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ELEMENTAL MICROANALYSIS OF BIOLOGICAL SPECIMENS

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Abstract

Although X-ray microanalysis in the electron microscope is the most common method for microanalysis of biological specimens, other methods of elemental microanalysis (electron energy loss spectroscopy, scanning Auger microanalysis, and proton, ion, and laser microprobe analysis) may provide important complementary information and help overcome some of the limitations of electron probe X-ray microanalysis. Despite differences in physical principles and instrumentation, the various microanalytical methods have much in common with regard to specimen preparation, quantitative analysis, and interpretation of analytical data. A common approach to microanalytical problems in the biological sciences, irrespective of the analytical techniques used, seems therefore indicated.

KEY WORDS: Electron probe X-ray microanalysis, electron energy loss spectroscopy, electron spectroscopic imaging, scanning Auger microanalysis, proton microprobe analysis, ion microprobe analysis, laser microprobe analysis, biological specimens, specimen preparation, elemental mapping.

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Introduction

The interaction of electrons with matter does not only allow visualization of minute structures in the electron microscope, but also provides information about chemical and physical properties of the specimen on a microscale. The most common method for microanalysis in the electron microscope at present is energy-dispersive X-ray microanalysis, in which the information from the X-rays excited in the specimen is used to determine its elemental composition. Biologists have now over 15 years of experience with this analytical method and during this period preparative and quantitative methods suited for biological specimens have been developed.

Simultaneously, other techniques for elemental analysis in the electron microscope have been developing to the point where they have become available to more than a handful of specialized laboratories. Electron energy loss spectroscopy and Auger microanalysis are now routinely carried out in the materials sciences. It is therefore reasonable to consider the application of these techniques to biological specimens.

Elemental analysis on a microscale is, however, not restricted to electron beam instruments. The proton microprobe uses the X-ray spectrum generated by a beam of high energy protons (Johansson et al. 1970). The ion microprobe uses the secondary ions from the specimen, generated by bombardment with a primary ion beam. The laser microprobe uses irradiation with a laser beam to ionize (part of) the specimen; the ions generated are analyzed in a mass spectrometer.

The basic principle of all these techniques is that a small part of the specimen is irradiated; changes in the primary irradiation or a secondary signal evoked in the specimen by the primary irradiation are used to obtain information on the elemental composition of the irradiated part of the specimen. This secondary signal can consist either of radiation from the sample (in the "non-destructive" techniques, e.g., electron probe or proton probe microanalysis) or of small fragments of the sample (in the "destructive" techniques, e.g., ion and laser microprobe analysis).
All microanalytical methods are different in physical principle, instrumentation and performance in terms of spatial and spectral resolution and sensitivity. This can be used to select the most adequate microanalytical technique for the particular problem at hand. However, the various techniques also have much in common, and experiences gained with one type of microanalysis can be fruitfully applied to other types. Good examples of such 'cross-fertilization' can be found in the literature, but the full potential of this view remains to be realized.

This paper is concerned with the application of microanalytical techniques to biological specimens. Since comprehensive recent reviews of the different techniques discussed are available, it is not our purpose to describe each technique in detail. Rather, we will attempt to present a unified concept of biological microanalysis, review the strengths and weaknesses of the various techniques for analysis of biological samples, and identify general and technique-specific problems of interest to biologists.

Features of Microanalytical Techniques

Spatial resolution

By definition, microanalysis deals with the analysis of small volumes (and should not be confused with trace element analysis, which by definition is the analysis of low concentrations irrespective of the size of the sample). The spatial resolution depends on the type of specimen, and the values given in Fig. 1 are valid for analysis under optimal conditions. In discussing spatial resolution one has to distinguish between lateral resolution and depth resolution. Lateral resolution depends on the diameter of the primary beam, and in some instances on scattering of the primary beam in the specimen. In some cases, technical problems prevent as yet the production of narrower beams, and technical developments in coming years may improve the lateral resolution of a particular technique. Such a development has, in recent years, taken place for the proton microprobe (Vis 1985, Malmqvist 1986). However, narrow beams may decrease the signal generated and negatively influence sensitivity. Spatial resolution and sensitivity cannot, therefore, be seen as separate characteristics of a particular technique. Depth resolution depends on the penetration of the primary beam in the sample and/or on the maximum escape depth of the secondary radiation. In general, basic physical principles (rather than technical problems) are involved and improvements much more difficult than in the case of lateral resolution.

Visualization

Although high resolution is the hallmark of a microanalytical technique, spatial resolution without the possibility of visualizing and identifying the microstructure analyzed is of little value. Visualization is dependent on (a) the instrumentation used to visualize the specimen during analysis and (b) the preparation of the specimen. In the electron beam-based techniques (X-ray microanalysis, EELS, Auger micro-

Fig 1: Lateral and depth resolution of microanalytical techniques. Data from Colliex (1986), Malmqvist (1986), Vis (1985), and Linton et al. (1988).
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Mapping

Related to the issue of spatial resolution, visualization, and sensitivity is the possibility to prepare elemental maps (Somlyo 1985). One way of preparing elemental maps is to have the beam scan over the specimen and to relate the analytical signal from the specimen to the position of the beam on the specimen. Elemental maps are an excellent way of obtaining a survey of elemental distribution at the cell or tissue level. In X-ray microanalysis, energy windows are set that correspond with the elements of interest. A digital elemental map can be prepared by determining the intensity of the signal in the energy window(s) of interest for each pixel. A basic complication is, that the energy window not only includes the characteristic signal, but also the background noise. Therefore, the accuracy of this method is highest at high peak-to-background ratios. For biological samples, where the peak-to-background ratio in electron probe X-ray microanalysis generally is rather low, elemental mapping has not reached the level of usefulness that this method has in materials sciences. However, with the advent of faster computers, it has become possible to correct more accurately for the background. Another problem is the long dwell time per pixel necessitated by the relatively weak signal: apart from the long analysis time needed, specimen stability may become a limiting factor. Nevertheless, the intrinsic value of mapping techniques is such that it is worthwhile to further develop their potential and the application of elemental mapping in electron probe X-ray microanalysis will increase. In proton probe microanalysis, where the peak-to-background ratio is much higher than in electron probe microanalysis, mapping should - albeit at lower spatial resolution - become a useful method. Here, technical difficulties associated with the scanning of the proton beam appear to be the main problem to be resolved.

In EELS, elemental mapping can also be carried out in STEM by acquisition of spectral data at each pixel in an image (reviewed by Leapman et al. 1985). Alternatively, a TEM instrument of the type used by Ottemeyer and coworkers (e.g., Ottemeyer et al. 1981), and which is now commercially available can produce elemental maps (electron spectroscopic imaging, ESI). The problems associated with elemental mapping in EELS are mainly due to difficulties in the determination of the background level and are discussed in more detail below.

A similar duality exists in ion microprobe analysis (Burns 1982). One type of instrument ('ion microprobe' uses a small diameter primary beam which is scanned over the sample. The second type of instrument ('ion microscope') uses a large diameter beam; the secondary ions generated are all directed to the mass spectrometer where they are separated on the basis of mass/charge, while still retaining the spatial configuration they originally had on the sample.

Specimen preparation

For all methods of elemental microanalysis, the aim of specimen preparation should be to retain the in vivo distribution of the element(s) of interest at the level of the spatial resolution of analysis. Much experience has been gained in studies using electron probe X-ray microanalysis and a number of critical comparative studies have been carried out. The present consensus in this field can be summarized as follows:

1) conventional fixation methods for light or electron microscopy leads to loss and/or redistribution of all but the very firmly bound elements from the tissue, and are therefore often unsuitable for analytical work (Morgan 1979). An exception to this rule is the identification of solid inclusions in tissue, which is frequently used in diagnostic pathology (Baker et al., 1985).

2) the best general methods to prepare specimens for microanalysis are cryomethods: rapid freezing of unfixed tissue, followed by cryosectioning, or alternatively, by freeze-drying or, possibly, freeze-substitution with subsequent embedding and anhydrous sectioning. Although cryosectioning in most cases is the preferred method, freeze-drying/ embedding and

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Quantitation

Although occasionally the qualitative demonstration of a particular element is sufficient (e.g., in the case of toxic heavy metals), there is an increasing demand for quantitative techniques. At present, the development of quantitative methods for microanalysis of biological specimens has reached different levels for different microanalytical techniques. In general, the signal from the specimen is compared with that of a standard containing the element(s) of interest in a known concentration. Corrections for differences in overall composition between specimens and standard ('matrix effects') have to be applied and may severely complicate quantitation. It is therefore useful to prepare the specimen in such a way that it resembles the specimen in relevant physical and chemical properties. Standards similar to those developed for electron probe X-ray microanalysis (Roomans 1979) can be used for other microanalytical techniques as well (Burns 1982, Forslind et al. 1985a, Vis 1985).

Electron probe X-ray microanalysis

When a target is irradiated with a high-energy electron beam, electrons in the inner shells of target atoms may be ejected, leaving an incompletely filled inner shell. This situation is unstable and may result in a transition of an electron from a higher shell to fill the vacancy. In this process, energy is liberated in the form of an X-ray with an energy equal to the energy difference of the shells between which the electron transition took place (Fig. 3). This energy is characteristic for the atomic species involved, and is used to identify that species. The background noise is due to retardation of the beam electrons in the field of the nucleus; this gives rise to a continuum X-ray spectrum.

In energy-dispersive X-ray microanalysis, the X-rays are detected by a Si (Li) semiconductor detector. Normally, this detector is shielded from the electron microscope by a thin beryllium window. This window absorbs low-energy X-rays and the lightest element detectable is Na (Z=11). However, windowless detectors are available with which also elements down to carbon (Z=6) and even boron (Z=5) can be detected. A method used less commonly with biological specimens, wavelength-dispersive analysis, uses crystal spectrometers with which the lightest detectable element is boron.

Analysis can be carried out on thin sections or, with sacrifice of resolution, on thick specimens. Except for the analysis of very firmly bound elements, cryopreparation methods are obligatory. The specimen is visualized before/during analysis in the electron microscope (generally SEM or STEM). Quantitative methods are relatively well developed.

Electron probe X-ray microanalysis has during the last decade developed into a near routine method in electron microscopy. Given the availability of a suitable electron microscope, the analytical equipment is only moderately expensive. A number of problems can be investigated using preparative techniques that are routine in the electron microscope laboratory. The simultaneous detection of all but the light-
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The sensitivity of the method is its low sensitivity. This is particularly evident in studies on, e.g., effects of heavy metals on cells and tissues, where the subcellular distribution of the (toxic) heavy metal has to be investigated. Generally, only those structures in which the metal is accumulated such as lysosomes (Cleton et al. 1986) or special storage granules in invertebrates (Morgan and Winters 1987) contain sufficiently high local concentrations for X-ray microanalysis; low levels in, e.g., cytoplasm or nucleus cannot be measured. Experimental studies often require the presence of unrealistically high levels of toxic metals in the surrounding medium. The level of physiologically important elements such as calcium is, in a number of cells or cell compartments, close to the detection limit of the analytical method.

Although the spatial resolution of electron probe X-ray microanalysis, especially in thin sections, is sufficient for many purposes, occasionally it may become a limiting factor when detailed analysis of small subcompartments or membranes is needed. Nevertheless, electron probe X-ray microanalysis appears today the overall most powerful technique for the combined study of structure and function. In particular in investigations on quantitative localization of the major cellular ions (Na, K, Cl, Ca, Mg) the possibility for direct correlation of X-ray microanalysis with other physiological and (bio)chemical methods is of great importance.

Electron energy loss spectroscopy

The energy loss suffered by the beam electrons in the process of excitation of target atoms (Fig. 3) is the basis of electron energy loss spectroscopy (EELS). The principles of EELS and its application to biology have recently been reviewed (e.g., Leapman et al. 1985, Shuman and Somlyo 1987, Jeanguillaume 1987, Simon and Heng 1988). The energy loss suffered by the beam electrons in the process of excitation of target atoms (Fig. 3). The energy loss spectrum is obtained (Fig. 4) where the X-axis is related to electron energy (loss). Generally, such a spectrum is obtained sequentially by scanning the dispersed electrons over a slit placed in the focal plane behind which an electron detector is placed. A newer and potentially more powerful technique is parallel detection of the transmitted electrons.

Equipment for EELS therefore includes, apart from the electron microscope (TEM or STEM) a spectrometer; the signal from the spectrometer can be processed in a conventional multi-channel analyzer, such as used for energy-dispersive X-ray microanalysis (Leapman et al. 1985). The spatial resolution of EELS is, as in electron probe X-ray microanalysis, dependent on the interaction volume of the electron beam with the target. An important requirement in EELS is that the high-energy electrons suffer maximally one inelastic scattering event on their way through the specimen. Multiple scattering gives rise to a very complicated pattern of energy losses from which the contribution of single scattering events can only be deduced with great difficulty, if at all. This means, that only very thin specimens can be investigated in EELS (for biological specimens a thickness below 40 nm is optimal), and as a consequence, the spatial resolution of analysis is high.

The requirement for thin samples poses, however, considerable technical problems. It is extremely difficult to prepare dry-cut cryosections of unfixed, uncryoprotected material with a thickness of 40 nm or less and only exceptionally has success been claimed. Plastic embedded material can be sectioned to the required thickness, if the sections are cut on water. However, for meaningful analysis of diffusible ions, plastic-embedded (freeze-dried or freeze-substituted) material has to be cut with a thin knife, and under those conditions sufficiently thin sections can, in our experience, not be prepared with presently known techniques. Wet-cut sections of freeze-dried embedded material are, of course, not suitable for analysis of diffusible ions, even though Simon and Heng (1988) recently reported that calcium could be retained in such a procedure. A further problem is the presence of the Formvar support film that is used when cryosections are examined under the electron beam: the support film adds to the total thickness of the sample, and further decreases the thickness allowed for the cryosection itself. If possible, carbon films should be used. In addition, the support film may contain elements of interest (C, N, O). The development of computer programs to correct for multiple scattering (Swetye and Leapman 1982) may eventually help to solve some of these problems; with these techniques, combined with the thinnest possible cryosections, there appears to be at least some hope that EELS can be used for analysis of the distribution of diffusible elements in biological tissue (Leapman 1986, Ornberg 1986). At present, most of the EELS analyses of biological samples have dealt with the occurrence of the main elements occurring in the samples. According to Colliex (1986) a mass fraction of 0.5% is necessary to obtain an EELS signal for a particular element and this means that apart from C, N, and O, one can generally only expect signals for P and K in the EELS spectrum of biological samples, unless, of course, the element of interest locally exceeds the minimum concentration. On the other hand, Shuman and Somlyo (1987) recently reported that the sensitivity of EELS for Ca could be five times better than that of electron probe X-ray microanalysis, even though it was very difficult to achieve this. It is therefore not surprising that, apart from C, N, and O, most results reported for EELS of biological samples have dealt with the distribution of P and high local concentrations of Ca.

With regard to the analysis of C, N, and O-elements that cannot be detected by conventional electron probe microanalysis, although they can be detected with a windowless or thin window spectrometer - a major problem is the changes in
the concentrations of these elements occurring during the analysis by mass loss and/or contamination of the specimen. Comparison of the few quantitative EELS data on light elements in biological specimens published (Jean Guillame et al. 1983) with the known composition of the organic matrix of biological tissue shows marked differences. This also agrees with our experience. Analysis at low temperatures may be the solution to this problem, but some of the dedicated STEM instruments used for EELS cannot (yet) be equipped with a cold stage. At present, EELS data on light elements in biological tissue must be viewed with suspicion.

Elemental mapping by EELS, either in a STEM where an energy loss spectrum (or part of it) is accumulated for each pixel point (Lebman et al. 1985), or in a TEM configuration as applied by Ottenscheyer and coworkers (Ottenscheyer and Andrew 1980, Ottenscheyer et al. 1981, Ottenscheyer and Arsenault 1983), or by Shuman and Somlyo (1982) is evidently a potentially very interesting technique.

Because of the low peak to background ratio in the energy loss spectrum (Fig. 4), a single energy-filtered image (corresponding to a single energy region in an X-ray spectrum) cannot be used at all. To correct for the background, two principally different methods have been used: a two-image method (one 'background' region) (Ottenscheyer et al. 1981), and a three-image method (two 'background' regions) (Jean Guillame et al. 1978). As argued in several publications (e.g., Jean Guillame et al. 1983, Lebman et al. 1985) the three-image method is to be preferred on theoretical grounds.

To compare the two methods on the same sample, it was attempted to make a phosphorus distribution map of a sample in which EELS analysis could not detect significant amounts of phosphorus. The two-image method of Ottenscheyer et al. (1981) shows an apparent phosphorus map (Fig. 5) whereas only a random distribution of points could be obtained by the three-image method, as was expected from the measured absence of phosphorus from the analyzed part of the sample. Comparison with the Z-contrast image and the dark-field STEM image leads to the conclusion that the two-image method is sensitive to local differences in mass and may mistake mass distribution for elemental distribution.

Scanning Auger Microanalysis

The inner shell ionization in atoms of the specimen caused by irradiation with high energy electrons can result either in the production of a X-ray (used in X-ray microanalysis) or in the emission of an Auger electron, leaving the atom doubly ionized (Fig. 3). The energy of the Auger electron is characteristic for the element from which it is emitted. Since the energy of the Auger electrons is low, only those produced at shallow depths can escape. As is also evident from Fig. 1, Auger analysis is a typical surface analysis. However, since modern Auger microscopes are provided with an ion beam source, with which a surface layer can be sputtered away with a controlled speed, depth profiles of elemental distribution based on the Auger spectrum can be measured. Auger microanalysis permits analysis of all elements from Li (Z=3) on (Fig. 4).

Auger microanalysis is mainly used in the materials sciences with wear and fracture studies as the dominant type of application. Its use in biology is at present mainly anecdotal (Janssen and Venables 1979). Auger analysis can be carried out on sections of embedded biological tissue, placed on a small copper electropolished or rhodium disc; the contact between section and disc should be good to prevent charging. Contrasted sections provide an acceptable SE/BE-image for localization of the analysis (although the contrasting, of course, may compromise the chemical integrity of the specimen). If surface contamination is suspected, one can try to remove the surface layer. The method described here was used in a study by Hofsten et al. (1984) to analyze nitrogen distribution in a nitrogen-fixing blue-green alga Anabaena cylindrica, and in analysis of eye lens crystals (Fig. 4).

Proton Microprobe Analysis

In the proton microprobe, the specimen is irradiated with a high energy proton beam generated in an accelerator. Characteristic X-rays are produced and detected in much the same way as in electron probe X-ray microanalysis. However, the continuum produced by proton irradiation is much lower than that produced by electrons. The improved signal to noise ratio makes for a much more sensitive technique. Drawbacks of proton microprobe analysis are its lower resolution (although some instruments may reach the 1 µm level) and the difficulties in visualization. The difference in analytical resolution is less serious if one compares proton and electron microprobe analysis of thicker samples. Since the proton beam spreads much less than the electron beam, the analytical resolution of the two techniques is comparable if e.g., histological sections or mounted whole cells are analyzed. If a 1 µm proton beam is used, the resolution of the proton microprobe with thick specimens is even better than that of the electron microprobe.

Generally these sections or cells are mounted on or between thin plastic films that do not contribute to the spectrum. For analysis of diffusible elements cryomethods have often been applied: the low resolution of analysis in most instruments has allowed the use of sections cut on a conventional cryostat, which is a fairly simple method. Most studies have dealt with the distribution of (toxic) trace elements e.g., metals. Recent reviews by Vis (1985) and Malmqvist (1986) show the impressive possibilities of the method as applied to biological problems. Elements such as Al, Ti, Cr, Mn, Cu, and Zn, which often cannot be measured by electron probe X-ray microanalysis (because their concentration in the tissue is too low) can routinely be determined by the proton probe. Experimentally or environmentally induced heavy metal concentrations can also be measured much easier with the proton probe than with the electron probe.
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Fig 4: (a) EELS, (b) EDX, and (c) Auger microanalysis of crystalline inclusions in rabbit eye lens. Conventional (glutaraldehyde) fixation and embedding in epoxy plastic; ultrathin sections cut on water. All analyses were carried out under standard operating conditions for each instrument.

It should be realized that the proton microprobe is a relatively new instrument and that most proton microprobes available were developed around 1980. Not all proton microprobes available are used for biomedical work. However, as more instruments become available and experience with this technique increases, it may supplement electron probe analysis and even replace it for low-resolution analysis. Also the prospects for quantitative analysis are good. As in electron-induced X-ray production, the characteristic intensity is dependent on the amount of a particular element in the irradiated part of the sample. To convert amount to concentration, a measure of local mass is needed, which may be obtained from the Bremsstrahlung background originating from secondary electrons, from the number of backscattered protons, or from the proton energy behind the target. Although much work still needs to be done, initial results indicate that proton microprobe analysis can be as accurate as electron probe microanalysis (Forslind et al. 1985a). Absolute quantitation can be achieved with the help of standards with similar properties as those used in electron probe microanalysis (Vis 1985, Forslind et al. 1985a).

Fig 5: Comparison of two methods to obtain EELS elemental images: (A) P image obtained by recording and subtraction of two inelastic images (Ottenmeyer et al. 1981), (B) P image obtained by recording of three inelastic images (Jean-guillaume et al. 1978, 1983), (C) Z-contrast image, and (D) annular dark-field STEM image. Specimen phosphorus-free region (as ascertained by EELS) of crystalline inclusions in rabbit lens.

Ion Microprobe Microanalysis

In secondary ion mass spectrometry (SIMS) a primary ion beam is focused on the specimen surface, resulting in the production of, among other fragments, positive and negative ions from the surface layer of the specimen. The secondary ions are extracted from the specimen and analyzed in a mass spectrometer. Not only elements, but also isotopes can be distinguished. As mentioned previously, mapping is possible and a two-dimensional ion image of the specimen can be obtained. In addition, by sputtering away subsequent surface layers, a depth profile of the sample can be obtained. This can be used in studies where one wants to investigate the penetration of a substance from the surface into the tissue (e.g., Lodding 1983). However, the sputtering yield is not homogeneous over the specimen surface and this may complicate the interpretation of depth profiles (Linton et al. 1980).
Many ion microprobe studies have dealt with mineralized tissues (Lodding 1983, Galle et al. 1983). However, specimen preparation presents little problems. Most studies with ion microprobe analysis on soft biological tissue have used conventionally fixed and, e.g., resin-embedded or paraffin-embedded material (Linton et al. 1988), either because the problem investigated lent itself to the use of such material, or because the authors overlooked the disadvantages of the conventional specimen preparation regime. Elemental maps showing the distribution of Na in aldehyde-fixed tissue impress with the sensitivity of the technique but not with their biological relevance. Comparative studies of specimen preparation techniques for ion microprobe analysis (Stika et al. 1980) confirmed the superiority of cryotechniques for analysis of diffusible ions, which had already been firmly established in electron microprobe analysis. Recently it was demonstrated that ion microscopy of frozen-hydrated biological samples (cell cultures) was feasible (Chandra et al. 1986).

Some practical difficulties may be encountered in the analysis of cryosections (e.g., it is necessary to ensure good contact between specimen and substrate), but Chandra et al. (1986) successfully carried out SIMS of 2 µm cryostat sections. In addition, Burns (1982) has demonstrated the usefulness of sections of freeze-dried and embedded material, so there should be a definite possibility of carrying out ion microprobe analysis without compromising the tissue by unsuitable preparation methods. The problem of visualization remains one of the great stumbling blocks especially in instruments where the specimen has to be viewed through low-power light optics. The use of secondary electron image (Levi-Setti et al. 1985) is a very promising development in this area.

Even though ion microprobe analysis in itself is a fairly sensitive technique, it may be possible to further increase its ability to detect low levels of elements in biological tissue by low temperature ashing of the specimen, which increases the surface concentration of the elements of interest (Brenn and Morrison 1984). Because of the complex physical processes taking place during sputtering of the samples, quantitative formalisms are not well developed. Originally it was reported that with standards containing known amounts of the element of interest (similar to those used in electron probe X-ray microanalysis) useful standard curves could be obtained (Burns 1982). However, it appears that the matrix effects are so severe that it may be difficult to compare e.g., gelatin-based standards to tissue (Burns et al. 1986).

**Laser Microprobe Microanalysis**

The laser microprobe (LMP) is akin to the ion microprobe, but a laser beam instead of a primary ion beam is used to generate the secondary ions. Originally, laser microprobe analysis was exclusively applied to thin sections, but later instruments are developed that also could analyze bulk specimens. Several instrumental configurations with somewhat different properties, but all based on the same principle are commercially available: LAMMA (Laser microprobe microanalysis) (Kaufmann et al. 1979), LIMA (Laser ionization mass analysis) (Southon et al. 1984), and LPMS (Laser probe mass spectrometry) (Chamel and Eloy 1983). The high sensitivity of the technique has provoked an emphasis on studies on the distribution of trace elements or toxic metals occurring in quantities lower than those that can be measured by electron probe microanalysis. Specimen preparation should follow the principles laid down for other analytical techniques: use of cryopreparation techniques is indicated unless the element of interest is very firmly bound. Since LAMMA analysis of cryosections has not been successful, freeze-dried or freeze-substituted, embedded, and sectioned tissue appears to present the most suitable type of specimen (Meyer zum Gottesberge-Orsulakova and Kaufmann 1985). An extensive review of LAMMA as applied to biological problems can be found in Verbeken et al. (1985). Visualization is a problem, since the specimen has to be viewed by low-power light optics during analysis, with a resolution of no better than about 2 µm. One can, however, 'reconstruct' the analysis by studying the position of the holes left by the laser beam. In a few instances where quantitation has been attempted, standards of the same type as those used in the electron probe X-ray microanalysis have been used.

**Other Microanalytical Techniques**

In this review it has been attempted to cover the most commonly used microanalytical techniques and their application to biological problems. However, in recent years other analytical techniques have been developing a potential for high-resolution analysis. Experience with some of these techniques is as yet very limited, mainly because of limitations in the number of instruments available. Mention should here be made of the fact that now subcellular resolution (albeit in large cells) can be obtained by nuclear magnetic resonance (NMR) (Aguayo et al. 1986).

Synchrotron radiation provides a high-intensity stable source of X-rays, and permits the selection of a desired bandwidth of X-rays. By scanning a biological sample with a sub-micron spot of monochromatic soft X-rays (1-10 nm wavelength), and using the abrupt changes in absorption coefficient of a particular element (absorption edge) an elemental image with sub-micron resolution can be obtained (Kenney et al. 1985, Panessa-Warren 1986). Although a number of problems needs to be solved, the fact that this technique can be used on hydrated samples under atmospheric pressure, makes absorption-edge imaging with synchrotron radiation a potentially very useful technique for microanalysis of biological samples. Harder X-rays focussed to relatively large spots (about 10 µm) allow very sensitive microanalysis by X-ray fluorescence.
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Conclusions

The review demonstrates that a single basic strategy with respect to specimen preparation and quantitation (in particular of standards) is adequate for many different types of microanalysis in biology. This also opens the possibility for correlative microanalysis, where several techniques can be used for analysis of one sample (see e.g., Kupke et al. 1984, Malmqvist et al. 1984). Application of techniques that overcome the limitations of conventional electron probe X-ray microanalysis in biomedical research is at present limited by (1) the inadequacy of specimen visualization techniques in relation to analytical resolution, (2) communication problems between biomedical researchers and physicists specialized in using the analytical techniques, and (3) the limited availability of instrumentation for biomedical research.

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Discussion with Reviewers

K. Malouf: You conclude that the use of other techniques than electron probe X-ray microanalysis (EPXMA) is limited by, e.g., specimen visualization. Do you think that new instruments combining electron microscope technique and any of the other techniques would be feasible to construct?

W. Jacob: You say that one can "reconstruct" the analysis by studying the position of the holes left by the laser beam. Because of the destructive nature of the LAMMA method, this seems a rather difficult task. Is examination before and after LAMMA and combination with cytochemistry not a better way to interpret the analysis morphologically?

Authors: To a certain extent even EPXMA is limited by specimen visualization, in particular in those cases where analysis is performed on uncontrasted (cryoprepared) sections. The problems are, however, even more serious in techniques such as proton, ion and laser microprobe analysis. In proton and ion microprobe analysis the use of the secondary electron signal may allow much better visualization that the present use of light optics. Whether an instrument combining laser microprobe analysis and electron microscopy would be technically feasible is difficult to say. Examination of the section in the electron microscope before and after the analysis, combined with a very accurate coordinate system to determine the exact location to be analyzed might be a more practical way to go. The feasibility of examining the sections before the analysis may be limited by contrast problems (if diffusible ions are to be analyzed the sections cannot be stained or contrasted) and by possible radiation damage during the preliminary examination. Also the use of serial sections, of which one would be used for analysis and the other would be stained or contrasted may be considered.