Resolution in soft X-ray microscopes

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X-ray microscopes are able to image thick, wet biological specimens at 50 nm or better resolution, to perform elemental analysis at 0.1–10 μm resolution with as high as ppm sensitivity, and to analyze surface composition at 0.1–1 μm resolution. We concentrate here on the use of soft X-rays, where the wavelength is typically > 1 nm, although mention of some techniques and results at shorter wavelengths is also made. We discuss the physical principles behind X-ray microscopes, the types of optical systems used and their achievable resolution, resolution limitations imposed by radiation damage, and recent application of X-ray microscopes to the imaging of biological, materials science, geological, and other specimens.

1. Introduction

Because of the short wavelength of the “light” used, X-ray microscopes have long been considered for imaging at a resolution well beyond the limit of the wavelength of visible light [1,2]. For biological applications, soft X-rays with wavelengths between the oxygen and carbon absorption edges (at 2.2 and 4.3 nm, respectively) have had particular appeal, because at these wavelengths organic materials absorb strongly compared to an overlying water layer [3,4]. In recent years, several types of soft X-ray microscopes have been used to image μm thick, wet biological specimens in absorption contrast at sub-100 nm resolution [5] and soft X-ray microscopes have also been used for materials science studies [6–9]. At shorter X-ray wavelengths, where the spatial resolution is typically a few μm at present but where the sample can be considerably thicker, fluorescence microprobes are able to image trace elements with concentrations in the ppm range [10–14].

X-ray microscopy has been the focus of an increasing number of researchers in recent years, and monographs [2,15], review articles [16–18] and conference proceedings [19–23,5] provide complete overviews of X-ray microscope optics and applications. This body of work provides a more complete picture of research in X-ray microscopy beyond the narrower scope of the present article’s emphasis on image resolution.

2. Resolution in microscopes

Resolution is the ability to detect a spatial frequency rather than to detect a feature of a given size. For example, the unaided human eye can detect light from individual bright stars which subtend angles on the order of nanoradians when viewed from earth, but it cannot resolve stars which are separated from each other by angles less than about a milliradian. From this we see that spatial resolution is a somewhat more subtle concept than the simple ability to “see” a certain feature.

We shall consider X-ray microscope systems as falling into one of two categories: one in which diffraction by the specimen plays an important role in the imaging process, and one in which X-ray diffraction by the specimen is assumed to be negligible. In the former case, the Abbe theory of image formation applies [24], so that for bright-field transmission imaging, the wavefield diffracted by the specimen is collected by an
objective lens and projected to form a real image of the specimen. We then Fourier-decompose the specimen transmittance into a collection of sinusoidal diffraction gratings with various periodicities, orientations, and absorptive/phase-shifting components. We also consider the objective lens in terms of its numerical aperture \( NA = n \sin \theta \), where \( n \) is the refractive index of the medium and \( 2\theta \) is the full angle subtended by the objective. If the objective lens is placed in the far field of the specimen, it will receive the Fourier transform of the complex optical wavefield leaving the specimen. The finite NA of the objective thus limits the spatial frequencies which can be captured by the optical system, for specimen “gratings” with a sufficiently fine period will diffract light to angles beyond the NA and thus escape detection. Lower spatial frequencies will be collected by the objective and Fourier-transformed to form a real image (assuming freedom from aberrations). We can summarize the response of the optical system to different specimen “gratings” or spatial frequencies in terms of a modulation transfer function or MTF [25]. If the Fourier-decomposed specimen transmittance is assumed to have a component \( a \sin(2\pi x/f) \) of amplitude \( a \) at the periodicity \( \Delta = 1/f \), and the imaging system reproduces only part of that amplitude \( b \) at the spatial frequency \( f \), the value of the modulation transfer function at \( f \) is given by \( MTF(f) = |b/a| \). For coherent imaging systems, one can also speak of the coherent transfer function \( CTF(f) = b/a \), where both \( a \) and \( b \) can be complex quantities.

This diffraction-based imaging process can be simplified by considering the imaging of a point object. Such an object is treated mathematically as a delta function \( \delta(x,y) \), which has a Fourier transform \( F(\delta(x,y)) \) that is a constant at all spatial frequencies. This is then truncated to zero outside the boundaries of the numerical aperture \( NA \) of the lens (i.e., it is multiplied by the pupil function \( P(f_x, f_y) \) of the lens) before the lens transformation to the image plane is performed. We can thus characterize the diffraction-based optical system based on the type of illumination used and the pupil function of the objective. In the case of fully coherent plane wave illumination (note that perfect spherical wavefronts also qualify as coherent illumination) of a specimen \( [1 - \delta(x,y)] \), the pupil plane wavefield becomes a \( \text{circ}(x,y) \) function [25] of \( (1 + i0) \) up to a cutoff spatial frequency of \( f_{coh} = (2NA)/\lambda \). In the far-field limit, the complex point spread function is the complex Fourier transform of the pupil function \( \text{circ}(x,y) \), and the modulation transfer function is the modulus of the Fourier transform of the point spread function, or \( \text{circ}(x,y) \) once again. With incoherent radiation, wavefronts incident upon the specimen from different angles have different phase gradients, so that one observes only the intensity (rather than the complex amplitude) of the point spread function of the lens. Because the phase of the point spread function is not preserved, the Fourier transform of the point spread function no longer restores the \( \text{circ}(x,y) \) function. Instead, the modulation transfer function is given by \( (1-f/f_{coh}) \), where \( f_{coh} = (4NA)/\lambda \). This doubling of the cutoff frequency between incoherent and coherent imaging can perhaps be better understood conceptually by considering the finest observable transverse grat-

\[
\text{Coherent}
\]

\[
\text{Incoherent}
\]

Fig 1 A schematic illustration of the cutoff spatial frequencies of incoherent versus coherent imaging. In the coherent case, rays can be diffracted up to an angle of \( \sin^{-1}(NA) \) by periodicities in the specimen, whereas in incoherent imaging, rays incident upon the specimen from an angle of \( \sin^{-1}(NA) \) can be diffracted through an additional angle of \( \sin^{-1}(NA) \) yet still be collected by the objective.
The relationship between the Rayleigh and Sparrow resolution criteria and the modulation transfer function for objectives with and without a central stop is shown in Fig. 2. The longitudinal or depth resolution $\delta_z$ of diffraction-limited optical systems is less frequently discussed than is the transverse resolution. The equivalent of the Rayleigh approach would be to define the longitudinal resolution as the distance between the focal plane and the first longitudinal minimum of the three-dimensional distribution of light near the focus. This approach gives $\delta_{z,R} = 2\lambda/NA^2$. Another approach which may be more consistent with the eye's perception of longitudinal resolution considers equivalent Ewald sphere conditions [26] or equiphase contours in a resolution element [27] to obtain the result we shall use in further discussions of $\delta_z = 1.22\lambda/NA^2$. Thus, as one increases the numerical aperture, the transverse resolution improves only linearly, while the longitudinal resolution improves as the square. For a circular lens, one can also express the longitudinal resolution $\delta_z$ in terms of the transverse resolution $\delta_t = 0.61\lambda/NA$ as $\delta_z = (4/1.22)(\delta_t^2/\lambda)$, so that if one were to compare X-ray and visible light microscopes with identical $\delta_t$, the visible light microscope would have a smaller $\delta_z$ than the X-ray microscope. As a numerical example, note that a microscope operating with a transverse resolution of $\delta_t = 30$ nm would have a depth resolution of $\delta_z = 0.7 \mu$m at 4.3 nm, and $\delta_z = 7 \mu$m at 0.4 nm wavelength. Whether small $\delta_z$ is desirable or not depends on the sample to be imaged, for good depth resolution corresponds to poor depth-of-field, and vice versa.

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**Fig. 2** Several resolution criteria for incoherent imaging (a) The Rayleigh criterion states that two points are resolved when the image of one point is centered on the first minimum of the diffraction-limited image of the second point. (b) The Sparrow criterion states that two points are resolved for separations as small as the one at which the second derivative of image intensity becomes zero halfway between the centers of the two point images. (c) Optical transfer functions and Rayleigh and Sparrow resolution points for coherent and incoherent imaging, and for incoherent imaging with a lens with half its diameter obstructed by a central stop. Optics with central stops are frequently used in X-ray microscopes to isolate a desired focal order.
Fig 3 Effect of partial spatial coherence on the resolution of scanning X-ray microscopes. Contours of modulation transfer function MTF = 0.10, 0.20, and so on, are shown for an objective optic with a half-diameter central stop, a normalized spatial frequency of \( f = 1 \) corresponds to a feature size of \( \lambda / 4NA \). The source phase space area \( \phi \) is defined as \( \phi = 2d \sin \theta \), where \( d \) is the diameter of the pinhole aperturing the incoherent source, and \( \theta \) is the angle from the center to the edge of the objective lens as seen by the source. The phase space parameter is thus \( p = 2d \sin \theta / \lambda \).

In microscopes which require coherent illumination the resolution is usually degraded by some degree of partial spatial coherence. We consider the example of a scanning microscope with a diffraction-limited objective lens, where the X-ray source is effectively demagnified onto the object plane. The point spread function of the instrument is then given by convolving the diffraction-limited point spread function of the objective with the geometrical image of the source. If a sufficiently small source is used, the objective lens will be fully coherently illuminated and the point spread function will be unchanged. As the source is increased, the point spread function will grow until it becomes dominated by the image of the source. If an incoherent illumination source is used, increased flux comes at the expense of lower resolution. An example of this tradeoff is shown in Fig. 3, which shows the modulation transfer function for a circular lens illuminated by an incoherent source apertured by various pinhole sizes. The flux/resolution tradeoff becomes a matter of choosing the accepted illumination phase space \( \phi = p \lambda \). The parameter \( p \) is given by \( p = 2d \sin \theta / \lambda \), where \( d \) is the diameter of the pinhole aperturing the incoherent source, and \( \theta \) is the angle from the center to the edge of the objective as seen by the source. The diameter-angle product of the Airy disk of a coherently illuminated objective is \( 2\delta (2NA) \), or \( 2.44\lambda \), so that choosing \( p = 1.22 \) only slightly reduces the scanning microscope MTF.

The above discussion applies to cases where diffraction by the specimen is assumed to play an important role in the imaging process. In the case of features of size significantly larger than \( \sqrt{\lambda d} \), the wavefield a distance \( d \) beyond a specimen is essentially just the shadow projection of the specimen transmittance. The resolution of such an image may also be affected by the degree of monochromacity of the illumination (knife-edge diffraction with a first minimum at \( \approx \sqrt{\lambda d} \) will tend to be washed out in white light illumination), by the degree of spatial coherence of the illumination (again contributing to fringe washout, although highly spatially incoherent illumination will produce penumbral blurring of the image), and by the resolution of the detector. If the specimen is placed at an intermediate distance between a point illumination source and a 2D detector, then the shadow image of the specimen will be magnified. In systems where diffraction is considered negligible, a single distance value for resolution is usually given, an equivalent of a modulation transfer function is usually not provided.

3. X-ray Interactions

While many X-ray microscopes use optical configurations familiar to visible light or electron microscopists, the nature of the sample-probe interaction is somewhat different. As has been described by Hubbell [28] and Michette [15], photoelectric absorption and elastic or Thomson scattering are the dominant cross sections for sub-10 keV X-ray interactions with low-Z elements. Incoherent or Compton scattering begins to dominate at somewhat higher energies. Furthermore, the atomic cross section \( \sigma_{\text{coh}} \) for coherent scattering is significantly lower than that for photoelectric absorption \( \sigma_{\text{a}} \), so multiple scattering is not expected to be problematical in X-ray
microscopy (The total scattering cross section for a microscope’s resolution element [18,27] or a microdiffraction specimen [29] can, however, become appreciable because of coherent superposition of atomic scattering amplitudes.) Based on this, Henke [30] has chosen to characterize sub-10 keV X-ray interactions solely in terms of a coherent scattering cross section $\sigma_{\text{coh}}$ and an absorption cross section $\sigma_a$ of

$$\begin{align*}
\sigma_{\text{coh}} &= \frac{8}{3}\pi r_e^2 (f_1^2 + f_2^2), \\
\sigma_a &= 2r_e\lambda f_2,
\end{align*}$$

(1)

where $r_e$ is the classical radius of the electron, and the complex number of electrons per atom $(f_1 + if_2)$ are equivalent to $(f' + if'')$ used in X-ray crystallography [31]. These scattering factors, which have been tabulated by Henke et al for elements 1–92 in the 100–10000 eV X-ray energy range [32], can also be viewed as describing the index of refraction $n$ of the specimen as $n = 1 - \delta - i\beta$, where

$$\begin{align*}
\delta &= \frac{r_e\lambda^2}{2\pi}n_a f_1, \\
\beta &= \frac{r_e\lambda^2}{2\pi}n_a f_2,
\end{align*}$$

(2)

where $n_a$ is the number of atoms per unit volume. A wavefield $\psi_0$ travelling through a thickness $t$ of material is thus modulated as

$$\psi(t) = \psi_0 \exp(-k\beta t) \exp(-ik\delta t),$$

(3)

so we are able to predict accurately the primary optical interaction between an X-ray and amorphous matter. In general, $|f_1 + if_2| \approx Z$, and the ratio $\delta/\beta$ increases from $\sim 1$ at $\gtrsim 10$ nm wavelengths until one reaches the sub-1 nm wavelength range, where phase shifts $\delta$ become the dominant interaction. Secondary interactions include the emission of characteristic fluorescent X-rays, Auger electrons, lower energy secondary electrons, and the creation of radiolytical products.

For microscopy, we are interested in predicting the contrast of a specimen so as to estimate the required photon flux and corresponding radiation dose for forming an image. This was first studied in 1977 by Sayre et al, who considered X-ray and electron amplitude contrast transmission imaging of protein cubes embedded in water layers of varying thickness [4]. Using the Rose criterion [33] of requiring a 5:1 signal-to-noise ratio in the image, they concluded that minimal dose imaging of hydrated biological specimens would be accomplished using 2.2–4.3 nm “water window” X-rays, where the dose for 50 nm resolution imaging would be in the $10^4$ Gy range compared to $10^7$ Gy for TEM or STEM. While this dose is certainly high enough to cause cell death, Sayre et al speculated that ultrastructure would be preserved at these dose levels (a point which will be discussed later). In 1986, Schmahl and Rudolph proposed the use of phase contrast in X-ray microscopy [34]. When compared in calculations similar to those of Sayre, phase contrast is predicted to offer slight dose reductions in the “water window” wavelength range, and it also appears attractive at 0.3–0.6 nm wavelength [18,35,36]. As an example, we present in fig 4 the radiation dose calculated under the Rose criterion for amplitude and phase contrast imaging of 50 nm protein features in 0.2, 2, and 10 $\mu$m of water. This figure shows that the radiation dose varies only slightly over the entire “water window” range. If the specimen is kept in an atmos-
pheric environment, one may choose instead to run at a wavelength of 3.2 nm (the long wavelength side of the N K absorption edge), where the 1/e attenuation length in air is > 3 mm, while differential absorption analysis of calcium concentrations is conveniently done using the Ca L\textsubscript{III} edge at 3.5 nm. Finally, London et al. have suggested that a wavelength of 4.5 nm is optimal for coherent imaging of small protein structures in very thin water layers [37], although their conclusions may be modified when one considers thicker water layers or carbon chemical binding shifts in the magnitude and exact spectral location of anomalous dispersion features.

As has been noted, X-ray microscopes can also be used to map the concentrations of certain elements. Considerations of contrast and excitation efficiencies for trace element analysis in biological materials have suggested that, with the exception of EELS studies of sufficiently thin specimens, X-ray microscopes should deliver the lowest dose for detection of a specified elemental concentration [38,39]. For Z \leq 20, differential absorption analysis (where one takes the logarithmic ratio of two images taken on either side of an absorption edge) should offer the lowest dose, while X-ray-induced fluorescence appears superior for higher Z elements (Similar calculations indicate that cross-edge phase contrast images should offer sensitivity similar to differential absorption images.) These calculations are summarized in Fig. 5, which shows the dose calculated for detecting various elemental concentrations in a sample composed of half protein and half water. Practical results in elemental analysis will be discussed below.

4. Sources, optics, and detectors

4.1 Sources

Rontgen made his discovery of X-ray radiation using an electron impact source, advanced versions of this same type of source are frequently used today, and water-cooled rotating anode X-ray generators are able to deliver copious X-ray fluxes. However, most X-ray microscopes benefit from highly collimated beams emerging from relatively small spots, lower power microfocus X-ray tubes are often superior to rotating anode sources in this regard. The figure of merit for X-ray sources is usually either spectral intensity (photons per time per solid angle per spectral band-
width) or spectral brightness (photons per time per phase space area per spectral bandwidth), as will be discussed below.

The availability of synchrotron radiation from particle physics storage rings and, more recently, storage rings dedicated to the production of synchrotron radiation, has led to enormous increases in available X-ray spectral intensity and brightness [40,41]. Dipole or bending magnet sources offer good spectral intensity and flux over a wide wavelength range, and X-ray monochromators can be used to deliver tunable, monochromatic light to an experiment. Higher intensity and brightness can be obtained by using periodic magnet structures called undulators and wigglers located in the straight sections between dipole magnets in a storage ring. In the weak field limit, these devices are called undulators and they deliver well-collimated, quasimonochromatic X-ray beams somewhat in the fashion of a coherent array of dipole sources. When stronger magnetic field strengths are used, the effective source size and X-ray beam divergence both increase, but significantly higher photon energies can be obtained on a storage ring with a given electron beam energy. The spectral brightness of undulator sources in particular is affected by the “emittance” or size-divergence product of the electron beam in the storage ring, and it is in recognition of this that a number of new low-emittance storage rings are being built worldwide. Many of these facilities have now or will have in the future beamlines dedicated to delivering X-ray beams for microscopy. The current situation is summarized in Fig. 6, which shows the time-averaged spectral brightness for a variety of synchrotron sources. The advent of storage rings dedicated to X-ray production (such as the 2.5 GeV X-ray ring at the National Synchrotron Light Source at Brookhaven National Laboratory) has led to a million-fold increase in brightness over what was available from X-ray tubes (typically $10^6-10^9$ in the above units). Undulators which are now in operation (such as the X-1 undulator at the NSLS, and the PMU-2 undulator at the Photon Factory, Japan) and which are being built for third-generation storage rings such as the ESRF (European Synchrotron Radiation Facility, Grenoble), similar rings are under construction in the USA and Japan) and the ALS (Advanced Light Source, Berkeley), similar rings are under construction in Italy, Taiwan, Brazil, and other places) offer further increases in brightness. These sources have proven crucial to the development of today’s X-ray microscopes.

For many studies, it would be ideal to have a pulsed X-ray source so as to take flash images of biological specimens. Laser-produced plasma X-ray sources have been used in several laboratories for flash contact microradiography of biological specimens [42–44], and both contact and transmission X-ray microscopes have been operated using plasma pinch pulsed sources [45,46]. As these sources continue to improve, they will allow imaging beyond the shot noise limit for microscopes which require quasimonochromatic illumination. X-ray lasers have demonstrated very high peak brightness in sub-nanosecond pulses at wavelengths in the 10–20 nm range, and work in X-ray laser microscopy is underway [47,48]. In addition, gain has been reported at wavelengths as short as 4.5 nm, and efforts are underway to produce sufficient single-pulse coherent flux for flash holography [49]. X-ray free-electron lasers have also been considered for X-ray microscopy [50].

4.2 Optics

At the time of his discovery of X-rays, Rontgen tried and failed to find a means to focus them by refraction. We can see the reason for his failure in the considerations of the above section, for any...
material thickness $t$ which will produce significant refraction due to phase shifts $\Delta \phi = - k \delta t$ also attenuates X-ray intensity according to $\exp(-2k\beta t)$. As a consequence, X-ray focusing can only be accomplished using reflection or diffraction.

Reflecting optics are typically based on total reflection at grazing incidence angles less than the critical angle $\cos \theta_c = n = 1 - \delta$. Fig. 7 shows that the critical angle $\theta_c$ for gold is 10–100 mrad through most of the X-ray wavelength range of interest, and that the reflectivity at $\theta_c$ is in general quite good. (Other materials have even higher reflectivities over certain wavelength ranges.) While small, these grazing angles are sufficiently large for soft X-ray grating monochromators to be fabricated with resolving powers approaching 10,000 [51,52], and diffraction efficiencies of ~15% [53]. Grazing incidence mirrors can also be used as high resolution imaging objectives in X-ray microscopes. Among the more widely used geometries (see e.g., Michette [15]) are Wolter and Kirkpatrick–Baez mirrors for imaging systems, and ellipsoidal optics for on-axis source demagnification in scanning systems [7]. These grazing incidence mirror systems have the desirable characteristic of being completely achromatic, and they give good performance at X-ray energies up into the keV range. One can also obtain good reflectivity at somewhat larger grazing angles over a narrow wavelength range by using grazing incidence mirrors coated with synthetic multilayers [11] (multilayers will be described more below). Spatial resolutions of 3.8 $\mu$m × 1.7 $\mu$m have been obtained with Kirkpatrick–Baez systems [54,11]. Obtaining higher spatial resolution in grazing incidence systems is made difficult by the requirement that the optical surface match the desired (frequently aspherical) geometric figure, by the alignment tolerances of multielement optics such as Kirkpatrick–Baez systems, and by the fact that optical surface roughness decreases specular reflection and increases scatter and flare at grazing incidence. Furthermore, such systems typically subtend a rather small solid angle from their foci, so their efficiency for collecting image photons tends to be low compared to what visible light or electron microscopists are used to.

As was noted above, synthetic multilayer structures can be used to produce mirrors with greatly enhanced X-ray reflectivities. These multilayer coatings typically involve sputter coating of alternate layers of a reflector and a spacer with a reflector–spacer layer thickness $d$ chosen to satisfy Bragg's law of $2d \sin \theta = \lambda$ (here $\theta$ is the angle of incidence relative to the multilayer surface). At wavelengths of about 10 nm or longer, W–C, Mo–Si, and other material combination multilayers with $2d = \lambda$ and typically 50 layers have shown normal incidence reflectivities of 60% or more [55]. Using multilayer-coated Schwarzschild objectives, scanning X-ray microscopes have demonstrated a spatial resolution of ~500 nm [56,8] and such optics have been used in $\lambda = 14$ nm X-ray projection lithography experiments to print 50 nm features in photoresist. Multilayer-coated Schwarzschild optics have also been used at shorter wavelengths [57], but obtaining equivalent performance will involve serious challenges as $\lambda$ decreases the figure tolerances on the fabrication of the optical surfaces become even more demanding, the required thickness of the multilayer coatings becomes non-uniform as the X-ray angle of incidence varies across the surface of the optic, and layer interdiffusion com-
Fig 8 A schematic of the X-IA scanning transmission X-ray microscope showing the exit window (EW) through which beam emerges from the beamline vacuum system, the zone plate (ZP) with 0 and +1 focal orders, the order selecting aperture (OSA), the specimen mounted on a scanning stage (Stage), and the proportional counter X-ray detector (PC). The zone plate diameter is 90 \( \mu \)m, and the first order focal length is 1.1 mm for the \( \delta_{r_1} = 45 \) nm zone plate used at present.

Fig 9 Theoretical first-order diffraction efficiency for phase zone plates made of several materials (a) The optimum efficiency when the zone thickness is allowed to be up to 0.2 \( \mu \)m (b) The optimum efficiency when the zone thickness is allowed to be up to 0.5 \( \mu \)m.
better [58] (where \( N \) is the number of zones), they act to focus light into a series of odd focal orders, however, a central stop and order sorting aperture can be used to isolate the \(+1\) diffraction order (this is shown in fig 8) In that case, zone plates with \( N > 100 \) zones behave effectively as lenses of equivalent diameter and focal length [15], except that the diffraction efficiency is only \( 1/\pi^2 \approx 10\% \) if alternate zones are opaque As fig 9 indicates, diffraction efficiencies of 20\% or more can be obtained by choosing zone material thicknesses which both absorb and phase shift an incident X-ray wave [59], and shaped zone profiles offer the potential for even higher diffraction efficiencies [60] The diffraction-limited Rayleigh resolution \( \delta_i = 0.61\lambda f/r \) of a zone plate can also be written in terms of the width \( \delta_{oN} \) of the outermost zone as \( \delta_i = 1.22 \delta_{oN} \) The spatial resolution which one can obtain using Fresnel zone plate optics thus depends not only on the finest outer zone width \( \delta_{oN} \) which one can draw, but also on the proper positioning of all zones to about 0.3 of their width [61] Zone plates with \( \delta_{oN} \) as small as 30 nm have been used with success in X-ray microscopes [62,63] (see fig 10), and \( \delta_{RN} = 25 \) nm zone plates have been tested with good results [64] The diffraction-limited performance of a scanning transmission X-ray microscope with a \( \delta_{oN} = 45 \) nm zone plate will be described below While the highest resolution zone plates have been fabricated using electron beam lithography systems [65,63,66,67], UV holography [68] and sputter-section techniques [69–71] have also been used with success This latter method (also called the “jelly-roll” method) offers a potential means for fabricating zone widths finer than the 10–20 nm linewidth which is the practical limit of conventional electron beam lithography systems using organic photoresists Finally, elliptical zone plates have also been used to compensate for astigmatic synchrotron beamline optics [72] 4.3 Detectors X-ray detectors include both detectors for photon counting with no spatial resolution, and 2D detectors for image recording Gas flow proportional counters can be used for near-100\% efficient single photon counting at rates [73,74] in excess of 2 MHz Solid state photodiodes can also be used for high rate photon counting or photocurrent detection [73,75,76] and Si(Li) detectors are widely used for energy-dispersive spectrometry with higher-energy X-rays Phosphors such as P31 can also be used to convert X-rays into visible light photons, this is useful both for observing the position of an X-ray beam by eye, and for quantitative detection using photomultiplier tubes [77] X-ray detectors with 2D resolution include photographic film, nuclear emulsion, microchannel plates, charge-coupled devices (either backside thinned [78] or phosphor-coated [79–82]), and photoresists Some characteristics of these devices are summarized in table 1, in general, their resolution is modest enough that they are usually used to record images already magnified by one of the X-ray optics described above An exception to this rule is provided by photore sist detectors, which are able to record soft X-ray images at a resolution in the 10–50 nm range although with relatively poor dynamic range [83]
Table 1
Characteristics of some commonly used 2D X-ray detectors

<table>
<thead>
<tr>
<th>Detector</th>
<th>Resolution (µm)</th>
<th>Pixels</th>
<th>DQE (%)</th>
<th>Dynamic range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photographic film</td>
<td>1–10</td>
<td>No limit</td>
<td>5–30</td>
<td>Good</td>
</tr>
<tr>
<td>Microchannel</td>
<td>20–100</td>
<td>~ 500</td>
<td>5–20</td>
<td>Poor</td>
</tr>
<tr>
<td>CCD</td>
<td>7–30</td>
<td>500–2000</td>
<td>10–40</td>
<td>Excellent</td>
</tr>
<tr>
<td>Photoresist</td>
<td>0.005–0.020</td>
<td>No limit</td>
<td>~ 10</td>
<td>Fair</td>
</tr>
</tbody>
</table>

DQE is the detective quantum efficiency of the detector, and the 2D pixel count of the detector is (Pixels)²

In photoresists, an X-ray irradiation pattern produces chain-scission in the resist polymer, and the heavily damaged areas are selectively etched when placed in an appropriate solvent, leading to a surface relief pattern corresponding to the X-ray image. The intrinsic resolution of the photoresist is thought to be limited by macromolecule size (typically 5 nm for polymethyl methacrylate [83]) and the nanometer range of secondary electrons produced after a primary X-ray absorption event. The secondary electron resolution limit has been thought [84] to be 5, 35, and 65 nm at 4.5, 0.84, and 0.46 nm wavelengths, respectively, although there have recently been suggestions that the transverse resolution may in fact remain quite good at shorter X-ray wavelengths [85]. Following development, the photoresist can be read out using TEM, SEM, or a scanning force microscope, or a surface replica can be made for TEM use [86,87]. Direct electron microscope readout of the resist is complicated by radiation damage and the need for thin resists and thin substrates (TEM), or poor sensitivity to gradually sloped surface topography (SEM).

5. X-ray microscope systems

Just as the term “electron microscope” fails to uniquely identify a single type of instrument, several types of X-ray optical systems are used for microscopy today. We briefly describe several types of X-ray microscope and their current spatial resolution.

Transmision X-ray microscope, or TXM This is the X-ray analog of the TEM or conventional optical microscope, where the wavefield leaving the specimen is imaged by an objective lens onto a 2D detector. The first and foremost examples of this type are the TXMs of the Göttingen group of Schmahl, Rudolph, and coworkers [88,89,46]. In the TXM, the spatial resolution is determined by the objective lens (or micro zone plate), although for incoherent bright field imaging, the numerical aperture of the condenser should be approximately matched with the numerical aperture of the objective. A major advantage of the TXM is that spatially incoherent illumination can be used, for each resolution element of the object independently serves as a coherent emitter illuminating the object. The TXM is thus well suited to use with synchrotron bending magnet [88] or high emittance undulator [90] sources, and several groups are exploring the use of intense, narrow linewidth radiation from gas discharge [46] and laser-produced [91] plasma X-ray sources for flash imaging in laboratory microscopes. Zernike phase contrast can also be obtained if the specimen is partially coherently illuminated [34] and a λ/4 phase plate is placed at the back focal plane of the objective. While partial phase contrast has been demonstrated using a synchrotron bending magnet source [89], this imaging mode will benefit significantly from the use of higher brightness X-ray sources. Finally, we note that radiation dose in TXM will be increased over the theoretical ideal by the diffraction efficiency of the objective zone plate and by the detective quantum efficiency of the 2D image detector.

Scanning transmission X-ray microscope, or STXM This is the X-ray analog of the STEM, where an X-ray source is demagnified by an objective lens to a small spot, and the radiation transmitted by the specimen is detected as the spot or sample is scanned. Assuming that the scanning stage operates with sufficient precision, the spatial resolution is again determined by the objective lens, which can be a zone plate or a focusing mirror. While the objective lens must be spatially coherently illuminated to obtain diffraction-limited resolution, when a photon detector with no spatial resolution is used, the imaging...
process is an incoherent one (this can be understood in terms of the non-collimated angular spread of the illumination to each sample pixel). This is in accordance with the reciprocity principle for bright field amplitude contrast imaging between TEM and STEM [92]. Darkfield imaging has also been demonstrated [93] and differential phase contrast is being explored [94]. The STXM is best suited to use with high-brightness X-ray sources such as undulators [74, 9], although bending magnet [95] and laser-produced plasma [56] X-ray sources have also been used. The STXM is also well suited to microanalysis using differential absorption, as was described above [96, 97], image ratioing is made easier by the fact that most STXMs are computer controlled. Finally, we note that by placing the objective lens in front and an efficient photon counter behind the specimen, the STXM is able to image specimens with the minimum radiation dose consistent with the intrinsic contrast and desired signal-to-noise ratio.

Point projection microscopy This technique first came to popularity in the 1950s, and General Electric actually produced a commercial point projection X-ray microscope for some time [98]. By using a modified SEM, X-rays are emitted from a spot of size $\delta_x$ produced by an electron beam focused onto a thin film target. A projection akin to an incoherent bright-field image is formed, and relatively short (~1 nm) wavelength X-rays are usually used to penetrate thick specimens. The image is magnified by a factor $M$ given by the source-to-detector distance divided by the source-to-specimen distance; detectors with moderate resolution can thus be used. The final spatial resolution is given by a combination of the X-ray spot size $\delta_x$ and by Fresnel diffraction $\delta_F = \sqrt{\lambda d} / M$, where $d$ is the distance from the specimen to the detector. X-ray spot sizes $\delta_x$ of 0.1–0.3 $\mu$m [99–101] (limited by electron scattering within the target film) have been reported, and images with a resolution of 0.1 $\mu$m have been obtained [99]. By rapidly switching the electron beam focus between two locations on the film, real-time stereo images can be formed, and fan- and cone-beam tomography can be performed on samples which are rotated in the beam [102]. No X-ray objective lens is used, and 2D image detectors for shorter wavelength X-rays can have favorable detective quantum efficiencies.

Contact microscopy or contact microradiography This is probably the most widely practiced X-ray microscopy technique, in it, a high resolution detector (typically the photoresist polymethyl methacrylate) is placed in contact with the sample to record the transmitted intensity distribution [103, 104]. The resolution is then limited by Fresnel diffraction over the finite sample and resist thickness $d$ to $\delta_F = \sqrt{\lambda d}$ (or perhaps slightly less [105]), and by the intrinsic resolution of the photoresist and image readout system. With sufficiently thin samples and photoresists, the technique may be capable of $\delta_i = 10$ nm resolution, although 50–100 nm is more likely to represent the practical limit of much work [86, 43]. If the X-ray source size is small enough that penumbral blurring is not significant, lack of spatial or temporal coherence is actually advantageous in that it minimizes fringing artifacts caused by Fresnel diffraction. As a result, contact microscopy can be performed using conventional electron impact sources [106], gas pinched plasma sources [45], and laser-produced plasmas [42–44], as well as synchrotron sources [104, 43]. No X-ray objective lens is required, although the radiation dose delivered to the specimen is increased by the lower detective quantum efficiency of photoresists.

Gabor holography In the Gabor (or in-line Fresnel) holographic geometry, the wavefield leaving the specimen is made to interfere with a planar reference wave, thus producing fringes resembling a zone plate pattern. Just as with a zone plate, the spatial resolution is determined by the finest fringe width that can be read out with little error on the location of the fringe. The highest resolution Gabor holograms have been recorded on the photoresist polymethyl methacrylate, enlarged using a TEM, and numerically reconstructed on a computer. While there are indications that the holograms contain information at the 16 nm level, it is believed that the 56 nm resolution limit observed in reconstructed images is caused by hologram aberrations introduced by field distortions in the TEM lens [107]. Another approach uses UV reconstruction optics, in which case a spatial resolution of 100 nm can be ex-
Gabor holography is a coherent bright-field imaging process, and both optical and computer reconstruction techniques can be used to form an amplitude or a phase contrast image of the specimen. The reconstructed hologram forms both a real and a virtual image which both lie on the optical axis, because one image is present in an out-of-focus form when the other is viewed. Gabor holograms suffer from so-called twin image noise. This noise can be reduced either by recording the hologram well into the far-field of the object \(\sqrt{\lambda d} \gg s\), where \(s\) is the size of the specimen, or by using phase-retrieval techniques in computer reconstructions [107]. Gabor holography can be used for flash imaging with suitable X-ray sources [49,50] and the image of extended objects can be brought into focus over an enormous field depth in the reconstruction stage [107]. Again, radiation dose to the specimen is increased by the low detective quantum efficiency of photoresists, and the hologram fringe signal must be detected as intensity variations on a non-zero background level.

**Fourier transform holography** In the lensless Fourier transform holographic geometry, the wavefield leaving the specimen is made to interfere with a spherical reference wave diverging from a point next to the specimen. The spatial resolution is thus determined by the resolution at which the amplitude distribution of the spherical wave source is known. Experimental results have been obtained using Fresnel zone plates to form this wave [109] and images with a resolution of about 60 nm (consistent with the finest zone width) have been obtained [82]. There have also been proposals to use scattering or reflecting spheres to form the reference wave [110,111]. Because of the spherical reference wave, the average fringe frequency decreases as a detector is moved farther from the specimen plane, so that relatively low spatial resolution electronic detectors (such as charge coupled devices) can be used. Again, a coherent bright-field image is formed in the reconstruction, and both optical and computer reconstruction techniques can be used to form an amplitude or a phase contrast image of the specimen. Because the reference wave source is located next to the specimen, the real and virtual images lie on either side of the optical axis, if the specimen fills only one half of the image plane, the two images are separated from each other. Fourier transform holography is well suited to flash imaging, for the energy of the X-ray pulse will be distributed over the large area of the low resolution hologram detector. Extended objects can also be imaged, although the highest resolution can be maintained only within a depth of field limited by the coherence length \(\lambda^2/\Delta\lambda\) of the illumination. As in Gabor holography, the signal must be detected as intensity variations on a background level, although the detective quantum efficiency of CCD detectors is higher than that of photoresists.

**Scanning fluorescence X-ray microscope**, or SFXM This is the X-ray analog to X-ray analysis in an SEM, except that X-ray fluorescence is excited by an X-ray rather than an electron beam (this results in considerably less radiation damage to the specimen [38,39]). Because of the limited fluorescence efficiency at low energies, scanning fluorescence X-ray microscopes are usually based on higher energy X-rays (\(\lambda < 0.5\) nm, or \(E > 2.5\) keV). The spatial resolution in scanning fluorescent X-ray microscopes is limited to that of the probe-forming optics, while keV X-ray focusing has been demonstrated using sputter-sliced Fresnel zone plates [70,71] and Bragg–Fresnel optics [112]. Most fluorescence X-ray microscopes use pinhole [113,10] or grazing incidence mirror [11,13,54] optics at 2–5 \(\mu\)m resolution. Finally, elemental concentrations in the ppm range can be mapped, even if the trace element is buried well below the specimen surface (e.g., trace element concentrations can be mapped in fluid inclusions in geological specimens [10]). The objective lens (if one is used) is placed in front of the specimen, so its efficiency affects only imaging time rather than radiation dose to the sample. High incident flux rates are required to obtain sufficient fluorescence signals, and Si(Li) detectors collect only about 0.1% of the emission solid angle. However, the more modest resolution (less flux per area) and greater X-ray penetration (less absorption per depth) work to keep the radiation dose to reasonable levels.

**Scanning photoemission microscope**, or SPEM
These microscopes collect photoelectrons emitted from a surface scanned through a focused X-ray beam. The focused X-ray beam can be produced using zone plates [6], multilayer-coated Schwarzschild objectives [8] or ellipsoidal grazing incidence mirrors [7]. One can either image total electron yield, or selectively image a photoelectron energy characteristic of one particular surface composition and state, thus the SPEM is used primarily in surface physics and materials science. The spatial resolution is determined by the probe-forming objective, and the energy resolution depends on the monochromatcity of the incident X-ray beam and the quality of the electron spectrometer used. The design of SPEM instruments is complicated by the fact that the X-ray objective and the electron energy analyzer must both be located on the same side of a solid sample. Compared to transmission microscopes, the radiation dose delivered to the sample is rather high in SPEM because only a small fraction of incident photons will lead to the emission of a photoelectron of a specified kinetic energy. However, when compared to its electron analog of scanning Auger microscopy or SAM, the radiation dose in SPEM is considerably lower in part due to the higher signal-to-noise achieved using X-ray excitation. A related instrument is the photoemission microscope or PEM, in which a surface area is flooded with VUV or X-ray radiation and electron optics plus energy filtering are used to form the photoemission image [114].

6. An example: the X-1A STXM

One X-ray microscope we have particular experience with is the scanning transmission X-ray microscope [74] we and others operate at the X-1A beamline of the National Synchrotron Light Source at Brookhaven National Laboratory. The beamline, which has been described in detail by Rarback et al. [53], uses an undulator X-ray source and a spherical grating monochromator to deliver a partially coherent X-ray beam to the microscope. The X-ray wavelength is tunable over a 2.2–9.0 nm range, and the degree of monochromaticity (100 ≤ λ/Δλ ≤ 1800) and spatial coherence can be adjusted using computer-driven monochromator entrance and exit slits. The X-ray beam is brought through a 200 × 200 μm² silicon nitride window into an atmospheric pressure environment, and the zone plate is mounted immediately behind this window. An order-sorting aperture is used to isolate the first-order focused beam from the zone plate with central stop (see fig 8), leaving a working distance of typically 250 μm to the focal plane. The sample is mounted on a scanning stage which is driven by piezoelectric translators with integral capacitance-based position sensors and feedback electronics used to deliver a linear scan field. This piezo-based stage can be driven in 5 nm steps over a 70 μm range, and it is in turn mounted on the stepping-motor-driven stage which can drive the sample with 0.5 μm steps over a range of many mm. Located downstream of the specimen plane is a high rate (when used in saturation mode, it is linear to > 1 MHz) 9 1 Ar CO₂ gas proportional counter that is used as the transmitted X-ray flux detector. A visible light microscope is used for specimen examination and prealignment when the proportional counter is retracted. The microscope is under the control of a VAX station 3200 computer workstation with a CAMAC interface.

Dry specimens such as those prepared on TEM grids can be immediately mounted on the stage of the microscope, prealigned with the visible light microscope, and aligned using X-rays in a few minutes. Wet specimen studies are somewhat more involved, depending on the type of wet sample chamber used (these will be described below). Quick, low-dose images are used to identify a desired sample region, and correct focus can be determined by viewing a focal series of line scans of a nearby feature as an X–Z rather than an X–Y image. A high resolution, higher dose image can then be taken of a sample which has not previously been exposed to a high flux of X-rays. The focused X-ray flux is typically 10⁶ photons/s, depending on the specimen transmission and the desired signal level, a 400 × 400 pixel image takes 1–10 min to acquire. Applications of the X-1A STXM will be discussed below.

The zone plates used in the X-1A STXM have been fabricated by E. Anderson of the Center for
X-ray Optics at Lawrence Berkeley Laboratory working with D Kern in the Nanolithography Laboratory at IBM Yorktown Heights. The highest-resolution zone plates we have tested have 400 zones over a 90 μm diameter, with an outermost zone width of $\delta r_N = 45$ nm (the zones are

Fig 11 A gold test object was used to measure the modulation transfer function of the X-1A STXM. The mark space features vary in period from 72 nm near the center, 100 nm at the first radial ring, 200 nm at the second radial ring, and so on. (a) The test object viewed using a BioRad MRC-600 scanning confocal optical microscope with a Zeiss Neofluor 1.2 NA water immersion objective, showing finite optical transfer to about 150 nm feature size (image courtesy of Barry Burbach, Neurobiology Laboratory, Howard Hughes Medical Institute, SUNY at Stony Brook, Stony Brook, NY), scale marker indicates 2 μm. (b) A portion of the center of the test object as viewed at 25 kV in a Hitachi S800 SEM with transmitted flux detector (“STEM”), image courtesy Erik Anderson, Center for X-ray Optics, Lawrence Berkeley Laboratory), the X-1A STXM (“STXM”), and the X-1A STXM following deconvolution of the theoretical point spread function (“deconv”). The 36 nm minimum width features are clearly visible in the deconvolved STXM image. From ref [74]
supported by a silicon nitride membrane) The 120 m thick Ni zones both attenuate and shift the phase of an incident X-ray beam, so that the zone plate shows an enhanced diffraction efficiency ranging from 9% to 13% over the 2.2–4.4 nm water window spectral range. We have recently measured the modulation transfer function of the zone plate in the X-1A STXM [74] by imaging a gold test object fabricated with the same electron beam lithography system used to produce the zone plate. Images taken of this test object with the STXM, a STEM, and a confocal optical microscope are shown in fig 11. The fact that the object is mostly binary allows us to ignore image contrast issues and assume that the STEM image represents the true object. We then denote STXM \( f_x, f_y \) = \( F \{ \text{stxm}(x, y) \} \), where \( \text{stxm}(x, y) \) is the real space image and \( F \{ \} \) is a Fourier transform. Identical notation shall be used for \( \text{object}(x, y) \) and the point spread function \( p(x, y) \) because

\[
\text{stxm}(x, y) = \text{object}(x, y) * p(x, y),
\]

(5)

(where \( * \) denotes convolution), we obtain

\[
\text{MTF}(f_x, f_y) = \frac{|P(f_x, f_y)|}{|\text{OBJECT}(f_x, f_y)|}
\]

(6)

The results of this procedure are shown in fig 12, which indicates good agreement between the measured and theoretical MTF for this zone plate. Since the STXM forms an incoherent bright-field image of the specimen, we can deconvolve the point spread function from a STXM image with the procedure

\[
\text{restored}(x, y) = F\left\{ \frac{F\{\text{image}(x, y)\}}{\text{MTF}(f_x, f_y)} \right\}^{-1}
\]

(7)

The results of such a deconvolution (which forces the MTF to be unity up to a resolution cutoff of \( \lambda/(4NA) \)) are shown in the “decon” image of fig 11b.

7. Radiation damage: limits to resolution

It has long been recognized that radiation damage limits achievable resolution. For example, Breedlove and Trammel concluded that the damage caused by multiple ionization events would preclude the imaging of isolated low-Z atoms by electron or photon probes [115]. In fact, radiation damage is a limitation even at lower resolution.

X-ray microscopes are able to image structural detail because X-rays interact differently with the features to be studied than with the surrounding material. This large difference in cross section yields large specimen contrast, so that on a per-particle basis, far fewer soft X-rays than electrons are required to image wet biological specimens at a given resolution [3,4]. Unfortunately, it is precisely because organic materials absorb soft X-rays more strongly than water does that these same soft X-rays are known to be very effective in damaging biological specimens. Using carbon Kα X-rays, Goodhead et al have found [116] that the LD\(_{50}\) dose for human diploid fibroblast cells is 0.25 Gy, and that doses above 2.75 Gy cause 1 chromosome aberration per human lymphocyte [117]. Bennett et al have found that a soft X-ray dose of \( 3 \times 10^3 \) Gy will stop contraction in active myofibrils [118] (although preliminary investigations indicate that chemical treatments can be used to increase myofibril radiation tolerance).
Clearly the large intrinsic contrast comes at a large cost.

For microscopists, the crucial parameter is the resolution attainable before radiation damage compromises interpretation of the experiments. Though the electron microscope offers sub-nm resolution for some material specimens, studies of biological specimens have shown that radiation damage limits the available resolution to 0.3 nm for hydrated crystals at liquid helium temperatures [119,120], while individual features in dehydrated biological specimens imaged at room temperature show a resolution limit of closer to 2 nm [121]. Since X-ray microscopes offer a modest dose advantage relative to electron microscopes for the imaging of dehydrated specimens, one may assume that radiation damage will not limit attainable resolution in such cases in the foreseeable future. Experience at the 50 nm resolution level indicates that dried specimens are extremely robust, with no radiation damage observed after many $10^4$ Gy images of the same area.

One of the principal advantages of X-ray microscopes is their ability to image thick, wet specimens, but under these conditions radiation damage plays a more visible role. Gilbert et al. have demonstrated [122] that initially live fibroblasts isolated from chick dorsal root ganglia appear structurally intact for more than 0.5 h following a 95 nm resolution soft X-ray image with a dose of $2 \times 10^4$ Gy. Furthermore, multiple $10^4$ Gy images of whole, glutaraldehyde-fixed fibroblasts and rat sperm show little mass loss per image and no grossly observable structural changes (mass loss becomes significant at $10^6$ Gy doses). However, experiments performed on fixed hydrated $V$ faba chromosomes imaged in the presence of air at 50 nm resolution using the X-1A STXM have more clearly shown radiation damage effects, albeit at time scales longer than the 1–10 ms time required to image neighboring pixels [131]. To retain 90% of the initial mass in a second image of the same chromosome, the first image dose must be less than $3.4 \times 10^3$ Gy, even then, the chromosome is severely shrunken as a result of its initial exposure to radiation. These chromosomes are among the most sensitive specimens examined thus far in this STXM, and it is known that their mass loss rate is sensitive to buffer conditions such as ionic strength. Studies now underway are exploring the degree to which radiation damage can be reduced by adding sulfhydryl reagents as free radical scavengers [123], and by excluding oxygen from the environment near the sample during the imaging process.

The results for chromosomes agree with the postulate that the time scale for biological damage resulting from the diffusion of radiolysis products will be in the millisecond range [124]. A 1–10 ms per pixel dwell time may be acceptable at the current resolution level of scanning X-ray microscopes when radiation damage is limited to these short-range effects. However, longer-range damage events and cell movement are clearly observed while imaging initially live dorsal root ganglia neurons and rat spleen acinar cells [125].

The desire to study more sensitive living specimens, and to push to higher resolution, prompts the need for higher data acquisition rates such as are available from undulators on low emittance storage rings. These and other considerations also make the possibility of flash imaging using pulsed X-ray sources attractive [126,124].

8. Recent applications

Historically, the development of X-ray microscopes has been driven by the desire to image biological structures in something akin to their native state: unfixed, hydrated, and unsectioned. In fact, prior to this decade there have been relatively few instances in which this ideal has been approached [45,127,9,128]. In sub-μm resolution images with further technological advances in X-ray sources, optics, detectors, and sample chambers, this situation has begun to change. Many recent applications in biological imaging are described in the proceedings of the 1990 international conference on X-ray microscopy [5], they include the imaging of muscle myofibrils [118,9] (see fig. 13), zymogen granules and acinar cells [125], fibroblasts [129,122] and C. thummi chromosomes [130] as well as imaging and quantitative mapping of chromatin condensation in $V$ faba chromosomes [131]. In several of these
Fig 13 Image of a single unfixed, unstained myofibril from the psoas muscle of a rabbit. The myofibril was kept hydrated in an environmental specimen chamber while imaged at 3.26 nm wavelength using the King's College STXM at the Daresbury 5U2 undulator beamline. The specimen was examined as part of a series of experiments to assess the effect of increasing X-ray exposure on the ability of such myofibrils to contract when stimulated by adenosine triphosphate [118]. The dark bands in the image contain thick muscle filaments, while the light bands, which are bisected by the thin dark Z-line, contain the thin filaments. Figure courtesy of G Morrison, from ref [9].

cases, the studies have been concerned not only with observing ultrastructure, but with determining the radiation sensitivity as outlined above. Studies in progress with the X-1A STXM include those discussed above plus the imaging of rat and human sperm (by B. Loo, Jr., et al.), and neurons in culture (by J. Pine et al.). These wet specimen studies have been advanced considerably by the development of a variety of wet specimen chambers. While the Gottingen group uses a sandwich of several organic films to hold wet specimens in the vacuum chamber of their TXM [132], several other groups use wet sample chambers based on silicon nitride membranes used as windows. In some designs, the sample is immersed in a thin water layer trapped between two windows separated by 1–10 μm [125]. Samples can be introduced through thin capillaries, and the surrounding medium can be chemically altered while the sample is mounted in the microscope [125]. In other designs, samples are cultured on interchangeable windows and placed in a thin chamber in which the medium is exchanged with hydrated air before imaging [133,134].

X-ray microscopes are finding increasing use in elemental analysis. For example, differential absorption analysis in STXM has been used to map (at 0.1–0.5 μm resolution) mineralized calcium concentration in bone sections from patients with osteoporosis in Cinotti et al. [135] and in cartilage sections from patients with osteoarthritis by Buckley et al. [97] (see fig 14), and Budinger has examined what may be crystallized calcium spicules in mouse heart cells [136]. At higher X-ray energies, scanning fluorescence X-ray microprobes are able to map quite low concentrations of a variety of elements at 2–10 μm resolution, recent examples of this work can be found in such fields as geology [10], biology [137] and even in art forgery detection [138].

X-ray microscopes are also being used for materials science applications. Fig 15 shows a STXM image of a thin single crystal sample of a high-temperature superconductor, since the chemical composition of the material is known, images like this can be used to measure the thickness of the sample and provide information needed for infrared transmission spectroscopy measurements.
[139] Considerable attention is being focused on scanning photoemission microscopy or SPEM, as this technique allows one to image the chemical state of surface materials [6–8] with good detection efficiency and relatively low radiation damage. In addition, SPEM systems are amenable to future incorporation of X-ray fluorescence and photon-desorbed ion detectors.

9. Future directions

The field of X-ray microscopy has undergone rapid development over the past several years. One of our particular points of reference, the scanning transmission X-ray microscope at the National Synchrotron Light Source, has advanced from delivering 300 nm resolution images with hour-long scanning times in 1986 to delivering 50 nm resolution images in a few minutes today, and we expect to make additional improvements to this performance level in the near future. Similar advances can be described for other X-ray microscopes, and we expect that the newest generation of synchrotron radiation sources now under construction will propel further advances in the field (for example, faster exposure times may further mitigate radiation damage effects). Efforts aimed at the development of laboratory X-ray microscopes (such as those of the Göttingen group [46]) may dramatically change the nature of X-ray microscopy research by making the instruments available to an even larger research community.

At present, the spatial resolution of X-ray microscopes is usually limited by the objective lens used. Fresnel zone plates give the highest resolution performance, with a non-zero MTF measured [74] to 20 μm⁻¹ and with good performance obtained in zone plates with an outer zone width as fine as δrN = 30 nm [62,63] (see fig 10). Finest zone widths of δrN = 10 nm or even less may

Fig 14 Calcium deposits in human hip cartilage A new type of micro-crystal calcium deposit has been found close to the surface of hip-joint cartilage, and it is thought that these deposits may be linked to the onset of osteo-arthritis. The question arises, are these crystals found in the chondrocyte cells lacuna, or are they matrix borne? Answering this question will help in determining the likely origin of the deposits. Several cells from a number of 0.1 μm thick unstained sections were imaged with the STXM and were mapped for calcium. The X-ray transmission image on the left shows what could be a deposit inside the cell, however, when the calcium map is taken (right image) it is clear that the deposit or artefact is NOT a calcium crystal. This is a typical result from much data, and supports the theory that the crystals are matrix borne. The scale bar is 1 μm. Figure courtesy of C J Buckley (see ref [97]).
become possible, although considerable effort will be required to place the zones with the 3 nm accuracy required over a 50–200 μm zone plate diameter. Alternative approaches to high resolution include the use of photoresists in Gabor holography and in contact microscopy of ultrathin films.

Fig 15. (a) STXM image of a thin film, single-crystal high-temperature superconductor (Bi$_2$Sr$_2$Ca$_2$Cu$_2$O$_{8+x}$). The specimen is about 400 μm across, and shows clearly defined thickness variations. (b) The sample thickness across the center of the image as determined using the X-ray absorption coefficients tabulated by Henke et al. [32] The thickness variation is about 6 nm, or the thickness of two unit cells of the crystal. Note that although such a thickness variation is easily measured as a change in absorptivity, it is far smaller than the 3 μm depth of field of the microscope. Specimen provided by L. Forró and L. Mihály in connection with studies of the far-infrared transmission of these materials [139].
specimens, and scanning force microscopes are a natural tool for extracting the information contained in such photoresist images [44,107]. It has also been suggested that very high spatial resolution might be attainable in Fourier transform holograms recorded using ultrasmooth microspheres as reference scatterers [110]. Of course, all of these approaches have to contend with the limits radiation damage imposes upon attainable spatial resolution, unless flash sources are used to record images with sub-nanosecond exposure times.

An exciting development in microscopy has been the improved ability to form three-dimensional images of complicated structures. At higher X-ray energies, voxel resolutions of as fine as \((10 \mu m)^3\) have been obtained using 2D detection systems and sample rotation in both plane wave [80,81,140] and point projection [100] X-ray tomography, and \((2 \mu m)^3\) resolution pencil beam tomographs have also been obtained [141]. These systems are unique in their ability to image the 3D structure of thick, complicated, optically opaque specimens. At lower X-ray energies, computer algorithms have been used to deblur through-focus series taken in TXM [142] and stereo pairs have been used to obtain some depth perception in contact microscopy [106,86] and in STXM [143]. Ultimately, it may be possible to deconvolve through-focus series in a manner similar to that done in optical microscopy [144,145] although the desirable condition of \(\delta_r \rightarrow \delta_i\) will not likely be achieved in X-ray microscopy. Another approach would be to combine holograms taken from various viewing directions in the method of diffraction tomography [146] to reconstruct a 3D image with isotropic resolution, this latter approach would be particularly attractive if the multiple views could be taken simultaneously (before radiation damage could degrade the specimen in multiple images) in one shot of an X-ray laser [111].

X-ray microscopes have operated largely on the basis of forming incoherent bright-field images, or by imaging fluorescent X-rays of photoelectrons. Explorations of other imaging modes have just begun, for example, Schmahl et al [89] have experimented with Zernicke phase contrast, and Morrison et al have begun to study the use of darkfield imaging [93] and differential phase contrast [94]. Furthermore, conventional brightfield imaging has already been used for differential absorption microanalysis of elemental compositions [147,96,97] (see fig 14), and spatially resolved information on specific chemical states might be obtained using absorption near-edge spectroscopy.

Just as X-ray microscopy may benefit from application of the wide variety of imaging modes employed in electron and optical microscopy, considerable advances may be achieved through the use of stains and biochemical labels. Os---mum-stained muscle myofibrils have been imaged with good contrast in the King's College/Daresbury STXM [9] and immunogold-labeled tumor cells have been imaged with contact microscopy [148]. We have experimented with the use of X-ray induced visible light fluorescence from biochemical dyes with encouraging results, this technique may also be applicable to materials studies in a way akin to cathodoluminescence in the SEM. These and other developments may reduce the radiation dose necessary for imaging at a given resolution level, and they may allow the imaging of sites of biochemical activity and thus supplement the imaging of structure.

10. Conclusions

It has been only recently that several X-ray microscopes worldwide have been able to operate at a resolution of 50 nm or better for the transmission imaging of thick, wet biological specimens. In addition, new capabilities in elemental mapping (using fluorescence, differential absorption, and photoemission) have been established for biological, geological, and materials science specimens. As long-anticipated resolution and sensitivity/contrast goals are realized, and as several synchrotron X-ray sources begin operations, we expect that the already large number of structural research programs using X-ray microscopes will continue to increase.
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Note added in proof

More recent results in soft X-ray microscopy, including 0.1 μm resolution photoemission microscopy using multilayer-coated Schwarzschild optics by Ng et al., can be found in the proceedings of a conference held July 1992 [149]. Another recent application is the development of spatially resolved X-ray absorption near-edge spectroscopy for the study of thin film polymers and biological specimens [150].

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