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APPLICATION OF NUCLEAR MICROLOCALIZATION
TECHNIQUES TO BIOMEDICAL PROBLEMS*

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Abstract

Ion beams at the Brookhaven 3.5 MV Research Van de Graaff accelerator have been used for elemental analysis and distribution in biomedical samples. This paper will present results from several collaborations. Both collimated and uncollimated charged particle beams are used for elemental analysis by measurement of characteristic x-rays (PIXE). A collimated proton beam, using a pinhole collimator ($\sim 20 \mu\text{m}$) has been used as a particle microprobe in the laboratory ambient. Thick, essentially unprepared, samples can be measured with general elemental sensitivities of $< 10 \text{ ppm}$. The spatial resolution and elemental sensitivity have proven adequate for many samples of tissue sections and cell clusters. Specimen damage by charged particle beams is discussed and results of cell irradiations by triton beams are presented.

Deuterium localization has been carried out in cell uptake studies using the $^2\text{H}(^3\text{H},n)^4\text{He}$ reaction initiated by the triton beam at the accelerator. Alpha particles from the reaction register the deuterium distribution in a plastic track detector. This technique suggests that ^2H may be replaced by the stable isotope ^1H in tracer studies. Studies have included the detection of nonexchangeable ^2H in oocytes and the uptake of deuterated thymidine in blood cells.

Proton Microbeams

The usefulness of highly collimated beams of heavy charged particles, mainly protons, with beam spot dimensions approaching one micron, was first demonstrated by Cookson¹ and has been recently well summarized.² The technology of proton microbeams has been further developed by many active groups,²⁻⁹ several of which have made significant demonstrations of the utility of the proton microprobe to the life science community. Horowitz and Grodzins^{3,6} have been particularly motivated by biological problems because they do use an external beam of modest current density in a convenient mode of operation. The development of the Melbourne scanning proton microprobe by Legge and Mazzolini¹⁰ illustrates growing interest and dedication to biological problems.

Many samples from the life sciences in which detection or localization of specific trace or minor elements is of interest require extensive careful preparation for analysis by conventional techniques such as electron microscopy or atomic absorption. Techniques in which the measurement is made in vacuum require sample dehydration by lyophilizing or critical point drying. Any sample preparation introduces the possibility of sample loss or contamination. The proton microprobe which allows the beam to exit the accelerator beam pipe and be available in the laboratory ambient circumvents many problems of target or sample preparation. A slight trade-off is made giving some sensitivity and spatial resolution for this advantage.⁸ Also, an external beam prevents some flexibility in beam scanning systems and beam movement has been provided by movement of the sample past an essentially fixed beam.^{5,6,8}

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The proton microprobe system we have used has been previously described.¹⁵ The proton beam emerges through a "pinhole" at the end of the beam pipe after being peaked in intensity by a quadrupole and momentum analyzing bending magnet. No window is used as vacuum is adequately maintained by differential pumping as shown in holes as large as $75\text{--}100 \mu\text{m}$. The surprise and distraction of window breakage common in early work using kapton windows is therefore avoided. Tests with collimators of diameters ranging from 12 to $100 \mu\text{m}$ indicate that vacuum conditions up to the $356 \mu\text{m}$ collimator can be maintained below 10^{-3} mm for collimators as large as $50 \mu\text{m}$ in diameter. External beam currents of $\sim 0.5 \text{ nA}$ for a $12 \mu\text{m}$ collimator, 3 nA for $25 \mu\text{m}$ collimator and $\sim 30 \text{ nA}$ for a $\sim 150 \mu\text{m}$ collimator (used with a window) are typical values which require more than $1 \mu\text{A}$ of beam incident on the final internal collimator ($356 \mu\text{m}$), about 5 cm distant from the Si(Li) detector.

A word should be mentioned about the pinhole itself, which is of importance to the low background response of the system. Heavy metal apertures, typically Mo and Pt, which have adequate thickness to stop the 2.5 MeV protons, are available from electron microscopy supply houses in a wide range of hole sizes. Unfortunately, sufficient beam is still present on the final collimator to produce copious K and L x-rays from the collimator edges and inner surfaces which are then scattered into the detector causing an enormous background. Collimators of lower atomic number, carbon and aluminum, have proved to exhibit low background and can be obtained from EM suppliers on special order. Graphite collimators (made by either drilling or spark-cutting) have not withstood beam fluence as well as aluminum collimators. Recently an aluminum collimator of $150 \mu\text{m}$ diameter, requiring a $12 \mu\text{m}$ aluminum window, has proved useful for coarse spatial resolution with high beam current and elemental sensitivity.

An optical microscope has proved useful as a simple and direct tool to view and position samples after the beam spot has been centered in the field of view by observing a thin ZnS scintillator inserted just in front of the sample. The elemental sensitivity using a fine collimator, $12.5 \mu\text{m}$, has been shown to⁸ be as low as $\sim 10 \text{ pg}$ or $\sim 10 \text{ ppm}$ for the most detectable elements having $17 < Z \leq 30$; however the sensitivity of the larger, $150 \mu\text{m}$, Al covered collimator is somewhat higher.

Further improvement in spatial resolution or beam current density of this system by the addition of beam focusing elements is believed to be unwarranted due to ambient vibration caused by the direct coupling of beam lines to the machine and the lack of solid support. In order to accommodate experiments and systems requiring better spatial resolution a dedicated low vibration beam line has been constructed and is shown in Fig. 1. The beam pipe is separated from the machine by a bellows at the analyzing magnet and is suspended from box beam "gantries" anchored to the building subfloor. Suspension from the gantries is by inflatable air bags* and the geometry as shown

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*Firestone Corporation, Akron, Ohio.

Figure 1. Low vibration beam line

keeps the center of suspended mass well below the suspension points. Additional acoustic damping in series with the air bags is provided by a honeycombed fiberboard-epoxy table that couples the beam pipe itself to the suspended frames. Two sets of precision tungsten defining slits are shown on either side of the vacuum system which can be used to define a parallel beam or an object for subsequent beam optics.

Near the end of the beam line the several cables carrying high voltage denote a BTL-type electrostatic quadrupole triplet¹¹ for demagnification of $\sim 10\times$ at a focal distance about 12 cm from the quadrupole element exit face. The construction details of this lens system have been simplified using "Macor"[†] machinable glass and stainless steel rods for elements while retaining the basic electrostatic design. Preliminary tests to date have produced a beam spot of $\leq 10\text{ }\mu\text{m}$ observed by video monitor on the final sapphire window with a beam current of 3 nA. Accelerator energy stability may not be adequate to permit demagnification to the few micron regime; however use of the lens will augment available beam current in the $10\text{ }\mu\text{m}$ region. Previous measurement of pressures in the differential pumping system suggests that pressures are low enough ($< 10^{-3}\text{ mm}$) to permit an external beam from an aperture following the electrostatic lens.

Specimen Damage

The trend of focused proton microprobes to higher beam current densities together with the generally accepted requirement that the sample be in vacuum places increasingly great stress on the integrity of the sample. Electron microscopists have a general awareness of the state of the sample from preparation and introduction into the sample chamber to its condition during and after measurement. Consideration of sample condition has been expressed in recent publications;^{2,10,12} however more attention will be required as deleterious beam effects could well be the limiting parameter on the sensitivity and usefulness of the proton microprobe for many biological samples.

Cookson² has reviewed a typical case reported by Talmon and Thomas¹³ of a single red blood cell placed on an aluminized formvar support film. The calculation of sample heating was made for a small area bom-

barded with electrons in vacuum but transposed to protons by Cookson by changing the specific ionization. For a beam current of 100 pA "scanned uniformly" and presumably focused to cellular dimensions, it is found that negligible heating occurs in either the cell itself or the entire cell holding system. However this result relies critically on the thermal conductivity of the 100 Å aluminum evaporation which is unusual for many sample preparations. If no metallic conductor is used on a $\sim .0003$ in thick organic film (nylon, kapton), an overall temperature rise to a target on a suspension system of 100 μm in diameter is 59°K and would be greater for larger sample suspension diameters (often $> 1\text{ mm}$). Beam current densities for these remarks are estimated from the given 100 pA beam current and a beam area of $\sim .1$ that of the $8\text{ }\mu\text{m}$ red blood cell ($5(\mu\text{m})^2$) to be $20\text{ pA}/(\mu\text{m})^2$. Even greater heating effects would be expected for "thick" targets due to the energy loss increases and poor thermal conduction to the target backing.

Hansen et al.¹⁴ report a novel method for determining temperature rise in a typical PIXE target system for protons and alphas with beam current densities of only $.01\text{ pA}/(\mu\text{m})^2$ and $\sim 5 \times 10^{-4}\text{ pA}/(\mu\text{m})^2$. They report sample temperatures of 225°C and 72°C which are probably due to the large area, thin, organic sample support.

Legga and Mazzolini¹⁰ discuss the effects of beam heating and radiation damage primarily from the point of view of ionization effects on chemical bonding. They show elemental yields as a function of time from their scanning system in an unscanned, high beam current density mode ($\sim 90\text{ pA}/(\mu\text{m})^2$) as used on a thin $5\text{ }\mu\text{m}$ thick biological section. In this example some loss of chlorine, buildup of Ca and negligible mass loss (by bremsstrahlung) was observed; however definitive views on the subject of beam damage were deferred. A scanning system might be expected to deposit considerably less energy per "pixel" or resolution element, dependent on the scan rate; however the beam fluence per resolution element must integrate to that of an unscanned system in order to achieve the requisite elemental sensitivity.

Maggiore⁹ has summarized the range of beam current densities available from the several operational proton microprobes at this time. Beam current density is not the whole answer to the very complicated problem of damage effects, of course, but does serve as a means to estimate the facility's potential utility. Values ranging from 30 to $190\text{ pA}/(\mu\text{m})^2$ are mentioned for focused probes which compare with $\leq 5\text{ pA}/(\mu\text{m})^2$ for that reported for a simple collimated $12.5\text{ }\mu\text{m}$ diameter beam.¹⁶

Several studies have been made on the effect of heavy charged particle beam damage to the morphology of typical cells by Slatkin and Jones,^{15,16} which are relevant to the problem of beam damage. The motivation for these studies was indeed not to address the problems of proton microprobes but to estimate the sensitivity for deuterium micromapping by the $\text{D}(\text{T,n})^3\text{He}$ reaction which will be discussed in a later section. Cells studied included human erythrocytes, leukocytes and mouse oocytes. Irradiation was carried out with 200 keV tritons in ~ 0.3 atmosphere of helium, which give a somewhat higher radiation dose (in Rads or LET) than 2 MeV protons; however it is a more representative exposure than a comparison with electrons.

The cells were presented as a monocellular layer on a standard glass slide which was well heat-sunk to a thicker aluminum backing; the beam was diffused by a thin foil to $\sim 2\text{ cm}^2$ in area. For these reasons, sample heating was not believed to play a role and the

[†]Corning Glass Works, Corning, N.Y.

observations are attributable primarily to ionization-induced bond breaking.

Figure 2 is a representative result from¹⁶ showing electron and light micrographs of many erythrocytes with a central leukocyte: (a) initially, (b) after 2×10^{16} T/cm², (c) 4×10^{16} T/cm² and (d) 6×10^{16} T/cm². Several conclusions can be drawn, the first being that damage effects as observed by the gross

Figure 2. Blood cells before and after triton bombardment

physical morphology are different for the erythrocytes (which become only slightly "mottled") and the leukocyte that appears completely disintegrated, except for several shreds of nuclear material. Secondly, the cytoplasmic membrane of the leukocyte is extremely sensitive to beam damage as compared to its nuclear membrane and material (possibly due to the expected resistance of the included DNA) and to the erythrocyte. Micrographs of the oocytes were recorded and studied before and after 1.4×10^{16} T/cm² and little, if any, damage to either of the cells was visible from the incident 230 keV triton beam which is stopped in the ~ 50 μ m thick cell. Again, visible morphological damage on the cellular level is dependent on the cell type.

These results do not imply that beam damage has not affected the cell in other important ways at lesser fluences in which morphological damage is not observed. Damage to membranes may occur at lower fluences, for example, which can alter the elemental distributions throughout the cell. The observations reported here represent a gross upper limit to the fluence under which a certain cell remains a morphologically identifiable entity.

If one assumes that the ~ 200 keV triton energy is absorbed in the cell and is equivalent (not necessarily equal to) to the energy loss by microprobe protons, one can convert these fluences to beam densities and use the micrographs to suggest effects from the proton microprobe. For example, 4×10^{16} particles/cm² is 4×10^8 particles/(μ m)² which is $\sim 60 \frac{\text{pA-sec}}{(\mu\text{m})^2}$.

Therefore, a one second exposure by a well focused microprobe (~ 60 pA/(μ m)²) would be expected to cause nearly total destruction of leukocytes as shown, and some morphological damage to erythrocytes and probably even the oocyte. High current density microprobes may not have as great utility in microanalysis as anticipated. Certainly more detailed studies of beam damage effects in biological materials are necessary.

Applications

Several representative and believed important applications are presented. The challenge for micro-analytic applications is now not so much in demonstrating technological capability but in demonstrating the application to important biological or physiological problems.

1. The role of zinc in bone formation is well recognized¹⁷ but continues to be a developing science. The high sensitivity for Zn measurement in unprepared samples and good spatial resolution allow the study of several skeletal sites that are expected to exhibit different utilization of elemental zinc. Scans have been made of animal Achilles tendon which ranges from cartilage or very rudimentary "bone" to fully mineralized tissue at the bone end of the structure. Figure 3 is a micrograph of a typical rabbit Achilles tendon

Figure 3. Rabbit achilles tendon and rat femur showing microprobe spots.

studied by point measurements in representative areas labeled by points A, B and C. In young rabbits having predominantly uncalcified tendons, the Zn levels (at A) were found to be only $\sim 10\%$ of that of the bone. However in older animals having a greater degree of tendon mineralization, the zinc concentrations were found to be 31.5, 78.3 and 90% of the bone levels as one approached the bone end of the tendon (A to C).

Figure 3 shows a micrograph of a section of normal young rat femur with labels A through F specifically indicating: A, the epiphyseal growth plate; B, the zone of provisional calcification; C, trabecular bone; D, marrow; E, compact cortical bone and F, the cartilage of the articular surface. The zinc concentration in the compact bone was in this section found to be 238 ppm; however the level in the region of provisional calcification where the ratio of mineral to organic matrix is even higher than in compact bone was found to be 367 ppm. At the epiphyseal growth plate A where mineralization is largely complete, the level was only 166 ppm. The role of zinc in the mineralization of growing bone is thus directly illustrated. Although the zinc levels mentioned are not at the ppm level, they are less than can be detected by conventional electron microprobes and the proton microprobe has proved to be ideally suited to this study.

2. Fibers teased from biopsies from paraspinal muscles of patients suffering from scoliosis have been examined for trace element anomalies by several analytical techniques including the proton microprobe, more standard proton induced x-ray fluorescence (PIXE) and x-ray induced fluorescence (XRF).¹⁸ The bulk PIXE and XRF measurements indicated that muscle fibers from the concave side of a patient having severe spinal distortion contained measurable amounts of selenium

(~ 10 ppm); whereas those from the laterally opposite muscle (the "convex" side) had very nearly unmeasurable selenium as did a "control" sample from the gluteus maximus. Selenium was observed despite quantities of arsenic fixative. Proton microprobe scans were made of these individual fibers, along the fiber, at a time when selenium was unexpected and occurred by an anomalously high collimator background; further work indicates however that the proton microprobe has adequate sensitivity to scan for Se in muscle structures. The fibers which were sandwiched in formvar and kept wet by a thin bubble of buffer solution remained intact and viable and essentially unaltered by this technique, giving impetus and enthusiasm for further wet tissue section studies. Selenium was also found in unheparinized (unchelated) whole blood samples from several scoliotic patients as measured by XRF and not found in either the normals studied or any samples which had been treated with heparin. Further work is in progress on blood analyses as there is substantial enzymatic rationale to believe that selenium plays a role in idiopathic scoliosis.

3. Calcium is a key element in muscle function and muscle cell development. Its role in the profound myopathic aspects of Duchenne's muscular dystrophy (DMD) is beginning to be appreciated by ultrastructural studies demonstrating focal defects in the outer muscle membrane which may enhance the influx of extracellular calcium to the muscle fiber. Cellular uptake of anomalously high available calcium may lead to serious impairment of muscle function, symptomatic of DMD.

Proton microprobe examinations have been made of living tissue cultures prepared and grown on standard kapton target slides or frames to search for variable, high calcium levels. Cultures of human DMD skeletal muscles were grown and coupled to mouse spinal cord complex. The test culture was sandwiched between Kapton with nutrient included and studied with the proton microprobe. Calcium was detected at very low levels (10 ppm) in the Duchenne culture and the spinal cord complex but not in the adjacent nutrient or target system. This experience illustrates the advantage of direct manipulation for on-off sample signal to background determination and the requirements for good sample viewing and manipulation over areas in the 100 μ m range. Spatial resolution below 10 μ m is not required for tissue culture studies nor is high beam current density a virtue for biological samples. The tissue cultures remained essentially intact and living following the irradiation; however slight effects on cell membranes was noticed in subsequent transmission electron micrographs. The ability to discern intracellular calcium with the proton microprobe opens a wide range of studies of cultures subjected to many regimes, sequestering agents, etc.

Deuterium Micromapping Using the $D(T,n)^4\text{He}$ Reaction and Plastic Track Detectors

It is appropriate in this review to describe and further illustrate a technique for the measurement and localization of small amounts of deuterium (or tritium) using unique features of the $D(T,n)^4\text{He}$ nuclear reaction¹⁹

This method combines the measurement of very small quantities of these hydrogen isotopes with microlocalization capable of distinguishing deuterium-labeled cells from nonlabeled cells, for example. The method was developed for a study in which several compounds, particularly thymidine, were to be labeled with a stable organic isotope rather than the more common practice of using a radioactive tracer, with certain obvious attendant disadvantages. Because of its low natural isotopic abundance ^2H is a prime candidate for

the stable organic label. The potential for the use of deuterated thymidine has been described by Slatkin et al.²⁰ who suggest that significant labeling of DNA in humans may be accomplished by the cellular interaction with administered deuterated thymidine. Biopsy specimens might, therefore, be used to determine if cells from certain tissues contained higher than normal amounts of the ^2H label indicating an extraordinary activity involving the cellular DNA. Another suggestion for application concerns the genetic hazard caused by nonexchangeable tritium in the egg cells (oocytes) of female progeny by a mother who has ingested tritium.²¹ The extent of tritium residence and transfer to the progeny is not known but can be studied without perturbation by the radioactivity of tritium if the stable ^2H label is used. Many other applications ought to suggest themselves with further description of the technique. The method produces a degree of spatial localization which is comparable to the proton microprobes being developed; however the localization is performed not by the beam but by the detector, a plastic track detector which records the tracks of one of the reaction products.

The total cross section of the $D(T,n)^4\text{He}$ reaction in the laboratory system for deuteron bombardment approaches 5 b over a broad resonance between 100 to 200 keV and the reaction is characterized by a 17.6 MeV Q-value. Often the 14.4 MeV neutron is of interest; however the ^4He receives ~ 3.6 MeV and will have a longer particle range than the low energy, 100 keV, incident tritons or deuterons that initiate the reaction. Therefore, a beam of tritons (or deuterons onto a tritium-containing target) can bombard a thin sample containing deuterium which can be separated from a ^4He detector by a thin, ~ 6 μ m absorber necessary to prevent the detector from responding to the incident beam. The experimental situation is shown schematically in Fig. 4 which indicates a plastic track detector, e.g., cellulose nitrate as the ^4He detector (Kodak Pathe nitrocellulose film LR-115). Plastic track

Figure 4. Deuterium micromapping system and results with human erythrocytes fixed in deuterated formaldehyde (a)(b) and human lymphocytes labeled with deuterated thymidine (c)(d).

detectors have, in general, responses which are just sensitive to the specific ionization of alpha particles of a few MeV and are much less sensitive to lesser specific ionizations. However, the interposed absorber is still the critical element in the experiment because it must be thin enough to stop only ~ 200 keV tritons, withstand large fluences and contain minimal background deuterium (or tritium). Thin, ~ 6 μ m or 12 μ m sheets of Teflon have been used successfully and withstand up to ~ 10^{16} t/cm² at a power density of 10 mW/cm².

Although the reaction is "symmetric" with respect

to the D or T beam-target relationship, the most interesting application has been to enable the "radiography" of deuterium-labeled compounds or organisms as opposed to the common methodology of tritium labeling. Tracer studies with a stable nuclide have many virtues for the life science experimentalist and also utilizes the research Van de Graaff's triton beam. Deuteron bombardment of tritium-labeled samples might also be useful, however, to detect a tritium label promptly rather than wait long periods (weeks or months) for the normal radiographic exposure.

Figure 4(a)(b) shows the comparison between the optical micrograph of several human erythrocytes fixed with deuterated formaldehyde and 4(c)(d) human lymphocytes labeled in vitro with deuterated thymidine. One notes the "star burst" affect of the track pattern, which if all tracks were traced to the origin by straight lines could localize the source to within 5 μ m. One notes the lack of deuterium uptake in the upper lymphocyte and the generally higher background (lesser relative deuterium) in the lymphocytes. The deuterium observed in Fig. 4 for example was calibrated by simultaneous irradiation of a 3.5 μ m thick strip of "Kinfol", having known hydrogen content. An average of 36 tracks per cell was observed, corresponding to $\sim 300 \times 10^{-15}$ gm of deuterium per cell, which is an enhancement over natural deuterium by a factor of about 600. Similarly, the observation of the lymphocyte tracks shown corresponds to about 6×10^9 atoms per cell.

Ultimate sensitivity of this technique is determined by the triton fluence possible on the target without destruction of the sample morphology necessary for recognition and examination and the ability of nonhydrogen-containing absorbers to remain intact and protect the plastic track detector from the incident beam. As mentioned previously, measurements made by Jones and Slatkin¹⁶ have shown significant deterioration of cell morphology after triton fluences between 1 and 4×10^{16} t/cm². Experience with absorbers also show failures after $1-2 \times 10^{16}$ t/cm² which indicate a lower limit to deuterium detection at about 10^8 atom/cm². Given the extensive technology for deuteration and tritiation of many experimental compounds, this sensitive technique should find more application than we are as yet aware.

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