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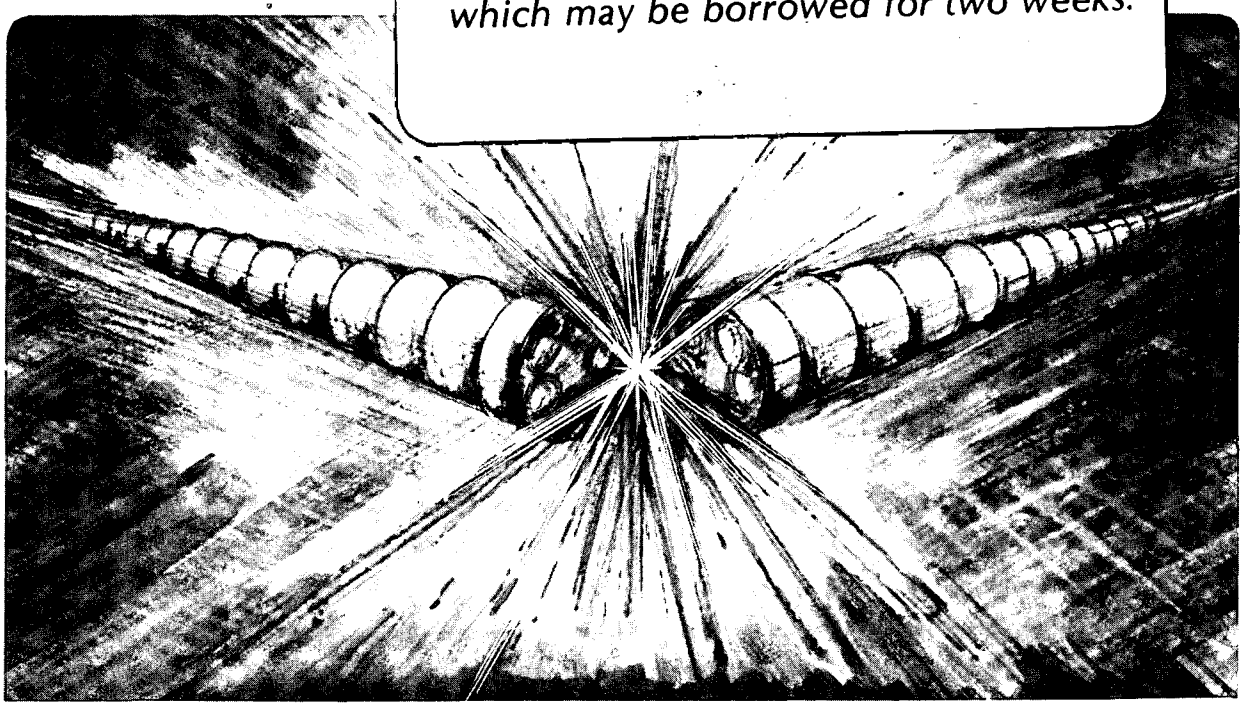

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SOME IDEAS ON THE ADVANTAGES OF SOFT X-RAYS AS IMAGING PARTICLES

by

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INTRODUCTION

The three main structural methods used in the life sciences are visible light microscopy, electron microscopy and x-ray diffractive techniques including crystallography. All of these have been enormously successful within their domains of application and together have supported the great advances in the understanding of cellular processes which have been characteristic of modern biology. However the capabilities of the above three methods are not so complete that there is no longer room for any new ones. In fact, analysis of the structural studies that have been done reveals that there has always been a need to compromise between fidelity and resolution. Thus the light microscope has the best ability to study natural, even living material but the most severely restricted resolution capabilities. Conversely electron microscopy has outstanding resolution but requires samples to be in non-biological form so that constant attention must be paid to the issue of fidelity in interpreting the features seen in micrographs. One can also make somewhat similar arguments about x-ray diffractive techniques which give the best high resolution information for the class of materials (crystals) that are furthest from natural, biological material.

THE SOFT X-RAY AS A BIOLOGICAL PROBE

We believe that the overall capability of this family of structural techniques can be enhanced by the introduction of soft x-ray methods which occupy an intermediate position in the spectrum of the fidelity-resolution trade-off. Thus, x-rays in the spectral range 10-50 Å can make images which have improved resolution compared to the light microscope and improved fidelity compared to the electron microscope. Indeed, such x-rays can image samples which, apart from the fact of illumination by an x-ray beam, are in their natural state, in an aqueous environment and in atmospheric pressure air, just as they would be on the stage of a visible light microscope. The resolution of such images enables much of the structure in the size range .05-1.0 micron to be imaged at the present time with prospects of improvements toward .01 micron in the reasonably near future.

The physics of the interaction of soft x-rays with matter is different from that of the other probes and this leads to some advantageous imaging properties. The spectral region between the Ok edge (23 Å) and the Ck edge (44 Å) has the special property that carbon and nitrogen containing materials give absorption contrast whereas water is relatively transparent. This is the basis of the claimed ability to study objects in an aqueous environment. Soft x-rays also have about the right penetrating power to interact with an intact cell and provide a measurable transmitted or diffracted signal. Fig 1 shows a comparison of both the contrast properties and the penetration of soft x-rays and electrons in the respective energy region in which they normally used for imaging.

OVERVIEW OF X-RAY MICROSCOPE TECHNIQUES

In this review we will limit our consideration to those x-ray microscopes which have demonstrated resolution superior to the visible light microscope. This limits our consideration to four soft x-ray schemes; contact x-ray microscopy [1], imaging x-ray microscopy [2], scanning x-ray microscopy [3] and x-ray holography [4]. These methods are all patterned after well-known optical techniques: contact printing, conventional optical microscopy, scanning optical microscopy and visible light holography. Where lenses are required, Fresnel Zone Plates [5] are used and the technology of these devices is one of the factors that favors the use of fairly soft x-rays in high resolution x-ray imaging. The resolution of a zone plate lens is roughly equal to the spacing of the finest (outer) zones of the plate and thus the zone spacing must be made small. This sets a practical limit to the thickness, so that zone plates work best for x-rays below about 1 keV and only poorly for x-rays greater than about 5 keV. There are other ways to focus hard x-rays and there are some higher energy techniques; especially microtomography [6] and the scanning x-ray microprobe [7] that we hope will soon enter the suboptical regime.

The capabilities and special characteristics of the four methods listed above are summarised in Table 1 which is intended to give some idea of the stage of historical developement of these technologies. All of the methods can be configured to provide imaging of wet, unfixed, unstained, unsectioned, biological objects up to about 10 microns in thickness. The achieved resolution at present is in the range 200-750 Å for all of the methods.

At the present time progress in three dimensional imaging by any of the methods has only just begun [8], although the issue is the subject of considerable study. The imaging and scanning microscopes can try to make

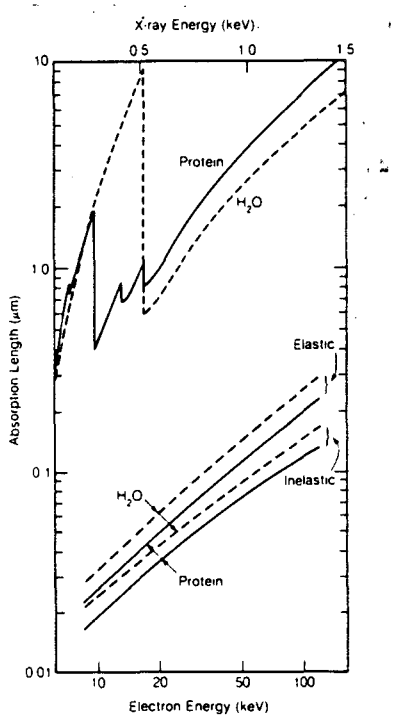


Fig1. Comparison of the interaction crossections expressed as a radiation length for soft x-rays and electrons over the range of energies that are typically used in imaging. The greater penetration and stronger contrast between water and biological material are the advantageous properties of soft x-rays for imaging life science specimens.

TABLE 1
CHARACTERISTICS OF SOFT X-RAY IMAGING TECHNIQUES

CHARACTERISTIC	CONTACT	IMAGING	SCANNING	HOLOGRAPHY
Achieved res'n with good contrast sample (Å)	200	500	750	500
Dose at above res'n imaging C & N (Mrads)	50	10-60	1	200
Exposure: bend magnet undulator	5 min few sec	10-100 sec	1 hour 2 min	1 day 1 hour
Coherent source needed ?	no	no	yes	yes
$\lambda/\Delta\lambda$ needed	3	300	300	1000
Type of contrast normally	amplitude	amplitude	amplitude	amplitude and phase
Arrangement to get phase contrast	not possible	done by frequency plane filters[9]	potentially doable by split detector [10]	happens naturally
Potential for quantitative microanalysis	poor	potentially doable by differential absorption	done by differential absorption[11] potentially doable by flourescence	poor

"optical sections" [8] just like their optical counterparts and holography is known to have a potential for three dimensional images. The difficulty in all cases is the poor numerical aperture (NA) of the experiments. The transverse resolution is 0.61 λ/NA while the depth resolution is 1.22 $\lambda/(NA)^2$. Thus we see that when NA<<1 the depth resolution is not useful and the image becomes two-dimensional. At present, with NA values around 1/20, the achievement of useful three dimensional imaging is marginal and improvements can be expected only when the transverse resolution improves. However, we should be encouraged to note that the depth resolution improves rapidly (like the square) with improvement to the transverse resolution.

The poor numerical aperture (spatial frequency bandwidth) of the experiments translates to a resolution that is many times the x-ray wavelength. The apparent similarity of all the methods in this respect can be traced to a common root: the dependance on the properties of x-ray resists, especially

polymethylmethacrylate (PMMA) [12]. Resist is used in all presently-favored methods of zone plate manufacture and is directly involved in contact microscopy and holography. The intrinsic resolution of this material is best for x-rays around 50 Å wavelength [12] and is limited by secondary electron range on the high energy side and diffraction on the low energy side of this value. The ultimate, useful resolution of PMMA is probably about 100 Å and so this sets a fairly hard limit to the resolution one could ever expect to achieve with these methods. We shall see later that one can arrive at a very similar conclusion using radiation damage arguments.

ANALYSIS OF THE USEFULNESS OF X-RAYS IN IMAGING AND MICROANALYSIS

A number of powerful microanalytical tools are presently existing and it is in the context of the capabilities that they offer that the usefulness of x-rays must be evaluated. The dominant instrument is the electron microprobe (EMP) but instruments for electron-energy-loss spectoscopy (EELS). proton-induced x-ray emission (PIXE) and scanning Auger microscopy (SAM) are also used and have their special advantages. These instruments all use charged particle probes. For many years x-rays have also been used as probes of various objects using the somewhat related techniques of x-ray-fluorescence specroscopy (XRF),electron spectroscopy for chemical analysis (ESCA) and differential absorption analysis (DAA). Most of these techniques have well developed commercially made equipement available.

For our purpose we need to consider just a few aspects. Firstly we note that the issue in most biological experiments will be the sensitivity or smallest detectable element concentration as a function of spatial resolution and radiation dose to the sample. Secondly and significantly we note that among the most sensitive trace element techniques and also reputedly those with the lowest dose are the x-ray techniques, XRF and ESCA. This comparison is not a legitimate one, however, because as normally practiced, XRF and ESCA have spatial resolutions of the order of a millimeter while the respective competitive techniques EMP and SAM have resolution of around 1 micron and .1 micron. A legitimate comparison between ESCA and SAM has been made [19] and it then appears that the x-ray probe still has a dose advantage of about two orders of magnitude.

The situation we are now faced with is that the circumstances that have traditionally prevented XRF and ESCA from having good spatial resolution, namely lack of an adequately bright x-ray source and lack of optics to focus it, have now changed. The availability of modern storage rings, high resolution Fresnel Zone Plates and reflective x-ray optical systems have now made it possible to implement all of the above-mentioned x-ray techniques with high spatial resolution. In anticipation of this situation a number of studies have been carried out [13-19] to determine the usefulness of the new x-ray methods. The broad conclusion of these studies is that in principle x-ray probes have advantages over charged particle probes for trace analysis of all elements in damage sensitive samples. The reason for this is generally that x-rays provide either a lower background of spurious events due to the absence of Bremsstrahlung and certain types of multiple scattering or that they provide improved contrast due to the availability of absorption edges. Either way a given microanalytical task can be carried out with less radiation dose.

Of course the details of these kinds of comparisons are complex and no one technique is always optimum even by the narrow criterion of lowest

dose. We do not try to reproduce all the arguments here. Instead we show two rather graphic comparisons taken from the literature that show how the use of x-rays can bring about lower background.

Fig 2 shows an Auger spectrum (made with an eletron probe) and an ESCA spectrum (made with an x-ray probe) taken from the study by Kirschner [18]. Both are taken with state-of-the-art equipment, have low spatial resolution and show peaks from the metal substrate materials. One can see that in the Auger spectrum the peaks are riding on a high background much larger than the signal while the ESCA peaks ride on a low background much lower than the signal. The Auger background is unavoidable and is mainly due to secondary electrons produced directly by the primary beam and inelastically scattered

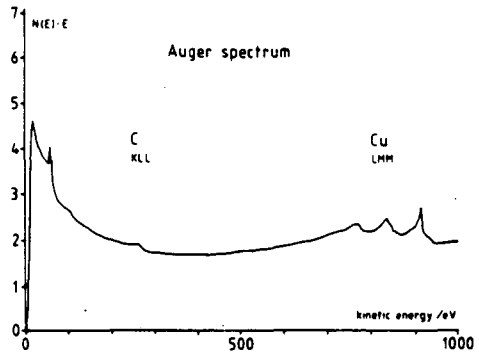
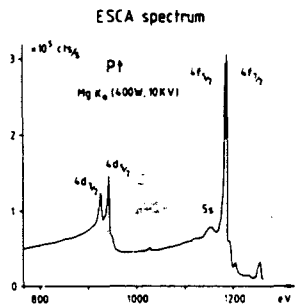
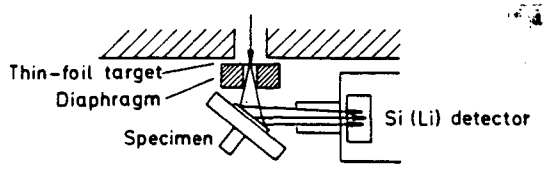


Fig 2. Comparison between typical Auger and ESCA spectra taken from Kirschner [18]. The high background and poor signal-to-noise ratio of the Auger spectrum compared to the ESCA one is very evident.





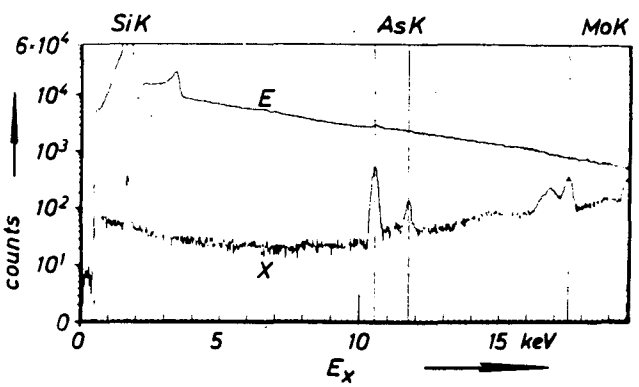


Fig 3. Comparison between an electron excited (E) and x-ray excited (X) x-ray fluorescence spectrum taken from Reimer [20]. The sample for both curves is a silicon wafer doped with 100 ppm of As. The x-ray curve was obtained using a converter foil as shown in the upper diagram.

primary electrons. These processes do not occur for an x-ray beam. The scattering process that does occur for x-ray exitation; scattering of the photoelectron is closely similar to what happens to the Auger electron.

Fig 3 shows two x-ray fluorescence spectra taken from the book by Reimer [20]. The spectra are from the same sample which is a silicon substrate doped with 100 parts per million of arsenic. The upper curve is the spectrum excited by an electron beam while the lower one is the spectrum excited by converting the same electron beam into molybdenum K x-rays by means of the foil converter shown in the upper part of the figure. The improvement in signal-to-noise ratio obtained by this strategy is spectacular. The high continuum background in the electron excited curve is mainly due to Bremsstrahlung, a process that does not occur for x-rays.

RADIATION DAMAGE

There is very little experimental evidence available concerning the effect of soft x-rays on biological material. What we would like to understand is the degradation of the image that we measure <u>at the resolution that we are using</u> and in the context of what we hoped to learn from the image. In the absence of direct evidence on this point we must turn to the evidence available from other imaging methods particularly x-ray crystallography and electron microscopy coupled with our understanding of the physics of the interaction of soft x-rays with matter.

The x-ray crystallography community have been illuminating protein crystals with x-rays for something in excess of half a century. Considering this extensive experience the amount of quantitative data on radiation damage is remarkably small. From our point of view, the main conclusion seems to be [21-23] that to obtain a usable data set one has to apply a radiation dose of at least 1-10 Megarads and that protein crystals in general are able to withstand such a dose and still provide 1.5-2.0 Å resolution data provided the dose is applied sufficiently rapidly.

For electron microscopy, values of the critical radiation dose for damage are tabulated, for example, in the reviews by Glaeser and Reimer [24,5]. The average value is about 0.01 Coulombs/cm^2 with a variation of around one order of magnitude in either direction for the range of materials that were measured. At 60 keV this is equivalent to 1000 Megarads. The end-points defining the critical dose in the above measurements were mostly loss of electron diffraction efficiency and mass loss. The onset of damage is a serious limitation to the electron microscopy of biological samples.

We must first recognise that these levels of dose are far in excess of the levels where major biological changes occur. We also note that this has not prevented electron microscopy and x-ray crystallography from making outstanding contributions to biological science. In fact important biological effects occur at the doses needed for imaging by all of the techniques mentioned so far. X-ray microscopy is no exception to this as can be seen from the table. The reasons that x-ray microscopy promises to provide some progress in the imaging of radiation sensitive samples is not

that it employs lower doses but rather that the lower background and improved contrast of x-ray images lead to a higher quality of information for whatever dose is permitted.

There are various ways to reduce radiation damage available to microscopists of all kinds and of course these could be used for the x-ray techniques discussed here. However, there is one additional trick that can be attempted using x-rays. It happens that there are x-ray "Flash Sources" available which can deliver enough x-rays to make an image in times of the order of a nanosecond or less. Many images have already been made this way [26,7]. Since damage processes are thought to take place on timescales related to the velocity of sound or slower, it appears that the image-bearing signals could be away from the sample before any damage had time to occur. If this is indeed true it could provide a way to circumvent the damage problem for some imaging processes and a powerful way to study it as well.

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