X-Ray Microscopy at Suboptical Resolution: Direct Observation of the 65-nm Periodicity in Collagen Fibrils

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Using a Fresnel zone plate we demonstrate for the first time the direct visualization by x-ray microscopy of suboptical regularity in a biological specimen, namely the 65-nm axial periodicity of tendon collagen. This resolution test demonstrates a resolving power of about 20\(\lambda\); a resolving power of <10\(\lambda\) is in prospect. © 1992 Academic Press, Inc.

X-ray microscopy is being intensively developed as an imaging method which fits in performance between the light microscope and the electron microscope.

Most attention has so far been given to the potential of x-ray imaging in biology for which the optimum soft x-ray wavelength lies in the so-called “water window,” for wavelengths between 2.4 and 4.4 nm, where biological material, e.g., protein, is much more absorbing than an equivalent thickness of water. Wet biological cells and organelles, mounted in an environmental cell, in air, and of total thickness up to about 5 \(\mu\)m, can be examined with a potential point-to-point image resolution currently of 50 nm, with every expectation that the resolution will be improved over the next few years toward 20 nm and indeed 10 nm.

X-ray microscopes working to this specification, and mounted on synchrotron sources, have so far been developed at three laboratories in the world. The laboratory/synchrotron combinations are Professor G. Schmahl of Gottingen: BESSY Synchrotron, Berlin; Professor J. Kirz of Stony Brook, New York: National Synchrotron Light Source, Brookhaven; and the King’s College London Microscope mounted at the SRS Synchrotron, Daresbury, U.K. (See Schmahl et al. (1); Kirz and co-workers (2); and Kenney et al. (3).)

We report here the first observation using the scanning x-ray microscope at Brookhaven of the well known macroperiod, or D-period, of periodically repeating structure along the fibril axis of rat tail tendon collagen. This periodicity, of about 68 nm in wet material, rather less when dry, is significantly below optical resolution on similar material (about 0.2 \(\mu\)m or 200 nm).

The resolution of a scanning x-ray microscope depends on the minimum width of a focused point beam of monochromatic x rays. The beam corresponds to the diffraction-limited image of a 100-pm-wide entrance aperture passing soft x rays from the monochromator (\(\lambda/\Delta\lambda \approx 500\)) forming part of the synchrotron beam line X1A at the NSLS. The imaging is carried out by a Fresnel zone plate lens. The structure
of the zone plate and the accuracy of its fabrication define the halfwidth of the focused x-ray probe at the specimen.

Considerable effort has been expended (4, 5) toward the fabrication of zone plates with suboptical resolution. Current zone plates have about 1000 concentric circular zones within a disc of 100 pm diameter, plotted by the methods of electron beam fabrication, as for integrated microcircuits, but to about 100 times finer spatial scale than for current commercial electronic applications. The Rayleigh resolution of a perfect zone plate is \( \delta = 1.22 \Delta r_N \) where \( \Delta r_N \) is the width of the outermost zone. For the zone plate we were using (4), the outermost zone was 45 nm and \( \delta = 55 \) nm. The zone plate pattern was drawn by the E-beam into photoresist and then transferred by methods based on reactive ion etching and electrolytic deposition into nickel, as a phase zone plate, with a first order diffraction efficiency within the water window of greater than 10%. The focal length of the zone plate for water window wavelengths is about 1 mm and higher order foci than the first are obscured from the specimen by a combination of central apodization of the zone plate and a diffraction order selecting aperture placed between the zone plate and the specimen. We have drawn zone plate patterns in carbon with \( \Delta r_N = 25 \) nm, \( \delta = 30 \) nm, and their transfer into metals of higher absorbing power and hence higher diffraction efficiency for soft x rays is in progress.

To resolve structure having a periodicity of about 65 nm with an optical system having \( \delta = 55 \) nm is to work under imaging conditions such that the contrast level to which even a binary structure can be rendered relative to background is low; the contrast level is even lower when the electron density fluctuations, as in the collagen D-period, are only a few percent about the mean value. Because of the expected very low contrast level, collagen fibrils prepared by blending rat tail tendon were first positively stained with a 1% solution of phosphotungstic acid (PTA) in water before being transferred to a 3-mm-diameter mount covered with a thin (50 nm) film of (soft x-ray transparent) silicon nitride. PTA staining is a technique frequently used in electron microscopy to increase contrast.

The specimen was mounted on a scanning stage, moved in a two-dimensional raster scan perpendicular to the stationary x-ray beam and with coarse and line shifts of 1 \( \mu \)m and 10 nm, respectively.

An x-ray image of a dry fibril from tendon collagen stained with PTA is shown in Fig. 1, taken at a wavelength of 3.5 nm. The contrast is due to differences in x-ray absorption through the fibril thickness and was optimized by through-focus series of images (focal increment 1 pm), as well as by selection from the images of several fibers showing banding at different contrast levels and for various orientations of the fibrils relative to the scanning direction of the specimen stage. As expected, optical Fourier transforms of the images show in the fiber axis direction only diffuse small-angle reflections corresponding to the first order of the D-period.

This work represents the demonstration of suboptical resolution by an x-ray microscope on a biological specimen, as distinct from a study of image resolution from the sharpness of the images of opaque edges of binary objects.

There is good reason to believe, from the relative speeds of exposure of x-ray diffraction patterns of wet and dry (but unstained) tendon collagen fibres, that the level of differential contrast corresponding to the density changes relative to background defining the collagen macroperiod is likely to be greater for wet fibrils than for dry. A
combination of wet fibrils and, certainly, a higher zone plate resolution is necessary for imaging of the collagen structure at increased contrast; such zone plates are being fabricated now.

Finally, it should be pointed out that future observations on wet collagen, as allowed by x-ray microscopy, at improved resolution (say 20 nm), will have relevance to the consideration of the molecular structure of the collagen “hole” region, within the description of the D-period structure model of Hodge and Petruska (6). In this model the “hole” and “overlap” regions divide the macroporid period approximately into two unlike halves.

It is thought that the hole region, i.e., the region of lateral molecular disorder, is the principal site for water uptake, for the uptake of positive stains such as PTA as used here, and for the location of calcification in collagen fibres. Studies of collagen structure by medium-angle x-ray diffraction (7) pick out preferentially the details of the ordered structure, i.e., the overlap region. The x-ray microscope, given the necessary resolution, and for wet fibril specimens, has the potential of yielding new information about the hole region. This is a topic we hope to address with improved zone plates.

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REFERENCES

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