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PNNL-34647

Imaging Intact Filamentous Fungi from Spore to Hyphal Tip on the Nanoscale

July 2023

1 James E. Evans
2 Eric Hill
3 Trevor Moser
4 Jory Brookreson

U.S. DEPARTMENT OF ENERGY

Prepared for the U.S. Department of Energy
under Contract DE-AC05-76RL01830

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Abstract

Previously, the Pacific Northwest National Laboratory was limited by a 3-order of magnitude gap in resolution for direct 2-D and 3-D imaging of chemical, biological, energy and materials science systems. Our imaging instrumentation could directly visualize 3-D ultrastructure without labeling at resolutions less than 10-nanometers or greater than 10-micrometers. To address this gap, we procured and commissioned a Sigray NanoFast Bio-Lambda laboratory-based x-ray nanotomography system. This equipment provides the capability to image through tens of micrometers of organic materials at down to 35-nanometer resolution to bridge the nanoscale to the mesoscale for biological systems, organic polymers, environmental and particulate samples.

Acknowledgments

This research was supported by Earth and Biological Sciences Directorate (EBSD) Seed funding, under the Laboratory Directed Research and Development (LDRD) Program at Pacific Northwest National Laboratory (PNNL). PNNL is a multi-program national laboratory operated for the U.S. Department of Energy (DOE) by Battelle Memorial Institute under Contract No. DE-AC05-76RL01830.

Contents

1.0 Introduction

X-ray nano-tomography is an advanced imaging technique that allows for three-dimensional visualization and characterization of samples at the nanoscale[1]. It utilizes X-rays to penetrate dense materials and generate detailed structural information without the need for staining or physical sectioning. X-ray nanotomography provides direct density images with sub-micrometer to nanometer resolution, and it enables the visualization of fine details and structures across scales from samples as small as individual cells to thicker tissues. Various wavelengths of X-rays can be used to interrogate different types of materials on the micro- and nanoscale. Hard X-rays (>6keV)[2,3] can provide nanoscale imaging of heavy inorganic materials hundreds of micrometers thick while soft X-rays (0.2-6keV)[4-6] can image organic materials several tens of micrometers thick.

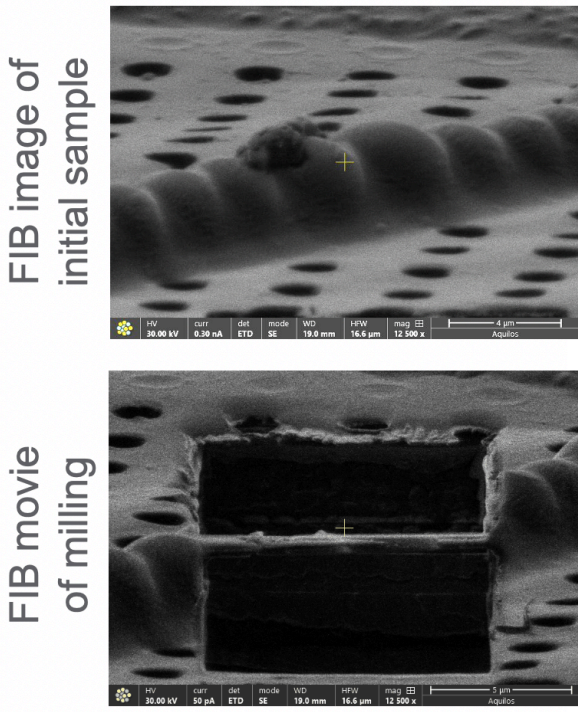

Figure 1: Current approach for nanoscale 3D imaging of cells and tissues that are too thick for TEM (method with finest resolution) is to mill the cells thinner (loses whole-cell context and statistics). This hinders our ability to probe how phenotype is determined and controlled by morphological and compositional changes.

X-ray nanotomography generates a series of 2D projection images from different angles, which are then reconstructed into a high-resolution 3D volume. This 3D representation provides a comprehensive understanding of the sample's morphology, spatial organization, and internal structures. This also enables the measurement of dimensions, volumes, and surface areas of substructures, facilitating quantitative characterization and comparison between different samples or experimental conditions[3,6]. This multiscale imaging capability is particularly valuable when studying complex biological systems or hierarchical materials. In addition, it can image both organic and inorganic components, making it suitable for studying matter with diverse compositions. Finally, X-ray nanotomography can be used in conjunction with other imaging modalities, such as fluorescence microscopy or electron microscopy to permit correlative imaging of structural information with functional and molecular data obtained from other techniques[1,7].

Previously, PNNL had imaging systems that directly visualize 3-D ultrastructure without labeling at resolutions greater than 1-micrometer (e.g., X-ray microtomography, Raman Microscopy) and less than 10-nanometer (e.g., Scanning Electron Microscopy and Transmission Electron Microscopy). To utilize the highest-resolution imaging modalities, samples would need to be cut

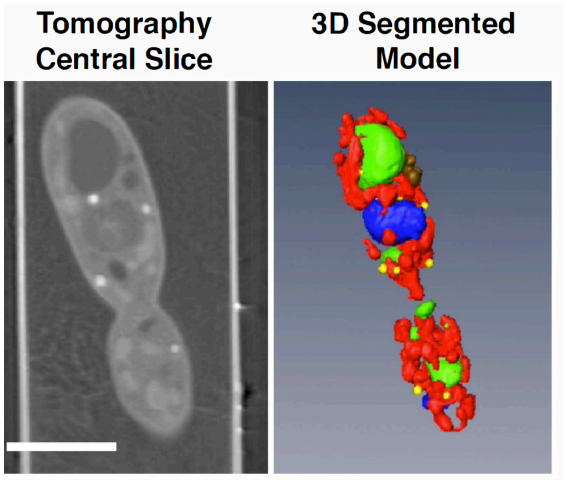

Figure 2. X-ray computed nanotomography (left) using synchrotron beamline and 3D segmented reconstructions (right) of static *Yarrowia lipolytica* (5 µm scale) with various subcellular organelles colored according to their linear absorption coefficients for label-free and unambiguous identification. Similar images and visible subcellular features are anticipated for the proposed laboratory-based microscope.

or milled to a compatible thickness (Figure 1) meaning the imaging area represents only a fraction of the true volume. However, synchrotron-based X-ray nanotomography systems[5] were able to bridge this gap (Figure 2). While we could not bring a synchrotron based light source to PNNL, a laboratory-based light source was possible.

2.0 Laboratory-based Instrument Solution

We sought to develop a laboratory-based x-ray nanotomography system at PNNL to allow structural analysis without physical sectioning from samples upwards of 100x thicker than currently possible with transmission electron microscopy and with spatial resolution of ~35 nanometer. Through a competitive bid process, we procured and then commissioned a Sigray NanoFast Bio-Lambda laboratory-based x-ray nanotomography (Figure 3) system that fit our technical needs. The instrument features: two beam energies (1.7 and 2.7 keV) each with absorption and phase contrast modes, operation under cryogenic or ambient sample conditions and preset low magnification and high magnification fields of view with optical microscopy correlative overlay via combined visible light and x-ray projection imaging for alignment and targeting.

Figure 3. Image of the Sigray NanoFast BioLambda nanotomography å commissioned at PNNL/EMSL.

3.0 Results

After installing the soft X-ray nanotomography microscope we began commissioning experiments. The first test was to demonstrate the resolution characteristics and imaging stability using the 2.7keV beam. Figure 4 shows the imaging performance for data collected/integrated over 1 minute and 1 hour. The inner most ring features of this standard star test sample are resolved even with only 1 minute data collection and the system does not suffer significant drift as demonstrated by the 1-hour image which has higher contrast and retains sharp features.

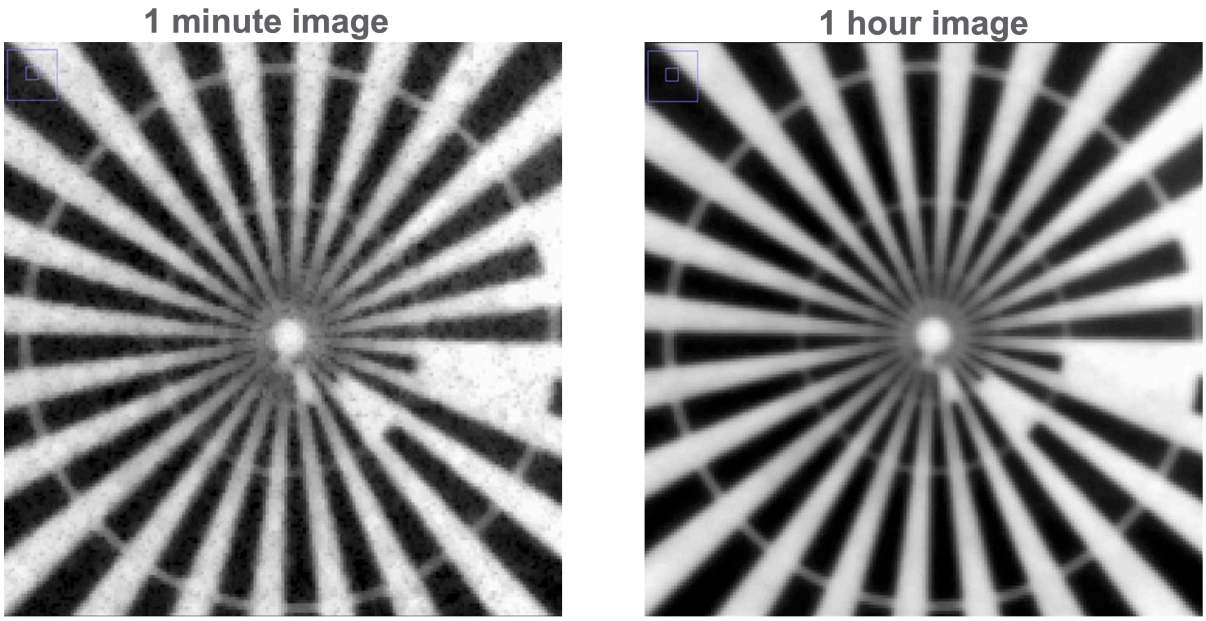

Figure 4. Absorption contrast already resolves 30nm spacings with only 1-minute exposures on standard testing sample. Inner ring shows spatially resolved 30nm spacings with only 1-minute exposures on standard test sample.

Next we confirmed that both absorption and phase contrast imaging modes were able to resolve all features on a standard metric calibration standard (Figure 5). These combined results demonstrated the functionality of the microscope but only in 2D.

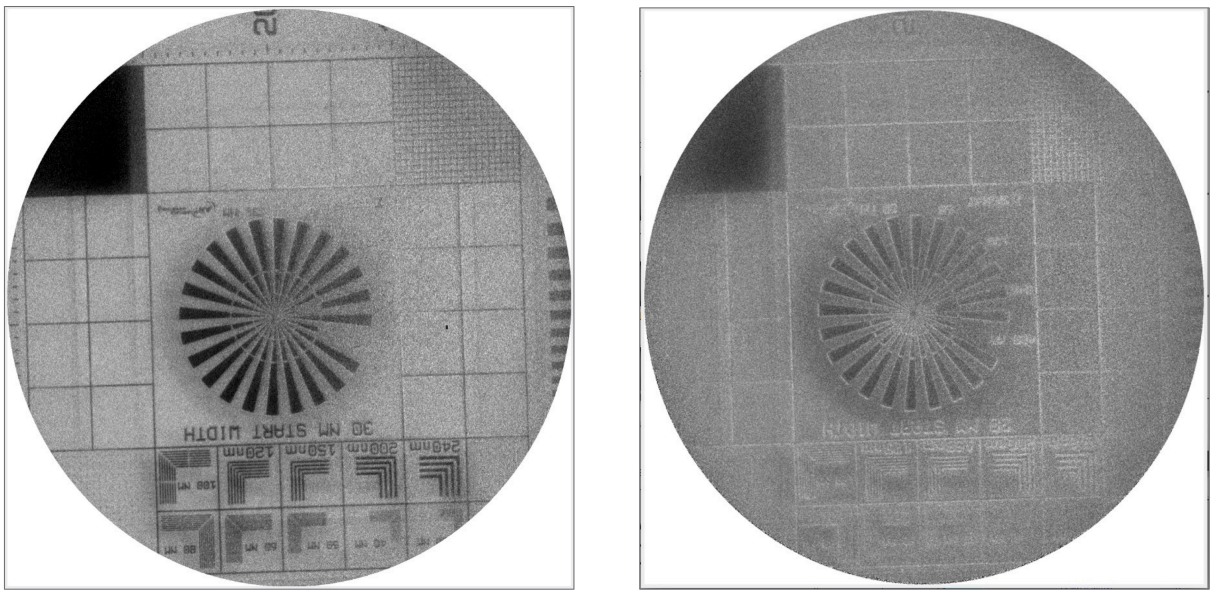

Figure 5. Demonstration of Absorption (left) and Zernicke phase contrast (right) at 2.7keV with standard testing sample. All features are detected in both modes.

To expand into 3D visualization, we performed Absorption contrast tomography of a diatom frustule. We collected a -60 to +60 degree tilt series in 0.5-degree increments and reconstructed the native structure. As can be seen in Figure 6, a roughly 35µm x 55µm x 20µm section of the frustule shell was reconstructed in full showing both the expected spines and surface roughness.

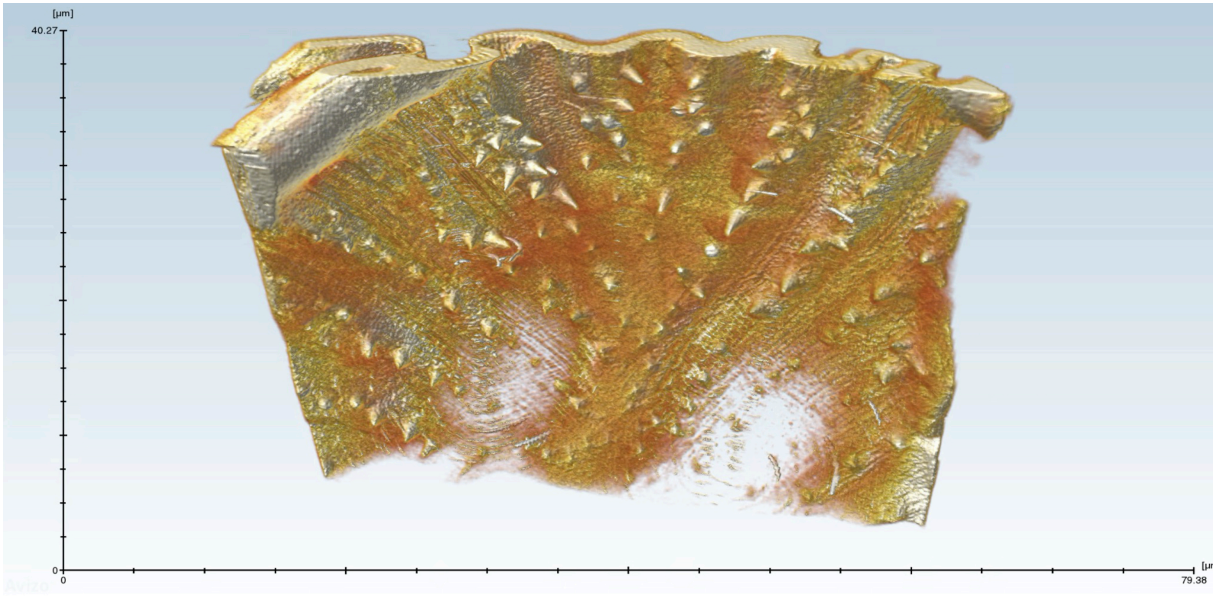

Figure 6. Absorption contrast tomography of diatom frustule at 30nm resolution. 3D tomographic reconstruction of *Campylodiscus hibernicus* frustule acquired on the Sigray Nanofast at PNNL.

After characterizing the performance in both 2D and 3D, we sought to compare imaging performance for our laboratory-based soft X-ray nanotomography system versus a synchrotron-based source. We therefore analyzed a biological system that had been previously imaged at a synchrotron under cryogenic conditions that had since been allowed to air dry (Figure 7). While we did not expect the performance to be identical, we did hope to see comparable information such as the ability to detect lipid droplet densities purely based on a linear absorption coefficient.

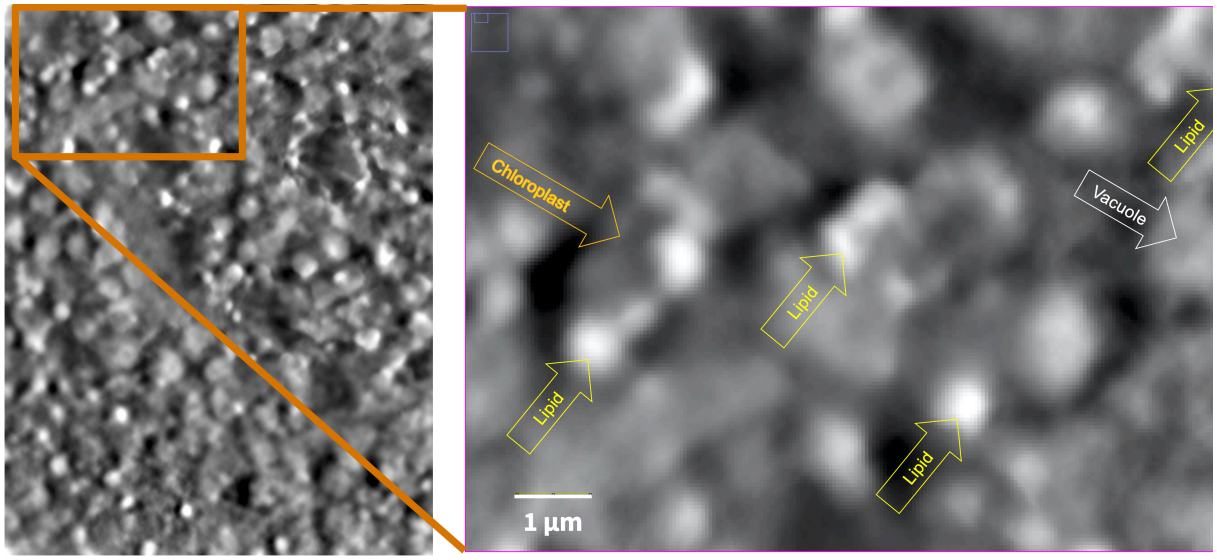

Figure 7. Absorption contrast soft x-ray nanotomography of crowded *Ostreococcus* biofilm showing cellular density that can be distinguished by linear absorption coefficient as showing lipid accumulation (yellow arrows), chloroplast (orange arrow) and vacuole (white arrow).

Excitedly, while the 3D reconstruction is at lower resolution compared to the synchrotron source, the overall outline of individual *Ostreococcus* cells was discernable, as was the location and size of chloroplasts, vacuoles, and lipid droplets (white arrowheads, Figure 8). The laboratory-based soft X-ray nanotomography instrument is therefore able to be applied as desired to either provide direct standalone imaging across scales, or it can be used as an initial local screening system to identify and optimize sample conditions such that the best or most variable samples that need even higher resolution capabilities are the only ones sent to synchrotrons for efficient utilization of those highly competitive and limited resources.

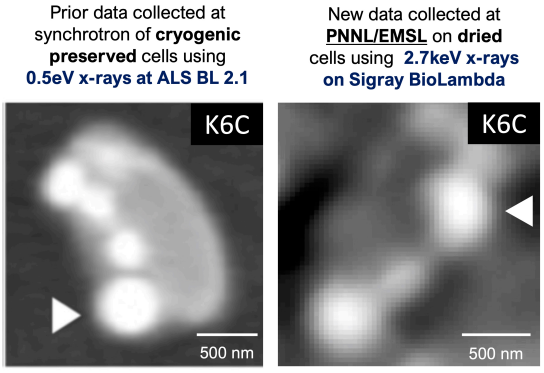

Figure 8. Comparison of *Ostreococcus* cells with visible lipid droplets acquired with synchrotron soft-x-ray nanotomography (left) and with the Sigray Nanofast lab-based system at PNNL (right) showing similar ability to detect the whole cell outline, chloroplast outline and presence of bright white lipid droplets (white arrowhead) based on linear absorption coefficient.

4.0 Summary

The specific goal of this project was to develop a new capability for visualizing thick biological samples while maintaining nanoscale spatial resolution to fill a gap in multiscale imaging at PNNL. This project commissioned a new Sigray Bio-Lambda soft x-ray nanotomography microscope to provide onsite three-dimensional imaging of intact organisms up to 30 micrometers thick with 35-100 nanometer spatial resolution. The new capability is synergistic with other imaging modalities at PNNL and can be used to visualize the native architecture of biological, organic, and inorganic samples.

5.0 Looking Forward

The soft X-ray nanotomography instrument commissioned by the above project is now available for use by any directorate at PNNL including EMSL users. The platform can be used to help maximize impact and access to synchrotron facilities via local sample screening at PNNL before an approved synchrotron session, or it can be used as a standalone truly laboratory-based microscope colocalized near other multimodal analysis capabilities within EMSL. The main impact of this work is the ability to link 3D ultrastructure from the nanoscale to the mesoscale for biological systems, organic polymers, environmental and particulate samples, and energy material.

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