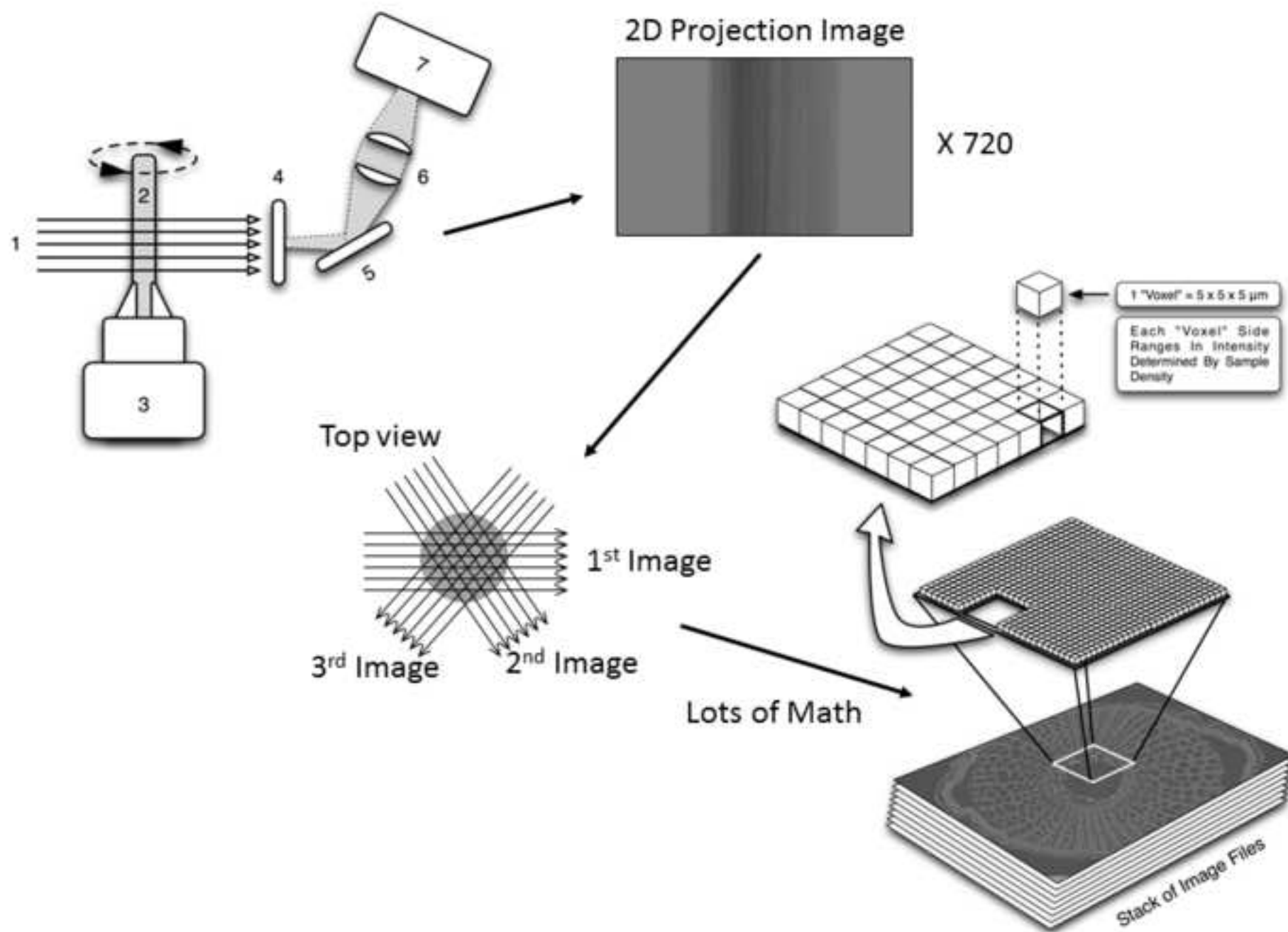
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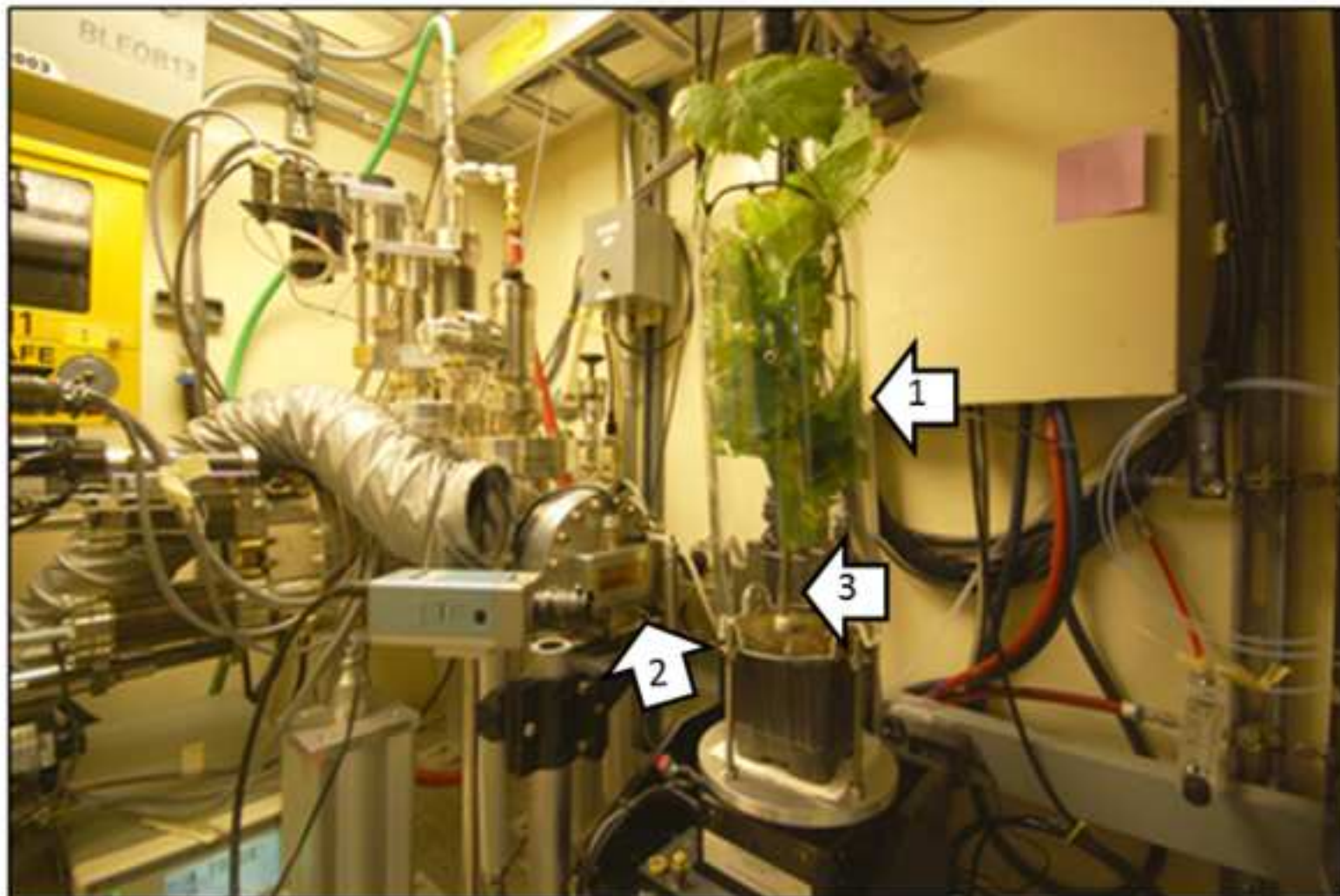
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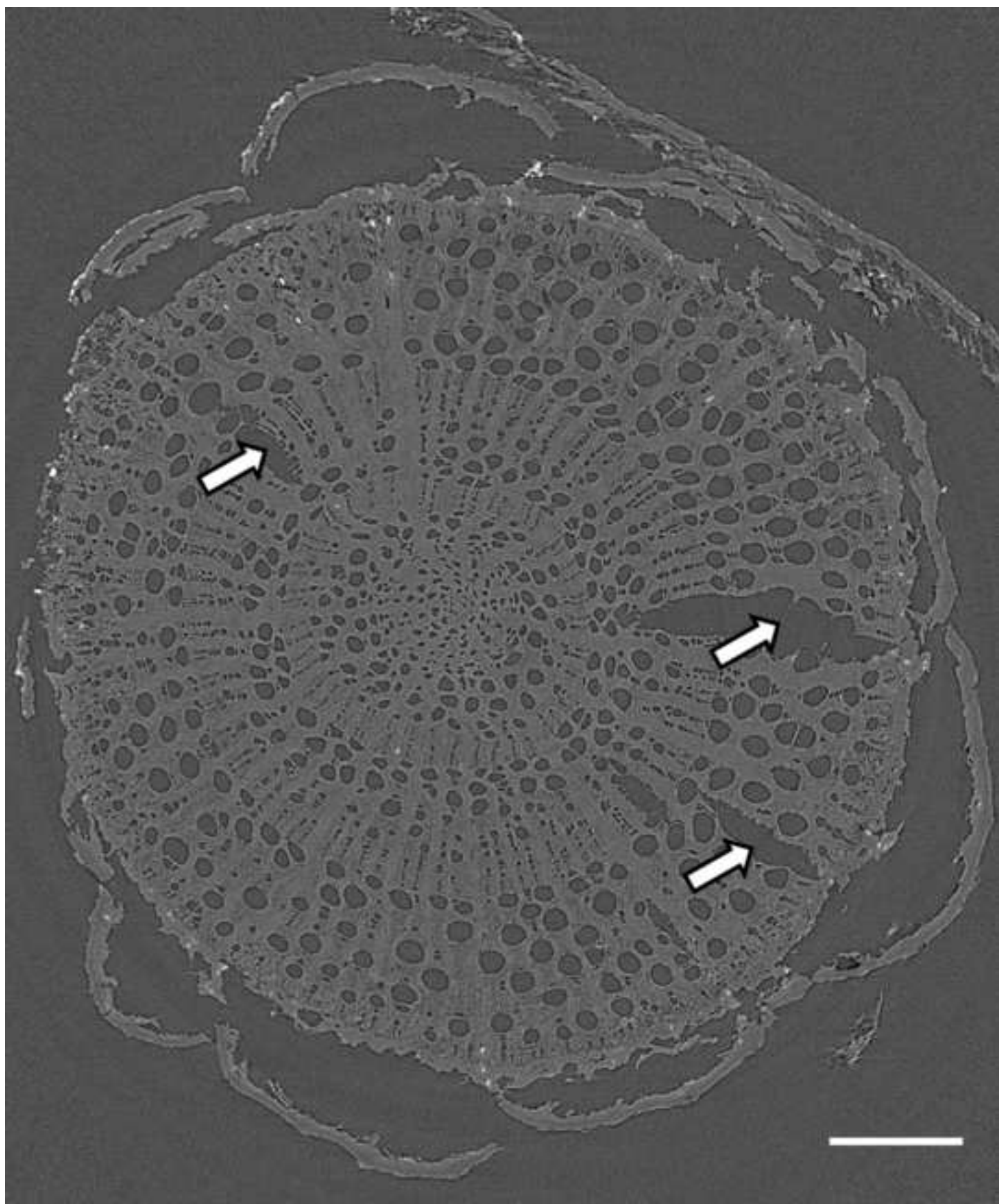
*Figure1
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*Figure2
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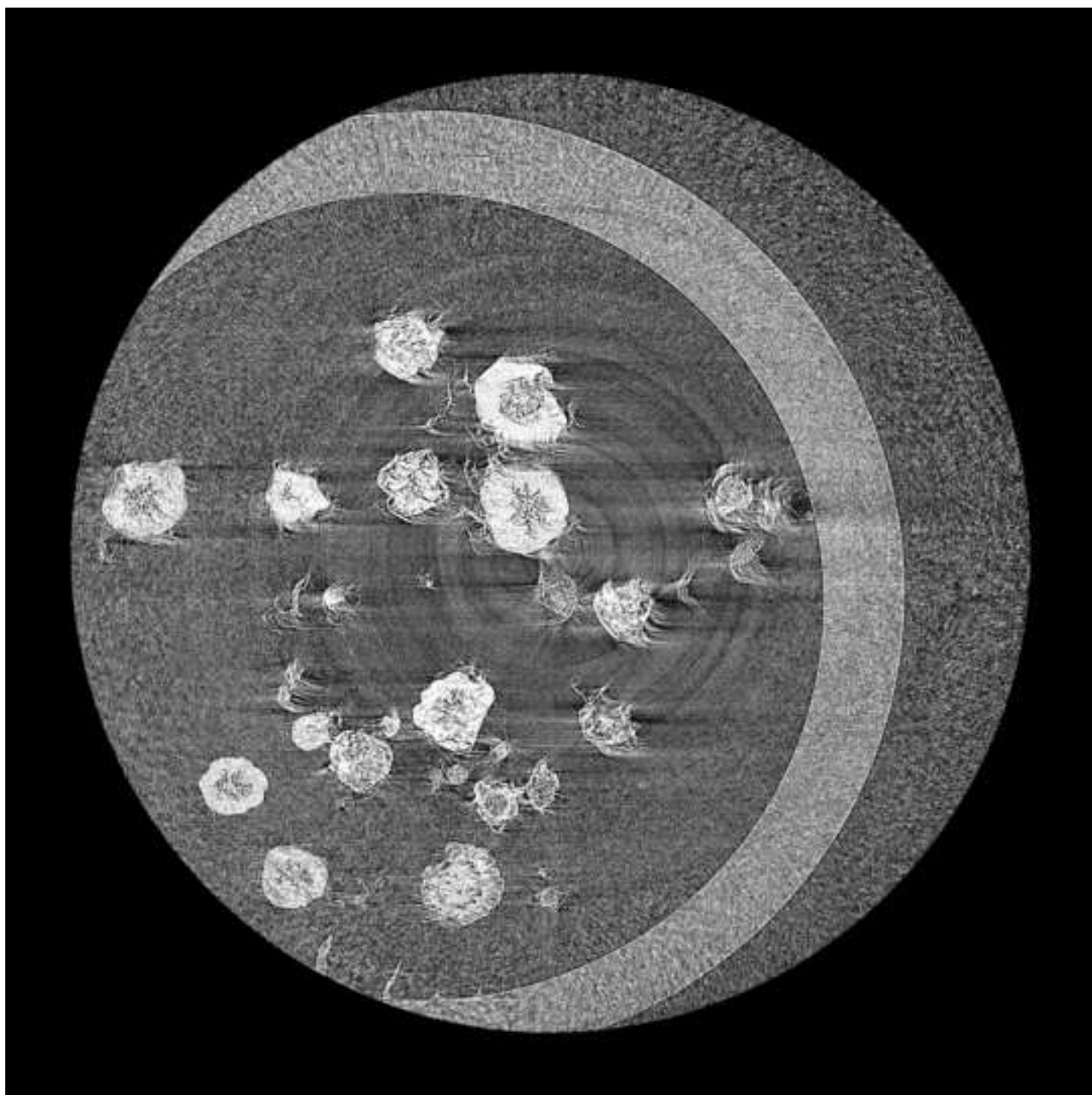


*Figure3
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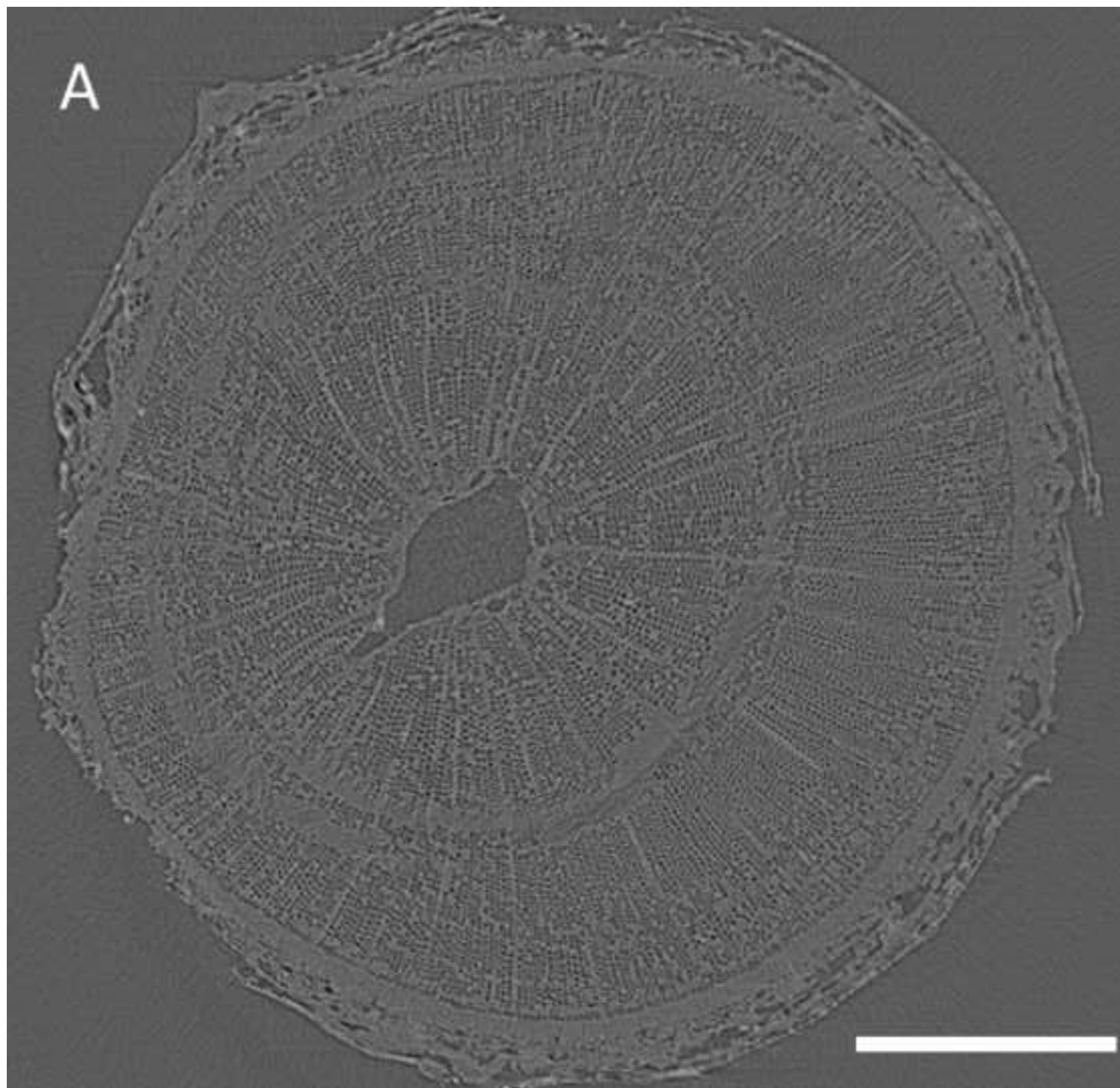


*Figure4

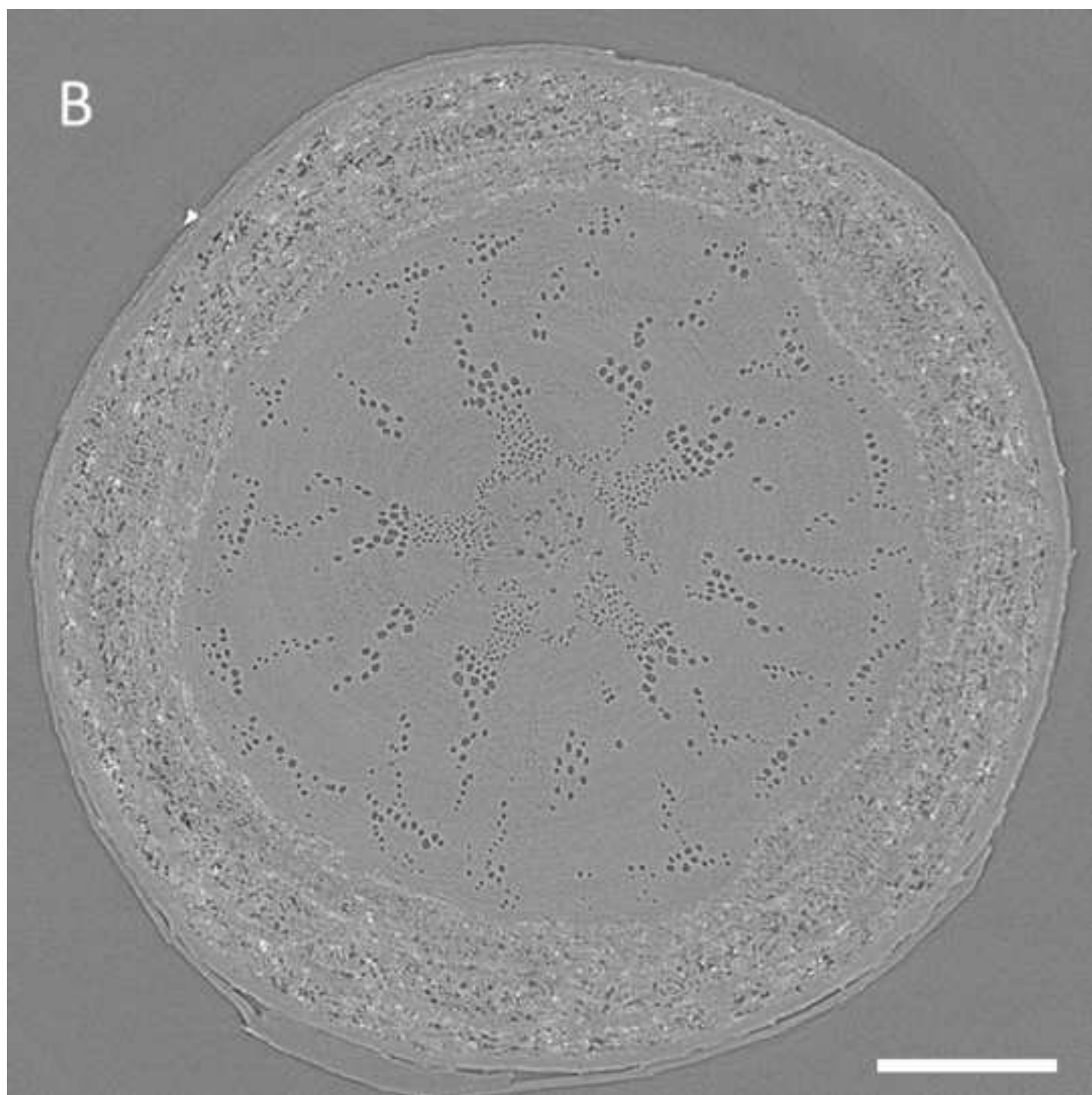
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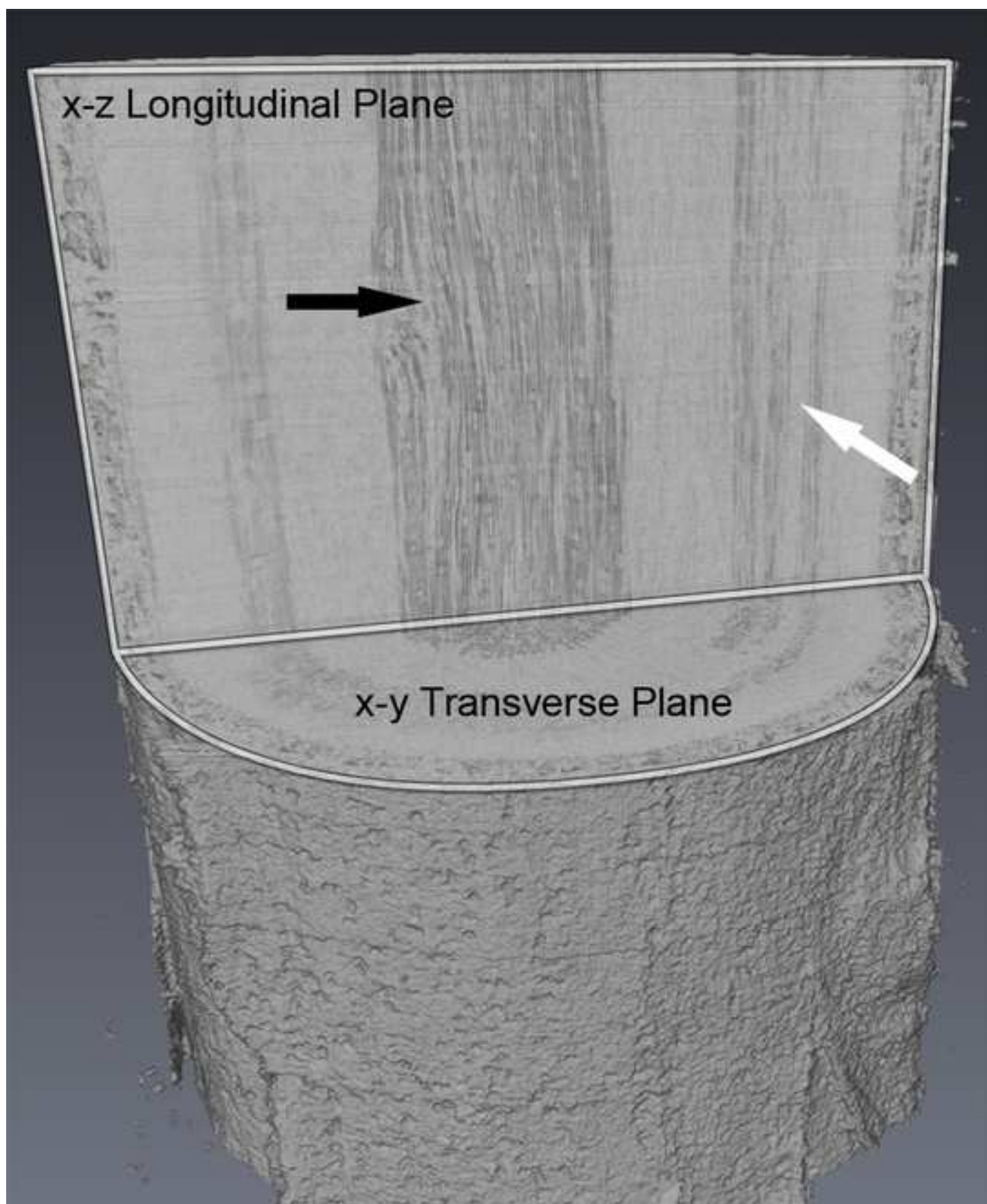
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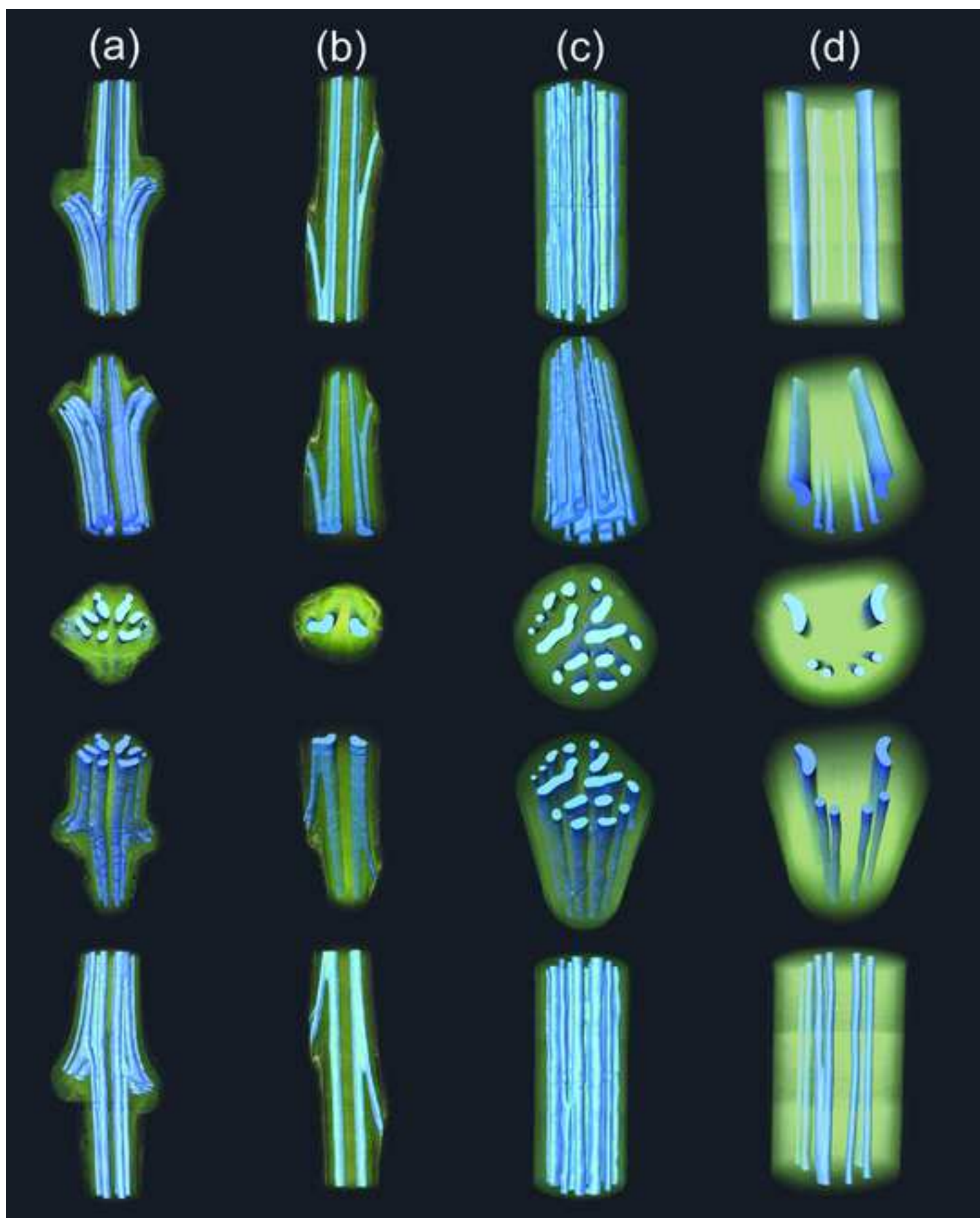
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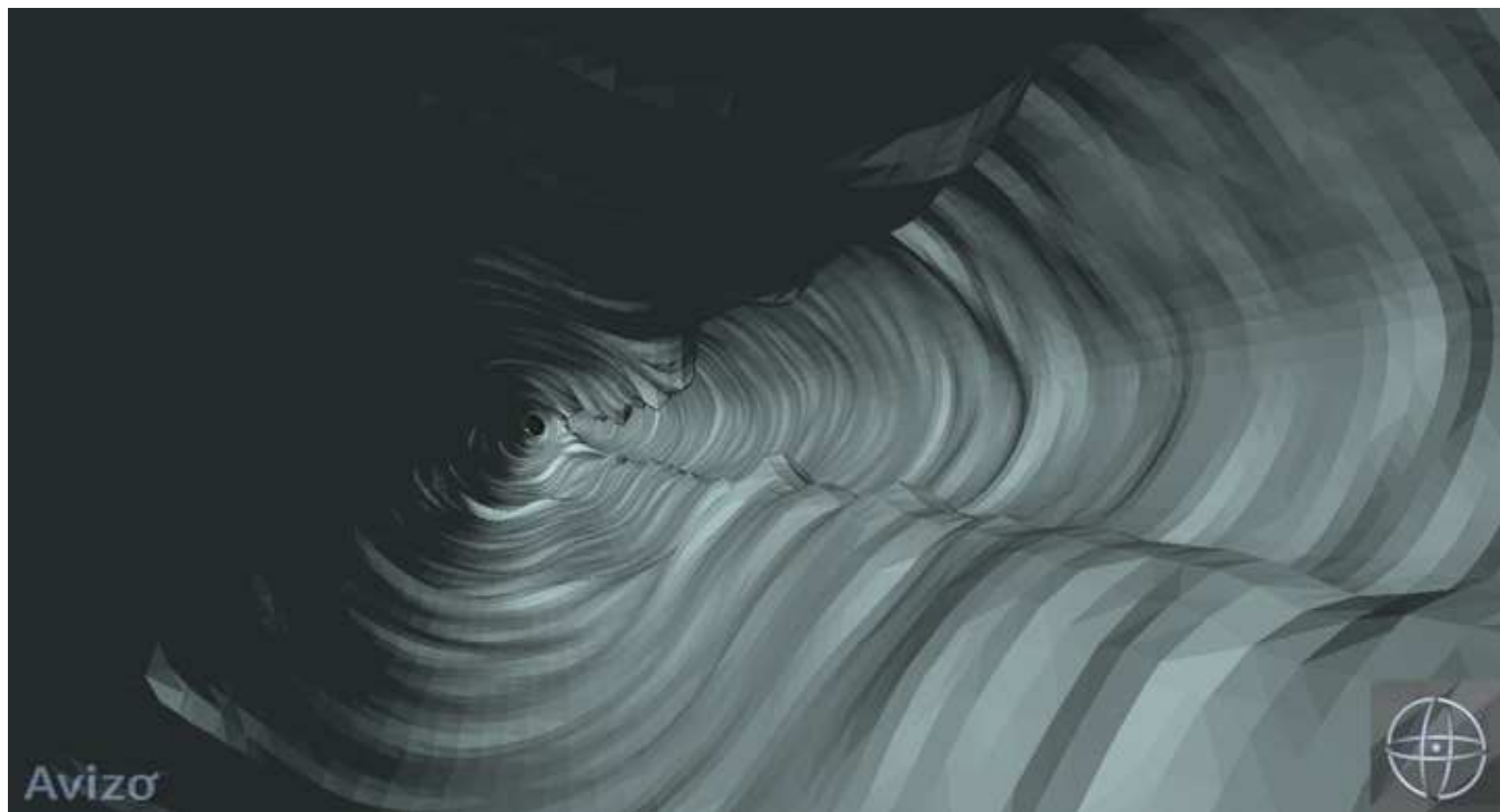


*Figure6
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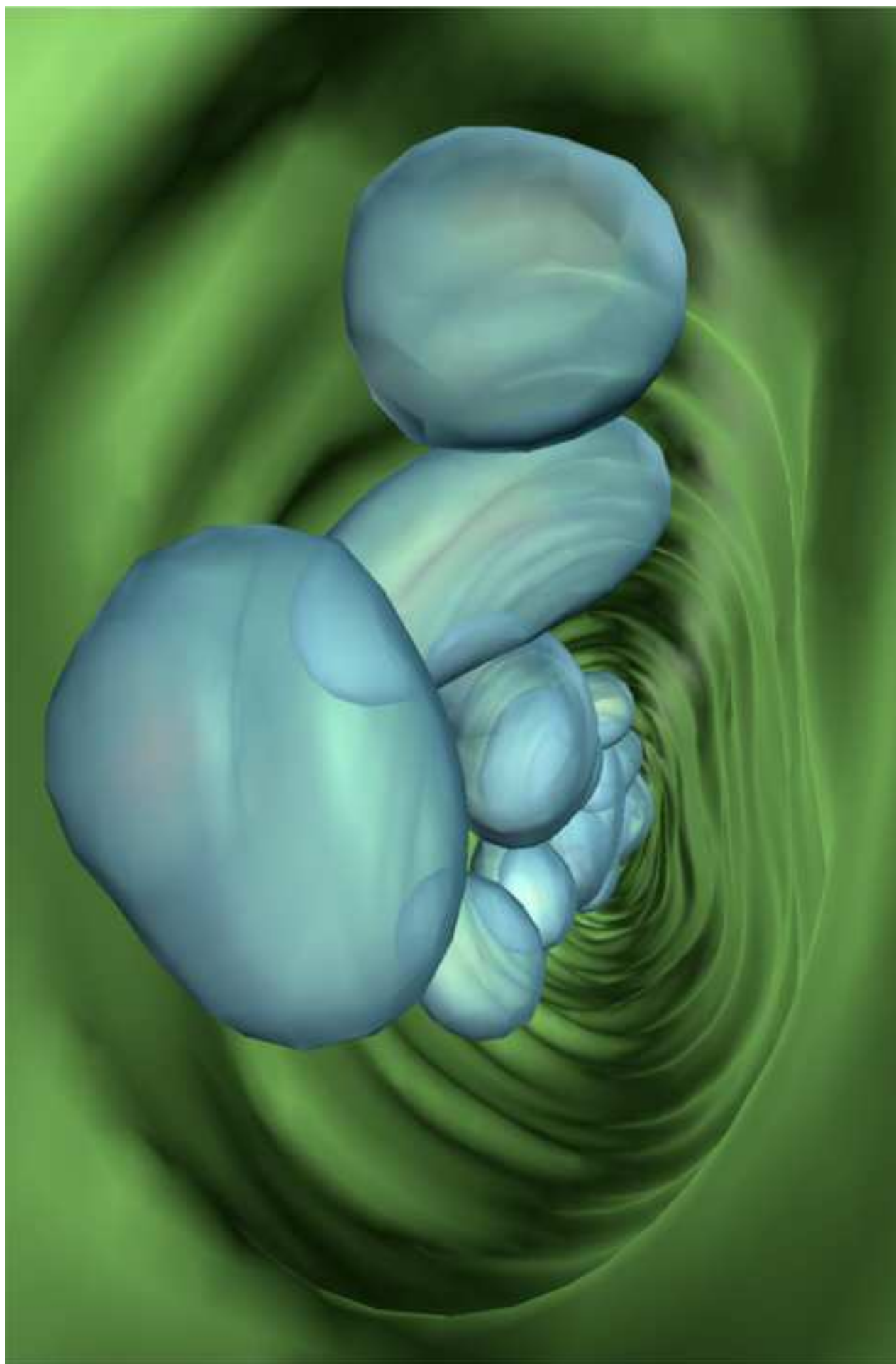
*Figure7
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*Figure9

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*Table of Reagents/ Materials Used
[Click here to download Table of Reagents/ Materials Used: McElrone et al_ JoVE_Materials.xlsx](#)

Name of Reagent/Material	Company	Catalog Number	Comments
No Reagents	NA	NA	NA

See specific equipment that is
unique to the ALS 8.3.2
beamline as listed throughout
the manuscript

*Rebuttal Comments

[Click here to download Rebuttal Comments: JoVE_Comments.docx](#)

No Rebuttal Comments needed. Per your request, we highlighted the sections of the protocol that will be the focus of the video.

24 August 2012

Dr. Nandita Singh
Senior Science Editor

[Journal of Visualized Experiments](#)

Dear Dr. Singh:

Below please find a detailed list of our responses/revisions based on the reviewers' comments. Our comments are in bold text below each reviewer comment. We hope these now make the manuscript acceptable for publication in JoVE.

Sincerely,

Andrew J. McElrone

Response to Reviewers' comments:

Reviewer #1:

Summary:

The authors provided an overview of the utilization of HRCT to study vascular tissue in woody plants. Details of the protocols are given, and software options to produce 3D reconstructions are listed, being one of them Avizo. The images are illustrative on what is possible to image and the quantitative data that can be extracted from the reconstructions, i.e. cavitation.

Major Concerns:

I consider that the authors could compare the HRCT to other types of CT scans, or non-invasive technologies in plants. For instance Lee et al, 2006, Plant Cell 18:2145 showed 3D reconstructions of organs and gene expression labels, or Iyer-Pascuzzi et al Plant Phys, 2010, 152:1148 showed 3D reconstructions of rice roots. Also a comparison to other high resolution 3D scans could be done, i.e. Jahnke et al, 2009, Plant Journal, 59:634. This would give the readers the elements to assess the strengths and weaknesses of HRCT for research purposes.

The space constraints of JoVE (particularly the methods/protocol section) limit how much we can talk about the details of these other techniques. While our research group has conducted work on other imaging techniques (e.g. NMR visualization of plant vascular function), we were invited to submit a

manuscript based specifically on HRCT work at ALS- thus the focus of the current manuscript. However, we do acknowledge that there are numerous additional applications of advanced visualization techniques and have addressed the reviewer's concern by incorporating text referring to these other techniques in the Discussion section where it was appropriate.

Minor Concerns:

I would prefer the authors to stick to the international metric system. For instance, in the section Description of the ALS facility Beamline 8.3.2, in the second paragraph, it reads "on the 4" diameter?". The measurement should be given in centimeters.

Good catch- We have thoroughly proofread the manuscript again and have ensured that only international metric units are being used.

The link of Movie 2 does not play.

The text listed for the link was written in half regular text and half hyperlink text. The link does in fact work if the entire address is copied into a browser address bar. We have edited the text to ensure consistent formatting of the entire text. We assume that this will be taken care of during final manuscript preparation.

Reviewer #2:

Summary:

The invited Methods article "Utilization of high resolution computed tomography to visualize the three dimensional structure and function of plant vasculature" by McElrone et al. provides a detailed description of the use of HRCT to visualize the architecture of plant xylem vessels. It is a very specialized technique that cannot be employed in any laboratory in the world but needs specific facilities. Therefore it is not a drawback that the article describes the details of the techniques adapted in the authors' laboratory. The article is a welcome addition to the nice work of the authors' group published in other journals.

Major Concerns:

None.

Minor Concerns:

*) The imperative used in the protocol description is rather unusual, albeit, for this reviewer it is not disturbing.

We followed the format required by this journal

*) This reviewer could not find any structure in the order of the reference list. This should certainly adjust according to the guidelines of the journal.

We followed the format required by this journal

*) One article was cited as "in review". Also this needs to be adjusted in accordance with the journal guidelines.

This manuscript has been in review since late June. We assume that we will hear about the status of this manuscript soon and will provide the JoVE editorial staff with this info as soon as we hear. If the timing of publication of this article requires, we could change this to an “unpublished data” reference.

*) typo: "Figure.1." -> "Figure 1."

Good catch- This typo was corrected.

*) typo: "The vine is contained an acrylic tube (1)." -> Should read "The vine is contained in an acrylic tube (1).", no?

The reviewer is correct and the text has been edited accordingly in the legend for Figure 2.

*) Some movies and Figures were published by other journals before. This reviewer is uncertain about the copyright issues in these cases.

We were told by the JoVE editor that copyright issues for already published figures and movies will be taken care of prior to publication. We assume this is still the case.