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X-RAY IMAGING OF BIOLOGICAL SPECIMENS

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Abstract

I compare alternative techniques for x-ray imaging of biological specimens on the basis of (1) transverse and longitudinal resolution, (2) depth of field, (3) choice of recording medium, and (4) recording efficiency. I show that for all imaging techniques, the dosages suffered by specimens are so high that the living state cannot be preserved, nor can the structural integrity of the specimen be maintained in the usual sense.

Introduction

Many researchers are engaged in studies that may extend the art of microscopy into the extreme ultraviolet and x-ray range. The impetus is three-fold: (1) obtaining higher resolution imaging because the resolution of all forms of microscopy is ultimately limited by the wavelength of the particle beam used, (2) sharp changes in atomic cross sections owing to photoelectric edges or resonances in their vicinity offer contrasts in biological specimens that have been previously unavailable without staining, (3) in many cases the specimen dosage levels are lower than with electron microscopy; and (4) some techniques may reveal three-dimensional information that has in the past required laborious correlation of large numbers of thin sections. The short-wavelength techniques presently under investigation divide roughly into three categories: (1) contact imaging, (2) lens imaging, and (3) microholography.

Contact Imaging

Contact microscopy[1,2,3] or microshadowgraphy has been developed to a high level of sophistication. Shadowgrams are made by placing the specimen directly on a smooth recording surface and exposing the material to an x-ray source. The technique usually employs photoresist as a recording medium. Photoresist (frequently called x-ray resist in this wavelength regime) is a polymer used in the microlithography, which records the incidence of radiation by breaking cross-links in the polymer, which in turn renders the material locally less resistant to etchants. After developing the resist in etching solution, the micrograph is examined with an electron microscope. Usually viewed with a scanning electron microscope, the resultant etched resist resembles a contour plot of the local x-ray opacity of the specimen. Such 3-D plots of specimen transmission can reveal real quantitative data on the presence of specific elements[2] or even chemical bonds within the specimen.

Contact microscopy images only those features in intimate contact with the recording surface. If the features have transverse dimension δ, then diffraction limits the depth of field to δ^2/λ, where λ is the x-ray wavelength, i.e., the range of acceptable blurring is from the recording surface into the specimen a distance δ^2/λ. This is somewhat complicated by the character of the resist. As the radiation is diffracted around the features it enters the resist at angles deviating from normal, and the concomitant electron showers tend to converge or cross as they penetrate the resist. This suggests that more resolution could be obtained without loss of sensitivity by constructing the recording surface of multiple thin layers of resist[4] and computer unfolding features near the diffraction limit. This could also correct for the effect of transverse etching in the vicinity of regions of high exposure.

The ultimate resolution of resist is 5-10 nm,[1] and is a tradeoff between secondary electron range (made worse by shorter λ) and diffraction in the polymer itself (made worse by longer λ). The optimum is about λ = 5 nm, and this problem might also be mitigated by the multiple layer technique.

Lens Imaging

There are two distinct approaches to lens imaging: (1) an optical microscope analogue[5] in which the lens is used to project an image of the microobject onto a recording surface, and (2) a scanning technique[6] in which the lens is used to focus x rays from a synchrotron source into a small region of the specimen, the transmission being recorded by a proportional counter.

In the x-ray regime there are no practical refractive lenses. We must use diffraction by a Fresnel zone plate to obtain focussing. The transverse resolution obtainable by lens-imaging techniques is limited to the finest spacing of the Fresnel zone plate, regardless of the type of technique being used. This resolution can be refined somewhat by placing a pinhole at the focus of the zone plate, where it is practical and the pinhole can be fabricated to higher precision than the zone plate. Present microlithography techniques permit researchers to construct zone plates of minimum spacing[5] of about 500 Å, although "jelly-roll" sputtering techniques[6] may extend the art well beyond this resolution. The longitudinal resolution, which is

identically the depth of field, is S^2/λ where S is the finest zone spacing (or pinhole diameter). Unlike contact microscopy, the focal point may be set anywhere in the specimen.

Scanning has a distinct advantage over the optical microscope analogue: the specimen exposure can be much less. Not only can a high efficiency, large-solid-angle detector be used, but the inefficiency of passing through the specimen first and zone plate second is avoided. The technique may localize features by fluorescence[2] as well as transmission, adding to its versatility. Furthermore, by enlarging the focal spot we can achieve a trade-off between resolution and specimen dosage.

Microholography

Advantages of microholography are: (1) it can be lensless, (2) it permits reconstruction of three-dimensional information about the specimen, and (3) it avoids the confusion that results from the large depth of field of the lensing techniques. There are two basic types of holography: (1) Fresnel-transform holography, which uses planar reference waves; and (2) Fourier-transform holography, which uses curved reference waves.

As Gabor[7,8] originally conceived it, Fresnel-transform holography is an inherently simple technique: in its most rudimentary form it uses the same beam of radiation both to provide the reference waves and to illuminate the specimen. The Gabor technique, however, requires the recording surface to be in the far field of the specimen in order to suppress obscuration of the virtual image by the real image in reconstructing the hologram.[9] Thus the usefulness of Gabor holography is limited to high contrast specimens with internal features whose subtended area divided by the wavelength is small compared with the distance from the specimen to the recording surface.[10] The Leith-Upatnieks modification[11] of Gabor's original scheme entails projecting reference waves at an off-axis angle, which makes the virtual and real images spatially separated in reconstruction. Complete separation is obtained if the off-axis angle exceeds the wavelength divided by the specimen resolution. A disadvantage of Fresnel-transform holography is that the resolution of the micrograph is limited to about twice the grainsize, Δ, of the recording surface.[12]

Fourier-transform holography[13,14] circumvents this problem. Because of the curved reference waves, spacing between fringes can be indefinitely expanded by moving the recording surface away from the reference source. Thus ordinary film, photocathode devices,[15] and even wire chambers[16] can be used for recording the hologram. A difficulty with Fourier-transform holography is generating the reference waves. Ideally the reference waves are spherically diverging, i.e., generated by a Fresnel zone plate, in which case the transverse resolution of the hologram (as in the lens imaging technique) is limited by the minimum spacing of the Fresnel zone plate,[17] or to the size of the pinhole placed at the zone plate's focus. Similarly, the longitudinal resolution is S^2/λ, but fully three-dimensional information is recorded. Interpretable holograms will be generated with minimum exposure of the specimen if the intensity of the specimen's scattered waves is approximately the same as the intensity of the reference waves.[16] This is also true for Fresnel transform holography. To ensure phase matching, the same source is used to illuminate the specimen as to generate the reference waves, therefore it seems likely that one would use a Fresnel-zone-plate-and-pinhole arrangement. We can also use a reference scatterer[18] of arbitrary shape, providing we either know the detailed scattering properties of that reference scatterer,[12] or we are allowed to take separate exposures with the specimen and reference scatterer together, the specimen, and the scatterer.

A nearly distinct variant is the far-field crystallographic technique,[19] resembling holography in the sense that a reference scatterer and specimen are exposed simultaneously. However, the reference scatterer may be a periodic array, and we obtain the image by exposing the specimen and scatterer together and separately and computing the specimen's structure from the separate exposures.

Exposures

Because of the quantum nature of electromagnetic radiation, the process of inferring the detailed geometric arrangement of scattering and absorption within the specimen is inherently statistical. In the short wavelength region, scattering on the atomic level is small compared to absorption. For biological specimens scattering is mainly owing to diffraction about locally opaque structures.[15] This situation differs profoundly from the visible region, where most of what we see is owing to reflection and refraction. In the visible region the number of quanta scattered per resolution element is extremely large, and the quantum energy is about two orders of magnitude less than in the ultrasoft x-ray regime of interest. Therefore it is rarely necessary to damage the specimen in process of obtaining diffraction-limited resolution.

Two factors affect the exposure the specimen must suffer to obtain a desired image resolution: (1) the number of photons required to obtain a statistically significant contrast between the features we are trying to study and the medium in which they are imbedded; and (2) the recording efficiency for those photons.

The statistical aspects of contact and lens imaging have been studied extensively by Sayre,[20,21] et al., and the statistical aspects of Fourier-transform holography as well as the optimal adjustments of illuminator and reference intensities have been studied by Kondratenko and Skrinsky.[16] To obtain an image with statistical contrast roughly analogous to a 5:1 signal-to-noise ratio using a modified form of the criterion introduced by Rose,[22] we find the specimen will suffer a dosage

$$D = \frac{5h\nu}{f\rho_0 x\varepsilon} \frac{P_s + P_f}{(P_s - P_f)^2} (1 - \alpha\beta_s) \quad , \tag{1}$$

where $p_{s,f}$ are the probabilities of a photon impinging a sample resolution element then producing an event of the type we use to form the image. In the same spirit as Sayre, the subscript f refers to a feature we are trying to image and the subscript s refers to the homogenized "soup" that elementally comprises the specimen. We have assumed all constituents to be the same density, ρ_o, an average thickness of the specimen is x, and the recording efficiency is ε. If the principal mechanism of image formation is absorption, i.e., we are forming a shadow whether by lens or contact technique, then $p_{s,f} = \alpha\beta_{s,f}$ where $\alpha = \exp\{-\mu_s(x - \delta^2/\lambda)\}$ and $\beta_{s,f} = \exp\{-\mu_{s,f}\delta^2/\lambda\}$, and $\mu_{s,f}$ are linear absorption coefficients.[17] If fluorescence were being used, $p_{s,f} = 1 - \alpha\beta_{s,f}$, where $\mu_{s,f}$ are linear fluorescence coefficients.

In holography, the part of the interference pattern containing information about fine features of the specimen is found at large scattering angles.[16] Proper adjustment of the reference intensity can make these fringes high contrast even if the specimen is intrinsically low contrast. The diffractive scattering cross section of a semiopaque feature is approximately the geometric cross section multipled by the square of the x-ray extinction through the feature.[18] The dosage to the specimen when the reference intensity is optimally adjusted for fringe contrast[16] of the features we want to resolve is given by Eq. (1) with $p_{s,f} = \alpha\{1 - \beta_{s,f}\}^2/4$.

Table 1 shows the dosage suffered by various specimens in attempting to record an image to transverse resolutions of δ = 5, 10, 20, and 50 nm. The specimens are treated realistically and reasonably good estimates of their actual composition are used. They are meant to be representative of the types of things that might be of biological interest ranging over 12 orders of magnitude from viruses, through mycoplasma, through the simplest prokaryotes to the higher eukaryotes. The dosage assumes that the specimen in question is being imaged along its greatest dimension, i.e , presenting its least dimension to the x-ray illuminator. We assume $h\nu$ = 400 eV and that the features we are seeking to image are made of protein.

Dosages are given in rad (100 erg·g^{-1}), a popular metric for biological damage. In humans, whole-body doses of more than 100 rad are considered dangerous, 1000 rad is almost always fatal, and 10,000 can cause prompt incapacitation. There are some bacteria that can survive 10^6 rad.[23] Table 1 shows that extremely high dosages are required to image viruses, partly because of their size, but mostly because of the low contrast between protein and nucleic acid at 400 eV. For cells, the dosage remains fairly flat between masses of 10^{-14} to 10^{-8} g, within each resolution category, mainly owing to slow increase in specimen thickness, but also owing to increase in the water-to-protein ratio as cells get larger.

Microholography requires somewhat higher dosage to obtain the same resolution as contact or lens imaging, but the difference is not dramatic. The dosage decreases more rapidly with decreasing resolution because the scattering cross section of a feature increases as the square of the extinction.

Table 1 assumes 100% recording efficiency (ε = 1). This efficiency varies greatly from one technique to another; it is generally the product of (1) the quantum efficiency of the recording medium, and (2) the x-ray gathering efficiency of the imaging optics.

For contact imaging, the gathering efficiency is unity, but the quantum efficiency of a single layer of resist is limited by diffraction. X rays are diffracted around the fine features of the specimen so that the image from even those features in intimate contact with the resist is blurred at a distance δ^2/λ inside the resist. The blurring is exacerbated by the divergence and variation in initial direction of the secondary-electron shower, and also by diffraction in the resist itself. Further complication occurs in the etching process, because unexposed resist will be etched in all three dimensions, albeit at a slower rate than exposed resist. Considering diffraction around specimen features only, the recording efficiency for a single-layer resist is less than

$$\mu_r\delta^2/\lambda \quad , \tag{2}$$

where μ_r is the linear absorption coefficient of the resist. For PMMA this efficiency is about 5% with δ = 10 nm and λ = 3 nm. This suggests an advantage accrues to a resist with large μ_r. The problem could in principle be eliminated by using multiple layer resist, and etching the layers separately, but this would require very thin layers and much computation for reconstruction.

The optical analogue x-ray microscope has a poor gathering efficiency; from solid angle arguments it is less than

$$\tfrac{1}{2}[1 - \{1 - (\lambda/a)^2\}^{\frac{1}{2}}] \quad , \tag{3}$$

assuming diffuse illumination. A condenser lens can considerably enhance this efficiency, but not more than an order of magnitude. If a = 50 nm and λ = 30 nm, the gathering efficiency is less than 2×10^{-3}, however, essentially unit-quantum-efficiency photographic film could be used to record the image. In minimizing dosage to the specimen, scanning clearly has the advantage.

The fringe pattern of a Fresnel-transform hologram has a three-dimensional character that will expose the resist along columns that are not necessarily normal to the surface. Unlike the case of contact microscopy, however, the angles of penetration of the fringes are more dependent on the geometry of the apparatus than on details of the specimen. Since we know the geometry of the apparatus, we can compensate for the varying angles of penetration, and, in general, the variation in angle will change along the resist surface on length scales of the same magnitude as the length scale of the apparatus. If the normal distance from the specimen to the planar recording surface in a Gabor-type holograph is d, and the characteristic dimensions of the specimen are small compared to d, then the angles of penetration of the fringes into the resist are given by

$$\theta = \tan^{-1}[Y - \sqrt{Y^2 + 2(X - \sqrt{X^2 + Y^2})}] \tag{4}$$

Table 1. Dosage to Obtain Transverse Resolution (δ) in Various Specimens

Specimen	Mass (g)	Least Dimension (cm)	Greatest Dimension (cm)	Dosage (rad) Assuming ϵ = 1 and $h\nu$ = 407 eV: Contact or Lens Imaging, δ = 5 nm	10 nm	20 nm	50 nm	Microholography, δ = 5 nm	10 nm	20 nm	50 nm
Tobacco necrosis virus	10^{-18}	7×10^{0}	2×10^{1}	1×10^{14}	--	--	--	1×10^{14}	--	--	--
Escherichia coli phage qβ174	10^{-17}	2×10^{1}	2×10^{1}	1×10^{14}	2×10^{12}	--	--	1×10^{14}	2×10^{12}	--	--
Herpes virus	10^{-16}	8×10^{1}	8×10^{1}	1×10^{14}	2×10^{12}	3×10^{10}	--	1×10^{14}	2×10^{12}	3×10^{10}	--
Vaccinia virus	10^{-15}	2×10^{2}	2×10^{2}	4×10^{15}	6×10^{13}	9×10^{11}	4×10^{9}	4×10^{15}	6×10^{13}	1×10^{12}	1×10^{10}
Mycoplasma pneumoniae	10^{-14}	3×10^{2}	3×10^{2}	2×10^{11}	3×10^{9}	5×10^{7}	3×10^{5}	2×10^{11}	3×10^{9}	6×10^{7}	6×10^{5}
Escherichia coli (immature)	10^{-13}	6×10^{2}	1×10^{3}	2×10^{11}	3×10^{9}	5×10^{7}	3×10^{5}	2×10^{11}	3×10^{9}	6×10^{7}	6×10^{5}
Escherichia coli (mature)	10^{-12}	8×10^{2}	2×10^{3}	2×10^{11}	3×10^{9}	5×10^{7}	3×10^{5}	2×10^{11}	3×10^{9}	6×10^{7}	6×10^{5}
Anthrax bacterium	10^{-11}	2×10^{3}	6×10^{3}	2×10^{11}	3×10^{9}	5×10^{7}	3×10^{5}	2×10^{11}	4×10^{9}	7×10^{7}	7×10^{5}
Red blood cell (human)	10^{-10}	2×10^{3}	8×10^{3}	3×10^{11}	4×10^{9}	7×10^{7}	4×10^{5}	3×10^{11}	5×10^{9}	9×10^{7}	9×10^{5}
White blood cell (human)	10^{-9}	5×10^{3}	1×10^{4}	2×10^{11}	3×10^{9}	5×10^{7}	3×10^{5}	3×10^{11}	4×10^{9}	8×10^{7}	8×10^{5}
Amoeba (dysentery)	10^{-8}	2×10^{3}	2×10^{4}	1×10^{11}	2×10^{9}	3×10^{7}	2×10^{5}	1×10^{11}	2×10^{9}	4×10^{7}	4×10^{5}
Smooth muscle cell	10^{-7}	1×10^{4}	5×10^{5}	2×10^{12}	3×10^{10}	6×10^{8}	3×10^{6}	3×10^{12}	4×10^{10}	8×10^{8}	8×10^{6}
Paramecium (protozoan)	10^{-6}	6×10^{4}	1×10^{5}	7×10^{19}	1×10^{18}	2×10^{16}	1×10^{14}	9×10^{19}	2×10^{18}	3×10^{16}	3×10^{14}

where $Y = y/\lambda$, $X = d/\lambda$, and y is the distance along the resist from the axis of symmetry. If the exposure of the resist is read with a transmission electron microscope, the nonlinearities introduced by the three-dimensional character of the fringe pattern can be compensated by tilting the resist according to Eq (4). An alternative is to shape the surface of the resist so that the columns of the fringe pattern are always normal. If the x axis is the axis of symmetry, then the angle of the normal of the resist surface with respect to the x axis is given by Eq. (4) with $X = x/\lambda$. If $x >> y$, then Eq. (4) becomes

$$\theta \cong \tan^{-1}\left(\frac{y}{2b}\right) \quad , \tag{5}$$

where b is the distance between the specimen and recording surface. This implies that the surface should be a spherical shell with radius 2b.

In Leith-Upatnieks holography, there is no axis of symmetry, so the angle of penetration is a complicated function of position on the recording surface, which can be derived by coordinate transformation of Eq (4). In the far-field case, however, it depends only on the geometry of the apparatus and can be accurately compensated by tilting in readout.

In Fourier-transform holography with spherical reference waves, the fringes penetrate radially in the far-field. A planar slab of resist could be easily interpreted by tilting at the angle from the specimen to the resist surface. If a spherical shell of resist were used, all the columns would be normal to the surface. Thus resist could be used with near-unity quantum efficiency, but because there is no grainsize problem, other high-efficiency detectors could be used as well.

Table 2 summarizes the features and recording efficiency of the various short-wavelength biomicroscopy techniques. When estimating the dosage given a specimen, the dosages given in Table 1 must be divided by the recording efficiences in Table 2.

Table 2. Summary of Optical Features and Recording Efficiencies

Type	Contact Imaging		Lens Imaging		Microholography	
			Optical Microscope	Scanning Illuminator	Fresnel Transform	Fourier Transform
Transverse Resolution (δ)	max $\{\lambda, \Delta\}$		max $\{\lambda, \Delta, a\}$		max $\{\lambda, 2\Delta\}$	max $\{\lambda, 2a\}$
Longitudinal Resolution	δ^2/λ					
Recording Medium/Detector	Single-Layer Resist	Multilayer Resist With Interpretation	Film	Proportional Counter	Single-Layer Resist with Variable-Angle Readout	• Film Resist • Wire Chamber • Photo-cathode
Recording Efficiency (ε)	$< \mu_r \delta^2/\lambda$	~ 1	$\frac{1}{2}[1-[1-(\lambda/a)^2]^{\frac{1}{2}}]$ or better with condenser lens	~ 1	~ 1	

Concluding Remarks

This paper has compared the contact imaging, lens imaging, and microholographic techniques for x-ray microscopy of biological specimens. Microholography is distinguished from the other techniques by the requirement that the image be formed from coherently scattered photons. The advantages of holography discussed in this paper are (1) it suffers less loss of resolution than the other techniques owing to the three-dimensional intensity patterns within the recording surface, (2) it provides, in a single exposure, the information that would require multiple exposures by other techniques, and (3) it eliminates the need for lenses, which are difficult to fabricate in the x-ray regime.

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