Is analysis of biological materials with nm spatial resolution possible?

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Abstract. Cells are bounded by a membrane, the plasma membrane, subcompartments within cells are also delineated by membranes, these membranes contain transporters that regulate the flow of ions across them. Fluxes of ions across the membranes underlie many of the basic properties of living material such as excitability and movement. Breakdown of membrane function ultimately leads to cell death. EM microanalysis has been instrumental in gaining understanding of how changes in element distribution affect cell behaviour and cell survival. The main problem that biologists face in undertaking such studies is that of specimen preparation. Cells consist mainly of water that needs to be either removed or stabilised before analysis can take place. Cryotechniques, fixation by rapid freezing followed by sectioning at low temperatures and freeze-drying of the sections have proved to be a reliable method for the study of intracellular element concentrations. These techniques have been used to show that elements are confined in different compartments within cells and produced results to support a new theory on the mechanism by which neutrophils kill bacteria. They have also shown that disturbance of the ionic content of mitochondria is one of the first signs in the pathway to cell death.

1. Introduction
The overall theme of this meeting is investigations at the nanoscale level. In this context I will address two questions, is analysis on the nanoscale relevant in biological materials and is it feasible? I will restrict my discussion to my own field of research that of cellular physiology, but this is not meant to ignore other areas such as biomaterials, the interface between a biological and a materials component such as occurs for instance in orthopaedic implants, where microanalysis is becoming increasingly important.

2. Relevance of microanalysis in cell physiology
Cells are the basic units of structure in the tissues that make up our bodies. Cells are bounded by a membrane, the plasma membrane, which is approximately 7nm in thickness, and acts as a barrier between the cell and the outside world. Transporters in the membrane regulate the flow of ions, and water, across the membrane and, in doing so, influence both cell function and cell volume. Intracellular organelles such as mitochondria, inclusion granules, and vacuoles are also membrane bound and also contain ion transporters so that these act as separate compartments within the cell. Microanalysis allows the investigation of elemental content in individual cells within a tissue and individual organelles within a cell without recourse to the usual methods of homogenization and cell fractionation that can cause disruption of the normal concentration gradients.
3. Specimen Preparation

Analysis at high spatial resolution requires the use of thin sections. Biological materials normally have a high water content (60 – 70 %), this water needs to be removed or stabilized before examination in the electron microscope. Specimens are usually fixed, dehydrated and embedded in resin before sectioning and the sections stained before viewing. These procedures do not usually affect components that are tightly bound within the cell, but remove the mobile ions, the elements that are of interest to physiologists (Fig 1). Cryopreparation techniques are used to overcome these problems[1]. Small pieces of specimen are fixed by freezing at rapid rates (> 5000º C/sec) so that ideally the intracellular water is vitrified without the formation of ice crystals that disrupt intracellular structure. These are then sectioned at low temperatures and usually freeze-dried before analysis in the electron microscope. The detail in sections obtained using cryopreparation methods is sufficient to allow analysis of different compartments within cells. Analysis of blood samples has confirmed that there is no redistribution of ions between plasma and red cells when these techniques are followed [2].

Fig 1. The effects of specimen preparation on elemental content in heart tissue. In (A) heart tissue has been prepared using chemical fixation followed by dehydration and embedding in resin. A typical spectrum taken from this material (C) shows the presence of Os, used as a fixative and U used as a stain. In (B) heart tissue has been prepared by cryofixation. Although the structural preservation is not as good as that achieved by conventional methods, the main features of the tissue including striations (z-lines) and mitochondria (M) can still be seen. A typical spectrum (D) shows the presence of the physiologically important elements Na, Cl, K. Marker = 2µm.
4. Applications

4.1 Analysis of neutrophils

Neutrophils are a class of white blood cells whose function is to destroy invading bacteria. For this purpose they possess granules that are membrane bound and which contain proteases, enzymes that are capable of degrading bacteria. In the granule these proteases are in an inactive form. X-ray microanalysis of individual granules shows that the elemental content of the granule differs substantially from that of the adjacent cytoplasm (Fig 2). When bacteria are engulfed by neutrophils the granules fuse with the bacteria and their destructive contents are released. In collaboration with workers at University College London [3] we have shown that the mechanism underlying this killing activity is pumping of K ions across the membrane into the vacuole containing the bacteria. The resulting change in ionic strength causes the release of the granule proteins allowing destruction of the bacteria.

Fig 2. A cryosection through a neutrophil is shown in (A). These cells contain a nucleus (N) with surrounding cytoplasm that is packed with dark granules (G) that contain proteases. Analysis of a granule (lower spectrum) and the adjacent cytoplasm (upper spectrum) shows that the two areas differ in elemental content. Marker 1µm.

4.2 The process of cell death

In animals cell death is important both in development and in adult life. Cells can die by one of two mechanisms necrosis or apoptosis. Necrosis is an uncontrolled method of death usually caused by external insult in which the cell swells and bursts, whereas in apoptosis, otherwise known as
programmed cell death, the cell goes through a defined pathway in which the nuclear material is broken down and eventually the cell shrinks. There is a great deal of interest in this process since the ability to control cell death could be useful in medical treatments. The shrinkage of cells during apoptosis implies a loss of control of cell volume. X-ray microanalysis has been used to study elemental content in cells in culture undergoing cell death [4,5]. The principle findings were that the cells lost K and Cl within minutes of UV-irradiation indicating that the mechanisms underlying shrinkage are stimulated very early in the process. Analysis of the individual cellular compartments nucleus, cytoplasm and mitochondria, showed that mitochondria were affected earliest. Loss of Cl and K from this organelle occurred well before any morphological changes appeared (Fig 3) and before enzymatic changes could be detected.

Fig 3. Changes in the elemental content in the mitochondria of U937 cells undergoing UV-induced apoptosis. The mitochondria (dark spots) of were analysed in a Zeiss EM10C in STEM mode using a 50 nm probe. In the mitochondria from control cells (A) spectra showed that the organelles possessed high levels of K, P and Cl (C). By 15 minutes after irradiation K was decreased and Cl had almost disappeared (D) even though the cellular morphology had not changed. Marker, 1µm.

5. Conclusions
The applications given above demonstrate the requirement for analysis at high spatial resolution in biological specimens. Both examples required information about the elemental content of intracellular organelles. Over 20 years ago Somlyo’s group showed that the generally used method of isolating organelles and then studying their elemental content lead to erroneous results [6]. In the examples given here (neutrophil granules and mitochondria of monocytes) the information provided by X-ray microanalysis could not have been obtained easily by the use of any other technique. However, when applying X-ray microanalysis to biological specimens it is essential to bear in mind that results
obtained are from the specimen **as it exists in the microscope** and these do not necessarily reflect the situation in *vivo*. It is essential to ensure that no movement of elements occurs during preparation procedures, and the onus is on the investigator to undertake sufficient controls to ensure that no such translocation occurs.

**References**

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