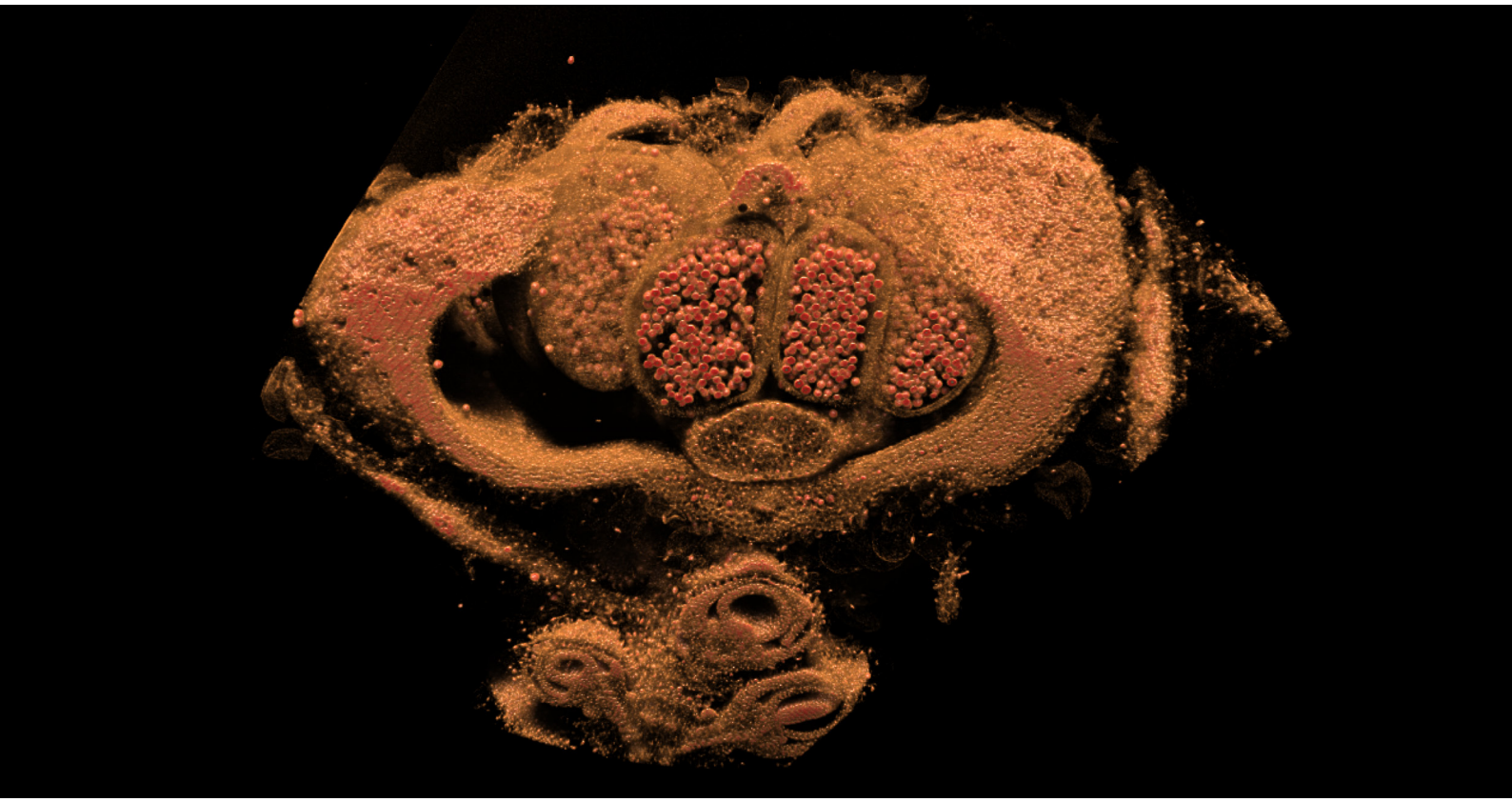
White Paper

Using X-ray Microscopy for High Resolution Multiscale 3D Imaging in Plant Biology

A valuable approach for exploring plant anatomy



ZEISS

Seeing beyond

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Unique high resolution 3D data of plant samples

Lab-based X-ray microscopy (XRM) is a powerful tool for tomographic imaging of plant samples, providing high resolution 3D image data that are not practical or possible with most light, laser, and electron microscopy platforms. Plant growth and development occur over many scales, and the ZEISS Xradia Versa line of XRM instruments provides multiscale imaging capability, particularly for intact plant samples that are large, delicate, complex or otherwise problematic for conventional tomographic systems, and allow high magnification cell-level scan data to be placed within tissue or whole plant context. Here we describe useful methods for preparing and imaging plant samples in the Xradia Versa series of XRM instruments for generating high resolution 3D volume data at cell, organ, tissue, and whole plant levels.

X-ray microscopy of plant samples

Plant tissues present unique challenges for high resolution imaging, particularly when 3D tomographic data are required. Plant (and fungal) cell walls, extensive vacuoles, and air-filled spaces present significant physical barriers for imaging and for penetration of fixatives, resins, immunochemicals, and affinity probes. Remarkable advances in sample preparation methods and in light, laser, and electron microscopy technologies have made high resolution tomographic imaging of plant samples possible, but samples are typically restricted to small and thin tissues that are isolated from their whole plant context, and sample preparation methods—particularly for volume electron microscopy (vEM)—can be complicated and require specialized instrumentation.

Basic X-ray tomography (XRT) was first used in the 1970s for non-destructive imaging to provide researchers with 3D volume data at moderate resolutions. In the intervening 50 years, advances in X-ray source design, detector sensitivity and resolution, and computational power have produced a wide range of XRT systems that provide richly detailed high resolution 3D volume data. In general terms, conventional XRT functions by passing X-rays through a sample onto a detector to generate a 2D digital radiograph, where variations in sample density cause differential absorption of X-rays, called attenuation. Differential attenuation of X-rays by the sample causes variation of grayscale values in the radiograph: the more dense the sample, the greater the attenuation and the higher the grayscale value. Samples are rotated during the scan—typically 360 degrees—and hundreds or thousands of digital radiographs are collected. The radiographs are then computationally reconstructed into a full 3D volume using algorithms that are often specific to the type of imaging system being used. This allows non-destructive imaging of intact samples over a range of sample sizes and complexity.

Conventional lab-based XRT can facilitate whole organ or plant 3D imaging, but magnification in most XRT instruments is restricted by interior cabinet size and the resulting source-sample-detector geometry. This limitation results in a trade-off in choosing higher resolution imaging of smaller regions/samples, or lower resolution imaging of large intact samples. The lab-based X-ray microscope (XRM) allows high resolution 3D imaging over multiple scales, providing cell-level resolution within tissue, organ, and whole plant context, effectively bridging the gap left by conventional XRT and light, laser, and electron microscopy systems. The XRM increases magnification by adding scintillator-coated objective lenses to the beam path, so X-rays that pass through samples strike the scintillator, are converted to light, and are then magnified by the lens onto the detector, typically a CCD camera. With the addition of a flat panel detector in the ZEISS Xradia Versa, a single intact plant sample can be imaged over multiple scales without removing it from the instrument, placing cell-level region-of-interest 3D scan data within the context of high resolution whole plant scans. The following sections describe methods and guidelines for effective plant biology imaging with lab-based XRM systems, as well as some of the existing XRM imaging research that has already been done with a variety of plant samples.

Sample fixation and contrast

X-ray attenuation is critical for generating good contrast tomography, but most plant tissues provide little differential density with which to form an image. As a result, chemical agents are often required to enhance contrast in plant samples to provide high resolution X-ray imaging. In addition, chemical fixatives may be needed for successful high resolution XRM imaging as longer scan times require samples to be stationary and stable throughout the scan. A variety of popular electron microscopy fixatives and contrast agents have been used to prepare plant samples for XRM (Staedler *et al*, 2013; Duncan *et al*, 2022).

Phosphotungstic Acid (PTA). One of the most versatile contrast agents for XRM is phosphotungstic acid (PTA). Prepared as ethanolic PTA (ePTA) at 1% (w/v) in 70% ethanol (v/v), it functions well as a combined fixative and contrast agent, and most plant

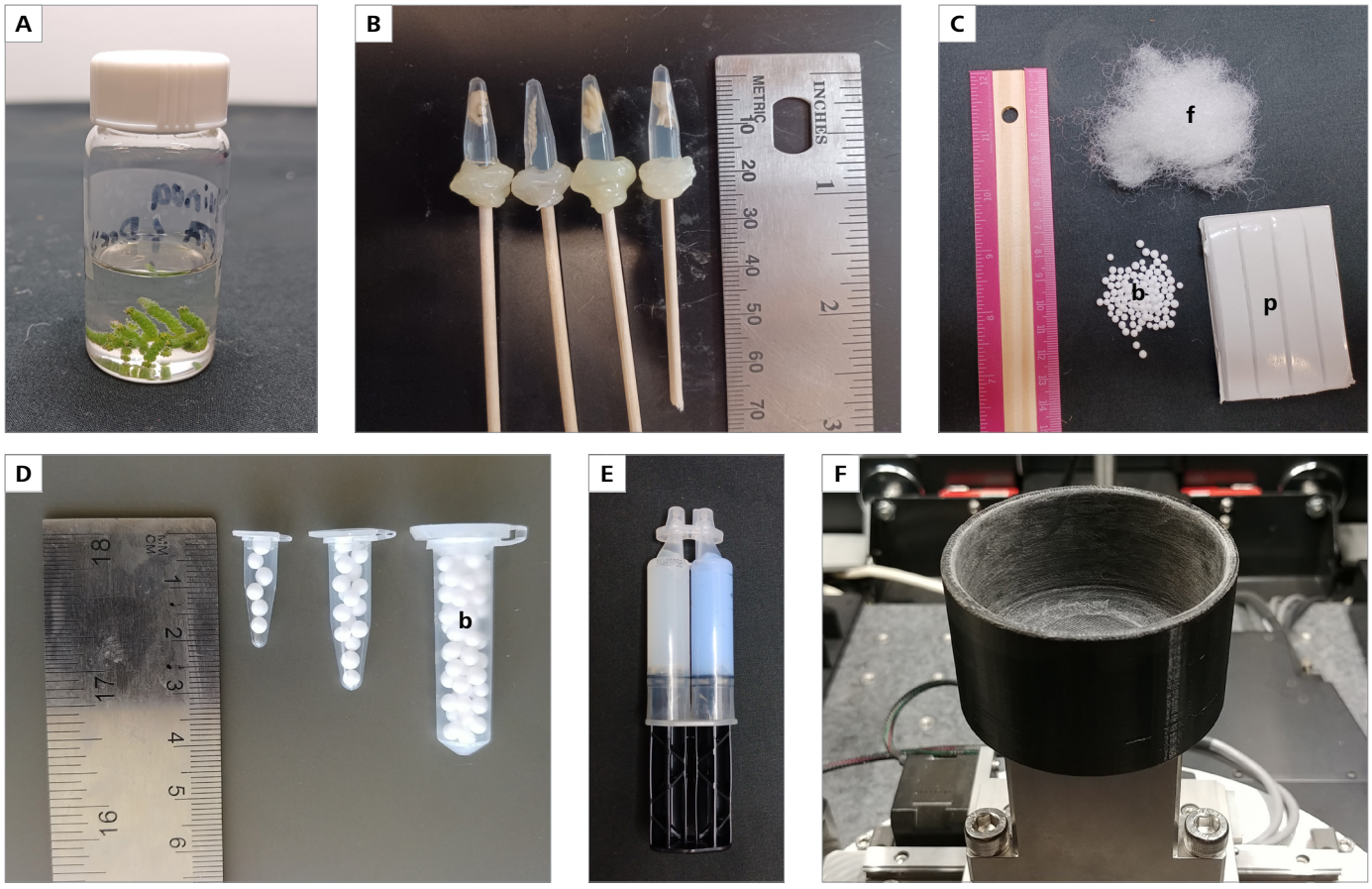

Figure 1 *Sample preparation and stabilization.* ***A)*** *Plant floral sample in ethanolic phosphotungstic acid solution for simultaneous fixation and contrast enhancement.* ***B)*** *Four different plant samples stabilized in low melting point agarose and affixed to wooden applicators for mounting in XRM sample holder.* ***C, D)*** *Polystyrene beads* ***(b)*** *and polyester fiber fill* ***(f)*** *are effectively X-ray transparent and useful for stabilizing samples inside tubes* ***(D)****; poster putty* ***(p)*** *is more dense but very effective for stabilizing more sturdy samples.* ***E)*** *Two-stage 5-minute epoxy gel allows easy mounting of samples on pins or wooden sticks, and also seals tubes to prevent desiccation.* ***F)*** *Custom 3D-printed holder for plastic pipes in which plants are grown for XRM imaging of intact root systems* in situ.

samples can be collected directly into ePTA (Figure 1A). The ethanolic component greatly assists fixation and penetration of the PTA, particularly with waxy hydrophobic samples or samples with extensive trichomes. Aqueous PTA solutions can be used but vacuum is typically required to assist penetration into plant samples. PTA has strong affinity for nucleic acids (nuclei) and phospholipids (cell membranes); see Hayat (1993) for a detailed review of PTA staining affinities. One drawback of ePTA is the time required for effective contrast, at least 14 days with two or three exchanges of fresh solution are a *minimum* requirement. However, with the use of microwave tissue processing, ePTA sample preparation time can potentially be reduced to days or even hours. Regardless, there does not appear to be an upper limit to incubation time in ePTA; delicate soybean flowers that were stored in ePTA for four years were scanned and showed no negative effects at lab-based XRM resolutions. Also, contrast with ePTA is durable and does not leach out when samples are stabilized in various media for imaging (see Sample Mounting section below).

Iodine. Iodine and iodine-containing solutions are also effective contrast agents for XRM. One popular iodine solution is Lugol's, which is available commercially or can be easily prepared in the lab. Lugol's is commonly prepared as 500 mL of a 4X stock solution by adding 50 g of potassium iodide to 400 mL ddH_2O and stirring until dissolved, followed by the addition of 25 g iodine and adding ddH_2O to a total volume of 500 mL. The 4X stock solution can be kept in a foil-wrapped bottle on the shelf for at least one year. Samples should be fixed in ethanol or a buffered aldehyde solution prior to rinsing and contrasting. Contrasting in iodine solutions is rapid compared to ePTA, with useful contrast possible in 24 hours depending on sample size, however the solution will leach out so care must be taken when mounting iodine-contrasted samples for XRM.

Osmium. Osmium tetroxide solutions are also useful for some plant applications as a contrast agent, particularly for tissue containing high levels of lipids or other oils. Osmium tetroxide is a hazardous chemical, however, so great care must be taken when working with osmium solutions. It is typically used for smaller samples as osmium tetroxide solutions do not penetrate easily into samples, although recent protocols have significantly improved this technique (Belanger *et al*, 2022), particularly for resin-embedded samples for correlative imaging workflows and vEM.

Nanoparticles, genetically-encoded expression systems. Additional strategies for imparting contrast for plant samples are possible using nanoparticles and nanogold. Lectins have specific affinity for a range of plant and fungal tissues, and nanogold conjugates are commercially available for most lectins. If sufficient quantities of the nanogold-lectin conjugate adhere to plant/fungal tissues, XRM may be used to visualize these structures as has been regularly demonstrated using electron microscopy. Nanoparticle chemistry has advanced significantly and plant- and/or fungus-specific nanoparticle conjugates could deposit sufficient electron-dense material to allow these structures to be visualized by XRM as well. Genetically-encoded expression systems have been used in animal tissues to generate compounds in specific regions for reaction with osmium tetroxide or silver/gold deposition for subsequent EM and XRM (Bayguinov *et al*, 2020). This has yet to be adapted to plant tissues, however improvements in cryo-based plant sample preparation techniques could allow expression-based contrast strategies to be used in plant and fungal systems.

Sample mounting

Presenting samples to the X-ray beam for 3D volume imaging is an important aspect of XRM. For most high-resolution imaging, relatively long scan times are required and samples must stay perfectly motionless the entire time (although see discussion of DeepRecon® below). A variety of methods for stabilizing plant samples for XRM imaging is available, and each different sample presents a new challenge in terms of mounting and stabilization. Regardless of the sample, it is most important that it be mounted in the center of the axis of rotation, while allowing the X-ray source and objective lens to get as close to the sample as possible without collision. Below are some examples of how plant samples have been prepared for XRM imaging, but researchers are encouraged to be creative with the tools and supplies they have at hand; there are many correct answers for solving sample mounting challenges.

Low melting point agarose. One useful method of stabilizing plant samples for XRM is in conical centrifuge tubes (*e.g.* PCR tubes) using low melting point agarose (Figure 1B). Despite the slight reduction in scan quality due to the presence of the agarose (as opposed to a sample in air), the agarose holds delicate and complicated samples in place with a minimum of physical distortion. This is ideal for samples fixed and contrast-enhanced in ePTA or osmium as the contrast agent remains in the sample and does not leach out into the agarose, whereas iodine-based contrast agents do leach into the agarose and negatively impact scan quality. Samples should be rinsed in ddH_2O for 5–10 minutes to remove excess ePTA. Low melting point agarose is prepared at 2% (w/v) and heated until bubbles first appear but the solution is not actually boiling. Using a glass pipette, melted agarose can be added to the bottom of the centrifuge tube—the smallest tube that will contain the sample—being careful not to introduce any bubbles. Fine forceps can be used to remove the sample from ddH_2O, and touch it carefully to a lab towel to wick away excess ddH_2O. The sample can then be submerged in the melted agarose in the tube. The tube is completely filled with melted agarose and allowed to solidify. In some cases, samples may float in the melted agarose. If this happens, just enough molten agarose can be used to contain the sample in the bottom of the tube and allowed to harden in place. Additional molten agarose can then be added to fill the tube while the sample remains locked in place in the narrowest part of the tube. When the agarose has fully solidified, the tube can be affixed to a wooden applicator stick in the XRM sample holder using a two-stage five-minute epoxy gel (Figure 1E).

Dry mounting strategies. For samples that are more durable and can withstand handling and mounting without physical damage (*e.g.* seeds, grains, stalk and stem segments), a variety of materials can be used to stabilize the sample. Polyester fiber fill, polystyrene beads, or poster putty are great examples of inexpensive and widely available materials that can be used to

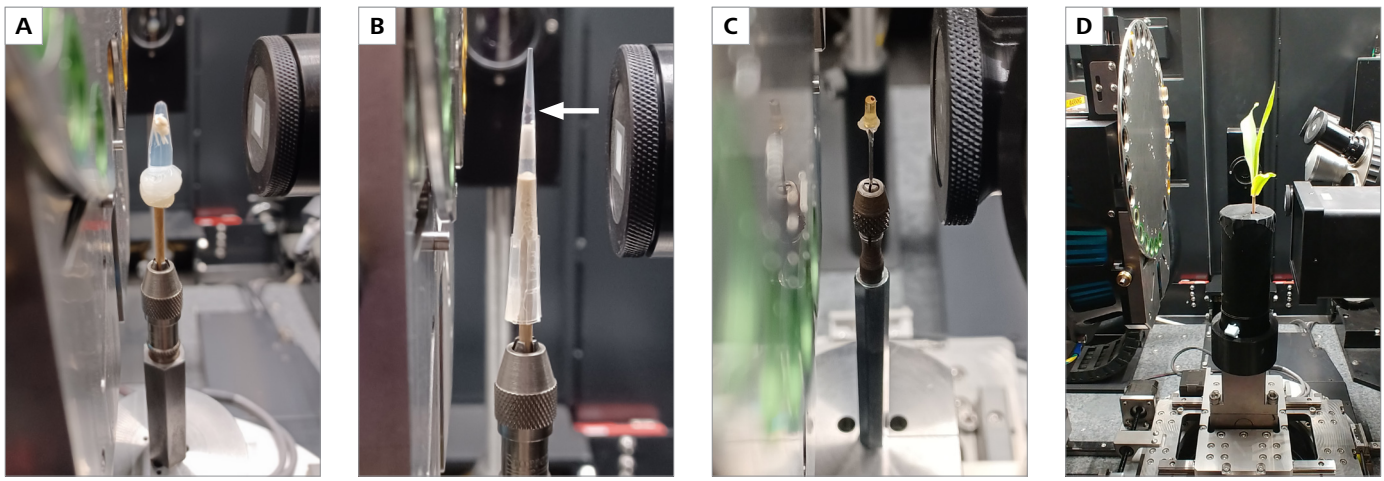

Figure 2 *Sample mounting.* ***A)*** *Soybean nodule stabilized in low melting point agarose in a PCR tube, affixed to a wooden applicator stick with two-stage epoxy gel, and mounted in XRM sample holder.* ***B)*** *Pennycress seeds* ***(arrow)*** *stabilized with poster putty in air inside a pipette tip, and affixed to a wooden applicator stick with two-stage epoxy gel.* ***C)*** *Resin-embedded osmicated plant sample mounted on flat-head pin with two-stage epoxy gel.* ***D)*** *Live maize seedling growing in a plastic pipe and stabilized in custom 3D-printed sample mount for XRM imaging of root system architecture* in situ.

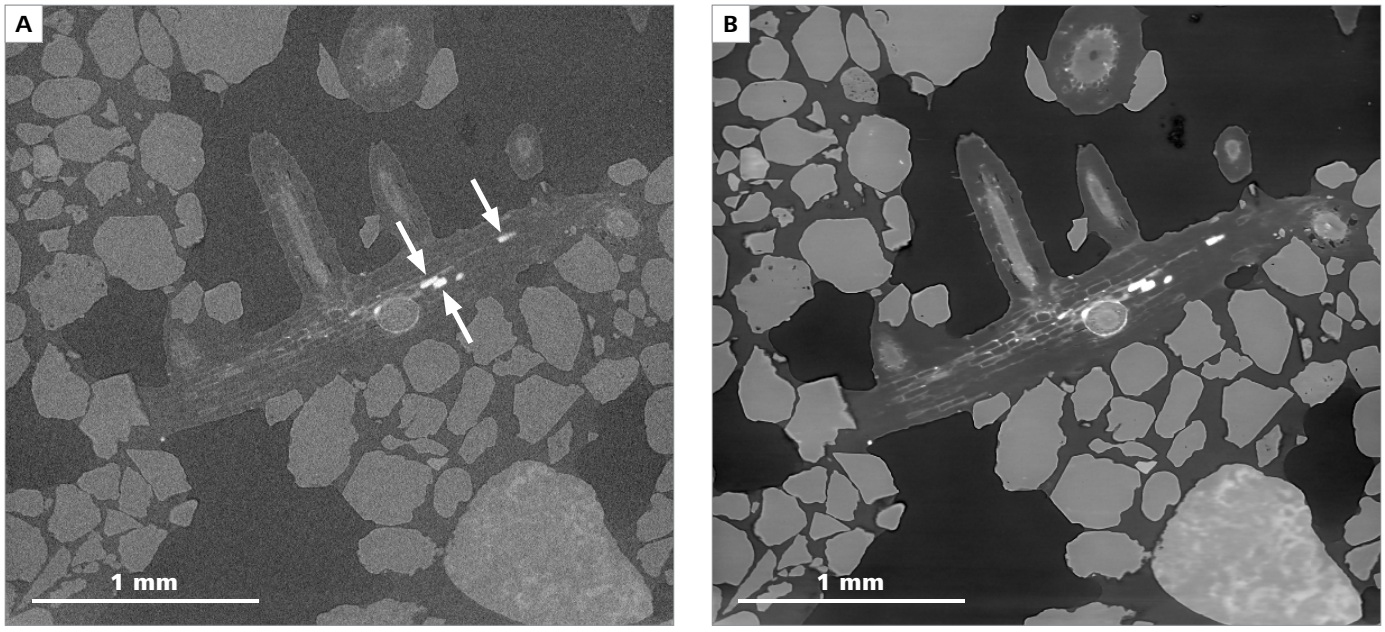

Figure 3 *DeepRecon® is a reconstruction functionality that uses deep learning to denoise existing XRM scan volumes, as shown here with a* Brachypodium distachyon *root colonized by a mycorrhizal fungus (*Rhizophagus irregularis*, arrows), visualized* in situ*. The original scan* ***(A)*** *was subsequently reconstructed a second time with DeepRecon®* ***(B)*** *using the Image Quality mode, with significant improvement in signal-to-noise ratio. DeepRecon® can also be used to reduce the number of projections required for high resolution and low noise XRM scan volumes, significantly reducing scan times.*

stabilize samples in tubes or pipette tips (Figures 1C, D). Moistened lab towels can also be added to maintain high relative humidity to prevent hydrated plant samples from drying out and moving during the scan. Samples embedded in resin can be mounted on wooden applicator sticks or flat-headed pins with epoxy gel.

3D printed holders. The flat panel detector of the Xradia Versa allows multiscale imaging of plant roots *in situ*, but it requires customized holders for proper mounting of the pot in the instrument. Using a handheld laser scanner to generate a 3D model of the XRM instrument's sample base, and then CAD software and a 3D printer, matching holders of different diameters that fit over the XRM sample base can be produced (Figure 1F). Plants can be grown in plastic pipes that fit each of the holders, the flat panel detector can be used to image the entire root system *in situ*, and then regions of interest can be selected for high magnification Zoom scans using the objective lenses, all without removing the sample from the instrument. Expanded polystyrene can also be used to craft specific holders/mounts for a range of tube sizes in which *in situ* root imaging is required. Figure 2 shows four examples of different samples prepped and mounted for XRM as described above.

Advanced methods for boosting imaging possibilities

Deep Learning Reconstruction. High resolution XRM scans typically take many hours due to the large number of projections that are required to increase quality of the final 3D volume. DeepRecon®, a software tool using deep learning that is integrated into the reconstruction workflow (see Villarraga-Gomez *et al*, 2022), was developed to address this. It functions in two modes: one designed to reduce scan times without compromising quality in the final volume, and the other generating reconstructions with significantly improved signal-to-noise ratio to improve image quality. Incorporating DeepRecon® into an XRM system can increase scan throughput while maintaining high quality 3D volume data. Figure 3 shows an example how DeepRecon® can significantly improve the signal-to-noise ratio from an existing scan. *Brachypodium distachyon* was grown in sand in a 20mL syringe barrel along with the mycorrhizal fungus *Rhizophagus irregularis*. When the roots were evenly colonized by the fungus, osmium tetroxide was used to fix and contrast enhance the entire system for an overview scan so that regions could then be identified for high resolution acquisition. Figure 3A shows a 2D digital section through the colonized roots *in situ* from the original scan, and Figure 3B shows the same region after a new reconstruction from the original volume was generated using DeepRecon®. The reduction in noise is significant and should greatly improve computational segmentation.

Phase contrast. Absorption-based XRM takes advantage of the difference in X-ray attenuation caused by differential density of sample features, typically as the result of exogenously applied contrast agents in the case of low-density biological samples. Propagation-based phase contrast imaging is an alternative method for imaging low-Z (low atomic number) samples without the use of contrast agents. Rather than relying on direct X-ray absorption with the detector placed close behind the sample, propagation phase contrast imaging moves the source and detector some distance away from the sample. This allows detection of the phase shift caused by the differential

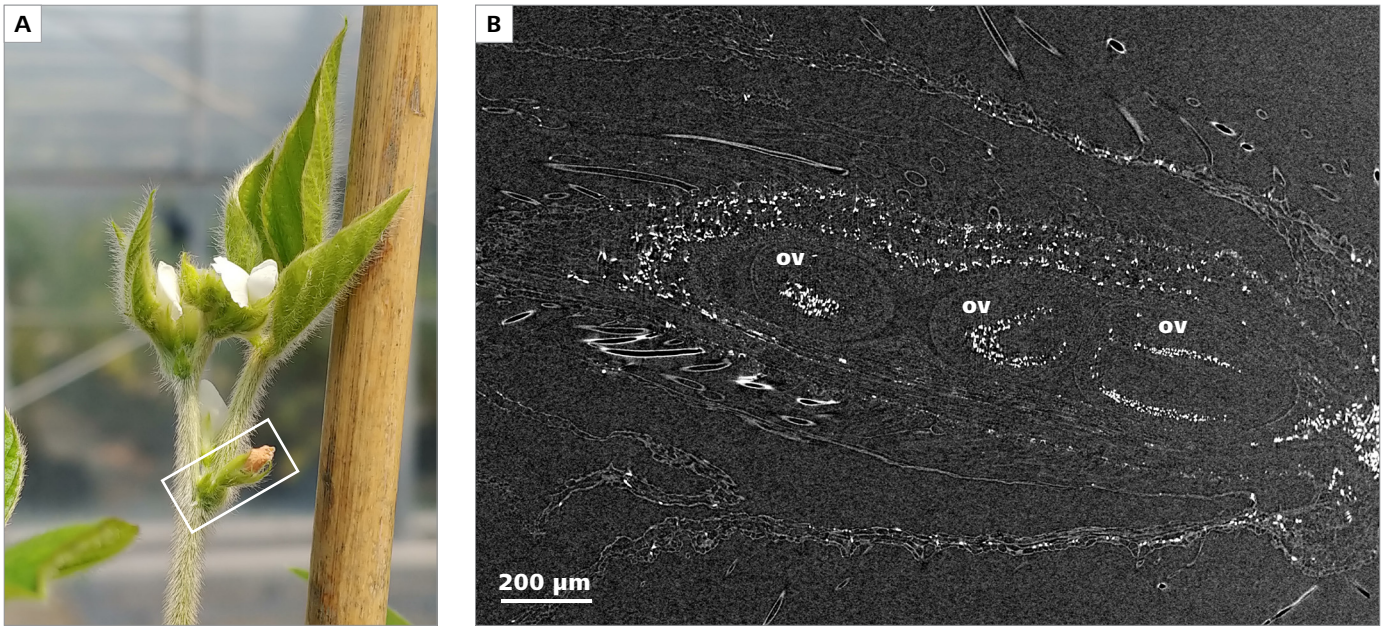

Figure 4 *Phase contrast imaging, XRM of plant tissue without contrast agents. A Single soybean flower (box) was fixed for 24 hours in ethanol, rinsed and mounted for XRM, and scanned using source-sample-detector geometry where phase shift resulted in a distinct 3D volume (B), where three developing ovules (ov) are visible. Phase contrast can be used in situations where application of contrast agents is not an option, and to significantly shorten the time from sample collection to final imaging volume.*

refractive index of the sample compared to air and enables detection and imaging of low-Z tissues, even without contrast enhancement. Figure 4 shows a fresh soybean flower that has been fixed for 24 hours in ethanol and then rinsed and mounted for XRM. A source-sample-detector geometry was chosen that optimized the phase shift to visualize important features of the developing ovary and ovules without the use of contrast agents. For the soybean flower example, compare the phase contrast XRM volume (Figure 4)—only 48 hours from living sample to completed XRM scan—to the equivalent sample fixed and contrast enhanced with ePTA (Figure 7) that required a minimum of 21 days incubation prior to imaging. Clearly the contrast-enhanced sample results in greater contrast in this example, but it is important to have the phase contrast option for situations where contrast agents may not be possible, or when rapid sample imaging is required. Further, deep learning-based tools like DeepRecon® can significantly enhance phase contrast scans, improving signal-to-noise and reducing scan times.

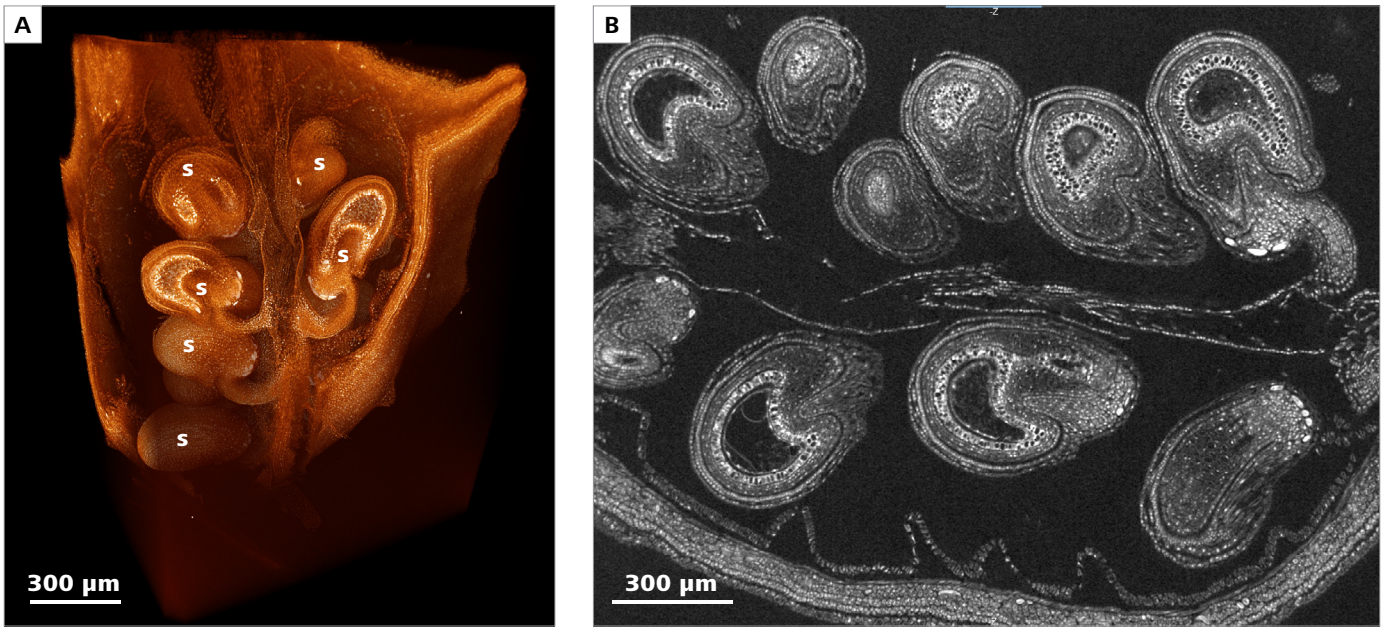

Figure 5 *Developing pod of the cover crop pennycress* (Thlaspi arvense). ***A)*** *3D volume rendering allows visualization of where developing seeds* ***(s)*** *are in 3D space within a single pod.* ***B)*** *2D clipping plane shows individual cross-section cell layers evident in developing seeds. Strong edge contrast in 2D planes should facilitate advanced image segmentation.*

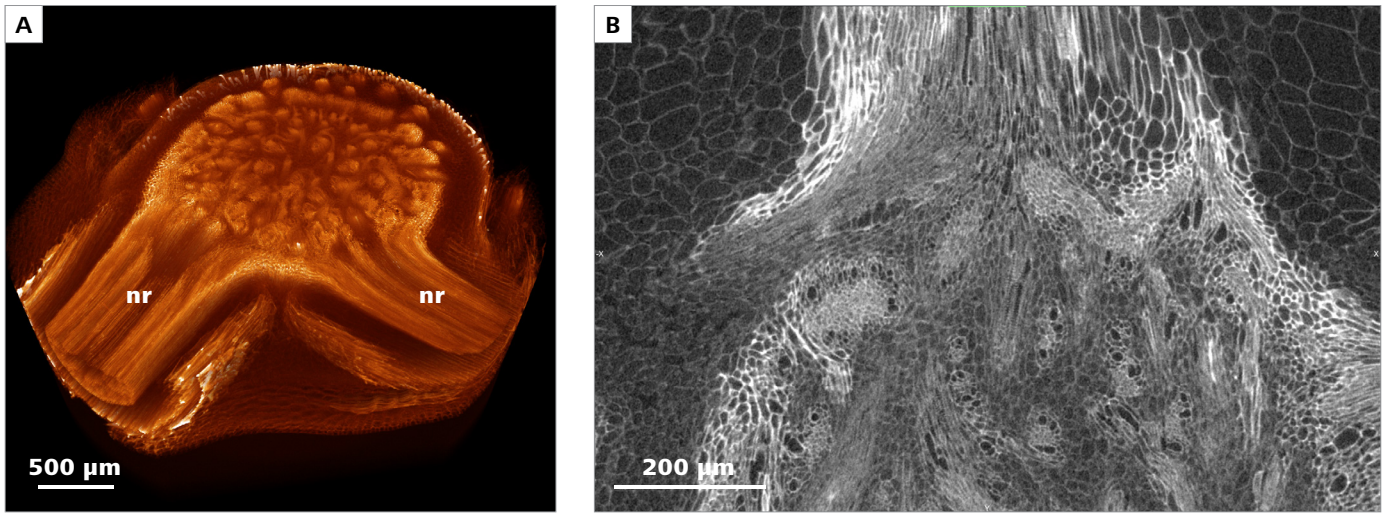

Figure 6 *Nodal plexus of an 8 day old maize seedling. This complex region of the plant is located at the soil line and contains the zone where root and shoot vascular bundles intersect, and where nodal roots* ***(nr)*** *are formed and emerge. The 3D volume rendering* ***(A)*** *and 2D clipping plane* ***(B)*** *illustrate this complex network of xylem and phloem tissue, responsible for transporting water and solutes throughout the entire plant. The higher density of vascular tissue provides a strong XRM signal and should facilitate computational segmentation to identify, map, and measure the entire vascular network.*

Using XRM as part of a Correlative Workflow

Electron microscopy (EM) can generate high resolution sub-cellular images of plant tissues, particularly with advanced sample preparation techniques like high-pressure freezing, freeze substitution, and low temperature resin embedding that combine to significantly reduce sample artefacts. Furthermore, improved methods of contrasting samples are expanding the range of plant tissues that can be successfully imaged using high magnification EM (*e.g.* OTO, see Belanger *et al*, 2022). Adding XRM imaging can dramatically increase efficiency and effectiveness in the vEM workflow, streamlining the difficult task of finding a specific region of interest in an opaque resin-embedded sample. For example, high pressure frozen tobacco leaf disks can be prepared for vEM, the entire resin-embedded leaf disk can be scanned with the 4X objective lens of the XRM to identify a region of interest, and then the 40× objective lens can be selected to scan the region containing chloroplasts. These detailed 3D data can then be used as a road map to direct vEM acquisition using techniques such as serial block face SEM, knowing in advance exactly where in the resin block we needed to section (Duncan *et al*, 2022).

XRM in action for plant biology specimens

The ZEISS Xradia 520 Versa XRM that is installed in Dr. Christopher Topp's lab at the Donald Danforth Plant Science Center has become a vital research tool. The lab has leveraged their plant

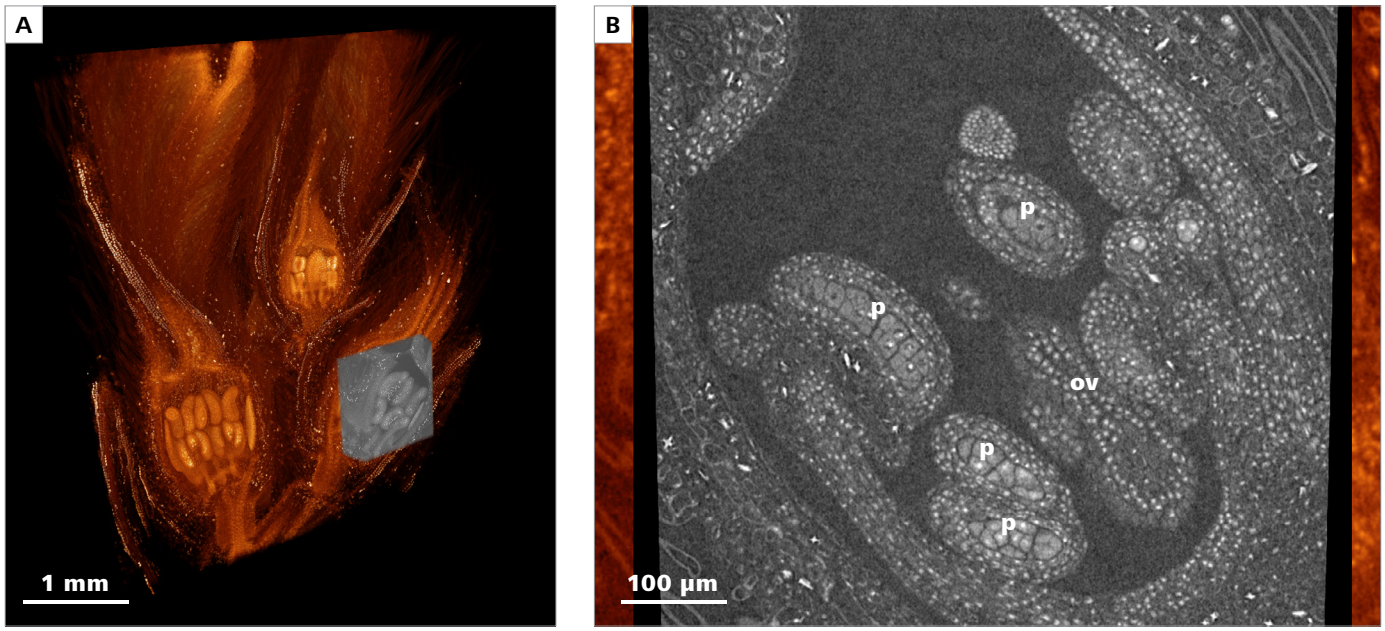

Figure 7 *Multiscale Scout & Zoom scan of a soybean axillary bud.* ***A)*** *3D volume rendering of a soybean axillary bud with multiple flowers that will self-pollinate and produce seeds in pods.* ***B)*** *2D clipping plane from a high magnification Zoom scan of a single soybean flower (grayscale region in* ***A****). Immature pollen grains* ***(p)*** *are developing inside anthers that are arranged around the stigmatic surface of the ovary* ***(ov)****.*

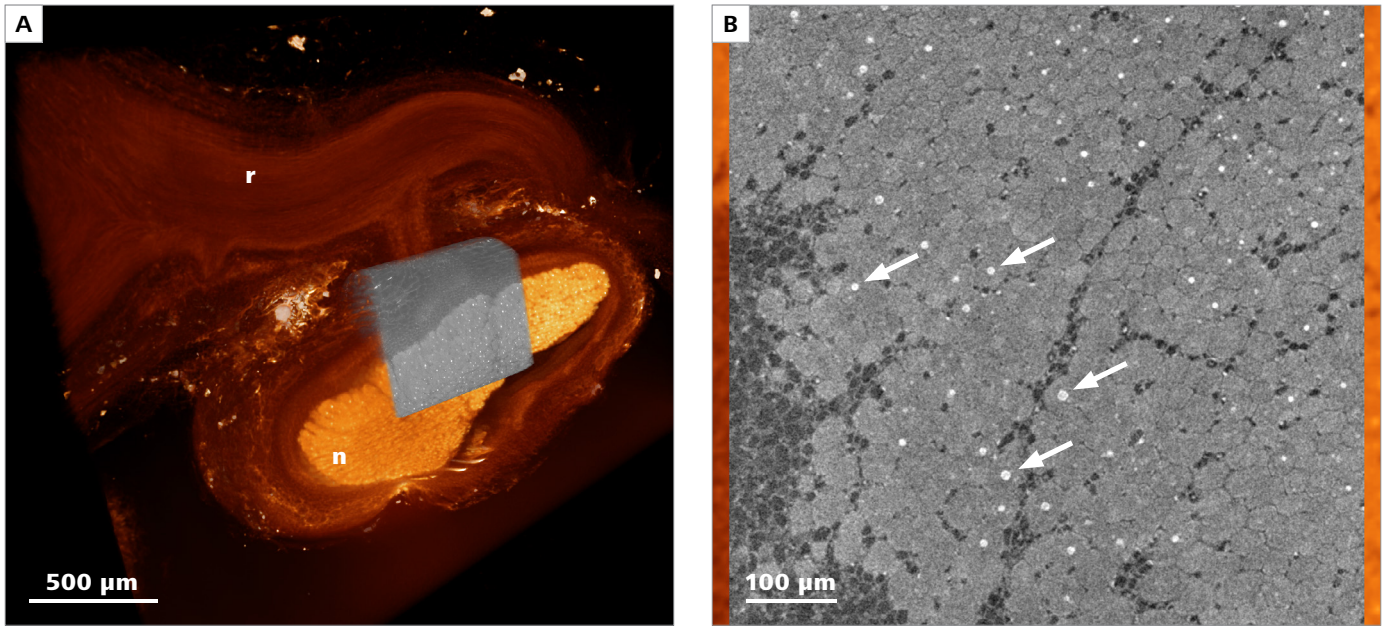

Figure 8 *Multiscale Scout & Zoom scan of a nodule formed by the nitrogen-fixing bacterium* Bradyrhizobium diazoefficiens *on a soybean root.* ***A)*** *3D volume rendering from a Scout scan of a soybean nodule* ***(n)*** *and associated root segment* ***(r)****.* ***B)*** *2D clipping plane from a high magnification Zoom scan within soybean nodule (grayscale region in* ***A****), where bright nuclei* ***(arrows)*** *of individual host cells that house bacteroids are easily visualized.*

biology-specific microscopy and imaging experience to incorporate XRM into the research program. Some highlights from their work involving XRM are included below. Fixation and contrast using ePTA is the most-frequently used preparation method for a wide range of plant samples and XRM scan data shown in Figures 5–8 were all prepared with this protocol. Specific instrument settings and scan parameters are listed in Table 1.

Pennycress—Ecosystem services and commodity crop. Soil health is an important consideration in developing modern agricultural systems that are efficient, effective, and sustainable. There is increasing research into the use of cover crops to improve soil health and also provide a commodity product for growers in addition to staple crops like corn and soybean within which cover crops can be integrated. Pennycress is a cover crop that is being studied for its ability to stabilize soil and enhance soil microbial populations, and also provide a commodity seed product high in oil and protein. XRM can be used to visualize pennycress pod and seed development to evaluate breeding efforts designed to increase yield (Figure 5).

Corn stalk—Mapping the connection between shoot and roots. Root systems are the most important part of the plant in terms of water and nutrient acquisition, so understanding 3D root architecture should provide insights into breeding root systems with superior agronomic qualities. The vascular system is responsible for moving water and solutes from the roots to the rest of the plant but visualizing this complicated network in large intact samples is problematic. XRM can be used to visualize the xylem and phloem cells of the vascular system at the nodal plexus, the region where the vascular networks from above and below the soil line intersect. Vascular tissue is denser than surrounding plant tissue and is readily distinguished with XRM imaging (Figure 6).

Soybean—Plant-based protein and beneficial bacteria. Soybean seeds are an important source of plant-based protein, and soybean oil has many valuable economic uses, so understanding soybean floral development should aid breeding programs aimed at increasing soybean seed yield. XRM has been used to follow growth and development of soybeans from floral tissue to mature seeds across scales, providing critical data on how soybean floral structures are positioned relative to one another in 3D space (Figure 7). XRM is also valuable for studying soybean roots and their symbiotic relationship with beneficial microbes. Soybean roots can be colonized by nitrogen-fixing bacteria that convert atmospheric nitrogen into forms that the plant can use, in exchange for sugars from photosynthesis, potentially reducing the need for chemical fertilizers. Bacteria induce specific host root cells to swell and divide forming a nodule, which then provides a protected environment for the exchange of nitrogen compounds from the bacteria for sugars from the roots (Figure 8).

Figure	Voltage	Power	Current	Objective	Filter	Source	Detector	Bin	Exposure	Pixel Size	Projections	Time
3	90 kV	8 W	89 µA	4X	LE1	30 mm	35 mm	1	7 sec	1.6 µm	4501	12 h
4	50 kV	4 W	80 µA	4X	Air	30 mm	30 mm	1	10 sec	1.7 µm	3201	11 h
5	50 kV	4 W	80 µA	4X	Air	16 mm	26 mm	1	5 sec	1.3 µm	4501	9 h
6	50 kV	4 W	80 µA	4X	Air	20 mm	20 mm	1	6 sec	1.7 µm	4501	11 h
7A	50 kV	4 W	80 µA	4X	Air	18 mm	20 mm	1	6 sec	1.6 µm	4501	11 h
7B	60 kV	5 W	84 µA	20X	Air	16 mm	13 mm	2	6 sec	0.7 µm	2401	10 h
8A	50 kV	4 W	80 µA	4X	Air	17 mm	17 mm	2	1.5 sec	3.4 µm	4501	5 h
8B	70 kV	6 W	86 µA	20X	Air	15 mm	12 mm	2	10 sec	0.7 µm	3201	10 h

Table 1 *ZEISS Xradia 520 Versa X-ray instrument settings for scans in Figures 3–8.*

Summary

Lab-based XRM offers a unique capability for generating high resolution 3D volumetric image data for a wide range of plant samples, filling an important imaging gap left by conventional X-ray tomography and light, laser, and electron microscopy. Many detailed methods for preparing and imaging various plant samples with XRM have been provided with the expectation that these examples foster a wider application of XRM in plant biological research. The use of XRM in a correlative workflow is especially exciting, providing a detailed road map of an entire resin-embedded sample to facilitate accurate and efficient vEM imaging of specific regions of interest, avoiding the uncertainty of sectioning blindly through a sample and hoping to find features of interest. The development of sophisticated deep learning-based reconstruction tools like DeepRecon® will increase scan quality and streamline workflows by reducing scan times without compromising image quality. A combination of the XRM instrument capability and sample preparation experience and optimization provided in this overview not only evidences the value of XRM in plant science research but also should assist those new to the technique in generating the same level of results in a wide range of plant tissues.

Acknowledgement

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Cover image

Quinoa (Chenopodium quinoa)

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