X-Ray Microscopy with Amplitude and Phase Contrast

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"The biologist's dream is to observe cellular structure and content by natural contrast mechanism and, if possible, to observe variations as a natural consequence of cell function" (1).

Two examples may give an impression, of what it is hoped can be done in this respect with x-ray microscopy; the three dimensional structure and the spatial arrangement in the nucleus of the cell are supposed to be of great importance for the function of chromosomes in mammalian cells. Our knowledge of these aspects is based mainly on very indirect imaging of chromosomes with either optical or electron microscopes. Because of the inadequacy of these methods our knowledge of the structure-function relationships on the level of the whole chromosome is rather poor (2, 3). Living cells contain molecular machines which differently convert free energy into motion. These machines are composed of macromolecules and have a size between 10 and 100 nm. The speed of movement is between 1 and 10 μm/s (4). Both cases require observation of the whole cell or of cell fractions in the natural state, i.e., in aqueous surroundings.

As will be shown in the following, x-ray microscopy provides a tool to observe biological material in the natural state. X-ray microscopy can be performed using amplitude or phase contrast. In amplitude contrast the best suited wavelength region is the so-called "water window", that is, the spectral region between the K-edges of oxygen (2.34 nm) and carbon (4.38 nm). Phase contrast allows extension of this region to shorter wavelengths of about 0.6 nm or even less. For soft x-radiation the atomic cross section of interaction between photons and matter depends strongly on the atomic number and on photon energy; this provides a natural contrast mechanism for organic and inorganic materials. In addition, the contrast is
rather sensitive to variations of density. The natural contrast of specimens enables investigation in the natural state without contrast enhancing manipulations, e.g., staining with dyes as in optical microscopy or with heavy metals as in electron microscopy. Nevertheless, it may be advantageous in special cases to use staining for contrast enhancement; in this respect, gold-labelling of special features of living tissue may be of particular interest. This method also allows drastic reduction of the radiation dose applied to living specimens during x-ray examination.

I. AMPLITUDE AND PHASE CONTRAST

As an example of biological specimens, Figs. 1 and 2 show contrast of the 50 nm features of protein and nucleic acid in water. For protein a composition of $C_{94}H_{139}N_{24}O_{31}S$ and a specific weight of 1.35 g/cm$^3$ and for nucleic acid a composition of $H_{16}C_{6}N_{4}O_{10}P$ and a specific weight of 1.70 g/cm$^3$ were assumed. The dashed lines indicate that amplitude contrast at nearly all wavelengths is less than the contrast based on phase contrast. The contrast, however, is not always the most important factor. For biological specimens the radiation dose is of crucial interest, especially when they are to remain alive during the examination. Figures 3 and 4 show the radiation dose for the same features assuming discrimination with a signal-to-noise ratio of three (5). As shown, the radiation dose is also less for phase contrast at nearly all wavelengths than for amplitude contrast. Of special interest is the fact that the dose remains nearly constant at the short wavelength end.

![Fig. 1. Contrast of a protein structure of 50 nm surrounded by water.](image-url)
AMPLITUDE AND PHASE CONTRAST

Fig. 2. Contrast of a nucleic acid structure of 50 nm surrounded by water.

Fig. 3. Radiation dose which is necessary to detect a protein structure of 50 nm in water with a signal-to-noise ratio of three.

To illustrate the advantage of gold-labelling of biological structures Fig. 5 shows the contrast of 20 nm gold structures in water. For an impression of the dose reduction using gold-labelling we calculated the number of photons necessary to identify a 50 nm protein structure of the above mentioned type and a 20 nm gold structure, each with a signal-to-noise ratio of three. Figure 6 shows the ratio $F$ of these numbers depending on wavelength for amplitude and phase contrast; this number corresponds to the dose reduction gained by gold-labelling. In the water window this reduction
Fig. 4. Radiation dose which is necessary to detect a nucleic acid structure of 50 nm in water with a signal-to-noise ratio of three.

Fig. 5. Contrast of 20 nm gold structures surrounded by water.

of radiation dose is between 10 and 100 and for wavelengths in the region of 0.6 nm it is about 100 for contrast based on phase contrast.

The data of Figs. 1–6 are calculated using the tables of the atomic scattering factor $f_1 + if_2$ from Henke et al. (6). Figure 7 shows transmission (1/e-value) of water as a function of the wavelength. It can be seen that in the water window the thickness of wet specimens should not be more than about 5 μm, whereas at wavelengths of 0.7 nm or less specimens can be
Fig. 6. Ratio $F$ of the number of photons necessary to detect a 50 nm protein structure in water and the number of photons necessary to detect a 20 nm gold structure in water (with a signal-to-noise ratio of three each).

Fig. 7. Transmission ($1/e$-value) of water as a function of wavelength.

much thicker. This makes a strong argument for using phase contrast in x-ray microscopy.

II. X-RAY MICROSCOPES

Figure 8 shows the x-ray optical arrangement of an x-ray microscope as it is operated with the synchrotron radiation of a bending magnet at the electron storage ring BESSY in Berlin. The polychromatic x-radiation meets the
condenser zone plate at a distance of 15 m from the source which is the point
tangent to the electron beam. The condenser zone plate with a diameter of
9 mm and a focal length of \( f = 304 \text{ mm} \) (for 4.5 nm radiation) generates a
reduced image of the source. The reduction factor is 48 at 4.5 nm. Together
with the free diameter of the object chamber the condenser acts as a linear
monochromator with a spectral resolution of \( \lambda/\Delta\lambda = 250 \).

The shadowed central region of the condenser is opaque to prevent zero
order radiation from reaching the image field and reducing image contrast.
The micro zone plate \( (f = 0.69 \text{ mm} \text{ at } 4.5 \text{ nm}, \varnothing = 55.6 \mu\text{m}) \) generates a
magnified image which is photographed; in addition, a micro-channel-plate
is installed so the image may be viewed directly. For more details see ref. 7.

A similar optical arrangement for phase contrast is shown in Fig. 9. A
phase plate is located in the back focal plane of the micro zone plate to
apply a phase shift of 90° to the direct light of the object. To keep this
phase plate as small as possible the object must be illuminated coherently
so that the direct light can be focussed as clearly as possible in the back
focal plane. Less coherent illumination requires a larger phase plate with
the disadvantage that not only the direct light but also light diffracted by
object structures of low spatial frequencies is phase shifted. In this case
only partial phase contrast is obtained \((5, 8)\). Coherent illumination of the
object requires a condenser/collector system of two optical elements (zone
plates) which is more lossy than for amplitude contrast (Fig. 8).
Figure 10 shows the x-ray microscope which is operated with synchrotron radiation at the electron storage ring BESSY in Berlin.

III. RESULTS

Most of the images to date were obtained with amplitude contrast due to the fact that no condenser/collector system for coherent illumination has yet been realized. Only preliminary experiments were made, using the

Fig. 11. Image of a part of a monkey kidney cell obtained with 4.5 nm x-radiation at the electron storage ring BESSY in Berlin.
arrangement of Fig. 8 with an additional phase-plate in the back focal-plane of the micro zone plate. With this illumination only partial phase contrast could be obtained.

Figure 11 shows an image of a part of a monkey kidney cell obtained in amplitude contrast with 4.5 nm x-radiation at the electron storage ring BESSY in Berlin.

IV. FUTURE DEVELOPMENTS

Until this time amplitude zone plates have been used which are realized in the form of gold rings on a polyimide foil transparent to x-radiation. The diffraction efficiency in the first diffractive order of amplitude zone plates is limited to about 10%; in practice, only 3-7% is obtained. To increase the efficiency of the optical components the opaque zones of amplitude zone plates can be replaced by a phase shifting material to obtain the added constructive interference from these parts of the plate. This increases the amplitude transmission of the device by a factor of two (absorption neglected) and the efficiency by a factor of four. In practice, absorption cannot be neglected. Figure 12 shows calculated values of efficiency for laminar nickel structures as a function of thickness with wavelength as a parameter. As the necessary thickness of material is independent of zone width, the aspect ratio of the zones becomes rather high for high resolution micro zone plates with outermost zone widths of 10-50 nm. Great effort is being devoted to micro structure fabrication of phase zone plates with aspect ratios of 20 or more. Use of these plates would allow an increase in the efficiency of the optical system by about a factor of ten.

Fig. 12. Diffraction efficiency of the first diffractive order of zone plates consisting of laminar nickel zones as a function of thickness $t$ of the zones for different wavelengths.
Using new generation electron storage rings with undulators which considerably increase the brilliance of the x-radiation will allow the realization of very efficient coherent illumination for phase contrast, especially at short wavelengths of 6 nm or less.

Further attractive aspects of x-ray microscopy are possible with the development of pulsed laboratory x-ray sources on the basis of plasma focus (9), laser induced plasma sources or even x-ray lasers (10). Especially, the fact that it may become possible to generate an x-ray image with only one short pulse from such a source is of great interest for biological applications.

SUMMARY

Amplitude and phase contrast x-ray microscopy is discussed. As examples, calculations of amplitude and phase contrast of 50 nm structures of protein and nucleic acid are given. In addition, the radiation dose necessary to detect such structures with a signal to noise ratio of three is given. The x-ray optical arrangements of x-ray microscopes for amplitude and phase contrast are discussed. Some images obtained with 4.5 nm synchrotron x-radiation of the electron storage ring BESSY in Berlin are shown. Future developments are discussed with regard to the possibility of higher diffraction efficiencies of zone plates, new generation synchrotron radiation sources (undulators) and laboratory x-ray sources.

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