Direct Ultrastructural Imaging of Macrophages Using a Novel X-Ray Contact Microscopy (44340)

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Abstract. **A compact, high-resolution, laser-plasma, x-ray contact microscopy method using a table-top Nd:glass laser system has been developed. This x-ray microscopy system was applied for the observation of macrophage ultrastructures. These images were produced using proximity imaging in which a 5-ns pulse of soft x-rays with wavelengths near and inside the water windows (23Å–44Å) produced by the laser-plasma were absorbed by the specimen and then registered on a photo resist. The x-ray images imprinted on the photo resist were then developed and analyzed with an atomic force microscope (AFM). Mouse thioglycollate-elicited peritoneal macrophages in suspension were examined by this new x-ray microscope. The x-ray images of the macrophages were compared with those observed by conventional transmission electron microscopy (TEM). The x-ray images showed no obvious organelles, including the nucleus and endoplasmic reticulum, as can be seen with TEM, but high- and low-contrast structures caused by mass distribution of carbon were observed. Thus, using the x-ray microscopy we visualized the first x-ray images of macrophage ultrastructures. The successful x-ray imaging of macrophage ultrastructure indicates that proximity x-ray microscopy may be of value in studying physiology linked to the dynamics of a cell.** [P.S.E.B.M. 1999, Vol 220]

X-ray microscopic techniques are well suited for the high resolution and sensitivity of microscopic analysis of biological specimens without the use of the conventional sample processing such as fixation and staining required for electron microscopy (EM) (1). The advantages of the x-ray microscopy technique are 1) high contrast is provided by the density of the elements in the specimen, thereby avoiding possible artifacts caused by standard processing of a specimen; 2) x-ray microscopy permits the observation of thicker specimens (up to a few μm in depth) than EM without special sample preparation or alteration; 3) the actual location of a specific element within a cell can be visualized when a differential absorption map is utilized; 4) three-dimensional observation is possible with a single exposure of x-rays. Therefore, this technique appears presently to be the most suitable method to exploit these advantages (1). Although x-ray microscopy has been developed and demonstrated by several groups, most of these microscopic methods have used synchrotron radiation sources, which are limited in availability (2). In the present study, we used synchrotron radiation sources, which are limited in availability (2). In the preset study, we used a compact table-top laser system for obtaining x-rays in the water window region (3). The advantage of a laser plasma x-ray source for x-ray microscopy includes the compactness and flexibility combined with the ability to capture an image in a brief time interval (<5 ns), freezing kinetic movement, and acquiring an image before any manifestations of radiation damage on the specimen.

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Although studies of the application of x-ray microscopy to biological specimens have been reported (1, 4–6), interior structural images of immune cells have not yet been studied. In the present report, a single frame x-ray image of mouse peritoneal macrophages was obtained with a laser-plasma x-ray microscopic system.

Materials and Methods

Cells. Thioglycollate-elicited peritoneal macrophages were obtained from 8- to 12-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME) as described previously (7). The peritoneal exudate cells contained 80%–85% macrophages as determined by microscopic examination with Camco Quik staining (American Scientific Products, McGaw Park, IL). The cells were fixed in 2% glutaraldehyde (Electron Microscopic Sciences, Ft. Washington, PA) in phosphate buffered saline (PBS; Sigma Chemical Co., St. Louis, MO) for 24 hr at 4°C, washed in PBS, and resuspended in PBS containing 0.2% sodium azide (Sigma).

X-ray Microscope. *Laser produced plasma source.* The laser-plasma x-ray microscope described in this paper used a pulsed laser producing 10J–20J of energy at 1064 nm wavelength in a 5 ns pulse as previously described (8). The pulsed laser beam was focused onto Yttrium targets to produce laser plasma x-rays. The irradiated area on the targets was approximately 100 μm in diameter. The intensity of laser power on the targets reached 5×10^{13} W/cm^2. The pulse width of the x-ray emission generated, in the water window region (23Å–44Å), was approximately the same as the laser pulse width, 5 ns.

Specimen holder. Since soft x-rays are absorbed in air, the sample holder and other elements of an x-ray microscope, except the laser itself, are installed in a vacuum chamber. Therefore, the sample holder was designed to keep wet specimens protected while in vacuum. The specimen was placed on an x-ray photo resist (PMMA) supported by a silicon nitride coated silicon wafer (Fastec Fabrication Services, Ltd., Northants, UK) and mechanically shielded with a thin silicon nitride window, 100 nm in thickness (Fig. 1). The specimen holder was placed 1 cm away from the target at an angle of 45 degrees from the target. A single x-ray exposure was made on each resist. The x-ray photo resists were rinsed with sodium hypochlorite solution to remove any remaining specimen from the resist, and then developed by dissolving the damaged material in a mixture of methyl isobutyl ketone and isopropyl alcohol (1:1, v/v) for 3–5 min. The development time was varied according to the laser energy and the thickness of the specimens to obtain the best images. The three-dimensional topological absorption image that resulted from the development was then observed with a differential interference microscope. An AFM (TopoMetrix, Santa Clara, CA) was used to reproduce the images from the developed photo resists.

Electron Microscopy. Macrophage monolayers on glass coverslips were prepared as previously described (9).

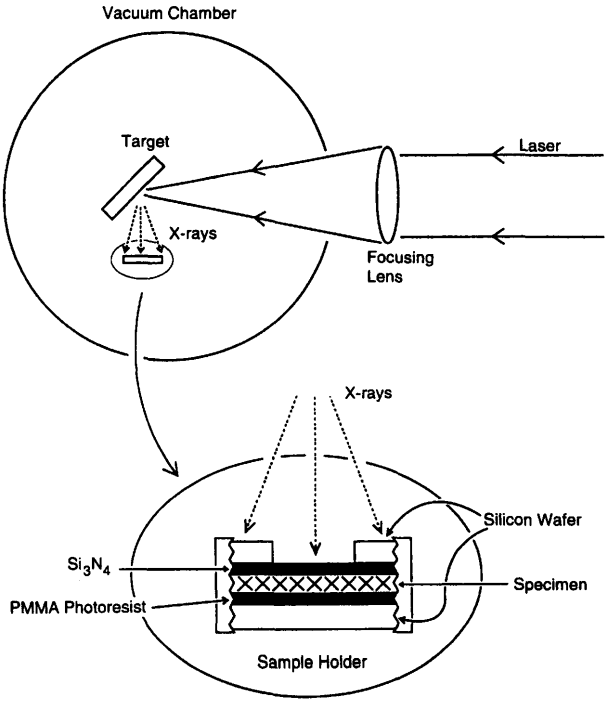

Figure 1. Schematic diagram of the x-ray microscope.

Using a standard method, the cells were fixed in 2.5% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.4) for 24 hr at 4°C. Postfixation was with 1% OsO_4 in 0.1 *M* phosphate buffer for 45 min at 4°C. The cells were then embedded in Epon 812. The coverslips were removed by quick immersion in liquid nitrogen, cut, stained, with uranyl acetate and lead nitrate, and examined with an electron microscope (Philips 301).

Results and Discussion

X-ray microscopy has many potential advantages over electron microscopy (10). Theoretically, the most favored application is the observation of living cells in water with the use of x-rays in the water window wavelength region (3) that has sufficient output energy for microscopy of a wide range of biological specimens (1). However, a hydrated specimen causes motion blurring and the application of x-ray microscopy with synchrotron has limitations due to long exposure time, such as a few seconds (1). In this regard, x-rays generated in a laser-produced plasma produce a short duration of <5 ns of pulse, which eliminates problems associated with a long exposure time. Huge laser facilities dedicated mainly for laser fusion programs have been utilized for laser plasma radiation (4, 11, 12), but they were largely inaccessible to biologists. In the present study, we utilized a compact table-top Nd:glass laser system for x-ray microscopy, since this table-top laser system generated 5×10^{13} W/cm^2 of intensity on the target in a 5-ns pulse duration, which is sufficient to produce laser plasma x-rays.

A macrophage suspension was placed on an x-ray resist supported by a silicon base and exposed to a parallel beam

of soft x-rays, usually 1–10 nm in wavelength and roughly normal to the resist surface. X-ray photo resists, such as PMMA and related polymers, are widely used for recording x-ray images (11, 13). The resolution of PMMA used in this study was as high as 5 nm and was adequate for analysis of macrophage interior structures, because the resolution of a photo resist is critical for the final spatial resolution of x-ray microscopy. The estimation of the number of photons on the resist was 1,219, since 20J of laser energy, 10% x-ray conversion efficiency (12), and 70% and 35% absorption of x-rays in 0.1-μm silicon nitride and water layer, respectively, were undertaken. The x-ray shadow of a specimen recorded on the resist was developed and observed by an AFM. An AFM is valuable for examining developed photo resists, since the relief on a nonconducting resist surface can be examined directly without coating or making a replica (4, 14).

Observations of biological specimens, including rat sperm nuclei, plant chromosomes, myofibrils, dry HeLa cells, and living sperm by x-ray microscopy have been reported by several groups (1, 4–6, 15, 16). However, x-ray images of the interior structure of immune cells including macrophages have not been studied. Therefore, in the present study, we examined the ultrastructure of macrophages by a newly developed x-ray microscopic technique with a compact table-top laser system. Figure 2 shows a representative x-ray image of a hydrated macrophage that has been fixed with glutaraldehyde, the same as for electron microscopy. In comparison with conventional TEM (Fig. 3), the x-ray images showed some structural components of the macrophages, but these seemed not rigid, which means not clear as compared with TEM images. The dissimilarities of x-ray images with TEM were apparent in the figures (Figs. 2 *vs.* 3). That is, no obvious organelles, including the nucleus and endoplasmic reticulum as seen in the TEM images, were observed.

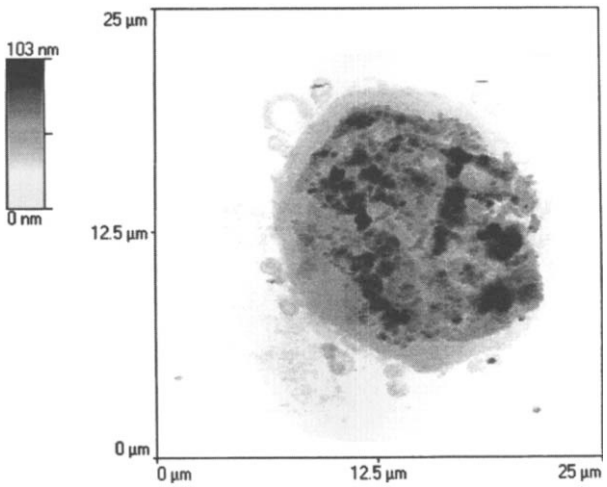

Figure 2. X-ray microscopic image of macrophages. Ultrastructure of a mouse peritoneal macrophage was examined and the x-ray images observed by an AFM.

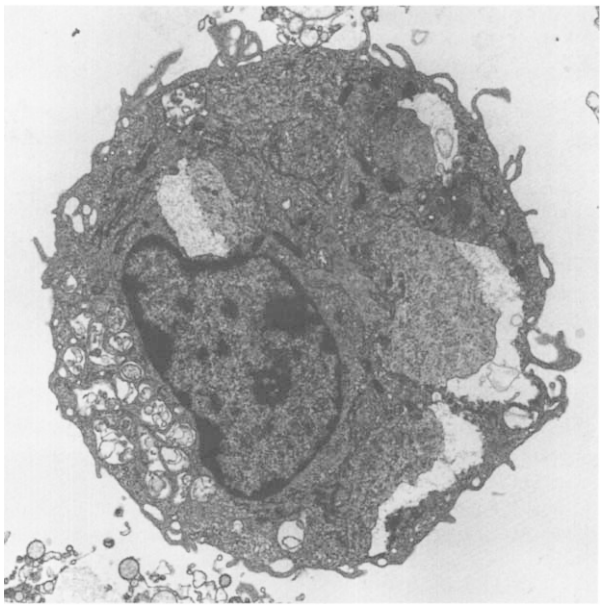

Figure 3. Electron micrograph of macrophages. Macrophages were fixed, sliced, stained, and then observed by TEM. Magnification, 2500×.

Unlike TEM specimens, the specimens for x-ray microscopy were not sliced or stained with heavy metals, such as lead citrate and uranyl acetate, which permit high contrast of specimens, but instead were whole cells. Furthermore, x-ray images are based on elemental density. That is, the elements of the specimen are the principal reaction sites. They act as x-ray photon removal sites and as particle emission sites emitting several classes of photons and electrons. In addition, each element (carbon, nitrogen, and others) has its own individual cross-sections for the removal and for the several emission behaviors, and these element cross sections are further individualized by the way in which they vary with the energy of the photons present and with the chemical state of the element (1). Therefore, images of a cell observed by x-ray microscopy may provide physiological rather than merely morphological information. Since, in the present study, we used fixed cells with glutaraldehyde for the initial study of x-ray microscopy applied to macrophages, it was not possible to examine the physiological state of the macrophages. However, we could observe viable microorganisms by x-ray microscopy (17) and have obtained preliminary data regarding viable macrophages without fixing cells. Thus, x-ray microscopy may be useful for analysis of the physiological state of cells. Furthermore, selection of an appropriate x-ray wavelength to specific elements, such as Ca, Fe, or S, may permit determination of localization of the element within a cell. This may be determined by selecting appropriate combinations of target materials and filtering as well as using monochromatic x-rays on either side of an element's absorption edge. Thus, it has been suggested that x-ray microscopy using a table-top laser system may provide an x-ray image of internal structures of macrophages, and this technique may provide a

new strategy permitting analysis of the dynamics of cell physiology.

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