

# SEM-HOSTED SOFT X-RAY MICROSCOPE FOR LIVE CELL IMAGING

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## ABSTRACT

Using soft X-ray radiation in the so called "water window" energy range allows visualizing of cell structure and provides information which is complimentary to information obtained by electron and optical microscopy. Till now all methods for x-ray generation with energies of several hundreds eV were based on synchrotron radiation sources or laser-pumped sources and cannot be used in laboratory environments. To overcome this limitation, an SEM-hosted soft X-ray microscope has been designed using a titanium target to produce 450eV radiation, which is well positioned inside the "water window" between the absorption edges of carbon (present in proteins), and water. Using a special specimen holder to keep the biological sample under normal environmental conditions allows imaging of living cells without any fixation or preparation. An X-ray camera with direct detection performs image acquisition. The suggested set-up was tested in two types of SEM with different electron beam density, showing the possibility of live cell imaging with submicron resolution.

Keywords: X-ray imaging, cell biology, soft x-ray, SEM

## 1. INTRODUCTION

So called "water window" energy range of soft x-ray radiation is very important for practical applications.

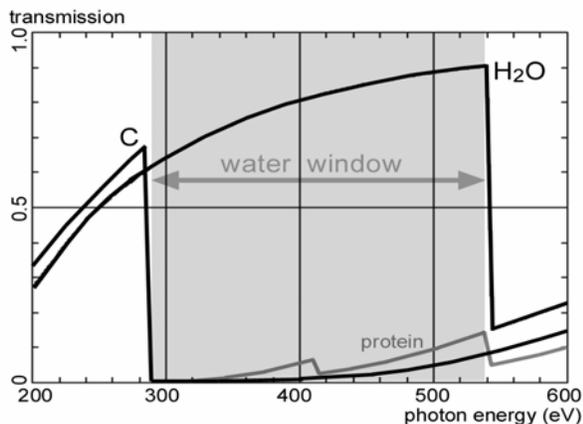


Fig.1. "Water window" X-ray energy range.

Using the energy window (Fig.1) between the absorption edge of carbon (present in cell structures and organelles) and the absorption edge of water, provides differential contrast where

water is practically transparent, but carbon containing objects can be seen with significant contrast even in submicron sizes. The main problems in using "water window" energies for practical imaging applications is connected to difficulties in generation of monochromatic soft x-ray radiation and its transmission and detection. There are two main types of source for soft monochromatic X-rays. The first is based on synchrotron radiation [1,2] with special vacuum beamlines to avoid absorption in air. The second is based on laser-pumped plasma [3,4] produced by a powerful pulsed laser beam focused on a liquid-jet [5] or solid targets including carbon nanotubes [6]. Synchrotron sources have limited availability. High flux in the soft x-ray beamlines requires keeping object at liquid nitrogen temperature and in most cases cannot be used for imaging in living cells. Laser-pumped soft x-ray sources are very complicated in construction with difficulties in target stability. The most compact of such sources can fit in a laboratory room, but are still very expensive and nonflexible. The clear advantage of both these types of soft X-ray sources is connected to the produced monochromatic (or quasi-monochromatic) radiation, which can be focused by different type of X-ray optical elements, mainly - by zone plates.

This paper demonstrates the possibility to use a conventional Scanning Electron Microscope (SEM) as the source of soft X-ray radiation in the "water window" range. A special wet cell maintains the specimen in normal environmental conditions without fixation or other sample preparation. A sensitive X-ray camera fitted in the wall of the SEM specimen chamber is used for acquiring a magnified object image. The setup described here does not contain any optical parts for focusing or magnifying x-ray radiation and produces direct geometrical magnification, which can be easily adjusted by choosing object position between the emission point and X-ray detector.

## 2. MATERIALS AND METHODS

### 2.1. X-ray source

The SEM hosted soft X-ray microscope uses an SEM focused electron beam to generate X-rays. The electron beam hits the titanium (Ti) target with characteristic energies 450eV/460eV (L-lines) and 4.51/4.93keV (K-lines). The 450eV line is well positioned inside the "water window" range; K-lines can be eliminated by choosing a low enough accelerating voltage for the electron beam. The calculated X-ray spectrum from a Ti target under a 5.5keV electron beam is shown in Fig.2. Notice that for 5.5keV electron excitation, the K-lines intensity is 15 times smaller than intensity of L-lines.

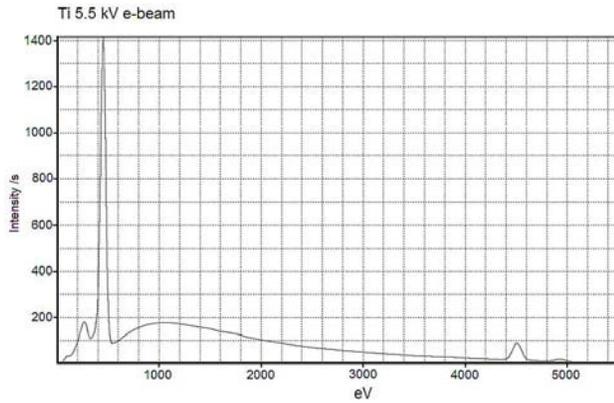


Fig.2. Calculated X-ray spectrum from Ti target.

This practical possibility to generate an X-ray line inside the "water window" range was verified by energy-dispersive X-ray analysis (EDX) using different accelerating voltages in the SEM. The measured emission spectra for 4, 5, 6, 8 and 10keV electron beam energies are shown in Fig.3.

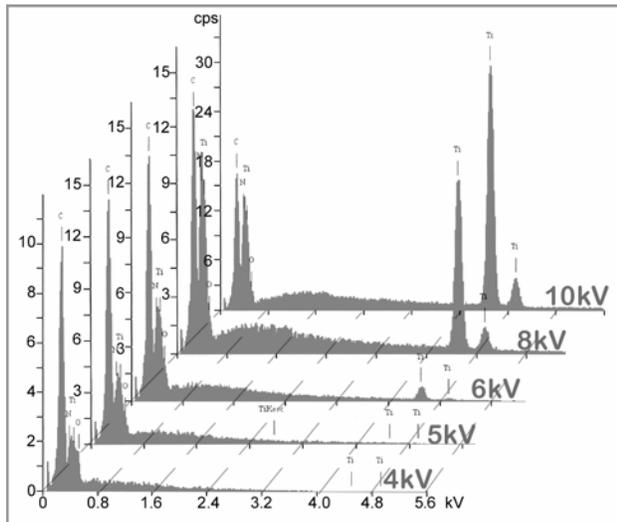


Fig.3. Measured X-ray spectra from Ti target for different accelerating voltages in SEM.

For excitation energies of 4 and 5kV the main part of the X-ray emission is produced in the 450eV Ti characteristic line and a carbon characteristic line from possible oil contamination of the target from the vacuum system of the SEM. The spectra with 8 and 10kV accelerating voltages show significant 4.51/4.93keV peaks, but at the same time the 450eV peak become larger compared to the low-voltage modes. For all accelerating voltages the number of counts per second (cps) for the 450eV peak is very low. Only 2 to 14 X-ray photons per second can be collected by a 10mm<sup>2</sup> EDX detector. In practical implementation for soft x-ray microscopy, such a low intensity will require a long exposure time to collect enough X-ray photons for imaging by a two-dimensional detector.

## 2.2. Object container

Significant absorption of soft X-rays in most gases and solids requires specific selection of materials for the object container.

For imaging living cells in air under normal environmental conditions, the X-ray beam should pass through a certain air layer. Calculated transmission for different thicknesses of air at "water window" energies is shown in Fig.4.

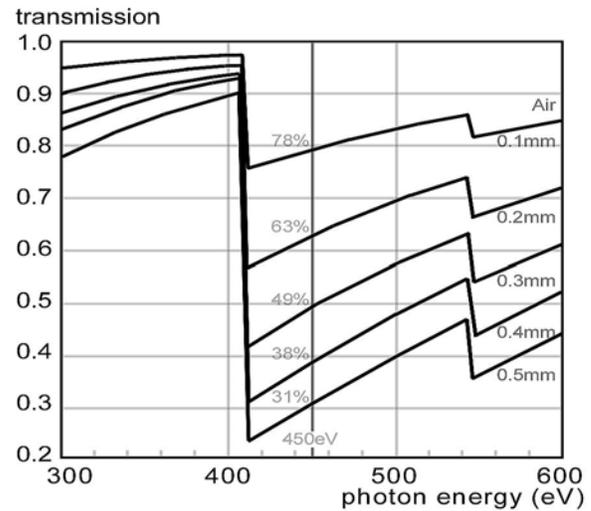


Fig.4. X-ray absorption in air layer.

As one can see, air layers thicker than 0.3mm will absorb more than 50% of x-rays with 450eV energy.

To separate an object in air from the vacuum, a Si<sub>3</sub>N<sub>4</sub> membrane can be used. Such membranes with thickness >50nm and a 0.5x0.5mm opening can withstand the difference between atmospheric pressure and vacuum. The absorption curves for different membrane thicknesses are shown in Fig.5.

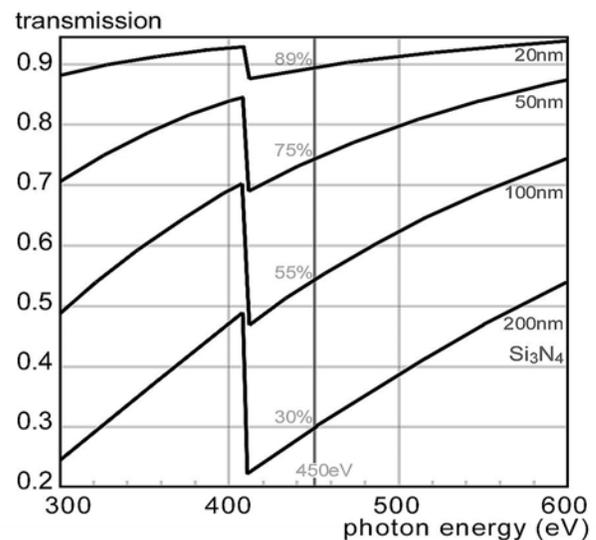


Fig.5. X-ray absorption in Si<sub>3</sub>N<sub>4</sub> membranes with different thickness.

The designed object container included two 50nm membranes with total absorption of 44% and an air gap 0.3mm with object in place, with absorption in the air close to 50%. Total transmission of the current object container design is 28% and can be improved by reducing the air gap for the object.

### 3. EXPERIMENTAL SETUP

An experimental setup for soft X-ray microscopy in SEM contains a Ti X-ray emitter, an object container and an X-ray CCD camera. It is shown in Fig.6.

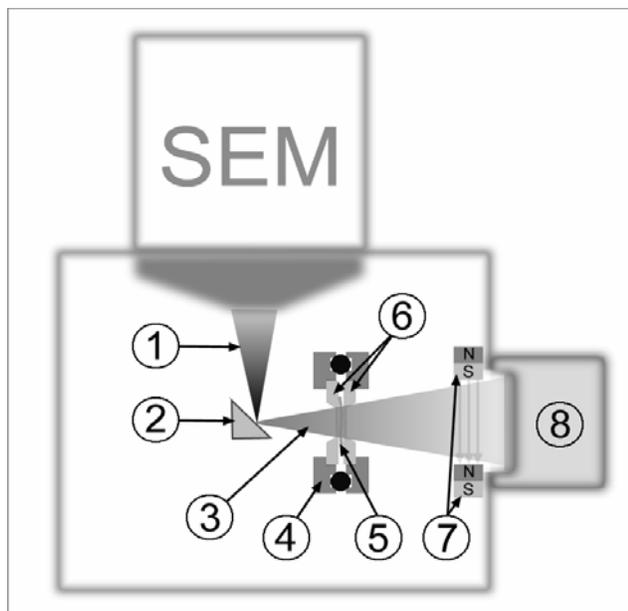


Fig.6. Experimental setup.

Inside the SEM specimen chamber a focused electron beam (1) hits the Ti target (2) to produce an x-ray beam (3). The x-ray beam passes through the object container (4) with the object (5) installed in the air gap and separated from the vacuum by two  $\text{Si}_3\text{N}_4$  membranes (6). The internal space of the container can be sealed or connected to air outside the SEM specimen chamber by a thin pipe. Behind the object container a special magnetic trap (7) deflects scattered and secondary electrons from the path to the X-ray camera (8). The X-ray camera works in photon counting mode. Collected photon distribution maps are accumulated in the computer connected to the camera.

The identical setup was used with two different types of SEM: JSM840A (JEOL, Japan) with W cathode in the electron gun and JSM7000F (JEOL, Japan) with an in-lens Thermal Field-Emission electron gun. The first one can produce a large electron beam current, second one can deliver very high beam current density.

The X-ray camera (PIXIS512, Princeton Instruments, USA) uses cooled back-illumination CCD for direct x-ray photon detection. Detection efficiency is better than 50% for energies from 300eV to 5keV. The camera is connected to the SEM beam blanking system. It interrupts the electron beam for a relatively slow readout from the CCD to avoid photon detection in the wrong position during information transfer through the CCD sensor.

Specially built software collects and analyses the acquired photon maps. It detects single-photon events, shows the full energy spectrum and image for all collected photons, allows selection of one or several energy windows and collects photon map images separately for every energy window.

### 4. FIRST RESULTS

First testing was done by imaging onion skin cells. The object container together with Ti target was mounted inside the standard 30mm specimen holder for JEOL instruments, which can pass through the airlock of the SEM. Using the JSM7000F SEM with a big specimen chamber, and special vacuum connections in the front of the camera with safety valve, limited the minimum distance from emission point to the CCD to 330mm. Big distance to the camera reduces the number of collected photons and require longer exposure for imaging. The average counting rate (only "water window" photons) was around 120 photons per second. Two images acquired using "water window" X-ray energies and different exposure times are shown in Fig.7. Pixel size is 800nm, 5.2kV accelerating voltage,  $6 \times 10^{-8}$ A beam current on the target.

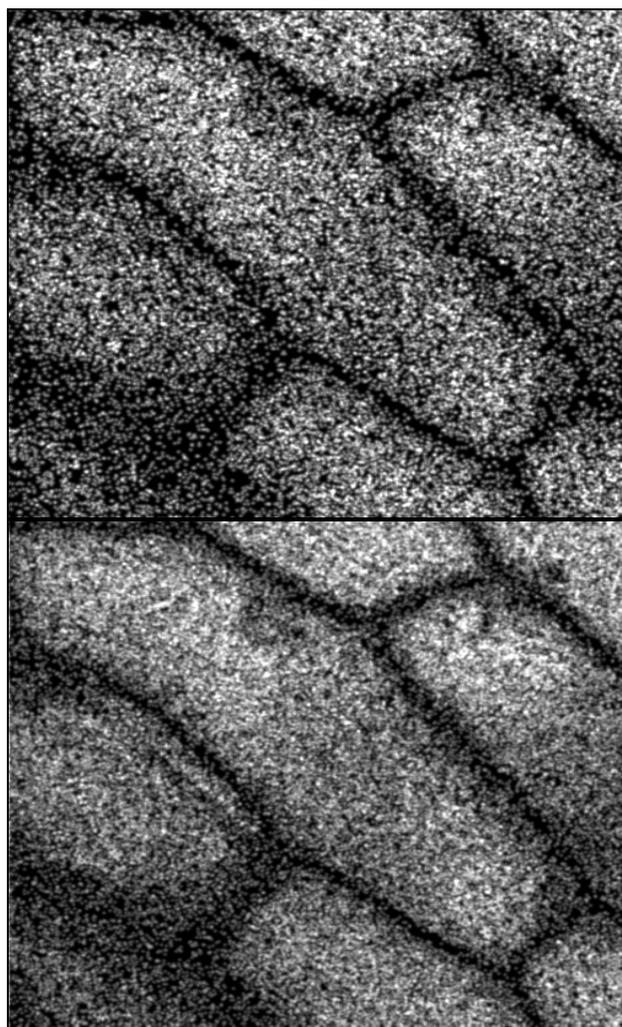


Fig.7. X-ray images using "water window" soft X-rays: 20min exposure time (top) and 60min exposure time (bottom).

For confirmation that the shown X-ray images of the cells are obtained by differential contrast between carbon and water absorption edges, the following images have been collected with different settings. Using 10kV accelerating voltage in the SEM electron beam generates all the characteristic lines from the Ti

target up to 5keV. By scrolling the energy window for selective photon detection one can choose a specific energy range for imaging. Fig.8 shows two images obtained using K and L characteristic lines correspondingly in the Ti spectrum. The energy window position is shown in the bottom of each image.

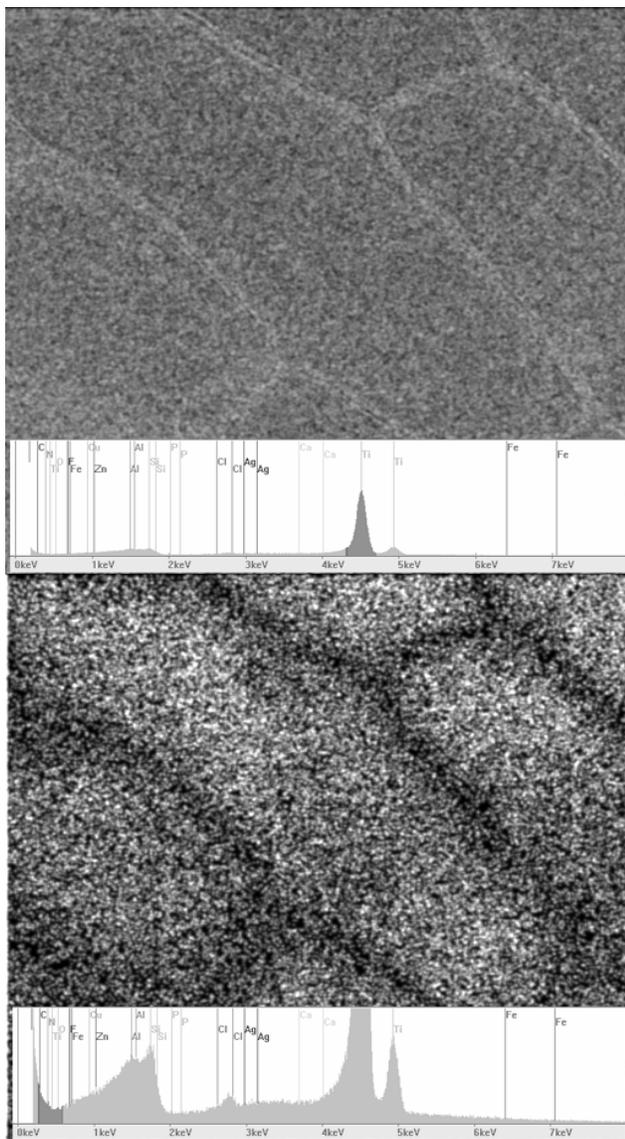


Fig.8. X-ray images using energy windows around 4.5keV (top) and 450eV (bottom).

The images shown demonstrate that photons with 4.5keV energy cannot produce any differential absorption contrast. Only weak phase contrast from the more dense cell walls can be seen in the high-energy image. But the image obtained by using 300-540eV photons shows significant differential absorption contrast typical for imaging in the "water window". According to Fig.3, using a 10kV accelerating voltage can increase the number of "water window" photons near twice compared to 5kV excitation. But comparison of the images in Fig.7 (5.2kV) and Fig.8/bottom (10kV) shows that the big accelerating voltage makes the "water window" image more noisy. This can be explained by the

increasing number of low-energy photons created by scattering of the strong high energy component.

For comparison, the same setup has been installed in the JSM840 SEM with a shorter distance from the emission point to the camera. A typical image obtained in this case is shown in Fig.9. It is less noisy because of more efficient photon collection with a shorter distance to the camera, but with reduced resolution due to difficulties in obtaining high current density in the SEMs with a W cathode.

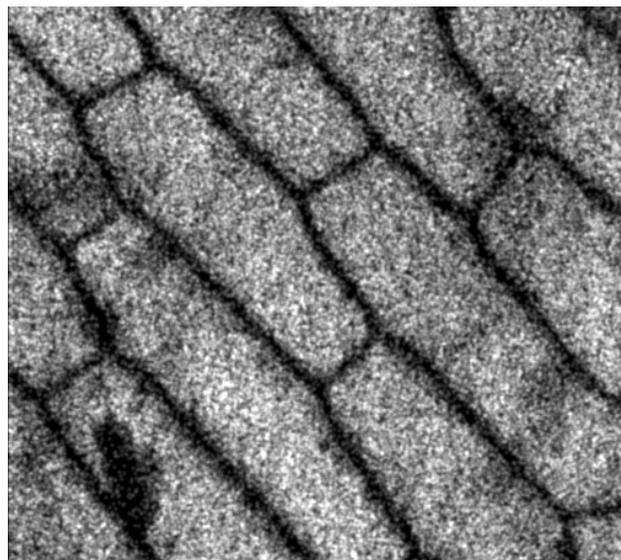


Fig.9. X-ray image obtained in the JSM840 SEM.

## 5. CONCLUSIONS

Modern x-ray techniques allow "water window" images of living cells to be obtained using an inexpensive setup for any conventional SEM.

## REFERENCES

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