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3D IMAGING OF X-RAY MICROSCOPY

Hiroshi KIHARA

Jichi Medical School, School of Nursing, Minamikawachi, Tochigi 329-04, Japan

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Abstract. Methods for obtaining 3D images by x-ray microscopy are reviewed; x-ray holography, contact microscopy, imaging x-ray microscopy with zone plate, x-ray microtomography, projection x-ray microscopy, scanning x-ray microscopy, micro x-ray scattering are introduced briefly, specially focussed on their capabilities of 3D imaging, real time imaging and spatial resolution of each method. Now, highest resolution is 50 nm except contact microscopy (20 nm). Comparison with electron and optical microscopies are also mentioned.

INTRODUCTION

In the preface of "X-ray Microscopy", edited by G. Schmahl and D. Rudolph (Schmahl & Rudolph: 1984a), it is described as, "X-ray microscopy fills a gap between optical and electron microscopy. Using soft x-rays, a resolution higher than with visible light can be obtained. In comparison to electron microscopy, thick, wet, unstained specimens can be examined. This is especially advantageous for biological applications".

Soft x-ray microscopy mainly utilizes its moderate absorptivity against electron or hard x-rays. It is the best for thick specimens such as several microns. It is specially advantageous in seeing inside of the thick specimen. Thus, 3D imaging is one of the most promising field which should be challenged by x-ray microscopy.

Characteristics of x-ray microscopy in comparison with optical and electron microscopies are summarized in Table 1. X-ray microscopy is inferior to electron microscopy in spatial resolution. However the former is superior to the latter in the points; (1) If the observations are done between 2.4 nm (absorption edge of oxygen) and 4.5 nm (absorption edge of carbon), absorption coefficients are greatly different between biomolecules and water (so called "water window"). Thus, biological specimens can be seen in wet state. (2) Figure 1 shows atomic number dependency of absorption coefficients. Those of electron and hard x-ray are also shown in the same figure in comparison. This demonstrates that soft x-ray shows sharper dependency on atomic number, which implies that images with higher contrast can be achieved

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Table 1. Comparative table of three microscopic methods.

	EM	XM	OM
staining	required	not required	not required
thick specimen	impossible	possible	possible
west sample	almost impossible	possible	possible
living sample	impossible	possible(?)	possible
atomic No. dep.	insensitive	dependent	insensitive
resolution	< 0.1 nm (in principle)	2–4 nm (in principle)	150 nm (in confocal M.)
radiation damage	violent	serious	less harmful
opacity	opaque	not opaque	opaque
label method	no	element analysis	fluorescence
spectroscopy	no	EXAFS, XANES	spectroscopy
observing place	surface	inside	partly inside
focal depth	very sharp	sharp	not sharp

EM; electron microscopy, XM; x-ray microscopy, OM; optical microscopy.

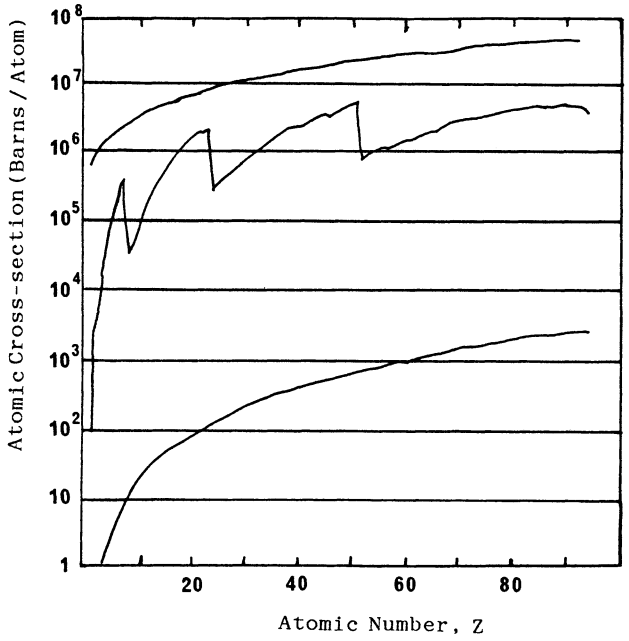

Fig. 1. Cross-sections of imaging reactions, versus atomic species Z. The upper, middle, and bottom curves relate respectively to electron microscopy, x-ray microscopy, and x-ray structure analysis. (Upper curve: total scattering of 100 keV electrons. Middle curve: photoelectric absorption of 2.4 nm photons. Lower curve: coherent scattering of 0.15 nm photons.) (Sayre: 1987b)

even without staining. (3) Absorptivity of soft x-ray is most suitable for the specimen of several microns thickness. Thus it is promising to see unsectioned (i.e. living) cells, as the typical thickness of the cells are about several microns. In combining these three characteristics, soft x-ray microscopy is most suitable for unstained, wet, and then living cells of several micron thickness. The large difference in absorptivity below and after absorption edges of elements can be used for the detection of spatial distribution of atomic elements.

When we compare characteristics of x-ray microscopy with optical microscopy, on the other hand, advantage of x-ray microscopy is, first, its high resolution due to its shorter wavelength than optical microscope. Now the resolution of confocal microscope is 0.2 μ/1.4 (Brakenhoff *et al.*: 1989), whereas practical resolution of x-ray microscopy at present is 50 nm for imaging x-ray microscopy and 20 nm for contact microscopy. Apparently, the two microscopes are not so much different in their practical resolutions. However, in theory, the resolution of x-ray microscopy is only limited by its wavelength (2 nm). When these resolutions limit are attained, the x-ray microscope will be very powerful in observing subcellular organisms and supramolecules in its acting state.

X-ray microscopy has another characteristics in comparison to optical and electron microscopies; opacity. With x-ray microscopy, insides of the specimens can be monitored, whereas with electron microscopy, usually only surfaces of specimens can be observed, and with optical microscopy, insides can be monitored only in limited cases, because of its transparency or large reflection at the surface. For these reasons, x-ray microscopy may have important applications in areas such as metallurgy, ceramics, geology, and thin-film technology besides biology and medicine (Kirz & Sayre: 1984). Moderate absorptivity of soft x-ray opens possibility to look into the inside of the specimen as mentioned above. It is especially advantageous in getting 3D image, which is the subject of this paper.

However, I should mention disadvantage of x-ray microscopy as well; radiation damage. To take an image, specimen should be irradiated by soft x-ray, which results the breakage of the chemical bond. Soft x-ray is much more harmful than visible or ultraviolet lights to living systems. At present we cannot avoid being pessimistic in expecting a cell alive after observing the living cells by soft x-ray microscope. Arguments were reported elsewhere (Sayre *et al.*: 1977a, 1977b, 1978).

METHODS OF X-RAY MICROSCOPY

Many types of x-ray microscopy have been reported. These are summarized in a review (Howells *et al.*: 1985), monographs (Schmahl & Rudolph: 1984b, Cheng & Jan: 1987a, Sayre *et al.*: 1988, Shinohara *et al.*: 1989) or proceedings of a symposium (Parsons: 1980) for x-ray microscopy. I shall review some of them, specially focussed on methods to get 3D imaging.

There have been many efforts in developing 3-dimensional imagings as;

(1) X-ray holography
(2) Contact microscopy
(3) Imaging x-ray microscopy with zone plate
(4) X-ray microtomography

(5) Projection x-ray microscopy
(6) Scanning x-ray microscopy
(7) Micro x-ray scattering

which will be described below by turns.

1. X-ray holography (Joueux et al.: 1988, Howells: 1984)

There is rich variety of geometries with which visible light holograms can be recorded. Only three of these are of interest for x-ray use: the Gabor in-line hologram (Gabor: 1949), the Leith-Upatneiks off-axis hologram (Leith & Upatneiks: 1962), and the Fourier transform hologram (Haddad *et al.*: 1988, Stroke: 1965a, 1965b), They are schematically shown in Fig. 2.

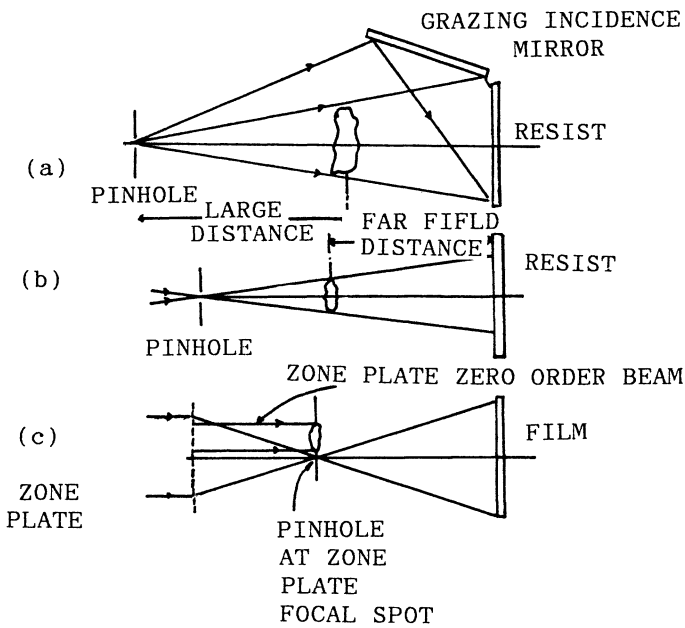

Fig. 2. Possible geometry for x-ray holography utilizing (a) Leith-Upatneiks geometry, (b) Gabor geometry, (c) Fourier transform geometry. (Howells: 1984)

Advantage of the Gabor hologram is that no optics are needed except a pinhole. The most obvious disadvantages are that the sample must be chosen to transmit suitably and there is no good solution to the problem of separating the wanted wave front from the confusing wave. This problem can, however, be much alleviated by placing the recording surface in the far field of the sample. The far-field or Fraunhoffer in-line geometry was the one used by Aoki and Kikuta (Aoki & Kikuta: 1972).

The Leith-Upatneiks hologram is similar to the Gabor but an off-axis reference beam is used. In this case, each point of the subject contributes an off-axis zone plate. The center may be off the recording area and so higher numbered, more

closely spaced zone plate rings are utilized. The advantage is that separation of the virtual image wave and the confusing wave can be achieved. The disadvantage is that a higher resolution detector and a source with greater coherence length are needed.

The Fourier transform hologram is a radically different approach. Here the reference is typically a point source and must be set at a distance from the hologram equal to the subject. The subject-reference distance is chosen about equal to the subject width which in microscopy would be small. The interfering beams thus have only a small angle between them so rather coarsely spaced Young's fringes are recorded on the detector. Consequently the resolution is not limited by the detector resolution but rather by the actual size of the nominal point source used as a reference. Detector resolution plays a role in limiting the field of view.

Recently, Jacobsen *et al.* (Howells *et al.*: 1987, Jacobsen *et al.*: 1988) and Joueux *et al.* (Joueux *et al.*: 1988) have succeeded in obtaining submicron resolution in reconstructed resist holograms. In Fig. 3, an example is shown (Jacobsen *et al.*: 1988).

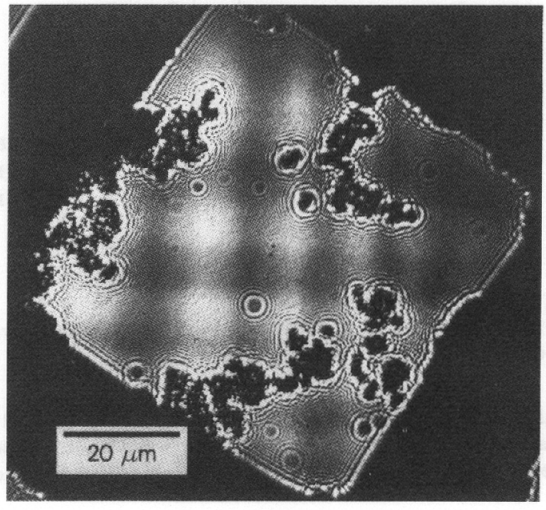

Fig. 3. Hologram of a zymogen granule taken at the NSLS undulator beamline ×17t and examined with a TEM. The hologram was taken at a working distance of about 400 μm, and are subfields of (200 μm)^2 total hologram areas. (Jacobsen *et al.*: 1988)

2. Contact microscopy

Figure 4 shows the principle of getting contact microscopic image (Sayre: 1987b). Putting specimen on x-ray resist, the specimen is irradiated by x-ray. The pattern of the specimen is thus stored on the resist. After development, it is observed by electron microscopy. As described before, highest resolution was obtained by the contact microscopy. The resolution becomes worse for thicker specimen. However, it is possible to get 3D image from the geometry shown in Fig. 5. Actually, it is

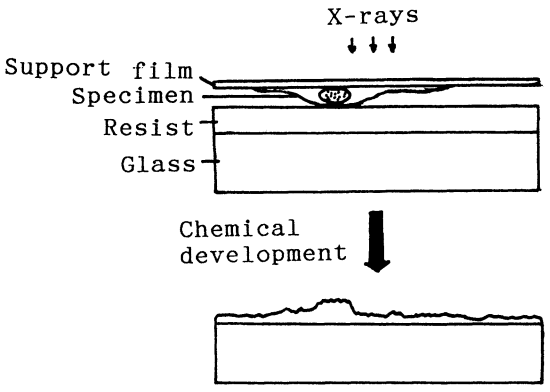

Fig. 4. X-ray contact imaging set-up. (Cheng & Jan: 1987)

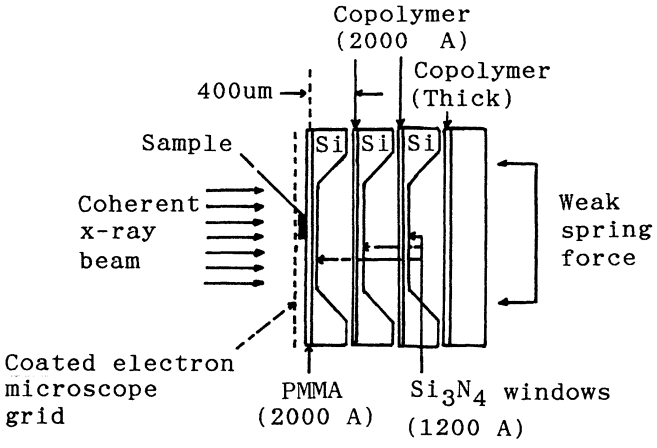

Fig. 5. Experimental arrangement for recording a contact micrograph and three holograms at distances of 400, 800, and 1200 μm from the sample. This setup was designed to allow three-dimensional reconstruction with better than 1000-angstrom transverse resolution. (from "Center for X-Ray Optics": 1986)

Gabor in-line holography mentioned above.

More conventional stereo view is available with contact micrography by taking two images of the sample by tilting (Feder & Sayre: 1980, Cheng *et al.*: 1987).

3. Imaging microscopy

G. Schmahl and his colleagues at University of Göttingen have developed imaging microscopy with two zone plates. Details of the method is described in another paper of this volume (Niemann, this volume).

Jochum in Schmahl's group has been recently developing a method to get 3D image by zone plate imaging system. She took a series of imagings in focus and a

little in front of and behind the focal point, and reconstructed a 3D image from them. Details should be referred to her original paper (Jochum: 1988).

4. X-ray microtomography (Flannery et al.: 1987, Sakamoto et al.: 1988, Kinney et al.: 1986)

X-ray computer tomography is widely used in clinical diagnosis. This method seems to be promising in getting 3D image of resolution of cells and micro-organisms, if the resolution in space is improved down to micron or submicron regions. Several attempts have been done to realize this resolution. In most cases, they use synchrotron radiation as parallel monochromatic source. Experimental arrangement is typically shown in Fig. 6. The resolution is limited by sizes of pixels of detectors. To improve it, magnification in front of detectors are usually employed; x-ray to photon conversion (Flannery *et al.*: 1987) or asymmetric diffraction (Sakamoto *et al.*: 1988) is used for this purpose. Up to now, the highest resolution reported is 2 μ by Exxon group.

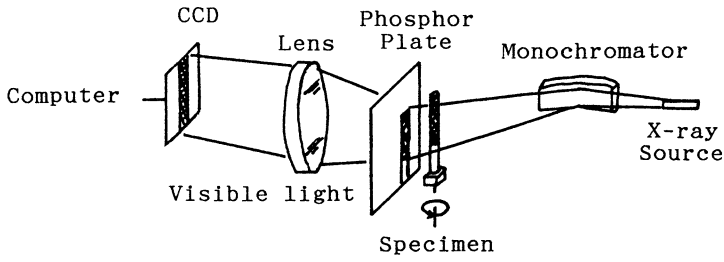

Fig. 6. Schematic of the x-ray microtomography device. A collimated beam of monochromatic x-rays illuminates a sample mounted on a rotatable stage. The intensity of the transmitted x-ray beam is recorded in a digital, panoramic, electro-optic detector. A phosphor face plate converts x-rays to optical radiation with high resolution. (Flannery *et al.*: 1987)

Tomography usually takes time to get sufficient data for the reconstruction of an image. Therefore real time imaging has been believed to be impossible for the measurement of tomography technique. However, de Beaucoudrey *et al.* reported that three-dimensional imaging of x-rays emitted by laser-induced plasmas could be performed by Coded Aperture Imaging (C.A.I.) using a "multislit" code and linear CCD detectors in order to provide real-time imaging (de Beaucoudery *et al.*: 1987). With this method, they obtained an image of a microballoon (diameter=80 μm) irradiated by two laser beams.

5. Projection microscope

This method is simple in principle and easy to set up in the usual laboratory. Samples are irradiated by x-rays injected from a point source, and the transmitted photons are recorded on a detecting film. Resolution of the projection microscope is limited by the beam size. Yada of Tohoku University reported that they could get an image with 0.3 μ resolution. Stereo view of the specimen is easily obtained by tilting specimens. In Fig. 7, a stereo image thus recorded is shown (Takahashi *et al.*: 1983).

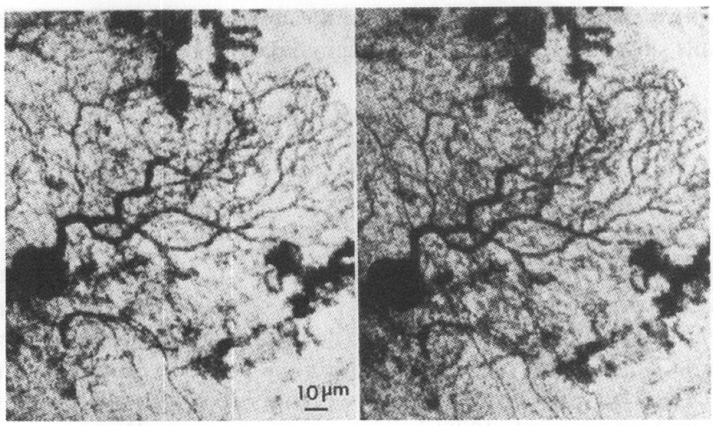

Fig. 7. A stereo picture taken by projection microscopy by tilting the specimen of sectioning of rat brain (200 μm thickness) (Yada & Takahashi: 1986)

6. Scanning microscope

Several groups have been developing scanning microscopes (Morrison *et al.*: 1988, Kirz & Rarback: 1985, Niemann *et al.*: 1988, Rarback *et al.*: 1988). Resolution so far attained is 70 nm (Kirz & Rarback: 1985). It seems a promising method, as all photons transmitted in the specimen are used for the imaging and thus it is superior to zone plate imaging microscopy in reducing minimal dose necessary for the imaging. Stereo image can be obtained by tilting specimens although the author is not aware who has done. Scanning tomography has been challenged by Elliott (Elliott & Dover: 1984), who achieved resolution of 15 μ.

7. Micro x-ray scattering

Diffraction-imaging microscopy constructs an image of the specimen from a recording of the coherently scattered, or diffraction signal from the specimen. Figure 8 illustrates the principle of the method. D. Sayre in IBM has done some recordings of diffraction imaging (Sayre: 1987b), but it should be understood that to date only a few such recordings have been made.

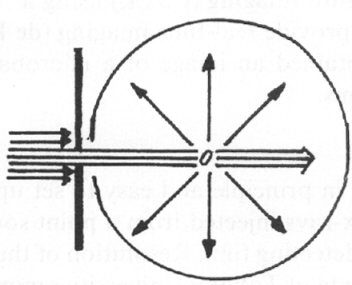

Fig. 8. Schematic of basic geometry for recording the micro x-ray scattering signal in the far field. (Sayre: 1987b)

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DISCUSSION

Q. Have you considered cooling biological specimen to reduce radiation damage? (Howard, C. V.)

A. We have not done it by ourselves. It will be promising. Cooling is also advantageous in slowing down biological reactions, which will be also necessary for real time imaging of X-ray microscopy. Probably, cooling below zero will be useful with the use of antifreeze as extensively investigated by Douzou (Cryobiochemistry, 1977 Academic Press). Such efforts were also made in time-resolved X-ray crystallographic study by Petsko (Structural Biological Applications of X-Ray Absorption, Scattering, and Diffraction, 1986, Academic Press, pp. 99–110).

Q. What is the optical depth of field in X-ray microscopy? Is it always larger than the specimen thickness? If so, then stereoscopic images can only be obtained as projection images with various specimen orientations. (Brakenhoff, G. J.)

A. Focal depth of X-ray microscopy was calculated by Guttman as

$$\Delta f = \frac{\lambda x_f x^2}{4 r_n^2}$$

which gives a result of 0.7 μm in Schmahl's group (P. Guttman: X-ray Microscopy [ed. G. Schmahl & D. Rudolph, Springer-Verlag, pp. 75–90]). According to the theory, Jochum tested imaging of thicker specimen with the aid of reconstruction mathematics as described in the main text.