10 keV X-ray Phase-Contrast Microscopy for Observing Transparent Specimens

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Hard X-ray phase-contrast microscopy has been performed with phase plates of tantalum using an X-ray beam from an undulator in SPring-8. The photon energy was set at 10 keV near the $L_3$ absorption edge of tantalum (9.9 keV) in order to increase the phase contrast. To demonstrate its capability, a transparent specimen was imaged clearly in the reverse contrast with phase plates that shift the phase by one-quarter and three-quarters of a period, while conventional absorption imaging showed little contrast. Further, an image contrast as high as approximately 40% can be obtained for the cell walls of another specimen.

KEYWORDS: X-ray microscope, hard X-ray, phase-contrast microscope, zone plate, synchrotron radiation, undulator radiation, biological specimens

Although hard X-ray microscopy cannot presently compete with electron microscopy and soft X-ray microscopy in terms of spatial resolution, it has several advantages for studying biological specimens such as the ability to image thick specimens in their natural living state, easy preparation of specimens and nondestructive observation under an atmospheric environment. Since biological specimens composed of light elements are transparent to hard X-rays, conventional absorption-contrast imaging is ineffective. For visible light, phase-contrast microscopy by Zernike’s method has been used in biology, crystallography, and other fields, to observe transparent objects.1) In the hard X-ray region, as expected from a comparison between the real and imaginary parts of the refractive index, the phase-shift cross section is almost a thousand times larger than the absorption cross section for light elements.2,3) Therefore, phase-contrast hard X-ray microscopy should be studied for applications to biological specimens by employing Zernike’s method. Several hard X-ray imaging microscopes have recently been attempted.4–8) However, its wide applications have not been made due to the common belief that the contrast of hard X-ray microscopy might be too low for biological specimens.9–11) The purpose of the present paper is to demonstrate a contrast sufficiently high to image transparent specimens by hard X-ray microscopy. The photon energy was set at 10 keV to serve biological specimens in their natural living state, easy preparation of specimens and nondestructive observation under an atmospheric environment. Since biological specimens composed of light elements are transparent to hard X-rays, conventional absorption imaging showed little contrast. Further, an image contrast as high as approximately 40% can be obtained for the cell walls of another specimen.

$I = (1 - \mu_{o}t) \left\{ \exp \left( -\frac{m_{o} \beta p}{\delta p} \pi \right) \pm 2 \exp \left( \frac{m_{o} \beta p}{2 \delta p} \pi \right) \cdot \sin \phi_{o} \right\}.$

where

$\phi_{o} = \frac{2 \pi}{\lambda} \delta_{o} t, \quad \mu_{o} = \frac{4 \pi \beta_{o}}{\lambda}.$

$\delta$ is the deviation of the real part of the refraction index from unity, $\beta$ the imaginary part of the refraction index, $\lambda$ the wavelength of the incident X-rays, $t$ the thickness of the object, $\phi$ the phase shift, and $\mu$ the linear absorption coefficient. Subscripts “o” and “p” are the corresponding parameters for the object and the phase plate, respectively. If both $\phi_{o}$ and $\mu_{o}t$ are small as expected for light elements in the hard X-ray region, eq. (1) becomes

$I = (1 - \mu_{o}t) \left\{ \exp \left( -\frac{m_{o} \beta p}{\delta p} \pi \right) \pm 2 \phi_{o} \cdot \exp \left( -\frac{m_{o} \beta p}{2 \delta p} \pi \right) \right\}.$

This relation shows that the intensity changes are proportional to the phase variations of the object apart from the small variation of $1 - \mu_{o}t$. The case when $m = 1$ and the sign is “+” is called positive contrast, while the case when $m = 3$ and the sign is “−” is called negative contrast. The positive contrast means that the larger the optical thickness of the object, the brighter the image, while the negative contrast has the reverse relationship. It should be noted that in the case of X-rays the optical thickness advances the phase because the refractive index in the X-ray region is less than unity. A one-quarter period phase plate ($m = 1$) produces positive-contrast images (+ sign), while negative-contrast images (− sign) can be obtained by using a three-quarter period phase plate ($m = 3$).12)

The case where the object exists in a homogeneous background material such as water, the contrast of the image, $C$, can be given by

$C = \frac{(\mu_{o}t - \mu_{o}t) \cdot \exp \left( -\frac{m_{o} \beta p}{2 \delta p} \pi \right) \pm 2(\phi_{o} - \phi_{b}) \cdot (1 - \mu_{o}t)}{(2 - \mu_{o}t - \mu_{o}t) \cdot \exp \left( -\frac{m_{o} \beta p}{2 \delta p} \pi \right) \pm 2(\phi_{o} - \phi_{b}) \cdot (1 - \mu_{o}t)}.$

where subscript “b” indicates the corresponding parameters.
for the background homogeneous material. In the process deriving eq. (4), \( \mu_a \), \( \mu_p \), \( \phi_0 \), \( \phi_b \), are assumed to be small. Figure 1 shows the contrast calculated for a 100-nm-thick protein feature in a water background by adjusting the thickness of the phase plate of tantalum so as to give rise to a phase shift of one-quarter or three-quarters of a period in the whole photon energy range. The negative contrast is higher than the positive contrast. This is due to the effect of absorption by the phase plate. The ratio of the second term to the first term in eq. (3) has the value \( \pm \frac{\phi_0}{\phi_b} \exp \left( -\frac{2\mu_p}{\mu_a} \pi \right) \); the more the phase plate absorbs direct light (central order), the more the image contrast is enhanced.\(^{1}\) Therefore, one can increase the image contrast by tuning the photon energy to the \( L_3 \) absorption edge of tantalum (9.9 keV) even though the absorption contrast is almost zero (namely, the object is transparent). At 11.5 keV, \( \mu \) of protein and water are the same, so that the absorption contrast approaches zero.

Figure 1 shows the contrast calculated for a 100-nm-thick protein feature in a water background (upper two curves). The elemental composition and the density of the protein are assumed to be \( \text{C}_{38}\text{H}_{139}\text{N}_{24}\text{O}_{31}\text{S} \) and 1.35 g/cm\(^3\).\(^{9}\) The optical constants, determined by Henke et al.,\(^{13}\) are used in the calculation. A solid line and a dotted line represent the negative and the positive contrasts, respectively. For comparison, the absorption contrast is also shown (bottom curve). The discrete structure near 10 keV corresponds to the absorption edges of tantalum used as a phase plate material. Image contrast of about 1% can be expected by tuning the photon energy to the \( L_3 \) absorption edge of tantalum (9.9 keV) even though the absorption contrast is almost zero (namely, the object is transparent). At 11.5 keV, \( \mu \) of protein and water are the same, so that the absorption contrast approaches zero.

The experiments were carried out at the undulator beamline BL24XU of SPring-8. Synchrotron radiation from the undulator is monochromatized by a silicon double-crystal monochromator. The operation photon energy was 10 keV so as to maximize the contrast as shown in Fig. 1. The microscope takes the critical illumination.\(^{1,9}\) The conventional absorption-contrast image can also be taken by removing the phase plate.

Figure 2 shows the contrast calculated for a 100-nm-thick protein feature in a water background by adjusting the thickness of the phase plate of tantalum so as to give rise to a phase shift of one-quarter or three-quarters of a period in the whole photon energy range. The negative contrast is higher than the positive contrast. This is due to the effect of absorption by the phase plate. The ratio of the second term to the first term in eq. (3) has the value \( \pm \frac{\phi_0}{\phi_b} \exp \left( -\frac{2\mu_p}{\mu_a} \pi \right) \); the more the phase plate absorbs direct light (central order), the more the image contrast is enhanced.\(^{1}\) Therefore, one can increase the image contrast by tuning the photon energy to the \( L_3 \) absorption edge of tantalum (9.9 keV). From Fig. 1, one can expect image contrast of about 1% even for 100-nm-thick protein in water that is usually detectable in experiments.

The experiments were carried out at the undulator beamline BL24XU of SPring-8. A hard X-ray transmission-imaging microscope using Fresnel phase zone plates as lenses has been developed in this beamline.\(^{14}\) The specifications of the optical system including the diffraction efficiency of the zone plates and the light source parameters were described in our previous paper.\(^{14}\) The magnification is limited to 9 \( \times \) at 10 keV by the finite space inside the experimental hutch. By employing an X-ray zooming tube with a spatial resolution better than 500 nm,\(^{15}\) high spatial resolution to image a 250 nm line-and-space pattern has been achieved with the total system shown in Fig. 2. For phase-contrast microscopy, an annular aperture made of 100-\( \mu \)m-thick tungsten was inserted in front of the illumination system and either a one-quarter-period or three-quarter-period phase plate with an annular shape was placed in the back focal plane of the objective. The phase plates are made of tantalum. Both the annular aperture and the phase plate have a similar ring shape so that the direct light (central order) in the back focal plane of the objective is exactly phase-shifted and the diffracted lights by the object (dotted lines in Fig. 2) do not pass through the phase plate. The outer and inner diameters of the annular aperture are 800 \( \mu \)m and 600 \( \mu \)m, respectively, while those of the phase plates are 77.7 \( \mu \)m and 58.3 \( \mu \)m, respectively. The thicknesses of the phase plates are 1.33 \( \mu \)m and 3.97 \( \mu \)m to exactly give rise to one-quarter- and three-quarter-period phase shifts at 10 keV, respectively, having transmissions of 59.2% and 20.8%. The pinhole is made of molybdenum with a thickness of 100 \( \mu \)m. According to Abbe’s theory,\(^{11}\) the diffracted orders and the phase-modulated central order interfere with each other and give rise to the phase contrast image of the object in the image field. For comparison, a conventional absorption contrast image was also taken without the phase plates. Because of the low diffraction efficiency of zone plates for higher energy X-rays and for higher diffraction orders, the effect of the higher harmonics from the monochromator is negligible in the image field.

Figure 3 shows X-ray micrographs of conidia of Aspergillus species. Since it was only placed on a thin polyimide film and mounted on the sample holder, its background was air. Several particles of Aspergillus species were observed. The positive- and negative-contrast images of the specimen are seen with a good reverse contrast, as shown in Figs. 3(b) and 3(c), respectively. The absorption contrast image, Fig. 3(a), showed a slight contrast of the features corresponding to those in the phase-contrast images of Figs. 3(b) and 3(c). This may be due to slight defocusing. The images demonstrate the high spatial resolution of the microscope because particle structures as small as approximately 1 \( \mu \)m can be clearly imaged.

In spite of the fact that the microscope can image a 250 nm line-and-space pattern,\(^{14}\) the image quality in Figs. 3(b) and 3(c) seems somewhat poor due to the low magnification of the zooming tube. By increasing the magnification of the zoom-
Fig. 3. X-ray micrographs of the conidia of Aspergillus species: (a) absorption contrast image, (b) positive-contrast image and (c) negative-contrast image. The photon energy used was 10 keV. The particle-like structures of the specimens are well observed in (b) and (c), while they can be seen with little contrast in (a). Further, the image contrasts between (b) and (c) are reversed as expected from eq. (3). Exposure time was 3 min. The total magnification of the microscope system was 450× (optical system 9×, image detector 50×).

Fig. 4. A montage X-ray micrograph of the conidium of Curvularia species. It was produced by increasing the magnification of the image detector to 200×, taking images separately, and then combining them using graphic software. The photon energy used was 10 keV. The intensity changes caused by the optical thickness variation in the specimen can be recognized. Exposure time was 5 min. The total magnification of the microscope system was 1800× (optical system 9×, image detector 200×).

with hard X-rays for improving transparent specimens.

We are now improving the exposure time and the spatial resolution. The exposure time was several minutes for the micrographs in Figs. 3 and 4. By introducing a condenser zone plate with optimized diffraction efficiency at 10 keV, the exposure time can be reduced to about one fifth of that of the present system. The spatial resolution will be improved to be better than 100 nm because, by improving the phase zone plate, we have already succeeded in imaging a line-and-space pattern as fine as 100 nm with 10 keV X-rays. Thus, the spatial resolution is approaching that achieved by soft X-ray microscopes.17) Our hard X-ray phase-contrast microscopy provides much higher penetration power that is essential for studying natural thick specimens without any sample preparation and for extracting information on the structural changes inside living specimens with the passage of time.

11) Very recently, differential interference contrast microscopy has been reported with a middle photon energy of 4 keV. This is another way to image transparent objects. T. Wilhein, B. Kaulich, E. Di Fabrizio, F. Romanato, S. Cabrinia and J. Susini: Appl. Phys. Lett. 78 (2001) 2082.