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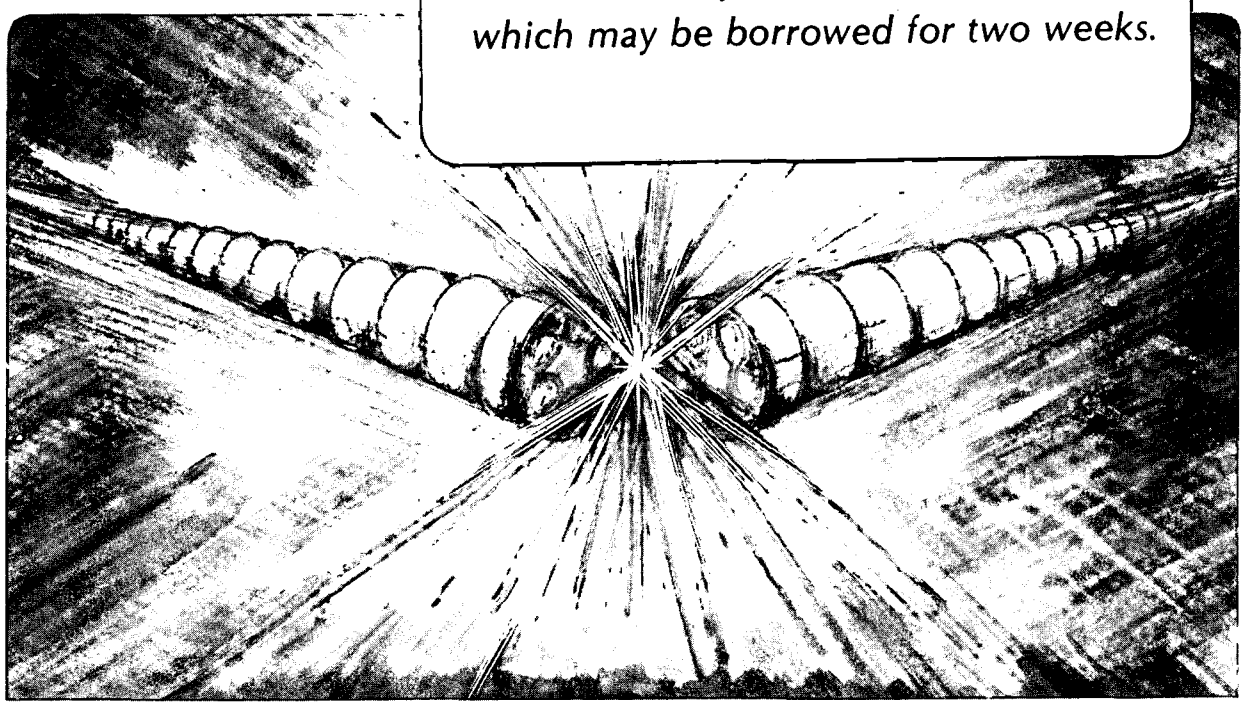

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PROGRESS AND PROSPECTS IN SOFT X-RAY HOLOGRAPHIC MICROSCOPY

by

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INTRODUCTION

The majority of x-ray imaging experiments today use the contact technique [1] or x-ray analogues of the optical microscope, either in direct imaging [2] or scanning [3] mode. However it is also possible to obtain sample information by exploitation of the diffracted field as is done in crystallography. To do this one must have a method for determining and using the phases of the diffracted wave. In the soft x-ray region, holography is one way to provide such a method. Other ways have been proposed by D. Sayre [4].

It was in 1948 that D. Gabor [5] first pointed out that it is possible to record both the amplitude AND phase of a wave using an intensity detector provided a suitably coherent phase reference wave is available to beat against the signal wave. He further showed how to use the recording as a diffracting structure to make a reconstructed image of the object emitting the original signal wave. X-ray holography seeks to use the information carried by the x-rays diffracted by a sample and to record it and interpret it using the method of Gabor. The use of such optical methods is possible in the soft x-ray spectral region and there is a long history, beginning in the early 1950s, of attempts to make x-ray holograms using x-ray tube sources and photographic film detectors. The hope was that one would be able to record the hologram with x-rays and reconstruct it with visible light thereby achieving a three-dimensional "microscope" with resolution superior to the light microscope and without the need to fabricate a lens with such resolution.

These hopes were never realized. Holograms of reconstructible quality [6][7] were obtained in relatively few cases and none gave images with resolution higher than the light microscope. As a result x-ray holography became a dormant field by the mid 1970's.

This general failure can be understood in terms of the poor coherence properties of the x-ray tube sources and the low resolution of the photographic film detectors that were used. Such an understanding gives insight into what is needed for successful x-ray holography, and forms the basis for our belief that current technological advances are opening the way to a productive future for x-ray holography. As we explain below, the use of high-coherence undulator sources of soft x-rays, high resolution resist detectors and digital image processing systems for making the reconstruction, is providing new capabilities for x-ray holography in the resolution regime between that of the optical and electron microscopes. Such approaches share the advantages of other soft x-ray imaging methods [8]; *viz.*, applicability to samples in water in an atmospheric-pressure air environment, sufficient penetration to image unsectioned cells, contrast without stains based on x-ray absorption edges, and freedom from many of the background noise processes that afflict charge particle probes.

In what follows we report some of the latest developments in x-ray holography experiments and make some speculations about the limits of performance of the approaches currently in use. We also make some suggestions about where the technique can (and cannot) go in the future.

THE NEW TECHNOLOGIES

One of the main goals of all soft x-ray imaging techniques is to improve on the resolution of the light microscope, and we consider the ways in which this may be achieved in holography. First, in Gabor (in-line) holography (Fig 1), the resolution is limited by the detector resolution [9]. This argues that we must turn to photoresist as the high-resolution alternative to photographic film. Such a strategy was first used by Bjorklund [10] and for polymethylmethacrylate (PMMA) resist, extends the detector limit to a value variously estimated [11][12] in the range 50-200 Å. Of course, resist is much slower than film but this is a necessary concomittant for such a substantial improvement in resolution. The real measure of the wastefulness of a detector, the detective quantum efficiency, is reportedly not much different for resist [11] than for film [13] in the soft x-ray region.

For the large jump in resolution involved in changing to resist it is obviously necessary to provide many more illuminating photons and correspondingly higher radiation dose to the sample. Some attention has been given [14][15][16] to the scaling law of the needed photon fluence (and therefore the dose) as a function of resolution. It is argued in [14][15][16] that the dose scales as the sixth power of the transverse resolution for a three-dimensional experiment: which means one where the sample thickness is significantly greater than the depth resolution. Otherwise, one has a two-dimensional experiment and the scaling law becomes a square law. The most popular way to provide an adequate flux of temporally and spatially coherent soft x-rays of wavelength 23-44 Å (which is the most appropriate range for making holograms of biological samples) is to use an undulator on an electron storage ring. A number of such devices are becoming available at the present time [17]. When operated on a storage ring that has a suitably low electron beam emittance (spatial-width, angular-width product), undulators provide near-diffraction-limited soft x-ray beams with many laser-like properties.

As we show later in more detail the use of an undulator source and a resist detector makes it possible to record holograms with much finer fringe detail than the earlier workers achieved. Such holograms are now being made in France, Japan and the United States [18][19][20]. The French group -- D. Joyeux, S. Lowenthal, F. Polack and A. Bernstein -- is based at the Institut d'optique and LURE and has used an undulator on the ACO storage ring to make phase holograms of diatoms and other objects on preexposed resist. They have chosen to develop an analogue approach to reconstruction that uses sophisticated optical correction methods to deal with aberrations. The procedure is optimised with regard to speed, convenience and signal-to-noise rather than resolution. One anticipates further improvements in the performance of this scheme when this program transfers to the new SUPERACO storage ring. The group in Japan is using an undulator on the PHOTON FACTORY storage ring in a natural continuation of the earlier work of S. Aoki, S. Kikuta (Universities of Tsukuba and Tokyo respectively) and collaborators, who achieved the most successful holograms of the 1970's. The experiments so far reported are in an early phase but recent improvements to the storage ring have made it a highly suitable source for the continuation of this widely-admired holography program.

A further step forward in holographic imaging can be achieved by using numerical processing as a means of reconstructing the final image [21]. The

availability and power of hardware capable of doing this is increasing at a rapid rate at the present time. Such an approach provides one way to avoid the resolution limitations normally involved in visible light reconstruction. It also gives much more flexibility in finding the focus, eliminating non-linearities in the record-develop-read-out-digitise sequence and in dealing with the twin-image problem that is inherent in the in-line holographic method. Furthermore, the end result in a computer reconstruction provides both the amplitude and phase of the image signal and this has potentially important applications. We give an example of the use of these digital methods in the next section.

X-RAY HOLOGRAPHIC EXPERIMENTS AT THE NATIONAL SYNCHROTRON LIGHT SOURCE (NSLS) AT BROOKHAVEN NATIONAL LABORATORY.

The present authors have been implementing a program of x-ray holography at the NSLS for some time and these experiments have recently begun to use an undulator source and resist as a recording medium. The optical system that we have used to record Gabor x-ray holograms is shown in Fig 2. The NSLS X-17t mini-undulator beamline [22] was used to provide a spatially and temporally coherent beam of 25 Å x-rays. The temporal coherence (about 1 micron coherence length) was achieved by means of a monochromator (spectral filtering) and spatial coherence by a pinhole (spatial filtering). The coherent flux was about 10^8 photons per second which enabled us to record a stack of holograms at 400 micron spacing (Fig 3.) with about a one-hour [19] exposure. The recordings were made on 2000 Angstrom thick layers of resist coated on to 1200 Angstrom thick silicon nitride windows supported on silicon frames. The resist [10] was either polymethylmethacrylate (PMMA) or a copolymer of 80% methyl methacrylate and 20% methacrylic acid (MMA-MAA). These are positive resists, which means that they are etched more quickly by solvents in regions of radiation exposure. After exposure the resist was "developed" by immersion in the solvent, methylisobutyl ketone diluted with isopropanol. The interference fringes formed by coherent superposition of the (roughly spherical) wave scattered by the sample and the plane incident wave were finally recorded on the resist surface as a relief pattern (Fig 4.). In order to give good contrast, the resist was shadowed with gold-palladium at glancing incidence and imaged in the transmission electron microscope (TEM), which produces a photographic negative. The final step of the experiment is to digitize the negative with a microdensitometer to produce the numerical data that forms the starting point for the analysis.

The analysis takes advantage of the fact that all holograms can give an aberration-free reconstruction if they are illuminated with the original reference wave [23]. This would not be a useful thing to do in the present case because the reconstructed image would not be magnified. However, we can mimic the same process in a computer and display the result to get any required magnification. This procedure also has the other advantages mentioned earlier.

The calculation [19] really consists of numerically simulating the propagation of the reference wave from the hologram to the real image in the Fresnel Approximation. This involves taking the Fresnel Transform of the data which are considered to represent a thin, amplitude hologram. The algorithm for doing this involves multiplying the data entries by a quadratic phase factor and taking the Fast Fourier Transform. On a MicroVAX II computer, it takes

about five minutes. The process of focussing takes place at this stage of the procedure and involves adjusting the propagation distance. We show an example of a reconstructed image in Fig 5. The samples were mounted on electron microscope grids and by reconstructing the image of the edge of one of the grid bars, we have determined that the system resolution, defined as the distance from 25% to 75% of the step-height, is around 500 Å.

These experiments were carried out with the dual purpose of developing the technique and studying the process of secretion, particularly as revealed by structural details of secretion granules. The granules used in our experiments were obtained from the pancreatic acinar cells of fasted rats, and are known as zymogen granules. They were fixed in 1.5% glutaraldehyde in 150 mM sucrose but were unsectioned and unstained. We are just beginning in our efforts to understand our data in terms of the morphology of the granules. For the moment we believe that we have shown that x-ray holography can be used to make interesting images of biological samples and can demonstrate resolution in the region of 500 Å.

THREE-DIMENSIONAL IMAGING

One of the main goals of holographic imaging is to provide three-dimensional information. The diffraction limits to this are the same as in imaging with lenses and are related to the numerical aperture (N.A.). The transverse resolution is $\lambda/2(N.A.)$ and the longitudinal resolution is $\lambda/(N.A)^2$. The N.A. in holography is not normally defined by the size of a physical aperture such as the edge of a lens but is determined in a complex way by the transfer function of the resist as a function of frequency (aperture angle), the power spectrum of the sample and the coherent x-ray exposure. These parameters determine the roll-off of the signal-to-noise ratio at high frequencies and hence the N.A. and the resolution.

One conclusion from this is that the transverse and longitudinal resolutions are related through the numerical aperture and that for experiments at low transverse resolution (low N.A.) the longitudinal resolution may be much larger than the sample thickness leading to an essentially two-dimensional image. To achieve three-dimensional imaging one has therefore to improve the numerical aperture and thus both types of resolution. For example the Brookhaven experiments reported above had a numerical aperture of about 1/40. If we could improve this to about 1/8 then we would have a transverse resolution of 100 Å, and a longitudinal one of 1600 Å, which would be quite useful. However, the dose required scales as the sixth power of the transverse resolution, so progressing toward 100 Angstrom would certainly imply that one would be dealing with extraordinarily high doses. For samples composed mainly of biological material it would be hard to imagine making more than a few exposures (say two for a stereo-pair, for example) if one pushed to the limit in this way.

Another approach may be exemplified as follows. Suppose we have an exposure at some given resolution and that we have a way to improve that by a factor of two for a hologram from a single view direction. This would give four times better depth resolution and sixty four times more dose. However, an alternative strategy, using the same dose, would be to forego the factor two resolution improvement and make sixty three more exposures at the same resolution with different illumination directions. This would provide greatly superior three-dimensional mapping and would lead us into a form of tomography

which is usually called diffraction tomography [24][25] because the wavefield emerging from the sample spreads out and propagates according to the laws of diffraction. This is in contradistinction to the conditions normally encountered in computerised axial tomography (CAT) scanning, which is based on the laws of geometrical optics.

The two choices discussed above represent opportunities to seek either the best resolution or the best three-dimensionality. We suggest that resolution is not the only criterion for useful imaging and that the advantage of being able to measure the full three-dimensional density map,(or other type of information such as chemical), may sometimes justify some compromise of the resolution.

The choice between the two approaches is partly an instrumental one. If the available source was an x-ray laser, then the intrinsic characteristics of the source (large coherence length and short pulse length) would allow large doses to be used on the argument that the damage processes are slower than the pulse, and this would favor pushing the limit with single shot imaging. With a synchrotron radiation source the diffraction tomography option would be somewhat more compelling.

The other issue is not entirely a scientific one. It has to do with how people process visual information and draw inferences from it. It is not easy to know what type of three-dimensional information a biologist needs to have in order to advance his or her understanding of a sample. We are accustomed to processing information about the surfaces bounding opaque objects because this is what perception based on visible light usually gives us. However, in the world of x-rays nothing is fully opaque and we could be faced with genuine three-dimensional density distributions, perhaps containing both phase and amplitude information. Such a distribution will often be difficult to view without technical aids such as the ability to cut sections and remove parts and will be highly unfamiliar to the observer. We are left with a software problem and a perception question. Is it necessary to map every tree in order to appreciate the essential nature of a forest or would a suitably three-dimensional picture from one viewpoint be enough?

A circumstance which makes it even harder to weigh the above two approaches against each other is our relative lack of experience in viewing single holograms of partially opaque objects even in visible light. Nonetheless, when looking at a reconstructed image of a good hologram of normal (fully opaque) objects one certainly has the feeling of having more information about the scene than the Ewald Sphere/information theory arguments would lead one to expect. Perhaps as so often in choices of method, each will prove to have their own particular merit. At the present time it seems that both deserve to be pursued.

In view of the unfamiliar nature of diffraction tomogaphy in both the soft x-ray and the optical microscopy communities, we review some of the basic ideas and provide some references where further information can be found.

DIFFRACTION TOMOGRAPHY

A diffraction tomography experiment could consist of illuminating the sample with monochromatic plane waves and measuring both the amplitude and phase of the diffracted field at some plane perpendicular to the illuminating direction and downstream of the sample. The same experiment would then be repeated many times for different illumination directions. Starting from an

understanding of CAT scanning one might suppose that by allowing the wave to spread and diffract, the information content would be compromised severly. However, it was shown by Wolf in 1969 [26] that the information may in fact be preserved and can be extracted by taking the complex two-dimensional Fourier Transform of the measured wavefield. Provided the measured wavefield is the same as the Born Approximation to the wavefield, this determines certain values of the three-dimensional Fourier Transform of the complex refractive index distribution of the scattering object at Fourier frequencies lying on the Ewald Sphere in frequency space. This is known as the Generalised Projection Slice Theorem. By making further measurements at different illumination directions one can fill in more data points on other Ewald Spheres that are rotated about the origin with respect to the first one. When a sufficient number of spheres of data have been accumulated, one strategy is to interpolate the data to fill the frequency space on an appropriate grid of points. A three-dimensional Fourier Transform then returns the desired values of the complex refractive index distribution of the sample.

This procedure has been applied using sound, radio waves, microwaves and seismic waves, but not, so far as we know, x-rays, presumably due to the difficulty in obtaining the phases in this case. The computational methods that can be used are not limited to those based on interpolation and the Generalised Projection Slice Theorem as described above. A good deal of effort has been devoted to designing very general and powerful algorithms [24] [25] which deal with limited amounts of data and noisy data and which allow prior knowledge to be used. We are interested in understanding the conditions under which this kind of approach could work with x-rays. At first sight it seems that the whole procedure depends on the use of the Born Approximation. However, cases where the Born Approximation breaks down can be treated by a device called the Rytov Approximation. [25][27][28] This method essentially provides an approximate way to calculate what the Born Approximation to the scattered field WOULD have been, had someone done a direct, forward calculation using it. From the point of view of implementation the Rytov method involves virtually the same amount of processing as the Born.

In Table I we provide some optical data for biological materials at 30 Å, which allow one to get a feeling for whether the Born and Rytov validity criteria are met in soft x-ray imaging experiments. The criteria for both are based on the idea of a linear approximation to the wave equation for "weak" scattering of the incident wave. The exact calculation is very intractable and little progress has been made with it. Both approximations usually require that the scattering potential (the refractive index distribution) should be expressible as the sum of a background term that is real and a sample term that may be complex but is small compared to the background. This is well satisfied in our case because the refractive index is always equal to unity minus small correction terms. Both also require that the scattered amplitude be small compared to the incident. This is also likely to be true for all soft x-ray diffraction experiments. The essence of the Born Approximation is that each element of the sample is assumed to be illuminated with the UNMODIFIED incident wave. This requires that the total attenuation be small as well as the scattering and this is certainly not satisfied by most soft x-ray imaging experiments. It is this difficulty that is resolved by the Rytov Approximation because it requires only that the attenuation and phase change PER WAVELENGTH should be small. This is a much easier requirement and as shown in the table is well satisfied for the kind of experiments that we envision.

The conclusion from this is that soft x-ray imaging experiments in or near the water-window spectral range (23-44 Å) do indeed satisfy the conditions for the use of the established procedures of diffraction tomography using the Rytov Approximation. This opens possibilities for new types of experiments in the future.

TABLE I

OPTICAL PROPERTIES OF BIOLOGICAL MATERIALS AT 30 Å

PROPERTY	WATER	PROTEIN	DNA	LIPID	CARBO-HYDRATE
Index real part(δ)*	.0010	.0015	.0017	.0012	.0017
Index imag part(β)*	.000047	.00057	.00076	.00038	.00039
Phase change per wavelength ($2\pi\delta$) (Radians)	.0063	.0094	.011	.0078	.011
Attenuation per wavelength ($4\pi\beta$)	.00059	.0072	.0095	.0048	.0049
Absorption length (microns)	5.1	.42	.32	.36	.61
Phase change per abs. length ($\delta/2\beta$) (radians)	10.7 (2.1/μm)	1.3	1.1	1.62	.2

* The complex refractive index is taken to be $1-\delta-i\beta$.

FUTURE DEVELOPMENTS

The experiments reported above demonstrate resolution values of about 500 Å with a dose of 200 Megarads. However, they do not provide a very complete basis for projections about the future. For one thing the microdensitometer data were smoothed to diminish the size of the data set and we do not presently know how much, if any, resolution was lost by this procedure. Secondly, we made no attempt to minimize the dose to the sample so we do not know if we could have obtained the same resolution with less dose. We can speculate with some optimism that we may have recorded information at about 200-300 Å which is what we calculate should be possible with the dose used. It is very difficult to estimate the ultimate dose that the sample can tolerate without loss of the interesting structures but the data in Riemer [29] and Glaeser [30] for the radiation tolerance of various organic materials in the electron microscope, suggest a value around 1000 Megarads with about an order of magnitude variation in either direction for different materials. The end-points used in these tabulations are not quite the same as in an x-ray imaging experiment but they represent the closest available measurements. The type of holography experiments we are considering are very much dependant on the

properties of resist and the resolution limit set by this consideration is in the range 50-200 Å.

Remembering the sixth power law referred to earlier, which gives the scaling of the needed dose with resolution for three-dimensional experiments, we can try to combine the above observations into a useful generalisation about what might be achieved in the future. We tentatively suggest that for a single hologram made with the currently-used technologies, the estimates of the ultimate, dose-limited resolution are in rough agreement with those of the ultimate resist-limited resolution and both are in the region of 100-200 Å. Thus we see that we may reasonably regard 100 Angstrom resolution as a goal. If 100 Å resolution were achieved then the depth resolution of single holograms would be useful and would be about 1600 Å for 25 Angstrom x-rays.

Some effort is presently being devoted to developing Fourier Transform holography (Fig 1) [31][32]. This approach does not depend on having a high resolution detector and allows devices such as charge-coupled devices to be used instead of resist or film. The achievement of good resolution is then dependant on providing a coherent reference source of sufficiently small size. The responsibility for resolution is thus shifted from the detector to the condensing optics. Less progress has been made in this direction than in Gabor holography because it is considerably more difficult. However there are some persuasive arguments that it will be rewarding when it is implemented successfully, particularly with regard to dose reduction.

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FIGURE CAPTIONS

1. Layout of the Gabor (in-line) and Fourier Transform holographic geometries.

2. Optical layout and parameters for the recording of Gabor x-ray holograms using the X17t undulator beamline at the National Synchrotron Light Source at Brookhaven National Laboratory.

3. Arrangement for simultaneously recording several holograms and a contact micrograph of a sample.

4. Electron micrograph of part of the glancing-incidence-shadowed hologram of several zymogen granules, recorded on copolymer. The portion shown has area 19 by 15 micron^2.

5. Numerically reconstructed image from the hologram shown in Fig. 4. The diffraction structure near the center of the hologram is seen to be due to a clump of granules which are resolved in the image. The pixel size is 490 Å.

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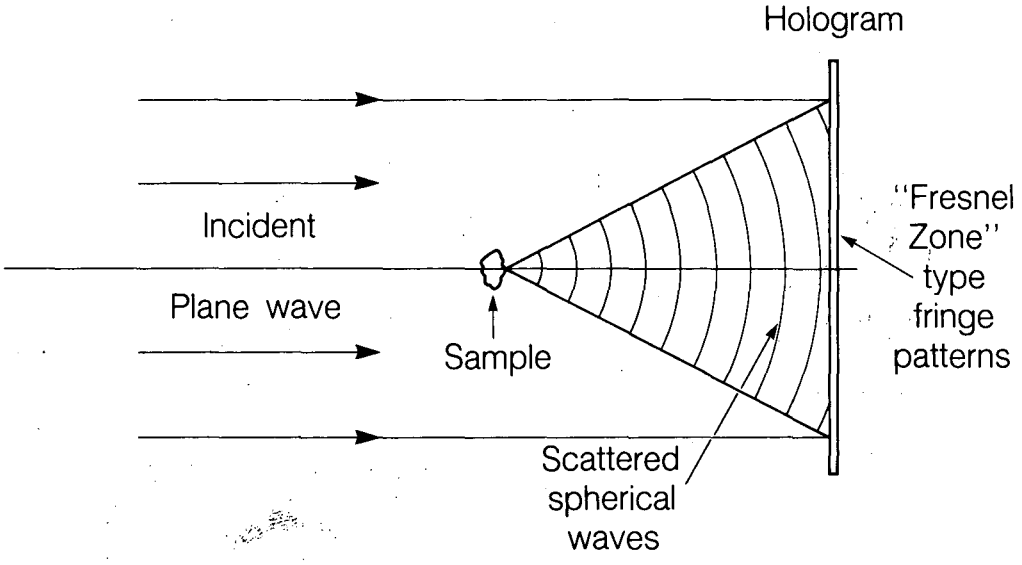
Gabor In-line Geometry:



Fourier Transform Geometry:

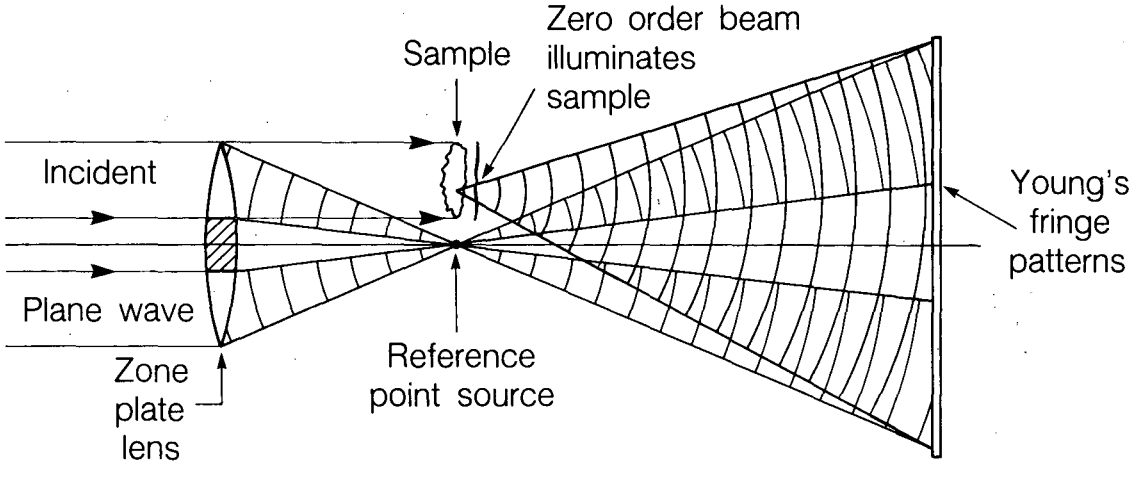


Fig. 1

XBL 886-8472

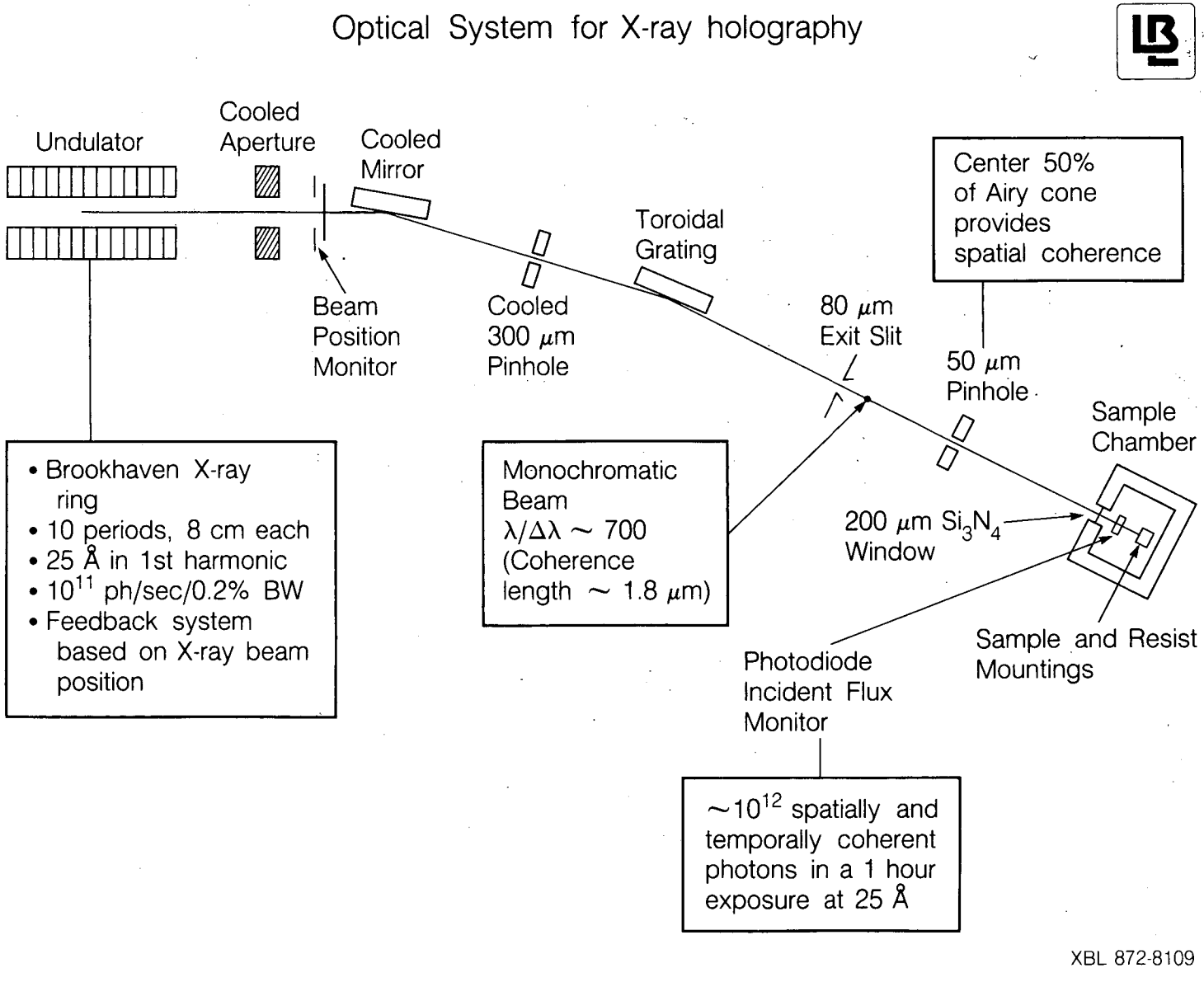
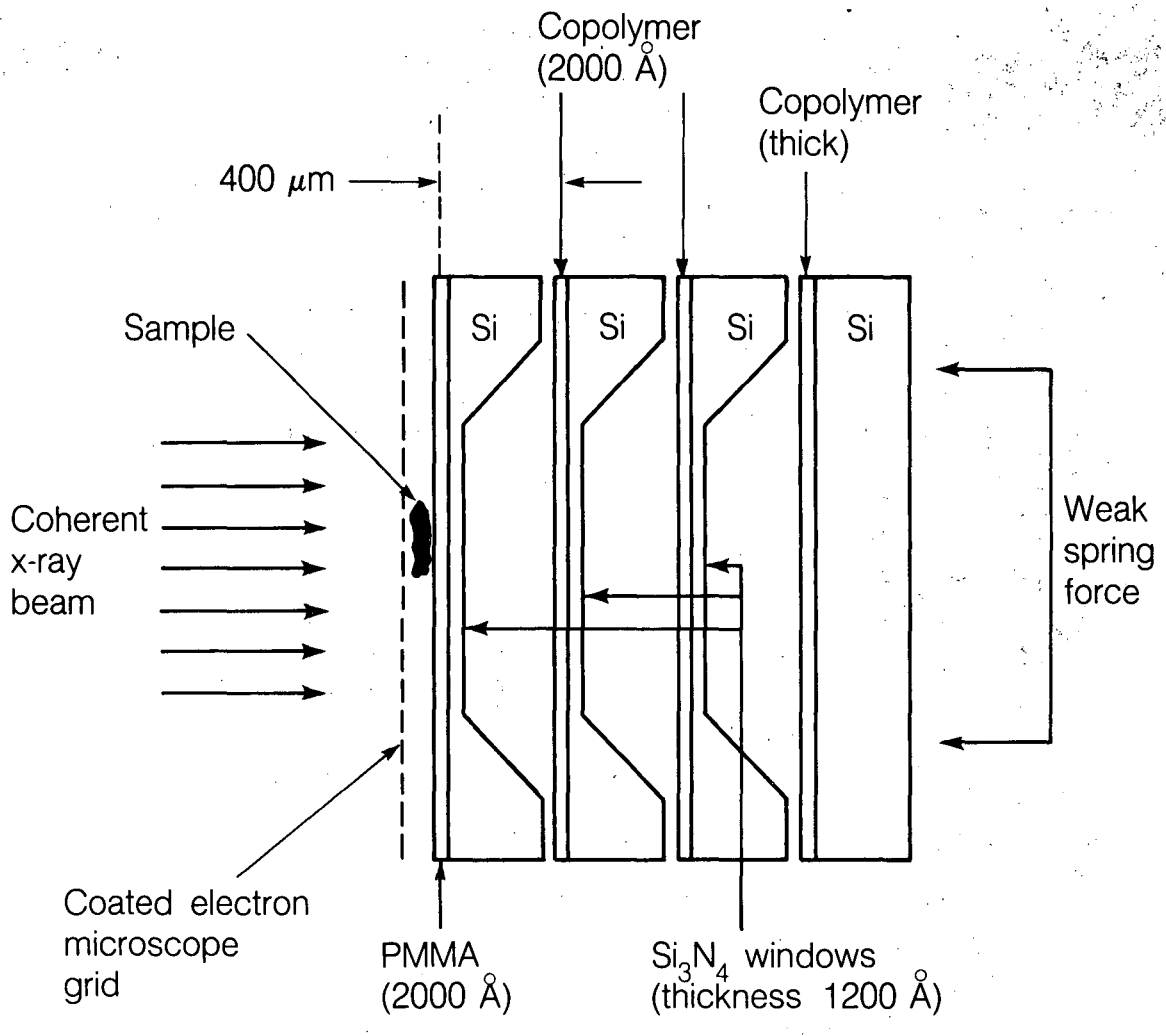

Optical System for X-ray holography
Undulator
Cooled Aperture
Cooled Mirror
Toroidal Grating
Center 50% of Airy cone provides spatial coherence
Beam Position Monitor
Cooled 300 μm Pinhole
80 μm Exit Slit
50 μm Pinhole
Sample Chamber
• Brookhaven X-ray ring
• 10 periods, 8 cm each
• 25 Å in 1st harmonic
• 10^{11} ph/sec/0.2% BW
• Feedback system based on X-ray beam position
Monochromatic Beam
λ/Δλ ~ 700
(Coherence length ~ 1.8 μm)
200 μm Si_3N_4 Window
Photodiode Incident Flux Monitor
Sample and Resist Mountings
~10^{12} spatially and temporally coherent photons in a 1 hour exposure at 25 Å
XBL 872-8109

Fig. 2

Arrangement of sample and resist detectors for x-ray hologram recording



XBL 871-271A

Fig. 3

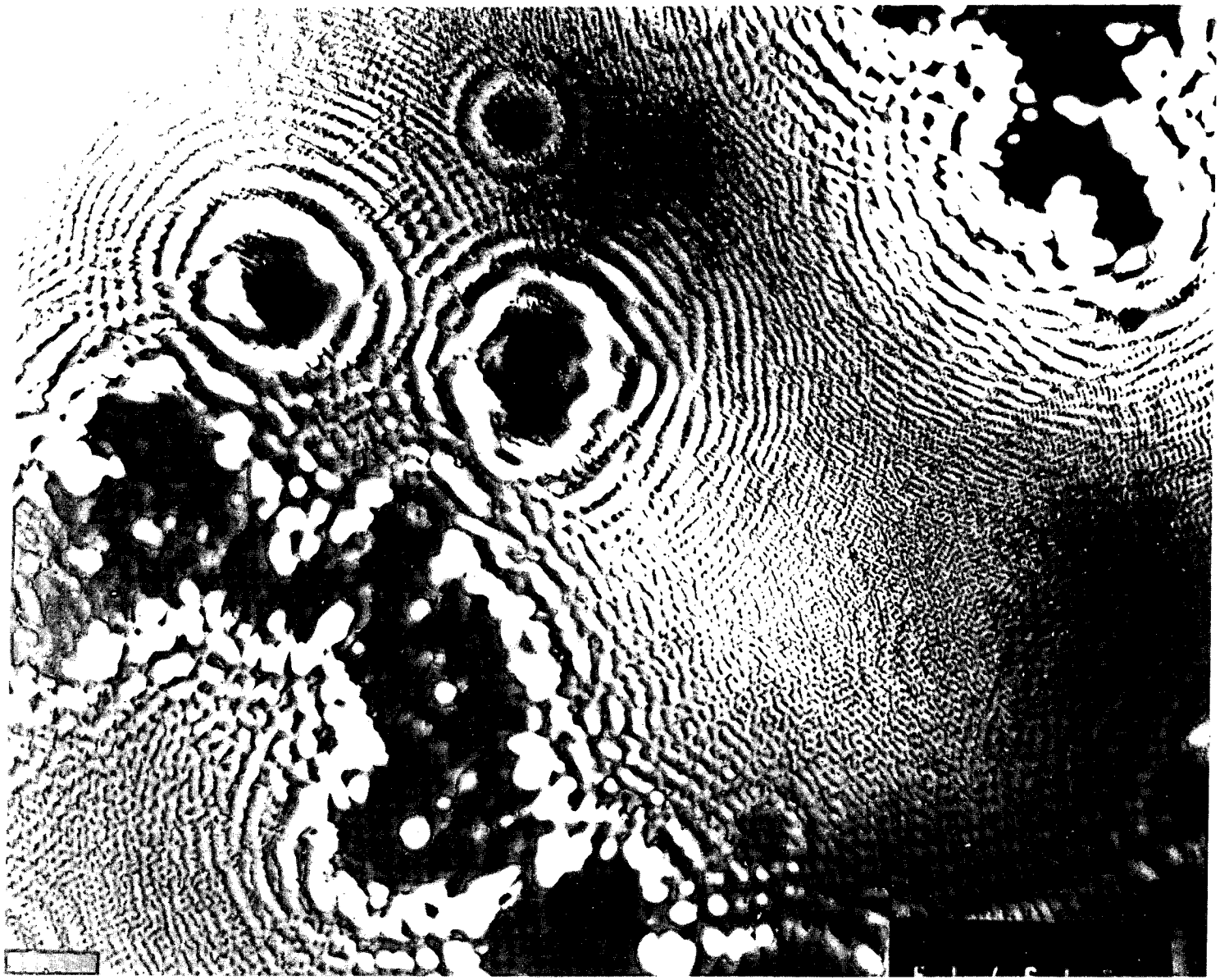
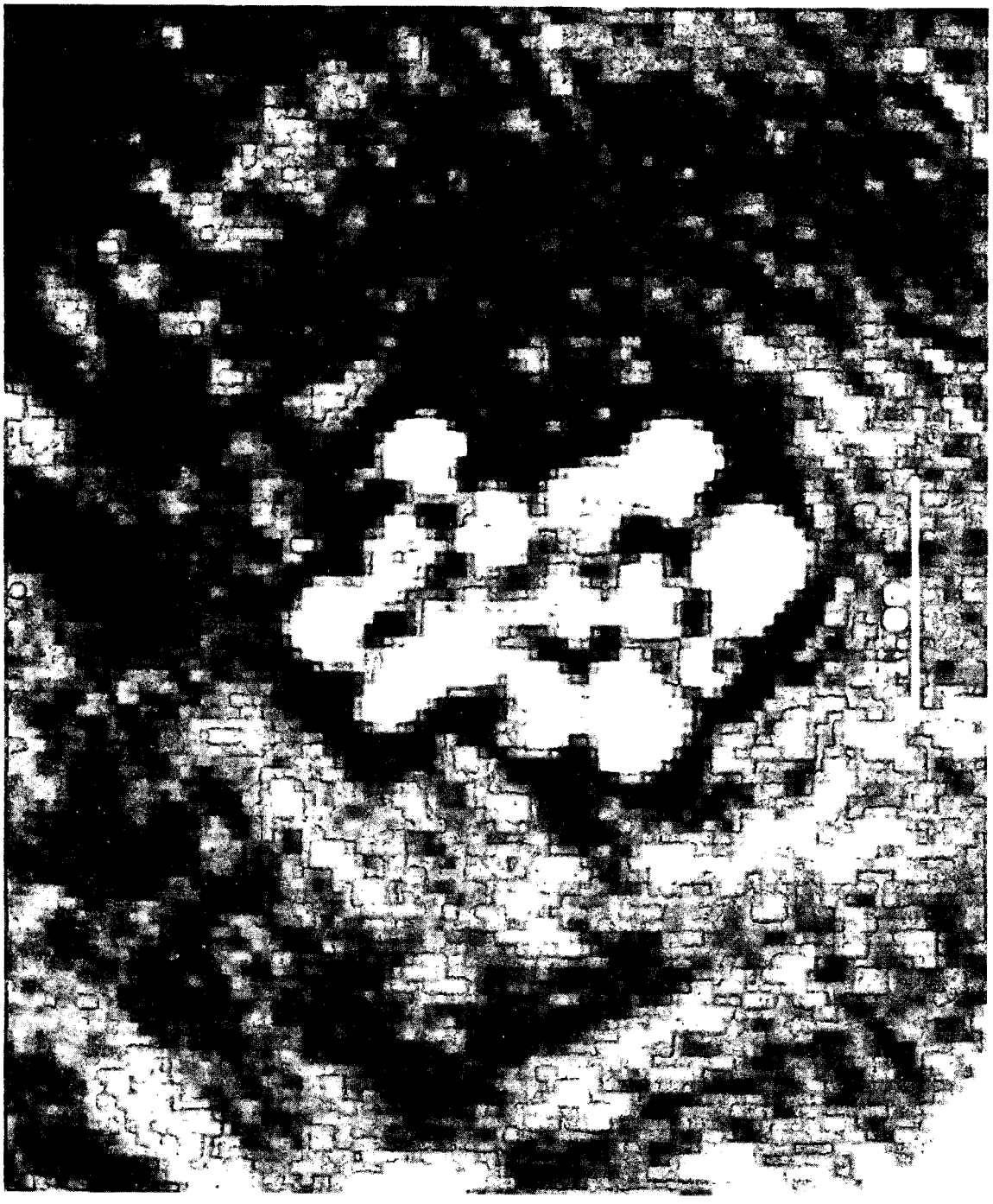


Fig. 4

XBB 871-421



XBB 884-2800

Fig. 5

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