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Author

Howells, M.

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Lawrence Berkeley Laboratory

UNIVERSITY OF CALIFORNIA

Accelerator & Fusion Research Division

Center for X-Ray Optics

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**X-RAY HOLOGRAMS AT IMPROVED RESOLUTION:
A STUDY OF ZYMOGEN GRANULES**

**M. Howells
Center for X-Ray Optics
Lawrence Berkeley Laboratory
University of California
Berkeley, CA 94720**

**C. Jacobsen and J. Kirz
Department of Physics
State University of New York
Stony Brook, NY 11794**

**R. Feder
Recently Retired from
IBM T.J. Watson Research Laboratory
Yorktown Heights, NY 10538**

**K. McQuaid and S. Rothman
Schools of Medicine and Dentistry
University of California
San Francisco, CA 94143
and
Center for X-Ray Optics
Lawrence Berkeley Laboratory
University of California
Berkeley, CA 94720**

Abstract

It has long been known that x-ray holography offered the possibility of three-dimensional microscopy with resolution higher than the light microscope and with special forms of contrast based on x-ray edges. In principle, the method is especially advantageous for biological samples if x-rays in the wavelength region between the C_K and O_K edges are used.⁴ However, until now the achieved resolution has not exceeded that of the light microscope and results have been generally disappointing. This can be understood in terms of the poor coherence properties of the x-ray sources and the low resolution of the detectors that were available. We now report that, using recently developed x-ray sources based on undulators on electron storage rings, and high performance x-ray resist, we have recorded a hologram at about 400 Å resolution. The experiment utilized 24.7 Å x-rays and required a one hour exposure of the pancreatic zymogen granules under study. This opens the way for extensive development of the holographic imaging technique using x-rays.

A number of theoretical studies^{1,2} have suggested the possibility of utilizing soft x-rays (wavelength 10-50 Å) to make three dimensional images of microscopic biological objects. One of the methods proposed in these studies is x-ray holography. The studies show that this technique is capable, in principle, of three dimensional imaging of biological samples with feature resolution in the 100-1000 Å range. Moreover, this capability should be applicable to cells and subcellular structures in something closely related to their natural state: that is, without dehydration, sectioning or staining.³

We have now verified some of these predictions experimentally. We report results which demonstrate that x-ray holograms of biological objects can be recorded on resist using 25 to 32 Å⁴ soft x-rays with reasonable exposure times and including information at the 400 Å resolution level. The significance of such a recording is that this has hitherto been the missing step in the sequence of operations needed to make a holographic image at that resolution level. We shall comment on the other steps in the sequence. They present some difficulties, but have all been demonstrated in other contexts.

The achievement of recordings of this quality has been prevented in the past by the poor coherence properties of available x-ray sources. We have addressed this in the present experiments by using one of the recently developed undulator sources.⁵ These devices provide a copious flux of spatially and temporally coherent soft x-rays and have many laser-like properties. Earlier sources such as x-ray tubes⁶ and synchrotron bending magnets⁷ have also been used for holography and there are a number of reports in the literature describing both the recording and reconstruction of x-ray holograms. However, we are not aware of any experiments that have

demonstrated resolution below about one micron and it is the recording of image information on a size scale much less than this that is our main reason for regarding the present results as an advance. We believe that the use of undulators will allow still further improvements in x-ray holography and in other x-ray imaging techniques that require spatially coherent x-rays.^{8,9} Some of these latter methods are already producing two-dimensional images with resolutions of 500-1000 Å and also are avoiding the most radical forms of sample preparation. This general picture leads us to believe that a new three dimensional form of x-ray microscopy is now within reach with improved resolution compared to the light microscope and improved fidelity compared to the electron microscope.

The holograms shown in this communication were recorded in November, 1986, using the X17T undulator beamline^{10,11} at the National Synchrotron Light Source x-ray ring. The best recordings are of rat pancreas zymogen granules,¹²⁻¹⁴ the enzyme containing components of the gland's secretory or acinar cells. The holograms are recorded on copolymer¹⁵ resist layers about 2000 Å thick which are deposited on a thin (1200 Å) silicon nitride substrate and placed 400 μm (or multiples thereof) downstream of the sample as shown in Fig. 1. The recording process was carried out using a spatially and temporally coherent (1.8 μm coherence length) beam of 24.7 Å x-rays. (Some holograms were also recorded at 32 Å x-ray wavelength). For the example shown in Fig. 2, the exposure lasted 80 minutes for 8×10^{11} photons which corresponds to a dose of about 200 megarads to the specimen.¹⁶ After exposure, the resist was developed for 1 minute in 50% MIBK : 50% IPA and then metallized with ~200 Å of 60% palladium - 40% gold at 7.2 degrees grazing

angle to enhance the contrast of the relief image. The micrographs shown in Fig. 2 and 3 were taken using a transmission electron microscope (hence the need for the thin substrate).

Each hologram consists of an interference pattern formed by coherent superposition of the approximately plane-wave incident beam with the roughly spherical-wave signals scattered by the sample. For the geometry that we are using (Gabor in-line geometry), the effect is to create patterns reminiscent of Fresnel zone plates, as seen in Fig. 2 and 3. Since we are using an axial reference wave which therefore has zero spatial frequency, the highest recorded sample frequency is simply equal to the highest recorded fringe frequency. Fringes are visible on the original negative out to a numerical aperture of approximately $1/25$, indicating that information is recorded at about $400 \text{ \AA}$ transverse resolution.

The x-ray source^{10,11} used for these experiments consisted of the NSLS X17t mini-undulator, which has 10 periods each of 8 cm length. Because of the low electron beam emittance of the NSLS x-ray ring, this undulator is approximately two orders of magnitude brighter than other available continuous soft x-ray sources. The undulator beam is relayed by a flat mirror to a $300 \text{ }\mu\text{m}$ pinhole, which removes most of the beam power and also acts as the entrance slit for the monochromator. The toroidal grating monochromator is operated in negative order and is able to obtain a slit width limited resolving power of ~ 700 with an $80 \text{ }\mu\text{m}$ exit slit. By using the center half of the Airy cone of a $50 \text{ }\mu\text{m}$ pinhole located downstream of the exit slit, we were able to coherently illuminate the sample at a distance of 1.74 m. Between the pinhole and the sample the $200 \times 200 \text{ }\mu\text{m}^2$ silicon nitride

(Si_3N_4) entry window¹⁷ of the experimental chamber was located.

Although the specimen chamber was operated at $\sim 10^{-2}$ torr for the work reported here, the window permits us to operate the chamber at any pressure up to one atmosphere. The difficult task of maintaining stable and reproducible alignment of the optical system was accomplished by a feedback system for steering the electron beam through the undulator.¹⁸ This system was based on signals derived from x-ray beam position monitors.

The reconstruction of x-ray holograms recorded in the above way poses unique problems not encountered in ordinary holography. For example, if reconstruction using a He:Ne laser were attempted using the photographic output from the electron microscope, one would have a wavelength ratio of 256 and a hologram scaling factor of 12,700. The holographic "lenses" in such a recording (shown in Fig. 2) have a focal length of about 250 m and are thus too weak to allow an optical system of manageable size to be used in reconstruction. By photographic reduction of the hologram, one can get down to a conveniently sized optical layout and calculations also show that aberrations can be made negligible. However, the twin-image problem must still be addressed because our holograms are not, in general, recorded in the far field. Moreover, we expect difficulties due to the complicated physics of the recording and readout processes. The latter comprise recording plus liquid development leading to a resist relief pattern followed by metal shadowing and electron microscopy. At least in principle, one can model the above processes and work backward from the photographic density to obtain the intensity of the wavefield. However, such allowances cannot be made if reconstruction is by a visible light laser. This limitation, coupled with the twin-image problem, which is compounded when the object is unknown, renders

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laser reconstruction rather unfavorable for this type of holography. To obtain a computed image, the reconstruction of the hologram would proceed by measurement of the electron microscope photograph using a scanning microdensitometer followed by a type of numerical processing that would be able to address the twin-image problem.¹⁹ Such an approach to hologram reconstruction has been described in the literature but is not trivial. We are now proceeding with the steps needed to obtain a reconstruction of this type.

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3. At the present time some form of fixation is used but we are hopeful that it will be possible to relax even this requirement.
4. The spectral region between the O_K edge (24 Å) and the C_K edge (44 Å) is of special importance for imaging natural biological samples. For these wavelengths, carbon containing materials give absorption contrast while water is comparatively transparent.
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12. The zymogen granules were fixed in 1.5% gluteraldehyde in 150 mM sucrose, but were unstained.
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15. Copolymer is 80% polymethyl methacrylate PMMA and 20% methacrylic acid. The mixture is spun on to the substrate in solution and then cured at 200°C for 1 hour. Some holograms were also recorded on PMMA.
16. It is desirable to understand the effect of such radiation doses in the context of microscopy at this resolution. Specifically, we would like to know the extent of degradation of the final image due to radiation damage. Dr. Thomas Ermak of the San Francisco Veterans' Administration Hospital has examined some of our holography specimens after soft x-ray exposure with the electron microscope. Unsectioned zymogen granules are too thick for effective use of ordinary transmission electron microscopy and they appear as black discs of close to perfectly circular shape. There were no discernible differences between exposed and unexposed granules viewed in this way. We plan further investigations of the damage problems in the future as well as efforts to reduce the dose required to make a hologram. We did not make any effort to minimize the dose in the experiments reported here.

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20. The authors wish to acknowledge valuable help and advice from M. Caldarolo, T. Ermak, J. Grendell, H. Rarback, and D. Sayre, as well as the generous assistance of the staff at the NSLS. We also acknowledge the support of the NSF under grant BBS-8618066 (C.J., J.K.) and the Department of Energy under contract DE-AC03-76SF00098 (M.H.). The NSLS is supported by the Department of Energy under contract DE-AC02-76CH00016.

Figure captions

1. Arrangement of specimen and resist detectors used for recording multiple x-ray holograms in Gabor (in line) geometry.
2. Transmission electron micrograph of an x-ray hologram of several zymogen granules. (A): A $62 \times 50 \mu\text{m}^2$ field of a $200 \times 200 \mu\text{m}^2$ hologram.
(B): The $19 \times 15 \mu\text{m}^2$ region indicated in A at higher magnification.

The recording is a shallow relief pattern in the x-ray resist and has been shadowed with heavy metal at glancing angle to give increased contrast (see text). The structureless outer areas of the field are grid bars while the irregular dark areas are the diffraction blurred shadows of heaps of granules which are too thick for the x-rays to penetrate. In places granules are found in groups of one or a few that can be penetrated. X-rays that pass through this sample material are diffracted by it and convey information about its three dimensional structure. The superposition of these diffracted waves with the straight-through beam forms the many interference fringes which compromise the hologram. The faint square grid pattern in A is the Fresnel diffraction pattern of the square entry window of the sample chamber. In B, two dark islands can be seen right of center. We believe these are each part of the diffraction pattern of a single granule.

3. A portion of a hologram recorded in a similar way to Figure 2 and centered on a single granule. The recorded diffracted signals do not have circular symmetry although we know the granules look like black circular discs in the electron microscope. This may be due solely to the inclusion of some

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wave amplitude diffracted from other objects in the field but may also be associated with diffraction by non-circularly-symmetric structures, internal to the granule, which were not observable by electron microscopy.

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XBL 871-271A

Figure 1

XBB 871-414

Fig. 2A

XBB 871-421

Fig. 2B

XBB 874-3139

Fig. 3

*LAWRENCE BERKELEY LABORATORY
TECHNICAL INFORMATION DEPARTMENT
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720*