3.9: Measurements of Total Calcium Concentrations

The measurement of total calcium in a biological sample can be made by any method sensitive only to the element and not to its particular chemical form. Atomic absorption spectroscopy is excellent as such a method. Obviously, the spatial resolution that can be obtained with this method is limited, and it is hard to imagine its application to elemental mapping of single cells. The techniques discussed in this subsection have been limited to those that permit a spatial resolution of at least 1 μm on samples usually prepared by sectioning the frozen biological specimens.

Electron Probe and Electron Energy-loss Techniques

When the electron beam in an electron microscope hits a thin sample, some atoms in the sample will be excited or ionized, and returning to their ground state will emit characteristic x-rays. The x-ray emission at different wavelengths may then be measured by a photon-energy-sensitive detector. This is the basis of electron probe x-ray microanalysis (EPMA). The electrons that pass through the sample, and that give the transmission image in electron microscopy, will suffer energy losses that depend on the nature (to some extent also, the chemical state) and distribution of different elements. The outcome of these phenomena forms the basis of electron energy-loss spectroscopy (EELS; see Figure 3.6).

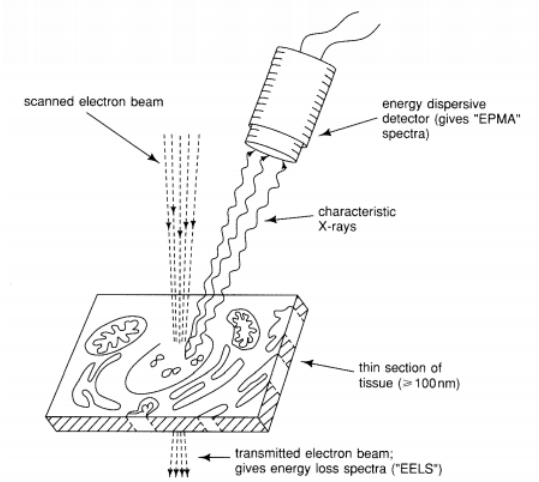

Figure 3.6 - Principles of electron probe microanalysis ("EPMA") and electron energy loss spectroscopy ("EELS"). A thin slice of a freeze-dried sample is exposed to a collimated beam of electrons that may be scanned across the sample. Atoms ionized by electron collisions will emit x-radiation at wavelengths characteristic of their nuclear charge (i.e., characteristic of each element). With the use of an energy dispersive x-ray detector, signals from different elements can be differentiated. Apart from the characteristic x-rays, a broad-spectrum background x-ray emission is also present because of inelastic scattering of the incident electrons. Some of the electrons that pass through the sample will have lost energy because of ionization of atoms in the sample. The energy loss is again characteristic for each element, and if the energy distribution of the transmitted electrons is analyzed, this will have "peaks" at certain characteristic energies. If the energy resolution is pushed far enough < 1 eV), the loss peaks even become sensitive to the chemical state of the element.

The EPMA technique as applied to calcium has been improved by Somlyo in particular.[30] Typically samples are rapidly frozen and sectioned at low temperatures (-130 °C) to preserve the *in vivo* localization of diffusible ions and molecules. Spatial resolutions of 10 nm or better have been attained on ≳100 nm thick freeze-dried cryosections. The minimal detectable concentration, which requires some signal averaging, is approximately 0.3 mmol Ca per kg dry specimen (i.e., 10 ppm). The calcium content of **mitochondria** and **endoplasmic reticulum** in rat liver cells has been studied by EPMA (see Table 3.1).[8]

The high calcium content of endoplasmic reticulum (ER) is consistent with the view that this organelle is the major source of intracellular Ca^{2+} released through the messenger inositol trisphosphate (see Section IV.C). Other EPMA studies have shown mitochondria to have a large capacity for massive calcium accumulation in cells where cytoplasmic Ca^{2+} concentrations have been abnormally high, for example, as a result of damage of the cell membrane.[30]

EELS is presently less well-developed than EPMA. Two of the major difficulties in the use of EELS for quantitative analysis of calcium and other elements are (i) large background, since it is a difference technique, and (ii) sensitivity to specimen thickness. The major advantage of EELS is that the spatial resolution is potentially much better than in EPMA, and can be 1 to 2.5 nm in favorable specimens.

Proton-induced X-ray Emission (PIXE)

A specimen exposed to a beam of high-energy (1 to 4 MeV) protons will also emit characteristic x-rays just as in EPMA. The advantage of using protons instead of electrons is that protons are more likely to collide with an atom, thus producing excited atoms emitting x-rays. The sensitivity in detecting a particular element is therefore much higher in PIXE than in EPMA or EELS. The PIXE technique, which was developed at the University of Lund, Sweden, in the late 1960s, was originally used mainly for studies of fairly large objects.[9]

In 1980 a group at Oxford University succeeded in focusing the proton beam to a diameter of 1 μm with sufficient energy (4 MeV) and beam intensity (100 pA/μm^2) to allow elemental mapping at ppm concentrations.[31] Similar beam performances (~0.5 μm diameter) are now also available at the University of Lund and other laboratories. Beam diameters of 0.1 μm are likely to be achieved in the near future. Like EPMA, the PIXE method allows the simultaneous observation of several elements in the same sample. The biological applications of the microbeam PIXE technique are limited, but it is clear that its potential is great. Some representative results obtained with the Oxford microbeam are shown in Figure 3.7. (See color plate section, page C-8.)

Ion Microscopy

Ion microscopy is another technique capable of detecting all elements at the ppm level. The basic idea is to expose a freeze-fixed, cryofractured, and freeze-dried sample, which has been put onto a conducting substrate in a vacuum chamber, to a beam of ions (e.g., D_2^+ or Ar^+). These ions will remove the top two or three atomic layers of the sample surface by sputtering. A certain fraction of the removed atoms will leave as ions. This secondary ion beam is accelerated into a double-focusing mass spectrometer, where the ions are separated according to their mass-to-charge ratio. The ion optics are designed to preserve the spatial distribution of the emitted secondary ions, and an element image of the sample can thus be produced with a spatial resolution of ~0.5 μm.[32] The ion-microscope technique can form images of a particular isotope of an element. In principle, then, one could perform isotope labeling or "isotope chase" studies and follow, say, the fate of isotope-enriched ^{43}Ca externally applied to a cell. The ion-microscope technique has not yet come into widespread use, but the quality of element (or ion) images obtained on single cells is impressive.[33]