Microsc. Microanal. 7, 211–219, 2001
DOI: 10.1007/s100050010069

Microscopy AND Microanalysis

Biological X-ray Microanalysis: The Past, Present Practices, and Future Prospects

Patrick Echlin*

Multi-Imaging Centre, School of the Biological Sciences, Cambridge University, Cambridge CB4 3DY, UK

Abstract: A brief description is given of the events surrounding the development of biological X-ray microanalysis during the last 30 years, with particular emphasis on the contribution made by research workers in Cambridge, UK. There then follows a broad review of some applications of biological X-ray microanalysis. A more detailed consideration is given to the main thrust of current procedures and applications that are, for convenience, considered as four different kinds of samples. Thin frozen dried sections which are analyzed at ambient temperatures in a transmission electron microscope (TEM); semithin frozen dried sections which are analyzed at low temperature in a scanning transmission electron microscope (STEM); thick frozen hydrated sections which are analyzed at low temperature in a scanning electron microscope (SEM), and bulk samples which are analyzed at low temperature in the same type of instrument. A brief outline is given of the advantages and disadvantages of performing low-voltage, low-temperature X-ray microanalysis on frozen hydrated bulk biological material. The article concludes with a consideration of alternative approaches to in situ analysis using either high-energy beams or visible and near-visible photons.

Key words: biological samples, X-ray microanalysis, low temperatures, sample preparation, analytical procedures, low voltage

Introduction

This article was the sole biological contribution to the 1999 Microbeam Analysis Society (MAS) symposium which celebrated 50 years of electron probe microanalysis. Under the circumstances, it is appropriate to provide a background to biological X-ray microanalysis and, accordingly, the article will consider first the contribution that Cambridge, UK, has made to biological X-ray microanalysis, followed by a broad overview of the biological applications of the technique. A brief review will then be provided of the current general approaches to the subject, including the methods and techniques currently being used by the author. Finally, we will consider the problems and limitations associated with biological X-ray microanalysis and suggest ways in which one might expect biological X-ray microanalysis to proceed in the future.

Received November 30, 1999; accepted January 12, 2000.

*Corresponding author, at Cambridge Analytical Microscopy, 65 Milton Road, Cambridge CB4 1XA, UK.

The Cambridge Contribution

Other contributors to this symposium have dwelt on the contribution that people in Cambridge have made to the understanding of the physics and optics of X-ray microanalysis, and to the development of the instrumentation

needed to carry out the technique. It had been long appreciated that dispersed within the organic matrix of biological structures is a variety of other elements whose local concentration could, in principle, be measured in situ by X-ray spectroscopy. This type of analysis presented quite a challenge because most biological samples are highly hydrated, beam sensitive, and of low density. Not surprisingly, much of the early work was largely qualitative and centered either on mineralized tissues in which the local concentrations of elements such as calcium and phosphorus were stable and present in relatively high amounts, or on sections of soft tissues prepared by methods which were designed to retain the macromolecular configurations familiar to transmission electron microscopists, but which, alas, caused most of the elements of interest to disappear down the sink during the sample preparation. Thirty years ago, biological X-ray microanalysis had a poor reputation that was dryly summed up by a distinguished scientist who stated, somewhat unwisely, that all the technique could do was to show that bones contained calcium.

The breakthrough came in 1968 with the publication of an article by Ted Hall, who was working in the Cavendish Laboratory in Cambridge, UK, in which he proposed a novel quantitative analytical algorithm that would provide quantitative analytical chemical data from sections of biological material (Hall, 1968). Like all good scientific ideas, the concept was disarmingly simple and combined the elements of the classical Castaing equation and an extension of the Kramer's relationship. The Castaing equation assumes that the intensity of characteristic radiation is proportional to the concentration of the element of interest present in the sample. The Kramer's relationship describes the proportionality between the mass thickness and the intensiy of continuum radiation as a function of the matrix atomic number. Since all bio-organic matrices have approximately the same mean atomic number, Hall realized that measuring the continuum radiation in a spectral region away from the characteristic emission line of the elements of interest, would provide a built-in monitor of changes in density and thickness from point to point in the sample. In this way, one has both a measure of the mass of the analyzed specimen region and the mass of a particular element in the same region from which local concentrations may be derived.

During the next 5 years, Ted Hall and his colleagues made further refinements to this algorithm; this resulted in what is now known as the Continuum-Normalization method (or, more colloquially, the Hall Technique) which is one of the most important algorithms for quantitatively analyzing the many diffusible ions and bound elements which are prevalent in thin sections of biological samples. A good summary of this pioneering work may be found in Hall (1979). The contribution Ted Hall has made to biological X-ray microanalysis cannot be overestimated.

At about the same time, the author of this article, working in the Department of Botany in Cambridge together with colleagues from the then Cambridge Instrument Company, were investigating the feasibility of carrying out scanning electron microscopy at subzero temperatures. Our article (Echlin et al., 1970) showed that it was possible to image biological specimens at liquid nitrogen temperatures. The development of the low temperature technology provided another important step forward in the X-ray microanalysis of soft biological material. Water is the most abundant building block of biological material and, when converted to the solid state, can provide the perfect matrix in which to study the structure and in situ chemistry of hydrated materials. By using low-temperature preparative techniques, it was proposed to circumvent the use of conventional wet chemical preparative methods which invariably resulted in massive loss of the diffusible elements ($Z = 11–20$) one wishes to analyze. Coincidentally, and quite separate from the work we were carrying out in the Department of Botany, Tim Appleton (Appleton, 1972) in the Department of Physiology was developing techniques for cutting 100–200 nm frozen hydrated serial sections from soft biological material. The sections were freeze-dried prior to analysis.

In 1971, Ted Hall, together with three colleagues from the Department of Zoology (Torkel Weis-Fogh, Brij Gupta, and Roger Moreton) and the author, made a successful application to the Science Research Council for funds to establish the Biological Microprobe Laboratory in Cambridge to study the distribution of electrolytes in undisturbed frozen-hydrated cells and tissue. The Laboratory was in full operation by early 1972, and we were joined in 1973 by Albert Saubermann, a National Institutes of Health (NIH) Post-doctoral Fellow from Harvard. By early 1974, we had established procedures that would allow us to prepare, examine, and analyze a variety of biological specimens maintained in a fully frozen-hydrated state using low-temperature techniques, and published a short paper (Moreton et al., 1974) in which we believed, at the time, that we were able to demonstrate the feasibility of the technique. With the benefit of hindsight, we were wrong in our supposition that we were dealing with fully frozen-hydrated material. Sadly, differences of opinion began to surface in

the laboratory concerning experimental protocols, image interpretation, an assurance of the fully frozen-hydrated state, and the validity of the analytical results. After a turbulent year and a half during which time Albert Saubermann and the author worked closely and productively together, Albert Saubermann returned to Harvard where he set up his own very successful biological microprobe laboratory based on the procedures he and the author had established in Cambridge. Shortly after, the author resigned from the Biological Microprobe Laboratory in order to continue his experiment work elsewhere. Ted Hall continued to refine the Continuum-Normalization algorithm, which by now was a fully quantitative procedure and, together with colleagues, successfully applied the methodology to a variety of biological problems. The Biological Microprobe Laboratory continued functioning until the late 1970s at which time it failed to receive further funding and was closed.

The next and last phase in the Cambridge contribution came in 1993 when Raymond Lund, from the Department of Anatomy, and the author, together with contributors from a number of departments within the School of the Biological Sciences, were awarded a substantial grant from the Wellcome Trust to establish an interdepartmental center for multiple imaging and in situ analysis at the cellular and subcellular level. The Multi-Imaging Centre opened its doors in 1994, and is a roaring success and fulfilling all its promises. The author retired from the University and the Directorship of the Centre in the fall of 1999, and his place has been taken by Jeremy Skepper who has been responsible for adding further analytical instrumentation to the Centre. The Centre, which serves the School of Biological Sciences and the Department of Archaeology has a many-faceted mission including transmission, scanning, and confocal microscopy, and, naturally, X-ray microanalysis and the low-temperature technologies. It is gratifying to report that Cambridge continues to make important contributions to biological X-ray microanalysis.

Biological Applications of X-ray Microanalysis

The following section is but a vignette of the types of samples, methods of preparation, and analytical algorithms commonly associated with biological microanalysis. No references will be given here because the information already exists in countless books, reviews, and research papers, and, doubtless, many more to come. The exigencies of space preclude a discussion of the advantages or disadvantages of a particular experimental approach.

Types of Analytical Problems

There are three main types: low Z elements in a low Z matrix, e.g., aluminum in plant cell walls; high Z elements in a low Z matrix, e.g., heavy metals in polluted marine organisms; and low Z diffusible elements in a low Z matrix, e.g., sodium and potassium in mammalian tissues. There is a wide range in the binding state of the elements of interest from the covalent bonds in mineralized tissue, the weaker bonds that predominate in many organic materials, to the hydrogen bonding which is a feature of the aqueous state.

Specimen Types

The specimens may remain intact or fractured, or sectioned to reveal their interior. Bulk samples may be polished and the sections may be ultrathin (80–100 nm), thin (100–500 nm), or thick (0.5–2.0 μm).

Sample Preparation

There are more ways to prepare samples than there are Portuguese recipes to prepare cod (365), although the procedures can be divided into the following main classes. Invasive, primarily wet-chemical methods, precipitation techniques which turn otherwise diffusible elements into insoluble products, low-temperature techniques such as freeze substitution and freeze drying, and low-temperature methods which center on preserving the specimen in its original (frozen) hydrated state.

Analytical Algorithms

For sectioned material, the Continuum-Normalization program and its derivatives continue to dominate the field. For bulk samples with smooth but unpolished surfaces, the Peak-to-Local-Background and the depth distribution of the production of X-rays (Phi-Ro-Z) algorithms are being used successfully to obtain quantitative data from a variety of specimens. The ZAF algorithm (where Z = atomic number, A = X-ray absorption, and F = X-ray fluoresence corrections), although not an ideal method for biological samples because of the difficulties with matrix corrections, can be used on polished samples that have a known organic chemical composition.

Instrumentation

Biological X-ray microanalysis may be carried out in transmission electron microscopes at between 80–200 kV, and scanning electron microscopes at between 3 and 20 kV, using both reflective and transmitted electron imaging. Derivative instruments such as the scanning transmission electron microscope (STEM), are also used. The X-ray photons are collected using both energy-dispersive and wavelength-dispersive spectrometers.

Current Procedures and Applications

There is now general agreement, among the leading practitioners of biological X-ray microanalysis, that the specimens which are invariably hydrated, of low-density and beam-sensitive, are the weakest link in the chain of processes between the living material and the X-ray spectrum. In light of these quite severe restrictions, there is only one sensible approach when using an analytical procedure that depends, critically, on the interaction of high-energy beam electrons with a labile specimen. The samples must, first, be prepared by the least-invasive preparative procedure and, second, irradiated with the minimum amount of energy in order to obtain the maximum amount of chemical (and structural) information. Low-temperature sample preparation, examination, and analysis are central to these processes. Specimen preparation will not be discussed here except to state that as far as bioorganic materials are concerned, low-temperature techniques are imperative (Echlin, 1992). In order to justify such an imperious statement, a very brief review of the work of four different research groups will, it is hoped, illustrate the range of approaches being used in modern biological microanalysis.

Andrew Somlyo and his colleagues have focused their experimental approach on using a combination of rapid freezing and cryoultramicrotomy to produce ultrathin frozen-dried sections which are examined and analyzed in a transmission electron microscope fitted with X-ray spectrometers. The X-ray analysis is carried out either in the spot mode or by compositional mapping. This approach has enabled them to measure, quantitatively, local concentrations of calcium at better than 10-nm spatial resolution. A recent article (Somlyo, 1998) provides a brief and constructively critical summary of their current experimental work together with a number of key references. A similar approach is being taken by Marie Wendt-Gallitelli and her colleagues in Germany (Schultz and Wendt-Gallitelli, 1998). By using freeze-dried sections less than 100-nm thick in a STEM instrument fitted with a Ge energy-dispersive detector, they are able to demonstrate an increase in the accuracy of calcium quantification in cardiac myocyte mitochondria by a factor of 2.7 compared with a silicon detector.

Peter Ingram and his colleagues use a combination of rapid freezing and cryoultramicrotomy to produce semithin frozen-dried sections which are examined and analyzed at low temperatures in a STEM fitted with X-ray spectrometers. They have developed a very comprehensive interactive computer program called "Programmed Beam Analysis" which, when used with a fine probe of electrons, can produce quantitative elemental maps either from sections or from cultured cells. Their experimental strategy is summarized in the first chapter of a recently published book (Ingram et al., 1999).

Richard Lopachin and his coworkers are making good use of the experimental approach developed by Albert Saubermann following his stay in Cambridge more than 20 years ago. Rapidly frozen samples are cryosectioned to produce 500-nm sections which are imaged and analyzed at very low temperatures. First, low-dose conditions are applied to the fully frozen hydrated state and then somewhat higher beam currents are used on the frozen-dried state at 20 kV in a scanning electron microscope fitted with a transmitted electron detector and X-ray spectrometer. The electron beam is rastered over regions of interest, and the water content of morphological compartments may be determined from the ratio of continuum counts in the frozen-dried and frozen-hydrated states. A recent article (Taylor et al., 1999) outlines the experimental approach and provides a number of key references.

Alan Marshall and colleagues use quantitative X-ray imaging of rapidly cooled, diamond-knife planed, frozen-hydrated biological bulk samples which have not been etched. Quantitative oxygen and carbon X-ray images can be used to distinguish morphological features, and the same procedure is used to map the elements of interest, thus removing the requirement of electron imaging. The samples are analyzed at 15 kV at very low temperatures in a scanning electron microscope fitted with an X-ray spectrometer. A recent article (Marshall and Xu, 1998) gives full details of this novel approach together with a large number of key references.

It is hoped that these four examples show how different instruments, sample preparation, and analytical procedures can be used to provide quantitative X-ray data from a va-

Table 1. Changes in the Beam-specimen Interactive Microvolume as a Function of Beam Voltage[a]

Voltage (kV)	10	9	8	7	6	5	4	3	2	1
Size (μm^3)	18.0	11.0	6.0	3.0	1.4	0.6	0.2	0.04	0.006	0.0002
Size over the size at 1 kV	90,000	55,000	30,000	15,000	7000	3000	1000	200	30	0

[a]Calculated size of the microvolume in a generic frozen hydrated bio-organic sample in which X-rays may be expected to be generated as a function of the primary beam voltage. The data is calculated using the Bethe range equation and assuming that the interactive volume is spherical.

riety of biological specimens. The common thread which binds these four examples and the approach adopted by the author, details of which now follow, is low temperature.

Low-voltage, Low-temperature X-ray Microanalysis

The advantages and disadvantages of low-voltage scanning electron microscopy and X-ray spectroscopy have been discussed recently (by Joy and Joy, 1996; and Joy, 1998), and provide the background to the physics of low-voltage microscopy and analysis. The good news is that, with bulk samples, as the primary voltage decreases, although the probe diameter becomes larger, the analyzed microvolume decreases, the specimen charging is diminished, and greater advantage can be made of the *L* and *M* X-ray energy lines. The bad news is that, paradoxically, beam damage may increase if the same beam current is put into a diminished specimen-beam interactive volume; the count rates may fall and the structural spatial resolution will decrease.

The following example reveals the dramatic decrease in the size of the primary beam/sample interactive volume that is a good indication of the volume from which X-rays are generated although not necessarily measured. If we assume a generic frozen-hydrated biological sample that has a stochiometric organic and water formula of $C_4H_6O_5N$ and a density of 988 kg/m^3, there is a decrease in size of the interactive volume as the beam accelerating voltage is lowered. Evidence for this is given in Table 1 and more dramatically, in Figure 1. This data shows, for example, that at a modest 5 kV, the interactive volume has decreased by a factor of 30 and, at 3 kV, the figure is 450 when compared with the size at 10 kV. In addition (see Fig. 1), the spatial X-ray resolution, i.e., the size of the microvolume in which X-rays may be generated, is the same in a bulk frozen-hydrated sample as in a 500-nm frozen-hydrated section. Although frozen-hydrated semithin sections are perceived to have many advantages for X-ray microanalysis, they are much less stable, difficult to prepare, are less resistant to beam damage and dehydration than bulk samples. The size of the ice crystallites is the only limiting factor to high structural spatial resolution. There is, thus, a powerful imperative to carry out X-ray microanalysis of bulk samples at the lowest possible voltage. The actual voltage to be used for a given application will be a function of the element(s) being analyzed, the sample density, which for most bio-organic materials is between 800 and 2000 kg/m^3, the estimated concentration of the element being analyzed, and the required accuracy of analysis. The beam current should be a compromise between that needed to obtain a statistically significantly count rate and that which causes beam damage.

It is useful to see what elements could be analyzed with a conventional atmosphere thin window (ATW) germanium energy dispersive (ED) detector with a resolution of 110 eV. Table 2 sets out the possible limits for the analysis of a number of elements that are have been found within bio-organic samples. The $K\alpha$, $L\alpha$, and $M\alpha$ X-ray energies are given and it is assumed that an over-voltage of 2.5 is required in order to obtain a statistically significant count rate. The elements are ordered into a series of bands corresponding to the accelerating voltage which would be needed to generate sufficient X-rays. The column at the right of the table gives some actual experimental results from a variety of biological and organic materials carried out by the author (Echlin, 1999a, 1999b, 1999c).

The analysis was carried out using the Peak-to-local-background (Oxford Instruments ISIS PB-QUANT algorithm) that earlier studies had shown to be as accurate as the classical ZAF technique (ISIS SEM-QUANT algorithm) when applied to flat surfaces (Echlin, 1999a). The criterion for including the experimental data is that the precision of analysis had to be within ±5% of the known value. The author is currently using low-temperature (123 K), low-voltage (5 kV) quantitative X-ray microanalysis with an ATW ED germanium detector to measure the local concentrations of aluminum, magnesium, and silicon in different

cellular compartments of young leaves from various clones of Assam tea plants which have been grown in soils of different pH. Preliminary evidence suggests that the normally phytotoxic aluminum is sequestered as an insoluble aluminum magnesium silicate in the epidermal cell walls away from the cytoplasm of the underlying photosynthetic tissues. Details of this work will be presented elsewhere.

Additional Approaches to Biological Microanalysis Using High-energy Beams

Richard Leapman and his colleagues at the NIH, Washington DC, are within an ace of mapping single atoms in biological structures using electron energy loss spectroscopy (EELS) (Leapman and Rizzo, 1999). It has been known for some time that light element analysis is improved by using EELS rather than X-ray microanalysis. For example, Leapman and Hunt (1991) were able to demonstrate that the sensitivity for detecting phosphorus and calcium is four times better in an EELS instrument than by electron probe microanalysis. Leapman and Rizzo (1999), using a nanometer-size probe available on a field emission-scanning transmission electron microscope (FE-STEM), are able to localize small numbers of phosphorus atoms at 1–2-nm spatial resolution in such well-characterized specimens as DNA plasmids and tobacco mosaic virus. Although the analyzed atoms may move a few nanometers due to beam-induced structural degradation, corrections for specimen drift helps to alleviate this problem. This "molecular microscopy," al-

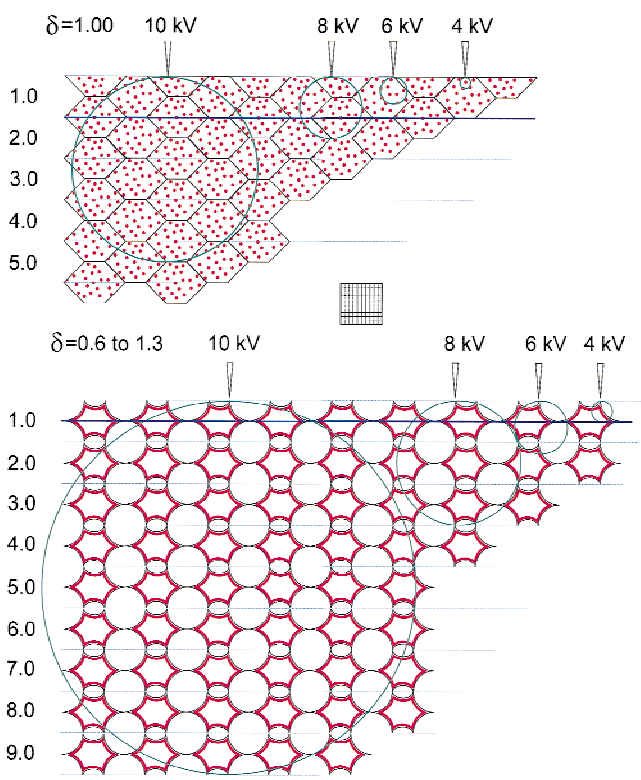

Figure 1. A schematic representation of frozen hydrated and frozen dried cells and tissues. The diagram is a schematic representation of cells and tissues in the frozen hydrated and the frozen dried state together with the size of the primary beam-specimen interactive volume. The fully frozen hydrated tissue is in the top diagram, the cellular compartments are represented as hexagons, and the elements within these compartments are represented as dots. Assuming a density of 1000 kg/m^3, the four circles of diminishing size represent the calculated beam-specimen interactive volume at the four accelerating voltages. The numbers at the left-hand side of the diagram represent the depth into sample in micrometers. The small square subdivided into smaller squares is a bar marker and each of the smallest squares represents an area of 100 nm^2 and is about the size of the ice crystals one might expect to find in the first 10 μm below the surface of a rapidly cooled bulk sample of biological material. This top diagram shows that one may confidently analyze subcellular compartments, including plant cell walls in frozen hydrated bulk samples at low kilovolts. Thus, at 6 kV, the interactive volume would remain inside a mitochondrion or a chloroplast and, at 4 kV, would be well within a plant cell wall. Note also that, at 6 kV, the beam-specimen interactive volume is just within the first 500 nm of the sample surface which is about the thickness of the semithin frozen hydrated sections which can be analyzed and imaged confidently in a scanning transmission electron microscope. The image on the bottom is a representation of what occurs in a frozen dried specimen. As the ice in the sample is removed by sublimation, the once dissolved elements are swept to the edges of the compartment and deposited on the now shrunken membranes. The original hexagonal shape of the cell outline is now deformed to an incurving hexagon. The density of the sample is variable and ranges from a high of 1300 kg/m^3 at the few places where the bio-organic material and attendant minerals are congregated, to a low of 600 kg/m^3 in the many places which were once occupied by the ice crystals. This decrease in average density has a profound affect on the size of the beam-specimen interactive volume which, on average, is five times greater for each of the comparable voltages. Thus, it would not be possible to analyze a single mitochondrion or chloroplast at 6 kV, although it should be possible to analyze a thick plant cell wall at 4 kV. Note the reduction in accelerating voltage needed to ensure that that beam-specimen fits within the first 500 nm of the sample surface.

Table 2. X-ray Emission Lines of Elements Associated with Biological and Organic Materials[a]

Voltage (kV)	Element	*K*α line (eV)	*L*α line (eV)	*M*α line (eV)	Experimental data
1	Carbon	262			
	Nitrogen	392			
	Calcium		341		
2	Oxygen	523			
	Chromium		571		
	Iron		704		
3	Copper		928		Bromine
	Zinc		1004		
	Sodium	1041			
4	Magnesium	1254			
	Selenium		1379		
	Bromine		1408		
	Aluminum	1487			
5	Silicon	1740			Silicon, aluminum, magnesium, sodium
6	Phosphorus	2015			Phosphorus
	Platinum			2051	Sulfur
	Gold			2123	
	Mercury			2195	
	Sulfur	2308			
	Lead			2346	
7	Chlorine	2622			Chlorine
8	Silver		2984		Potassium
	Cadmium		3133		
9	Potassium	3313			Calcium
10	Calcium	3691			

[a]Theoretical and experimental limits of quantitative X-ray microanalysis of elements of biological interest together with elements which are used during sample preparation, at voltages between 1.0 and 10.0 kV, assuming an overvoltage of 2.5 for the *K, L,* and *M* lines for each of the elements concerned. The column headed Experimental data includes the elements within a bulk organic matrix which have been quantitatively analyzed using energy dispersive spectroscopy to within ± 5% of the known value as measured by chemical analysis.

though a highly specialized form of analytical microscopy, offers great promise for the detection and imaging of specific atoms within macromolecular assemblies.

Another approach is being adopted by Nicole Grignon and her colleagues in France. They have been using NanoSIMS, a modern derivative of scanning ion mass spectrometry (SIMS) to study nitrogen transport in the leaves of higher plants. The technique of SIMS has been available for many years. The work of a student of Raymond Castaing, George Slodzian, was pivotal in the development of SIMS microscopy that involves bombarding samples, not with electrons, but with primary ions from such elements as oxygen or cesium. The secondary ions that are generated at the surface of the bombarded, thick or thin, sample are analyzed in a mass spectrometer and provide in situ chemical and structural information about the specimen. The technique works well with light elements and now offers the prospect of analyzing masses up to 10,000 D. Unlike either X-ray microanalysis or electron energy loss spectroscopy, SIMS can provide isotopic analysis. The spatial resolution is, at the moment, only 50 nm; that is not as good as the 10 nm of X-ray microanalysis and the 1–2 nm of the EELS technique. As with EELS and X-ray microanalysis, the way the biological samples are prepared is of critical importance to the quality of the analytical data. As yet, low-temperature techniques and instrumentation have not been applied to SIMS microscopy. Details of the instrumentation and applications of NanoSIMS can be found in two recent publications by Nicole Grignon and her coworkers (Grignon et al., 1999, 2000).

Alternative Approaches to In Situ Biological Microanalysis

The past 20 years have seen a marked change in the emphasis and focus of biological research. Microscopy and in situ X-ray microanalysis have moved from center stage and have given way to molecular biology as a means of obtaining chemical information about cells and tissues. The combined forms of carbon, hydrogen, oxygen, and nitrogen as molecules and macromolecules are generally of more interest than the individual elements. The techniques of immunocytochemistry, fluorescent and confocal microscopy, ratio imaging, and 3-D live time analysis are providing a wealth of chemical detail about biological systems. In some cases, these new techniques are more sensitive than X-ray microanalysis. For example, it is now possible to monitor, in real time, very low calcium level changes in cells with calcium-sensitive dyes in combination with laser scanning confocal microscopy. The need for high spatial resolution is considered less important than understanding the nature of dynamic processes.

The Way Forward for Biological X-ray Microanalysis

The future prospects for biological X-ray microanalysis can be considered both within the general future trends for X-ray microanalysis and as part of the perceived improvements which are likely to occur in biological sample preparation. There is every hope that there will be increased use made of hyperbaric cooling which we now know can produce the best preservation in hydrated biological samples. It is possible to vitrify hydrated samples to a depth of 200 μm and, with a little ingenuity, it should be possible to cut thin (100–150 nm) sections which could be freeze-dried and analyzed, and/or semithick sections which could be analyzed directly in the fully hydrated state. At the moment, ultrathin (30–80 nm) freeze-dried sections, which generally contain such small amounts of material, will only become a practical proposition when there is a further improvement in the collection efficiency of the X-ray detectors. Another possible way forward might be to use freeze-substituted samples which are proving so useful for immunocytochemical studies on small molecules, although great care will be needed to ensure the elements of interest stay at their natural location in the specimen.

The prospect of commercially available X-ray microcalorimeters will be a great asset for X-ray spectroscopy generally and biological X-ray microanalysis in particular. But in the meantime, other less grandiose, and much less expensive, improvements could be made to existing solid state detectors. Somlyo (1998) has outlined some of the hoped for improvements. These include improving the geometric efficiency of X-ray detectors interfaced to transmission electron microscopes, improvements to the way X-ray spectra from which the background has been removed are fitted to empirical filters and, finally, the quantification of the large number of X-ray counts originating from the organic background which forms the main part of biological samples. The author is of the firm belief that low voltages and low temperatures provide considerable promise for biological microanalysis. There is a need for further refinement of the analytical algorithms which are used in order that they are more applicable to light element analysis within a light element matrix. We also need a better understanding of the size and shape of the beam/sample interactive microvolume, although in this respect, David Joy, University of Tennessee (private communication) has provided the author with an algorithm that gives a measure of the size of the interactive volume, if not the shape. Finally, we need to understand more about the processes of radiation damage in bio-organic materials, particularly when using bulk samples. Unlike thin sections, through which, it is presumed, a large proportion of the beam energy passes without scattering, the bulk samples absorb all the beam energy which impacts onto the surface.

References

Appleton TC (1972) Dry ultrathin frozen sections for electron microscopy and x-ray microanalysis. *Micron* 3:101–105

Echlin P (1992) *Low Temperature Microscopy and Analysis.* New York: Plenum Press

Echlin P (1999a) Low-voltage energy dispersive x-ray microanalysis of bulk biological materials. *Microsc Microanal* 4:577–584

Echlin P (1999b) Low voltage energy dispersive microanalysis of bulk organic materials. In: *Microscopy and Microanalysis, Vol. 5, Suppl 2, Proceedings: Microscopy & Microanalysis '99, Portland, Oregon, August 1–5, 1999,* New York: Springer-Verlag, pp 312–313

Echlin P (1999c) Minimally invasive energy dispersive analytical spectroscopy (MIDAS). The golden touch for the in situ x-ray microanalysis of bulk bioorganic samples. In: *Microscopy and Mi-*

croanalysis, Vol. 5, Suppl 2, Proceedings: Microscopy & Microanalysis '99, Portland, Oregon, August 1–5, 1999, New York: Springer-Verlag, pp 574–575

Echlin P, Paden R, Dronzek B, Wayte R (1970) Scanning electron microscopy of labile biological material maintained under controlled conditions. *Scanning Electron Microsc* 1:49–56

Grignon N, Jeusset J, Lebeau E, Moro C, Gojon A, Fragu J (1999) SIMS localized measurements of the isotopic ratio. *J Trace Microprobe Tech* 17:477–490

Grignon N, Vidmarr JJ, Hillion B, Jaillard B (2000) Physiological application of the NANOSIMS 50 ion microscope: localization at the sub-cellular level of $^{N}15$ labelling in Arabidopsis thaliana. In: *Proceedings of the 12th International Conference on Secondary Ion Mass Spectrometry*. Brussels, September 5–10, 1999 (in press)

Hall TA (1968) Some aspects of the microprobe analysis of biological specimens. In: *Quantitative Electron Probe Microanalysis,* Heinrich KFJ (ed). Washington DC: Department of Commerce National Bureau of Standards Special Publication 298, pp 269–299

Hall TA (1979) Biological x-ray microanalysis. *J Microsc* 117:145–163

Ingram P, Shelburne JD, Roggli V, Lefurgey A (1999) Principles and instrumentation. In: *Biomedical Applications of Microprobe Analysis.* San Diego: Academic Press, pp 1–57

Joy DC (1998) The efficiency of x-ray production at low energies. *J Microsc* 191:74–82

Joy DC, Joy SS (1996) Low voltage scanning electron microscopy. *Micron* 27:247–263

Leapman R, Hunt JA (1991) Comparison of detection limits for EELS and EDXS. *Micro Microanal Microstruct* 2:231

Leapman R, Rizzo NW (1999) Towards single atom analysis of biological structures. *Ultramicroscopy* 78:251–268

Marshall AT, Xu W (1998) Quantitative elemental x-ray imaging of frozen hydrated biological samples. *J Microsc* 190:305–316

Moreton RB, Echlin P, Gupta BF, Hall TA, Weis-Fogh T (1974) Preparation of frozen hydrated tissue sections for x-ray microanalysis in the scanning electron microscope. *Nature* 247:113–115

Schultz M, Wendt-Gallitelli MF (1998) Improved quantitative electron probe microanalysis of biological cryosections using an EDS germanium detector and a super-atmosphere thin window (ATW). *J Microsc* 190:293–297

Somlyo AP (1998) The impact of biological microanalysis on analytical electron microscopy. In: *Microscopy and Microanalysis, Vol. 4, Suppl 2, Proceedings: Microscopy & Microanalysis '98, Atlanta, Georgia, July 12–16, 1998,* New York: Springer-Verlag, pp 170–171

Taylor CP, Weber ML, Gaughan L, Lehning EJ, LoPachin RM (1999) Oxygen/glucose deprivation in hippocampal slices: altered intraneuronal elemental composition predicts structural and functional damage. *J Neurosci* 19:619–629