Chemically sensitive 3D imaging at sub 100 nm spatial resolution using tomography in a scanning transmission x-ray microscope

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ABSTRACT

Computed angle scan tomography has been implemented for the first time using sample raster scanning in a scanning transmission X-ray microscope. The experimental apparatus, acquisition and data analysis procedures, and first results from fully hydrated river biofilm samples measured from 528-534 eV are reported. The multiple energy images are processed to 3-dimensional quantitative chemical maps of major biological components, such as proteins, polysaccharides and lipids.

Keywords: 3D chemical imaging, scanning transmission x-ray microscopy, biofilms, spectroscopy, tomography

1. INTRODUCTION

The biochemical processes of many biological and environmental systems take place on a sub-micrometer scale, thus there is a need for detailed chemical analysis at high spatial resolution. Most chemical imaging studies have been conducted as two-dimensional projections. In many cases, a three-dimensional image of a particular chemical compound would further our understanding of the biochemical processes.

Previously, x-ray micro-tomography experiments of biological samples[1-3] have used only a single photon energy. These studies have provided high spatial resolution, density-based images, but only limited chemical information. Near-edge x-ray absorption fine-structure spectroscopy (NEXAFS), performed by a soft x-ray scanning transmission x-ray microscopy (STXM) has the chemical sensitivity[4,5] required to look at samples in hydrated environments.[6-9] Thus, the combination of NEXAFS to achieve chemical sensitivity and tomography with sub-micron spatial resolution performed by a STXM shows great potential when applied to chemical systems, such as multi-phase polymer composites,[10] biomineralization, soil samples, biomaterials, etc.

We are developing new methods for applying computed angle-scan tomography in a scanning transmission x-ray microscope for chemical visualization. Our goal is not only to do x-ray tomography at 50 nm spatial resolution, but also to utilize the capability of STXM to perform 3D NEXAFS at very high spectral resolution in order to identify and quantitatively map the chemical composition of the sample in a spatial volume. In this study we present our experimental arrangement, sample preparation, measurement procedures and first results of STXM-based "chemical tomography". Chemical maps of river biofilms derived from the 3D volume are shown to illustrate the usefulness of this new technique.

2. EXPERIMENTAL

2.1 Sample preparation

Glass capillaries were used to hold the sample and keep it hydrated inside the scanning transmission x-ray microscope, at BL 5.3.2 of the Advanced Light Source. These glass capillaries are similar to the ones typically used in intra- and extra-cellular physiology as micropipettes to study electrical activity in cells and for injecting a variety of substances. To create a hollow tip with micrometer dimensions, the capillaries (initial diameter of ~1mm) were heated and pulled using a micropipette puller.

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For the STXM tomography experiment, the pulling parameters were chosen so the tip diameter would be 2-4 μm and have a uniform thickness over the imaged length, 10-20 μm. The thickness of the glass wall of the tip is a few hundred nanometers.

In this study, the glass capillaries were filled at the backend with cultivated biofilm (inoculated from the South Saskatchewan River, Saskatoon, Saskatchewan, Canada) containing a complex community of bacteria and algae in river water. The capillaries were then centrifuged for 5-10 minutes with a minimum of 6000 g to ensure that the water and the sample would move into the fine tip. The 1mm backend of the capillary were then sealed with silicone.

2.2 Scanning transmission x-ray microscopy and near edge X-ray absorption fine structure spectroscopy

X-ray imaging and spectroscopy were carried out using the scanning transmission x-ray microscope at beamline 5.3.2[11,12] of the Advanced Light Source (ALS). STXM is a synchrotron-based analytical microscopy[4,5] which achieves strong chemical sensitivity through near-edge X-ray absorption spectral (NEXAFS) contrast.[13] It can provide quantitative maps of chemical species at environmentally relevant concentrations (i.e., mg/kg global), with a spatial resolution of better than 50 nm.[7-9] In the implementation at the 5.3.2 bending magnet beamline used in this work, STXM provides excellent spectroscopy in the soft X-ray regions with more than 3000 resolving power over a photon energy range from 200 to 600 eV. It also has the ability to achieve a spatial resolution of better than 40 nm over the full range of accessible photon energies through the use of differential laser interferometers. Recording images at x-ray energies at which specific compounds absorb preferentially provides a means for locating specific compounds. A much more powerful technique is to record a full image sequence ('stack')[14] and then fit the spectrum at each pixel to a set of quantitative reference spectra to extract quantitative chemical maps.[6-9] When this type of image sequence measurement is carried out at a fine mesh of angles, in a computed angle scan tomography experiment, the resulting 4-dimensional data set (x,y,E,θ) can be used to generate a quantitative 3-dimensional map of each chemical species present in the sampled volume.

2.3 Tomography sample stage

Tomography requires collection of multiple 2D images over as wide a range of projection angles as possible to achieve a reconstruction without severe artifacts.[15] By using a capillary to hold the sample, the symmetrical cylindrical geometry the capillary allows the sample to be oriented over the full 360 degrees. A special sample stage was developed to rotate the glass capillary containing the sample perpendicular to the x-ray beam inside the STXM. The use of this stage required that the sample be repositioned and refocused for every angle. Work is currently under way to construct a eucentric sample mounting stage that can adjust the sample more precisely to the rotation axis, which will reduce or eliminate the need for repositioning and refocusing of the sample for each projection and thus speed up data collection.

2.4 Image and spectral processing

The initial image and spectral processing was carried out using the aXis2000[16] software, which can handle the 4-D data stacks (2D spatial, spectral, and tilt angle) acquired by the STXM. All images are usually transformed into an optical density (OD) scale, defined as $OD = -\ln(I/I_0)$, where I is the intensity of the transmitted x-ray and I_0 is the incident intensity of the x-rays before the sample.

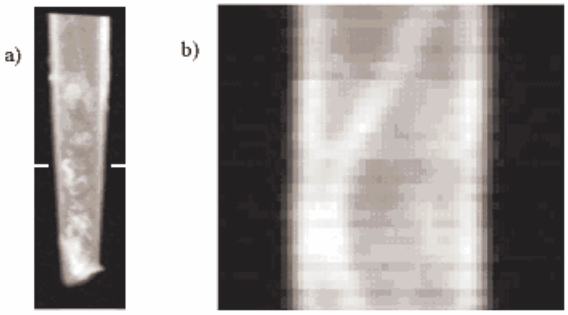

Fig. 1. (**a**) an example of one projection, an x-ray transmission image converted to an optical density (OD) scale, recorded at 532.2 eV and a tilt angle halfway through the 0 to 180 degree rotation. The sinogram (**b**), taken from the position marked in (**a**), shows that the 36 projections have been successfully aligned. The diameter of the capillary at the position of the sinogram is ~2 μm.

This transformation eliminates variation in the x-ray beam during data acquisition and enables better estimates of the sample thickness in various regions, see, e.g., Figure 1 (a). Data can be organized as a 3-D image projection series at a selected single energy or as energy difference maps.

After the initial processing in aXis2000, the projection series data is exported to the MRC file format which can be imported by many major electron microscopy tomography reconstruction programs. Currently, the IMOD software[17,18] package is being used, which is available free from the Boulder Laboratory for 3-Dimensional Electron Microscopy, University of Colorado. IMOD can do alignment of the projection series to a common axis of rotation by autocorrelation and/or alignment to fiducial markers. The alignment can be visualized as a sinogram, see Fig. 1 (b), which gives a good indication of the quality of the alignment. Finally, a 3-D volume reconstruction can be performed using filtered back projection.

3. RESULTS AND DISCUSSION

The cultured river biofilm examined in this study contains a complex community of bacteria and algae. The current sample preparation technique makes it hard to control which species end up in the viewable area of the capillary tip. The type of sample affects which photon energy (wavelength) region to use, which is further limited by absorption in the glass capillary. For this study, we chose to target the discrete region of the O 1s absorption edge (528-534 eV) where it is possible to distinguish between biological material (protein, polysaccharides, lipids), SiO2 in the glass wall of the capillary, and water. The O 1s spectra taken from the sample are plotted in Fig. 2. In the region below 533.6 eV, the glass of the capillary is sufficiently transparent to achieve a good signal to noise ratio.

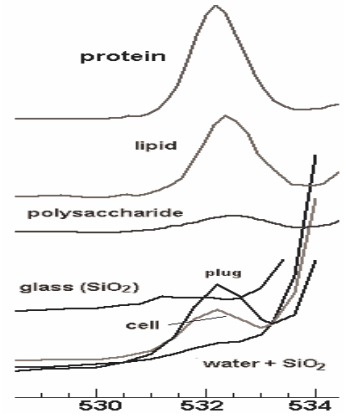

Fig. 2. O 1s spectra extracted from a 2-d projection image sequence corresponding to mainly protein, the plug, water and SiO2. Reference spectra of protein (albumin), polysaccharide (xanthan gum), and lipid (DOPC) are also plotted for comparison.

Data were collected from the capillary in the form of 8 μm by 18 μm images at 13 different energies between 528.0 to 534.0 eV to cover the O 1s edge. This was repeated for 36 tilt (rotation) angles of the capillary covering 0 to 180 degrees.

3.1 Two-dimensional chemical maps

NEXAFS data from STXM can be analyzed by energy difference maps between two energies (on- and off-resonance images of a strong feature associated with a specific component) or by spectral fitting of an image sequence using linear regression procedures such as single value decomposition (SVD).[19] Both methods intend to give a chemical map of one or more components in the sample and can be used on both two- or three-dimensional data. Figure 3(c) shows the protein map derived using energy difference maps, whereby the C 1s $\rightarrow \pi^*_{C=O}$ transition at 532.2 eV, associated with proteins, is specifically targeted (Fig. 2). Fig 3.(a) has the spectral contributions of all other substances (in particular, glass and water) removed by subtracting the image (Fig. 3(b)) taken below the absorption peak at 530.0 eV (Fig 2). Figure 3 is a single projection of the sample, giving only two-dimensional information on the spatial distribution of protein, thus no information on lateral location within the biofilm. In the next section, chemical maps are extended to three-dimensions through the use of tomography.

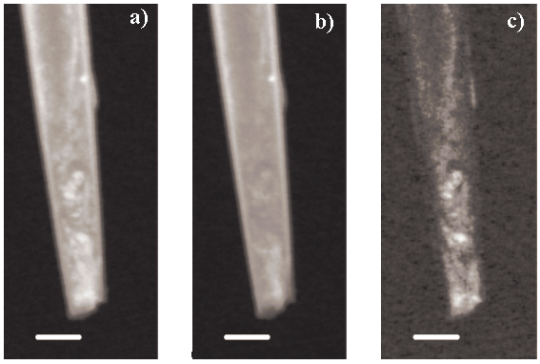

Fig. 3. The (**a**) on-resonance image (532.2 eV) and (**b**) off-resonance image (530.0 eV) used to produce the (**c**) 2D protein image difference map. (Scale bar 2 um)

3.2 Tomogram sections

Slices extracted from the tomographic difference map (532.2-530.0 eV) are presented in Fig. 4. The advantage of being able to arbitrarily select an image plane in the 3D volume is apparent in this comparison as some slices image through the centre of a cell, revealing cell envelop detail, while others image at the cell boundary. A more detailed NEXAFS analysis is in progress and is expected to yield substantially more detailed chemical maps.

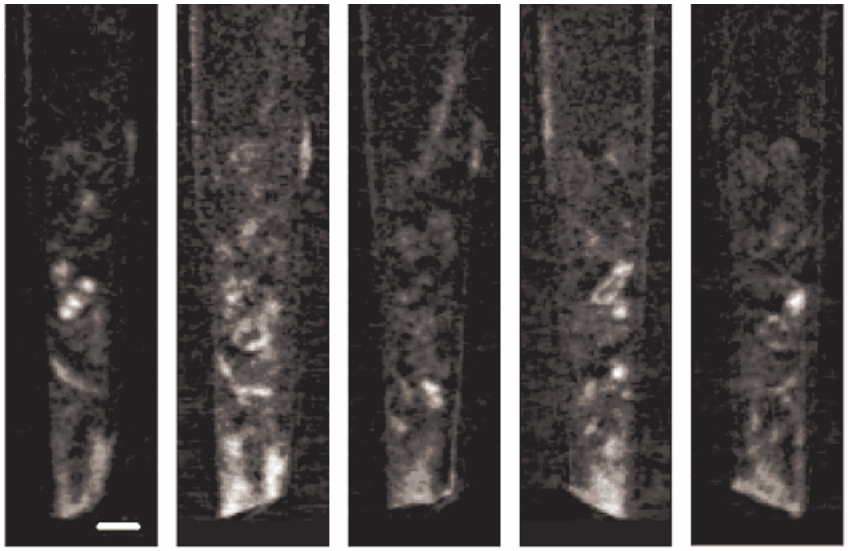

Fig. 4. Selected slices of protein ($I_{532.2} - I_{530.0}$) component maps in a 3D-volume. (Scale bar 1 um)

4. SUMMARY AND OUTLOOK

3-dimensional chemical mapping at high spatial resolution using angle scan computed tomography has great potential to add useful information about biological and environmental samples. An application area we are exploring is biomineralization, where the capability to examine both forming minerals, and the associated biological machinery may provide insight into the biochemical processes. The time to acquire a tomographic data set with STXM is somewhat longer than that in a full-field transmission x-ray microscope, although image stitching is not required. However, the dose used to acquire this particular sequence was ~200 Mgy (500 msec per pixel at a dose rate of ~400 MGy/s, in 1 msec 'acquisitions'), which is considerably lower than typically used in the corresponding full field tomography (typically 1-10 GGy). Thus excessive radiation damage of biological and environmental samples often experienced in full field tomography is expected to be reduced considerably with STXM tomography. We anticipate a further reduction in the radiation damage with implementation of a cryo system, although this will prove technically challenging if sample

scanning is retained as in this work. With a free standing sample of appropriate density (e.g. 1 micron thick at a solid density of 0.2, as in gels or foams) this technique can be applied even at the C 1s absorption edge, which would significantly further our understanding of the biochemical processes occurring in the nature. In the future we will extend the method to studies of 3-D structures of polymeric systems, which should be an improvement over our 3-D mapping of toners by serial section STXM.[10]

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