In situ observation of cellular organelles with a contact x-ray microscope

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Abstract. A contact x-ray microscope coupled with a highly intense laser plasma soft x-ray source has been developed and in situ observations of cellular organelles have been conducted. The soft x-rays were generated by irradiating a high power laser pulse onto a thin-foiled gold target and about 1.3x10¹⁵ photons/sr were obtained, which allowed the inner structures of live wet biological cells to be imaged. Single shot flash imaging is a key technique to image cellular organelles of live biological cells avoiding degradation of the spatial resolution of the images resulting from motion blur and radiation damage. The use of a fluorescence microscope to identify cellular organelles in conjunction with the soft x-ray microscope has allowed several cellular organelles to be identified precisely in the soft x-ray images. Combining the fluorescence microscope with the soft x-ray microscope will be very useful and will establish the technique as a powerful tool to analyze living function of biological cells.

1. Introduction
Laser plasma soft x-ray sources have advantages such as high intensity and short pulse duration and a contact x-ray microscope coupled with a laser plasma soft x-ray source has the potential to capture an x-ray image of biological specimens in a single shot flash imaging without any artificial treatment such as staining, fixation, and slicing. However in order to realize the single shot flash imaging of hydrated living biological cells extremely high intense laser plasma x-ray sources are required and success has been limited with the images only showing the surface structure of cells [1].

In order to realize the single shot flash imaging of wet live cells it is also very important to avoid reduction of x-ray photon flux caused by low efficiency of x-ray optics. In this respect, the contact type x-ray microscopy that requires no x-ray optics has an advantage over x-ray microscopes with x-ray optics. Hence, combining the highly intense x-ray source and contact-type x-ray microscopy allows in situ observation of cellular organelles of live biological cells to be realized. According to the estimation by Sayre et al. [2], a photon flux of more than 1x10⁷ photons/µm² is required on specimens to obtain 100 nm spatial resolution or more than 1x10¹⁵ photons/sr at the source.

It is also important to establish how to identify cellular organelles in x-ray images for biological applications since the inner structures of cells are complicated and it is difficult to distinguish each organelle in the x-ray images. We propose an in situ method of a soft x-ray microscope in conjunction with an optical fluorescence microscope to identify the cellular organelles of live hydrated cells in the soft x-ray images obtained of biological cells. Direct comparison of both the soft x-ray images and the fluorescence images allows cellular organelles to be identified in the soft x-ray images.
2. Flash imaging with highly intense laser plasma soft x-rays

The laser plasma x-ray sources are suitable for the single-shot flash imaging to observe live biological cells since they have advantages such as bright and short pulse duration. There have been several studies using a laser plasma soft x-ray source with an x-ray microscope [3]. However most of them did not achieve a single shot flash imaging due to the difficulty of obtaining enough photon flux [2].

The high power Nd:glass laser system, which can generate over 20 J output energy in a 600 ps pulse duration, was employed to generate enough photon flux to capture an x-ray image with a single shot flash exposure. The pulse duration of the soft x-rays emitted from the laser plasma source is also expected to be about 600 ps [4]. The 600 ps duration is shorter than the time scale of any artificial effect on image blurring including radiation damage and Brownian motion [5].

The laser pulse was focused onto a thin-foil gold target at a laser intensity of about 1x10^{14} W/cm^2. The x-ray flux was measured by a soft x-ray plasma camera with Fresnel zone plate [6] and as ~1.3x10^{15} photons/sr over the wavelength range 2.3 nm to 4.4 nm in the so-called “water window” [7]. Figures 1 (a) and (b) show the soft x-ray images obtained using the soft x-ray plasma camera of the laser plasma source with gold target thickness of 50 μm and 20 nm, respectively. It is clear that the x-ray intensity obtained using the 20 nm thick target in (b) is much brighter than that with the 50 μm thick target in (a).

![Figure 1](image1.png)

**Figure 1.** Soft x-ray images of the laser plasma source with target thickness of (a) 50 μm and (b) 20 nm. The image of the laser plasma with 20 nm target thickness is brighter than that with 50 μm target thickness.

3. In-situ observation with contact-type x-ray microscope

Leydig cells from mouse testicle were placed directly onto an x-ray photoresist and were cultured for two to three days to establish good contact onto the surface of the photoresist and the thickness of the cells were measured to be about 5 μm. Poly methyl methacrylate (PMMA) with thickness of 500 nm was used as the x-ray photoresist and it was coated onto a thin glass plate. The biological cells on the PMMA were covered with a silicon nitride membrane with thickness of 200 nm and were enclosed in a stainless steel holder specifically designed to protect the biological cells from vacuum. The cells were stained with fluorescent dyes of Phalloidin, Mito-tracker, and/or DAPI, placed in the specimen holder and observed by a fluorescence microscope to obtain images for the cellular organelles of cytoskeleton, mitochondria, and/or chromatin, respectively. After observation by the fluorescence microscope the specimen holder containing the cells was placed in the vacuum chamber for soft x-ray imaging. The time interval between the observation by the fluorescence microscope and the irradiation with soft x-rays was typically 10 minutes. After exposure to soft x-rays the PMMA was rinsed with sodium hypochlorite (NaClO) to remove the cells and developed with a mixed solution of methyl isobuthyl ketone (MIBK) and isopropyl alcohol (IPA). Before the development of the PMMA the surface of it was carefully observed by differential interference microscopy to make sure that no cells were left on the surface. Soft x-ray images of the cells were obtained by scanning the surface of the developed PMMA using an atomic force microscope. Shown in Figure 2 is the typical soft x-ray image.
image of the wet Leydig cells. Fine structures surrounding nucleus were clearly visible and structures in the nucleus were also visible. The structures surrounding the nucleus were identified to be mitochondria and the structures in the nucleus were identified to be chromatin. The identification of the structures was done using a fluorescence microscope, which is described in the next section. The spatial resolution was measured to be about 90 nm [8], which equates well to that estimated as achievable with an x-ray flux on the specimens of $4.4 \times 10^5$ photons/$\mu m^2$ [2].

![Figure 2. A typical soft x-ray image of wet Leydig cells. Fine structures surrounding nucleus are clearly visible and structures in the nucleus are also visible.](image)

4. Identification of cellular organelles using a fluorescence microscope

The soft x-ray images of Leydig cells were directly compared with corresponding fluorescence images and each cellular organelle was identified [9, 10]. Shown in Figures 3 (a) and (b) are the fluorescence image and soft x-ray image of the cells, respectively. As the cells had been stained with Mito-tracker to label the mitochondria, the structures that appeared red in (a) were identified as mitochondria. The same structures as in (a) were also found in (b). Lattice-like structures were also seen in the soft x-ray image, which were not seen in the fluorescence image. These structures were identified as cytoskeleton according to the previous results observing the cells stained with Phalloidin [8].

![Figure 3. Fluorescence image (a) and a soft x-ray image (b) of Leydig cells. The structures appearing bright red dots in (a) are mitochondria and the same structures are also seen in (b).](image)

Shown in Figure 4 is an enlarged area of the soft x-ray image shown in Figure 3 (b). Mitochondria and cytoskeleton were clearly recognized and those structures were found to be in close proximity. This is the first result where it was possible to identify the cellular organelles in the soft x-ray images obtained by in-situ observation of wet biological cells.
5. Summary
In summary a contact soft x-ray microscope with an intense laser plasma soft x-ray source has been developed and live Leydig cells were observed. Using a high intensity laser plasma source with the soft x-ray microscope made it possible to image wet biological cells with a single shot flash exposure to avoid motion blur and radiation damage. The specially designed specimen holder made it possible to obtain fluorescence images and soft x-ray images of the same cells in the same environment. Comparing the fluorescence images with the soft x-ray images for the same cells allowed cellular organelles in the soft x-ray images to be clearly identified for the first time and fine structures of the cellular organelles to be analyzed.

The soft x-ray microscope has been expected to be a powerful tool for life science research for a long time as it allows the observation of live biological specimens with high spatial resolution [8]. However the lack of the brightness of the x-ray source prevented the ability to image live biological specimens. The difficulty in identifying the inner structures of biological cells was also a barrier for use of the soft x-ray microscope for life science. In the present work, solving these problems using the high intensity laser plasma soft x-ray source and the hybrid microscope system combined with fluorescence microscope, in-situ observation of cellular organelles in a live cell has been realized.

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