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NEW APPLICATIONS OF THE NUCLEAR MICROPROBE FOR BIOLOGICAL SAMPLES

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Abstract

The continuing development of the Nuclear Microprobe (NMP) has opened up new fields of applications in biology and medicine. Quantitative multielemental analysis in small sections of samples can be performed routinely. The use of techniques such as scanning transmission ion microscopy makes imaging as well as mass normalization possible at submicron resolution. Recent medical applications include studies on thin cryosections prepared from autopsies and biopsies, as well as single cells grown directly on the backing foil used in the NMP analyses. The purpose of the single cell analysis is often pharmacological, e.g., testing of new drugs, their uptake and distribution. New applications, for instance, in food chemistry, ecology and evolutionary genetics, are also taking advantage of the high analytical sensitivity of the NMP in combination with its imaging capability.

Key Words: multielemental, microanalysis, microprobe, medicine, single cell, particle-induced X-ray emission (PIXE), scanning transmission ion microscopy (STIM), dermatology, retinal damage, micro-tomography.

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Introduction and Background

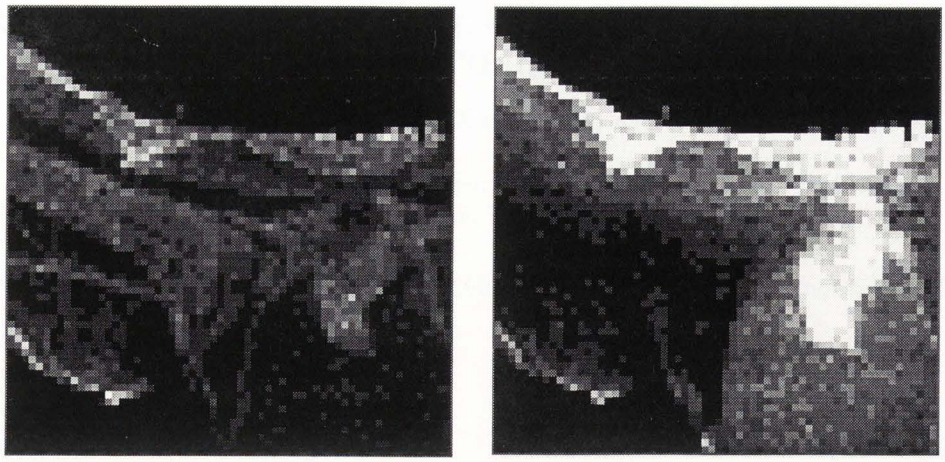
Among microanalytical techniques, the nuclear microprobe (NMP) has established itself as a powerful research tool for quantitative analysis of the elements (Watt and Grime, 1987; Vis, 1985; Johansson and Campbell, 1988; Tapper and Malmqvist, 1991). The applications in biology and medicine are numerous and new fields are ready to be opened (Lindh, 1993).

The main advantage of a nuclear microprobe is the possibility, using one instrument only, to obtain information on both the structure of a sample and its quantitative composition at a trace element level and at a reasonable cost.

The main drawback of the instrument - from a biologist's point of view - may be that it is a research instrument, normally operated by a physicist or technician, and that the number of available microprobes in the world is limited, at present about 30. An in-house instrument is thus not often available and for a researcher intending to use the NMP a close cooperation with the instrument specialist is needed.

As in other microanalytical techniques, many pitfalls exist for the inexperienced user. In order to avoid the many potential problems and to produce results of high quality, a set of quality controls in all steps should be in place: the selection of the relevant medical/biological problem, the choice of samples and their relevant numbers, the selection of suitable preparation techniques, choice of irradiation parameters to the final evaluation and interpretation of the data.

The NMP can offer quantitative maps of most of the elements, *nota bene* with varying sensitivity. Using Particle Induced X-ray Emission (PIXE) for the detection of elements heavier than Al (Na, Mg also possible) [see Johansson and Campbell (1988)], complementary methods such as back-scattering (Chu *et al.*, 1978) to detect carbon, oxygen and nitrogen, Nuclear Reaction Analysis (NRA) for selected elements, and forward scattering to measure hydrogen, virtually the whole periodic system is measurable. Scanning Transmission



Potassium Chlorine

Figure 1: The chlorine and potassium distributions used for imaging the sample. The scan size is 600 μm.

Ion Microscopy (STIM) may be used both for imaging and mass normalization (Lefevre *et al.*, 1987). New techniques such as ionoluminescence (IL) (analogous to cathodoluminescence) may contribute information on molecular status (Yang *et al.*, 1993).

Imaging the Sample

An important technical problem is the imaging of the sample in order to find the morphological structures of interest for irradiation. Histochemical techniques such as staining and similar methods, used to make structures in the sample visible, are normally incompatible with trace-element analysis, as other elements are added to the sample. In some cases it may be possible to go the opposite way; to stain after the analysis. However, the ion beam irradiation often breaks molecular bonds, leading to a smearing out of structures, thus obstructing the imaging. In addition, imaging after the analysis can only serve as a information of what was analyzed, but offers no possibility of feedback.

For some types of sample, it is possible to prepare parallel sections and use one section for element analysis with the NMP and the adjacent one for imaging under a (light) microscope. The limit to this method is given by the fact that the thickness of the section must be smaller than or at least comparable with the feature of interest, e.g., cells, structures of the cell, etc. It must also be put in relation with the spatial resolution of the NMP, see Discussion below.

A direct feedback is given if the ion beam itself is allowed to create the image. For some samples the distributions of the elements measured by the NMP can reveal some of the structure, i.e. potassium or phosphorus can indicate cell features (figure 1). Element mapping using the backscattering information on carbon, oxygen, nitrogen or a combination of these, often yields even better information, as normally a greater number of scattered ions than of X-ray photons can be detected from the sample. The backscattering map, apart from being a help for imaging, can be used to calculate the areal mass density, which in turn is used for concentration determinations (figure 2). Secondary electron imaging, as in electron microscopy, is also possible.

One of the methods that gives the highest contrast is STIM, where the energy loss of the ions as they pass through the sample is measured and used to modify a grey scale (Overley *et al.*, 1987; Lefevre *et al.*, 1987; Sealock *et al.*, 1987). Due to the small number of ions required, a beam resolution below 1 μm is easily achieved. From the STIM-image, conversion to areal mass density can quickly be done and used to calculate element concentrations (figure 3). A useful procedure is therefore to start the analysis by using STIM to evaluate the morphology, select a suitable part and then increase the beam current for PIXE analysis.

Medicine

Dermatology

The epidermis of the human skin is a stratified cellular structure, less than 100 μm thick. The stratified nature of the epidermis and its differentiating cells makes the use of ordinary physiological probes inappropriate for measuring its ion contents. Using freeze-quenching for fixation followed by cryo-sectioning and freeze-drying provides inert preparations for element analysis with particle probes. Electron microprobe

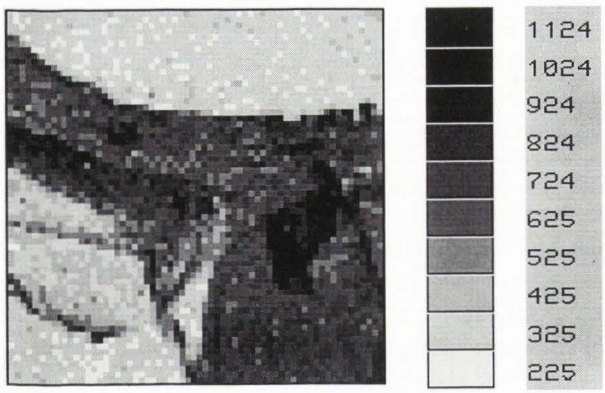


Figure 2: Backscattering used for imaging and areal mass density measurement. The scale displays the areal mass density in $\mu g/cm^2$. The scan size is 600 μm.

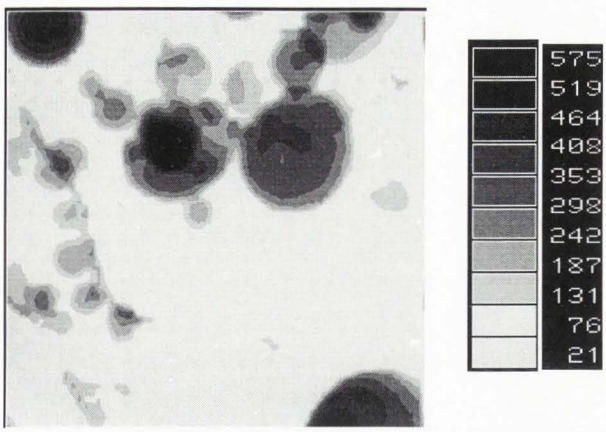


Figure 3: Using STIM to image a potato section, showing the starch granules. The scale shows the corresponding areal mass density (500 = 5 mg/cm^2). The scan size is 136 μm.

(EMP) analysis has made possible the analysis of some of the elements; for instance Na, P, S, Cl and K (Wei *et al.*, 1982; Forslind *et al.*, 1983). With the NMP it was possible to also determine the distribution of physiologically important elements such as Ca, Mg, Fe and Zn (Malmqvist *et al.*, 1984a). It was demonstrated that the two techniques (EMP and NMP), are indeed complementary (Malmqvist *et al.*, 1984b; Forslind *et al.*, 1984). From a practical and economical point of view, the EMP can be chosen for the study of Na, K, Cl, S and P. When detection of elements close to the detection limit of the EMP was required, or for trace elements such as Fe and Zn, the NMP was the best or only choice.

Early studies of elements in the normal skin revealed that the elements Ca, Fe and Zn were located mainly in the basal cell layer where cell division takes place. Above this region, the elements were not found in measurable amounts. However, preliminary and repeated studies on psoriatic skin revealed that (Zn) and Fe had an abnormal distribution with unexpectedly high values in the cell layers above the basal cells and even up in the stratum corneum (Lövestam *et al.*, 1988; Pallon *et al.*, 1993).

Brain damage

There has long been a controversy concerning the role of aluminum in relation to Alzheimer's disease. Watt and Landsberg (1993) report on the finding of alumino-silicates in many reagents. This colloid could become bound to the senile plaque, which is known to be sticky, and thus give a false signal of aluminum in the plaque. From the analysis of 80 unstained plaque cores using STIM, backscattering and PIXE, the results show no evidence of aluminum in the plaque cores at a detectable level of 15 ppm. From their work these authors suggest that the role of aluminum in Alzheimer's disease should be revised, taking into account the possibility of contamination from alumino-silicates present in most reagents.

Retinal damage

The nuclear microprobe has proven to be a sensitive tool for the detection of early retinal damage (Chen, 1993). It has been observed clinically on a number of groups of people that long-term exposure to strong (blue) light causes retinal damage. Among these groups are a) aged people, b) people who have had their natural lenses replaced by plastic ones, due to cataracts and c) eye surgeons operating under a microscope using strong artificial light sources. People with blue eyes as compared to those with brown, suffer more frequent retinal damage, while the group having implanted plastic lenses seems to be most sensitive, as the plastic lens does not have the same filtering capacity as the natural one (which is slightly yellowish).

The nuclear microprobe (PIXE) was used to investigate the redistribution of ions (potassium and chlorine) in the retina on research animals exposed to light in a controlled way (Chen, 1993). Retinal sections were analyzed using biochemical methods, while cryosections with a thickness of 15-20 μm were prepared and analyzed by the NMP. Thus it was possible to detect alterations in the distribution of other elements which might be related to retinal degeneration, such as calcium and copper. These can not be detected by particle probes other than PIXE due to their low concentration levels. This preliminary study, showed that there was an accumulation of chlorine, an indication of sodium accumulation, in the inner segments after exposure to blue light.

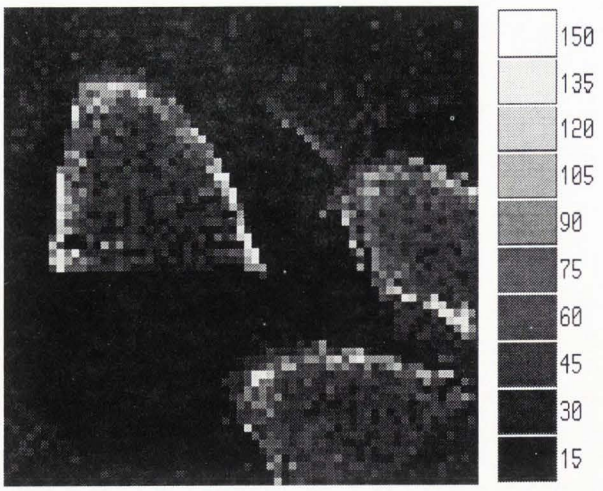


Figure 4: The aluminum distribution in thin sections of onion roots, cultivated in the laboratory. The aluminum is mainly located at the section borders. Scan size 0.8 mm.

This cellular damage was followed by photoreceptor edema and photoreceptor injury. The exact conditions for developing a retinal damage are still unknown, and thus no limits for exposure have been established. The energy-dependent Na/K-ATPase on the cellular membrane of the inner segment of the photoreceptors regulates ion gradients across the membrane. This gradient is important for retinal function. Inhibition of the cellular metabolism by exposure to blue light reduces the activity of Na/K-ATPase, redistributes the ions and causes cellular edema. A severe cellular edema leads to cellular degeneration, causing retinal damage.

Single cells

Many studies in experimental biology involve the human cell and are aimed at the study of the cell functions in vitro under normal conditions or the effect of molecular species on cell metabolism. Microanalysis of subcellular distribution of the elements is a field in which progress is called for. Detecting intracellular essential or toxic elements involves a compromise between spatial resolution and sensitivity. The NMP offers reasonably high sensitivity and resolution to make possible element analysis on the cellular or subcellular level.

Moretto *et al.* (1993a,b) discuss experimental procedures of cellular and subcellular quantitative mapping on cell cultures grown on thin Formvar film. Having managed to grow living cells on the film, the next step is to prepare the cells for vacuum analysis. The main purpose was to investigate the effect of pharmacological drugs on cancer cells, in this case cisplatin used in ovarian and testicular cancer. The drug was applied to an adeno-carcinoma cell line and compared to a selected drug-resistant sub-population. The point was to localize the cell nucleus in order to assess the spatial repartition of the drug between nucleus and cytoplasm. Also different molecules, known for their ability to bind to DNA were tested for nucleus labelling. For example, iododeoxyrubicin was compared to cisplatin. After incubation, platinum was found homogeneously distributed, while iodine was located in a narrow region assumed to be the nucleus. The potentials of PIXE microanalysis are extremely well suited to the analysis of isolated cells.

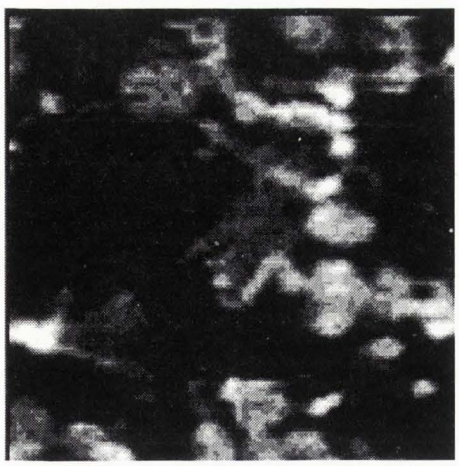
Another example of single cell NMP analysis is given by Cholewa *et al.* (1993). In the search for efficient drugs against AIDS, organometallic and inorganic drugs are being tested for their efficiency on cultured human T-lymphocytes. In addition to the light elements normally found in drugs, these drugs also contain atoms of heavy metals (Co and W). These atoms, apart from their biological function, can act as tags to identify the drugs. By NMP analysis on single T-lymphocyte cells the location of the drug can be assessed, whether it is taken up by the cell, is bound to the cell nucleus, or remains outside the cell. Due to the toxicity to normal cells the heavy metal concentration in the drug must be kept low, and thus the EMP can not detect these elements. In this first report it is shown how the nucleus can be identified by its content of phosphorus and that cobalt and tungsten are found inside the cells.

A beam size of 1 μm was used, with the beam intensity limited to 30 pA owing to the risk of radiation damage to the cells. The detected concentration of tungsten was much higher than the detection limit of the NMP, which is still a factor of 4 lower than the toxic level. The risk of radiation damage was demonstrated by first using STIM to yield an image of the cell, then by performing PIXE analysis using a more intense beam, and finally by imaging the cell again using STIM. In this procedure, the cell was found to have shrunk by about 10% during the PIXE analysis.

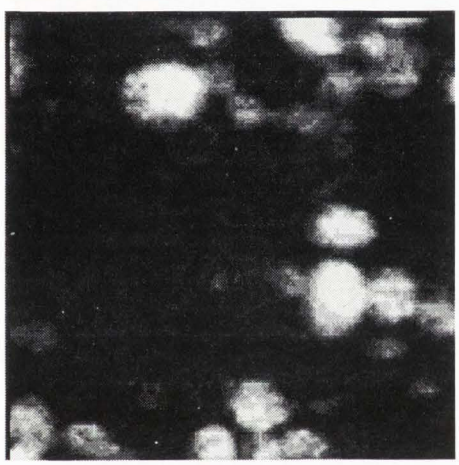
These two examples illustrate the possibilities of the technique in analyzing layers of single cells grown or deposited on thin media.

Botany

The decrease in forest growth and large scale forest dieback in central Europe is related to the increased emission of acidifying gases and other air pollutants. The vitality of the trees is most certainly affected by both direct exposure and changes in the soil chemistry



a



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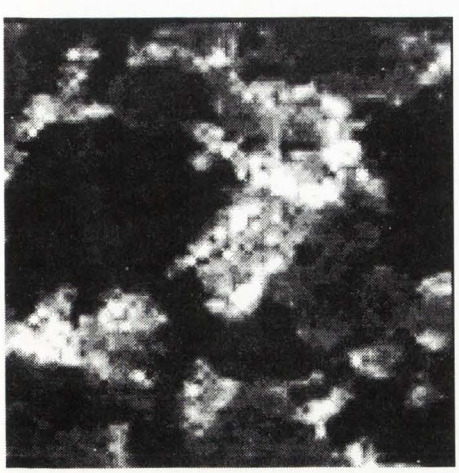

c

Figure 5a: The potassium distribution in a 20 μm thick section of potato. Note the high contents in the starch granules. **b**) The backscattering image of the same section, proportional to the areal mass density. **c**) The potassium concentration. Note that the concentration of K in the starch granules is not as high as indicated in figure 5a. The images in figure 5 are smoothed to give a more detailed look.

due to acidification. Aluminum is often pointed out as a possible correlator between soil acidification and inhibition of growth and reduced vitality of trees. The growth and vitality are affected by the ability of the roots to take up and process minerals. NMP analyses reported by Hult *et al.* (1992) on root samples from beech, cultivated in the laboratory in media containing $AlCl_3$, show that aluminum is taken up and distributed in an asymmetrical way in the root. Aluminum accumulated in the epidermis and the outer layers of the cortex. It also effectively depleted calcium in this region. There may be a genetic difference in resistance to Al, which is observed in some plants. In order to further investigate these phenomena, the location of elements in plant material has been investigated. In figure 4 the distribution of Al in sections of onion root tips, cut along the tip, is shown in which the accumulation of Al along the border of the root tip can be noted. A more detailed study in progress (in cooperation with the Swedish University of Agriculture, and with the Department of Genetics at the Lund University) indicates that aluminum actually accumulates in the extracellular space rather than intracellularly.

A relatively new technique associated with the NMP and especially with STIM is micro-tomography, which uses the highly focused ion beam to create three-dimensional images of small samples. These images not only display the 3-D mass density of a sample, but also the 3-D composition of major elements (PIXE tomography). The technique is an analogue to electron beam tomography used on samples of nm size. However ion-beam tomography can be used on samples with a diameter of 10-100 μm. In a recent study Schofield and Lefevre (1993) discuss the possibility of analyzing the uptake of different elements in the environment by selected insects, and thus being able to use them as biological monitors without having to section them. Using micro-tomography it is possible to see where in the insect the uptake has taken place. The tomography technique is time-consuming both concerning the time required for the analysis and for the computer reconstruction and should thus be restricted to selected cases.

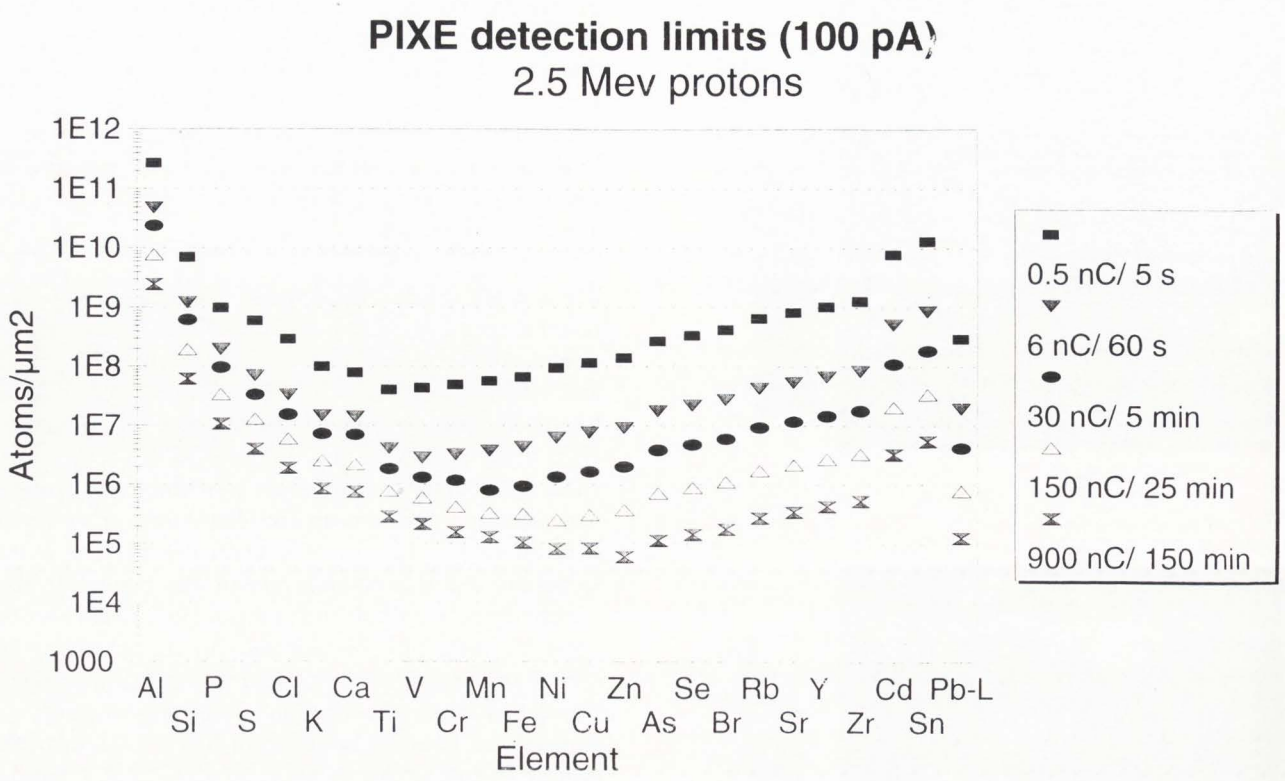

Figure 6: The PIXE detection limits of a NMP using 2.5 MeV protons at 100 pA with a 78 μm Mylar foil as X-ray absorber. All values except for Pb are based on K X-rays.

Applications in Food Science and Engineering

Elements in the ionic form such as K^+, Ca^{2+}, Mg^{2+}, Cl^- play an important role for the nutritional, textural and overall quality of vegetables. With the aim to identify the dominant modes of transport of small molecules through more or less intact biological material and the effect of different treatments (thermal, mechanical) on the transport, the NMP has been used in a pilot study (together with the Department of Food Engineering at the Lund Institute of Technology). Due to the low concentration of the elements, especially Ca, the NMP had to be used for the analysis. As a model system, potato treated by blanching was chosen. From the mass transport point of view, potato tissue may be considered as a multiphase structure consisting of vacuoles, cytoplasm containing the starch granules, cell walls and intercellular spaces. Preliminary results show a clear difference between starch granules and non-starch cell contents (figure 5). The amount of K and Ca is high in both areas, but the ratio of both K and Ca to total mass is much lower in starch than in non-starch, as expected. In samples treated with $CaCl_2$, the Cl content is significantly higher and located between the cells.

Discussion

Discussing the analytical sensitivity, one must remember the inverse relation between the sensitivity of the NMP and the resolution obtainable. The smaller the beam, the higher will the detection limits be. Thus, in relation to a given biological problem, a decision must be made based on the technical performance of the NMP, namely, the minimum beam size for which an analytically usable beam current can be produced. Is it 0.5, 1, 2, 5 μm or larger? This choice is closely related to the number of samples one will be able to process within a certain, limited time, which in turn is related to the possibility to answer the specific biological question.

Figure 6 shows the detection limits of PIXE at the Lund NMP, but these can be taken as typical of any NMP (factors of 2-4 may occur depending on detector solid angles). Data are taken from measurments on thin medical sections, with a 2.5 MeV proton beam, and using a 78 μm Mylar absorber in front of the X-ray detector. In the calculation, a beam current of 100 pA has been used- a value typical of what can be obtained

when producing a μm-sized beam. The curves are obtained from fitting spectra with the charges stated in the figure, i.e., between 0.5 nC and 900 nC. For each given charge, the corresponding irradiation time is also given, assuming a beam current of 100 pA. The time values can, of course, be recalculated assuming a different value of the beam current.

The inherent sensitivity can thus be used for the analysis of small areas containing fairly high concentrations, in a short time, small areas with low concentrations over a long time, or a combination of these.

The span in detection limits in figure 6 is typical of what is achieved for the scanning analysis of any single spot (0.5 nC) when the whole scanned area is irradiated with 900 nC. This means that, in each single spot, the detection limits will follow the 0.5 nC curve, but if all data collected, here corresponding to 1800 single point spectra, are merged together, the detection limit will follow the 900 nC curve. Of course a single spot can be given any charge with a non-moving beam, thus achieving low detection limits; on the other hand, the important strength of the NMP is the scanning mode. The detection limit roughly scales as the square-root of the accumulated charge.

To arrive at the optimum sensitivity at the point in the sample where the interesting information is located, one must first find that point, and then irradiate it with as much charge as possible without destroying the information.

In the study by Cholewa *et al.* (1993), one single cell was irradiated in the scanning mode with a one-micron beam and given a total charge of 200 nC. The detection limits achieved will be just below the 150 nC curve of figure 6, and it is interesting to express the limit as the number of atoms/μm^2 that can be seen, as the dimensions of the cell nucleus are of the order of μm. Scanning a cell for two hours will thus permit detection of 5.10^6 atoms/μm^2 of a foreign element added to the cell. Achieving this sensitivity using much smaller beams is not possible at present but, as discussed by Legge (1993), development of brighter ion sources in combination with optimized beam lines may in the future push this resolution limit down by a factor of at least 10, possibly more, which will open up new fields of applications. However, the question arises - what type of beam damage will occur in the sample, assuming that there is a corresponding increase in beam current density of a factor of 100? There is a risk that the dose given to the sample will set a limit to the beam density which is lower than the future development of the NMP would actually permit, thus restricting element analysis to above a given resolution.

Acknowledgement

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Discussion with Reviewers

G. Legge: For the detection limits of figure 6, what criterion has been used to determine the limit?
Authors: The limit is $3\sqrt{B}$ where B is the background in an energy interval equal to 2 full width at half maximum (fwhm) around the peak energy, or 10 pulses, whichever is the largest.

G. Legge: What is the geometrical efficiency (or solid angle) assumed for the X-ray detector in the calculation of the detection limits?
Authors: The solid angle of the detector is 0.3 sr.

U. Lindh: Have you tried to stain sections after irradiation? The trials I know of until now have failed.
Authors: Yes, but with no success.

U. Lindh: In your study of retinal damage you indicated an accumulation of sodium in the inner segments. Do you have indications that the accumulation was not due to artefacts in the specimen preparations?
Authors: This study included both exposed retinas and un-exposed controls, showing a statistically significant difference.

U. Lindh: You have found indications that aluminum is accumulated in the extracellular space rather than intracellularly in root tips. Have you discussed the possible biological consequences of this finding?
Authors: No, those preliminary results are based on too few samples to allow for biological speculations.

U. Lindh: What is meant by blanching?
Authors: It is a rapid heat treatment (70-80 °C) of the sample with the purpose of deactivating certain breakdown enzymes.

U. Lindh: If the beam current density will be limiting the use of nuclear microprobes in biology, development of brighter ion sources will not provide a solution. Are there other ways of improving the detecting conditions than developing more efficient detectors subtending larger solid angles?
Authors: This is not an easy task. The physics of the PIXE-process is given and can not be changed, the solution might be to accept beam damage of the sample but have it under control or at least monitored. As data acquisition in an NMP analysis often takes place in a time sequence (recording data event-by-event) this is technically possible. A brighter and smaller beam spot will permit thinner sample sections to be used, which reduces the amount of energy deposited thus counteracting beam damage. Beam blanking may also be incorporated to reduce the damage, this is especially important in the case of high count rates where a large dead time is likely to occur in the detector system.