

Contact Microscopy using a Compact Laser Produced Plasma Soft X-Ray Source

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Soft X-ray contact microscopy potentially allows imaging of wet living biological specimens at a spatial resolution higher than optical microscopy and without many of the constraints of scanning electron microscopy. In this paper, we present the development of a laboratory scale contact microscope that uses a laser produced plasma soft X-ray source. The source is based on a double-stream gas-puff target approach and it operates in the “water window” spectral range which enables to capture images of biological samples with a natural contrast. In the preliminary experiments the contact microscope system has been used for imaging of fixed and dried non-malignant HCV29 human bladder cell lines cultured on polymethyl methacrylate photoresists. The samples were exposed with 150 pulses of soft X-rays as an initial test to demonstrate the possibility of image formation. The soft X-ray contact images registered in the photoresists exhibit high resolution in the atomic force microscopy topography which indicates the potential application of soft X-ray contact microscopy in life science to examine small features as small as few tens of nm. The technique could also be used for living cell imaging with further optimization of the microscope system and development of a special specimen holder. The details of the soft X-ray contact microscopy technique and the experimental results are presented and discussed.

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1. Introduction

Soft X-ray contact microscopy (SXCM) is an interdisciplinary technique that has many applications in life sciences and materials technology. In the life sciences SXCM could be an efficient technique to image the fine structures of living biological specimens at a resolution superior to that of optical microscopy. In addition to that multistep sample preparation procedures (including cell fixation, staining and coating) required for electron microscopy could be avoided by using this technique [1]. SXCM enables the researchers to achieve a good contrast image of wet biological specimens by using soft X-rays in the “water window” (WW) spectral range, corresponding to 2.3 nm to 4.4 nm wavelengths, and having photon energies of about 280 eV to 540 eV, respectively. In the WW spectral range, the absorption of soft X-rays by carbon containing compounds (such as proteins and carbohydrates) is much higher than that of water [2]. Moreover, the higher penetration depth of soft X-rays through materials allows SXCM to easily observe wet specimens up to several μm thick [3].

SXCM is characterized by its simplicity and cost effectiveness. In such techniques images are formed mainly through an absorption contrast process without the need

for coherent radiation sources (such as synchrotrons) and involvement of complicated optics could also be avoided. Therefore, compact laboratory scale incoherent soft X-ray sources based on laser produced plasma can be used.

It is well known that ionizing radiations could damage the living samples [4]. In order to avoid the damage, the delivery of high photon flux in an extremely short time is imperative to image the living specimens before the ionization radiations could induce damage. Due to the long exposures needed for contact imaging using the soft X-rays generated by synchrotron radiation sources, the biological specimens required to be frozen and fixed [5]. Therefore the ultra-short pulse soft X-ray radiations generated by laser-plasma sources could be used to capture images of living biological specimens prior to the occurrence of the sample damage [6]. Several studies demonstrated the application of laser produced plasma soft X-ray sources for contact microscopy of living specimens in nanosecond time scaled single pulse [7]. These sources were based either on solid or liquid targets. The use of solid and liquid targets produce debris upon laser irradiation that could be avoided by using gas puff target. A contact microscopy employing a laser plasma soft X-ray source with a gas target has previously been demonstrated to examine human chromosomes at a high resolution of 50 nm [8].

In this paper, we present the development and application of a laboratory scale soft X-ray contact microscopy based on a compact laser produced plasma source us-

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ing a gas puff target approach. Although the ultimate goal of the study is to produce high-resolution images of wet and living biological specimens in a single shot exposure, fixed and dehydrated non-malignant HCV29 human bladder cell lines have been selected as biological models to provide an initial performance test of the microscope. This allows to estimate the number of photons that will be required to produce contact images of living biological specimens.

2. The soft X-ray contact microscope system

The contact microscopy system developed in this work uses a laser produced plasma soft X-ray source based on a double stream gas puff target approach. The system is comprised of a commercially available compact Nd:YAG laser generating laser pulses with energy of ≈ 740 mJ at $1.06 \mu\text{m}$ wavelength and 4 ns pulse duration with 10 Hz repetition rate and small vacuum chamber containing electromagnetic valve system for gas puff target, optical system for laser focusing, sample holder for dehydrated biological sample placement, and silicon nitride window. The double stream gas puff target was created using a double nozzle setup operated by the electromagnetic valve system. The gas puff target was composed of a working gas (argon) and a confining gas (helium). The principle and operation of the electromagnetic valve system is described elsewhere [9]. The laser beam was focused onto the gas target by a focusing lens of 25 mm focal length. As the soft X-rays could be easily absorbed in air, the sample holder, containing the biological specimens and the image recording medium (X-ray photoresist), was mounted in the source chamber at the closest possible distance. The source chamber was pumped to a pressure of about 10^{-2} mbar. A 500 nm thin layer of polymethyl methacrylate (PMMA) photoresist, deposited onto a silicon wafer (produced by ITME, Poland) was used as an image detector to record the contact images of the biological specimens. The specimen holder was placed at a distance of 2 cm away from the source, and a multi-shot exposure of about 150 pulses (15 s) was required to produce contact images of the specimens. However, further optimization of the number of pulses required for living cell imaging has to be established to achieve high resolution images. The photon flux and energy fluence on the specimens at the specified position were estimated about 4×10^4 photons/ μm^2 /pulse, and $0.25 \text{ mJ}/\text{cm}^2$ /pulse. The schematic view of the experimental arrangement of the contact microscopy system is shown in Fig. 1.

3. Preparation and imaging of dehydrated cells

The soft X-ray contact images of fixed and dried biological specimens were obtained employing the contact microscopy system. Non-malignant human bladder HCV29 cell lines were used as test sample in the preliminary experiments due to their elongated shape and well-known

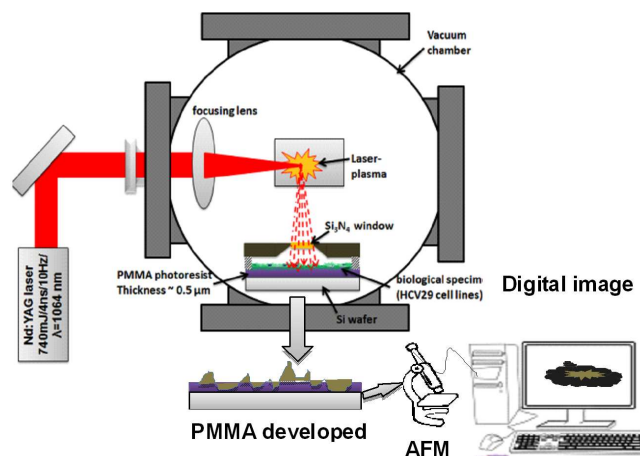


Fig. 1. Scheme for the experimental arrangement of laser plasma soft X-ray contact microscopy system.

morphological features. For the successful application contact microscopy in life science a careful sample preparation procedure is required. In this study, the samples were prepared in the biological laboratory in the Institute of Nuclear Physics, Polish Academy of Sciences in Kraków, Poland. The cells were suspended in RPMI 1640 culture medium and cultivated directly on the surface of PMMA photoresist for 24 h at 37°C in CO_2 atmosphere in the Petri dish.

Prior to soft X-ray exposure the cells were chemically fixed and dried. In the fixation process, the culture medium was removed from the Petri dish with embedded photoresist with biological specimens. Then, the photoresist was washed three times with PBS buffer only in every two minutes at room temperature. Afterwards, a 1–2 ml of 3.7% r-r paraformaldehyde fixative solution in PBS (phosphate buffered saline, pH 7.4, Sigma) was added for 15 min. Finally, the fixative solution was washed out from the Petri dish, followed by rinsing it with the PBS buffer only for 5 min. A drying protocol was applied after the fixation process was completed. In the drying procedure, 40–90% of ethylene alcohol and dehydrated alcohol were added to the sample for 10 min. Finally, the sample was kept in air to dry and to let alcohol to evaporate for 5 to 10 min.

For analysis of the structure of the cells, the biological specimens were placed directly on top of a silicon wafer spin-coated with PMMA that act as an image recording medium. The thickness of the PMMA coating was about 500 nm. The samples were exposed to soft X-rays through a $1.5 \times 1.5 \text{ mm}^2$ and 100 nm thick silicon nitride window that filtered out the longer wavelengths above the soft X-ray region. In the initial test experiments the samples were irradiated with 150 pulses of soft X-rays to get the imprint of cells on the PMMA photoresist. This could help to estimate the appropriate number of soft X-ray pulses required to produce an optimized images of living biological specimens.

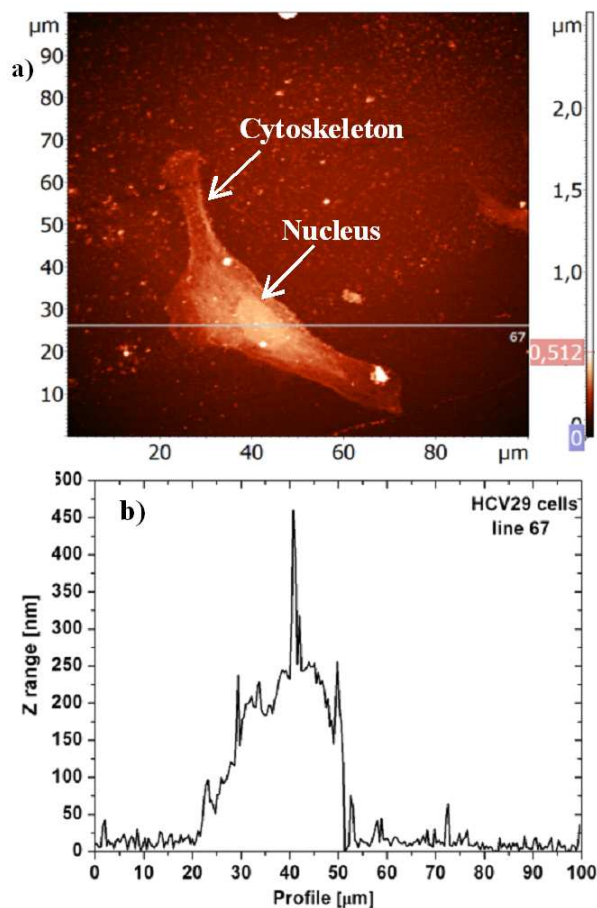


Fig. 2. AFM topography of SXCM image of fixed and dried non-malignant HCV29 human bladder cells a) and profile obtained along a selected line b).

After exposure to the soft X-rays the PMMA photoresist was rinsed with sodium hypochlorite (NaClO) solution for one minute to remove any remaining of biological specimens. The photoresist was then developed using a standard mixture of methyl isobutyl ketone (MIBK) and isopropyl alcohol (IPA) (1:3, v/v) for 60 s. Following the development process the photoresist was washed with pure isopropyl alcohol for 30 s in order to suppress further development. Finally, a compressed nitrogen gas was blown on the photoresist to let it dry.

A commercially available atomic force microscope (AFM) (NT-MTD, Russia) was used to reproduce the topography of the specimens imprinted on the photoresists (PMMA). The AFM measurement was made in a semi-contact mode using a rectangular cantilever equipped with a tip made of silicon with radius curvature 10 nm. Typical scan of 256×256 points per line was performed, which corresponds to a scan size of $100 \mu\text{m} \times 100 \mu\text{m}$. The distance between the tip and the photoresist surface was controlled by the feedback system of the AFM. From the AFM topography, the profile of the soft X-ray contact image of the specimen was obtained along a selected line

using Image Analysis software available from the AFM manufacturer.

An exemplary soft X-ray contact image of a fixed and dried non-malignant human bladder HCV29 cell lines and the corresponding profile are shown in Fig. 2a and b. In the AFM topography, the structure of the cell beneath to the cell membrane (cytoskeleton), the dense part close to the center of the cell (nucleus) and cellular organelles surrounding the nucleus are clearly visible. In addition to these cellular structures, small and unclear features appeared in the AFM topography. The main reason for this could be pollution of the photoresist surface by the chemical used to remove the remaining of biological specimens after exposure to soft X-rays. In order to avoid such problem the use of proper chemicals with well known concentration should be taken into account.

The result obtained from the preliminary SXCM experiments on fixed and dried biological specimens indicates the potential application of the technique in life science and it can also be used to observe organelles of living biological specimens as small as few tens of nanometer if only sufficient number of photons are delivered to the sample. According to Sayre et al. [10] estimation a photon flux more than 10^5 photons/ μm^2 on the specimen or 10^{15} photon/sr at the source and exposure time less than 4 ms are required to capture images of living biological specimens with a spatial resolution of 100 nm. The contact microscopy system developed in our laboratory could be further optimized to fulfill the entire requirement for living specimens imaging at high resolution.

4. Conclusion

We have developed a contact type microscopy system using a laser produced plasma soft X-ray source. The source is based on a double stream gas puff target approach and operating in the "water window" spectral range. As an initial test for the performance of the SXCM system fixed and dried non-malignant HCV29 human bladder cell lines were chosen for study. From the result obtained from preliminary experiment indicated structures of the cell including, the cytoskeleton that lies beneath the cell membrane, the nucleus which is located close to the center of the cell, and cellular organelles surrounding the nucleus are clearly visible. The potential application of the SXCM for high resolution imaging in life science will be demonstrated by optimizing the source for high emission of soft X-rays in the "water-window". Furthermore, a special specimens holder that can keep them alive in vacuum environment for irradiation will be designed and developed for detailed studies of different types of living specimens.

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