The Henryk Niewodniczański
INSTITUTE OF NUCLEAR PHYSICS
Polish Academy of Sciences
152 Radzikowskiego Str., 31-342 Kraków, Poland

www.ifj.edu.pl/reports/2005.html

Kraków, May 2005

Report No. 1963/AP

Development and applications of microanalytical techniques

Marian Cholewa

Habilitation thesis

Dedicated to my parents, wife Małgorzata
and my three children Katarzyna, Małgorzata and Paweł.

The good life is one inspired by love and guided by knowledge.

Bertrand Russell

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Papers which are included in this habilitation thesis could be divided into following categories:

<u>Category A:</u> Elemental and chemical microanalysis on single cell.

I. Cholewa, M., Legge, G.J.F., Weigold, H., Holan, G., Birch, C.
"The use of a Scanning Proton Microprobe in AIDS research".
Presented at 3rd Int. Conf. on Nuclear Microprobe Technology and Applications, Uppsala, Sweden, June 7-12, 1992, *Nucl. Instrum. & Meth*. **B77** (1993)282-286.

II. Cholewa, M., Legge, G.J.F., Weigold, H., Holan, G., Birch, C.J.
"The use of a Scanning Proton Microprobe to observe anti-HIV drugs within cells".
Life Sciences, **Vol. 54, Issue 21** (May 23, 1994)1607-1612.

III. Dillon, C., Lay, P.A., Bonin, A.M., Legge, G.J.F., Cholewa, M., Collins, T.J., Kostka, K.L.
"Permeability, Cytotoxicity and Genotoxicity of Cr(V) and Cr(VI) Complexes in V79 Chinese Hamster Lung Cells".
Chem. Res. Toxicol., **Vol. 11, No. 2** (1998)119-129.

IV. Dillon, C., Lay, P.A., Bonin, A.M., Legge, G.J.F., Cholewa, M.
"Permeability, Cytotoxicity and Genotoxicity of Cr(III) Complexes in V79 Chinese Hamster Lung Cells".
Chem. Res. Toxicol., **Vol. 13, No. 8** (2000)742-748.

V. Dillon, C., Lay, P.A., Cholewa, M., Legge, G.J.F., Bonin, A.M., Collins, T.J., Kostka, K.L., McCarthy, G.-S.
"Microprobe X-ray Absorption Spectroscopic Determination of the Oxidation State of Intracellular Chromium Following Exposure of V79 Chinese Hamster Lung Cells to Genotoxic Chromium Complexes".
Chem. Res. Toxicol., **Vol. 10, No. 5** (1997)533-535.

Category B: Damage introduced by the microprobe.

VI. Cholewa, M., Legge, G.J.F
"Temperature estimation of organic foil for particle beams".
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VII. Cholewa, M., Bench, G., Kirby, B., Legge, G.J.F.
"Changes in Organic Materials with Scanning Particle Microbeams".
Presented at 2nd Int. Conf. on Nuclear Microprobe Technology and Applications, Melbourne, Australia, Feb.5-9, 1990. *Nucl. Instrum. & Meth*., **B54** (1991)101-108.

Category C: Development of new analytical techniques.

VIII. Cholewa, M., Bench, G., Legge, G.J.F., Saint, A.
"Channeling Scanning Transmission Ion Microscopy".
Applied Physics Letters, **56(13)** (1990)1236-1238.

IX. Bench, G., Nugent, K.A., Cholewa, M., Saint, A., Legge, G.J.F.
"Sub-Micron STIM Tomography Reconstruction Techniques".
Presented at 2nd Int. Conf. on Nuclear Microprobe Technology and Applications, Melbourne, Australia, Feb.5-9, 1990. *Nucl. Instrum. & Meth*., **B54** (1991)390-396.

X. Jacobsen, F.M., Zarcone, M.J., Steski, D., Smith, K., Thieberger, P., Lynn, K.G., Throwe, J., Cholewa, M.
"Detection of Heavy Trace Impurities in Silicon".
Semiconductor International, June (1996)243-246.

Category D: Development of a single ion hit facility (SIHF) and new detector.

XI. Cholewa, M., Saint, A., Legge, G.J.F., Kamiya, T.
"Design of a Single Ion Hit Facility".
Nucl. Instrum. & Meth., **B130** (1997) 275-279.

XII. Kamiya, T., Cholewa, M., Saint, A., Prawer, S., Legge, G.J.F., Butler, J.E., Vestyck, D.J.
"Secondary Electron Emission from Boron-doped Diamond under Ion Impact: Applications in Single Ion Detection".
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PREFACE

Progress in experimental physics nowadays is achieved by team work. This is especially true in the area of applied physics, where development of modern experimental equipment as well as the application itself demands the effort from many people from different backgrounds in order to achieve significant goals. This habilitation thesis is not an exception. Even so, it is still important to write thesis like this, which emphasise the contribution of individual. However, I consider my contribution to the material presented as essential and have been leading all of the presented topics. During the realisation of a wide variety of work presented in this thesis I had the pleasure and privilege of collaborating with people whose contributions were also very important:

Prof. Dr hab. Jan Styczen – the head of the Department of Nuclear Spectroscopy in the Henryk Niewodniczanski Institute of Nuclear Physics, Krakow, Poland. I appreciate his help in organising a collaborative research with his Department and great support in preparation of this thesis.

Prof. Dr Andrzej Hrynkiewicz – who introduced me over thirty years ago to the world of analytical and microanalytical techniques. And with whom I had the privilege to discuss and to start many new projects.

Dr Zbigniew Stachura – the head of Applied Nuclear Spectroscopy Division within the Department of Nuclear Spectroscopy in the Henryk Niewodniczanski Institute of Nuclear Physics, Krakow, Poland. I highly appreciate all interactions with Zbyszek which led on many occasions to start of many new projects during the last twenty years.

Dr George Legge – the head of the Micro Analytical Research Centre (MARC) at the University of Melbourne in Australia. Under George's guidance I had the privilege of starting a series of new projects involving ion microprobe in Melbourne and overseas.

Dr Chris Birch – the head of the Virology Department in the Fairfield Hospital in Melbourne, Australia. For the very first time we were able to complete a five year long project on the distribution of new anti-AIDS drugs inside individual cells.

Prof. Peter Lay – the head of the Centre for Heavy Metals Research in the School of Chemistry at the University of Sydney in Australia. It took us almost ten years of continuous work on the distribution of chromium and other elements inside individual cells. This project is still being pursued by Peter and other groups around the globe.

Dr Peter Thieberger – the head of the Tandem Accelerator Division at Brookhaven National Laboratory in the USA. In the short time allocated to this project we demonstrated a usefulness of a new approach for the detection of low level impurities in silicon. This technique has been successfully utilised by many groups around the world.

Dr Tomihiro Kamiya – the head of the nuclear microprobe group at the Japanese Atomic Energy Research Institute (JAERI) in Takasaki, Japan. Together with Tomihiro we developed a new detector based on the Boron-doped chemical vapour deposited (CVD) diamond. We also started serious work on the development of the single ion hit facility (SIHF) for live cells irradiation. The work we started together in Melbourne has been continued during my visit at GSI, Darmstadt, Germany and at the National University of Singapore in Singapore.

Dr Bernd Fischer – from GSI, Darmstadt, Germany for his long collaboration on the design and development of the SIHF and diamond detector.

And thanks to many others, listed as co-authors on the attached papers, to which I am indebted for interdisciplinary cooperation. Those I have forgotten to mention here are certainly not forgotten.

ABBREVIATIONS USED in THIS THESIS

Abbreviation	Explanation
AAS	Atomic Absorption Spectroscopy
CAT	Computer Assisted Tomography
CDKN1A	p21 protein
CVD	Chemical Vapour Deposition
CSTIM	Channeling Scanning Transmission Ion Microscopy
cytotoxicity	Technique for assessing cell mortality after exposure to chemical compound
fg	femtogram = 10^{-15} g
FWHM	full width at half maximum
genotoxicity	Technique for assessing genetic changes inside cells after exposure to chemical compound
GLC	Gas-liquid chromatography
GSI	Gesellschaft für Schwerionenforschung mbH, Darmstadt, Germany = German National Laboratory for Heavy Ion Research
HIV	Human Immunodeficiency Virus - AIDS
ICNMTA	International Conference on Nuclear Microprobe Technology and Applications
LAMMA	LAser Microprobe Mass Analysis
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time-of-Flight mass spectrometer
MARC	Micro Analytical Research Centre
MDL	Minimum Detection Limit
MT2 cells	human T-lymphocyte cell line
nuclear microprobe	System with ion beam focused to micron or submicron dimension
PBL	human peripheral blood lymphocytes
permeability	Concentration of elements inside single cell after exposure to chemical compound
PIXE	Proton Induced X-ray Emission
ppm	parts-per-million
RBS	Rutherford Back-Scattering
SBD	Surface barrier detector
SEM	Scanning Electron Microscopy
SIHF	Single Ion Hit Facility
SIMS	Secondary Ion Mass Spectroscopy
SRIXE	Synchrotron Radiation Induced X-ray Emission
STIM	Scanning Transmission Ion Microscopy
STIMT	Scanning Transmission Ion Microscopy Tomography
TOF HIBS	Time-of-flight Heavy Ion Backscattering Spectrometry
V79 cells	Chinese hamster lung cells
XANES	X-ray Absorption Near Edge Spectroscopy

1. INTRODUCTION

There is a growing demand for analytical techniques with either high sensitivity or high spatial resolution or both. The author has been involved in developing and applying several such techniques, which cover several fields including biology and material science. This thesis displays a few examples from over 90 papers published by the author, such as:

- Distribution of different elements including anti-AIDS (***papers No. I & II***) and chromium compounds (***papers No. III-V***) inside individual cells. This work has been performed over many years in order to collect meaningful and biologically useful data. While there is no magic in elemental analysis with proton microbeam focused to 1 μm^2 the tedious task of analysing hundreds of cells individually produced astonishing results.
- Damage formation during the application of high current techniques including PIXE (Proton Induced X-ray Emission) as presented in ***papers No. VI & VII***. These two papers show the importance of good understanding of materials modification during the use of micro-analytical technique with highly focused ion beam.
- Development of new analytical technique called Channeling Scanning Transmission Ion Microscopy (CSTIM). This work is presented in ***paper No. VIII***. While at the University of Melbourne, the author had been involved in the development of the CSTIM technique, which appeared to be extremely useful in the characterisation of crystals which are very sensitive to radiation damage.
- Development and applications of high resolution Scanning Transmission Ion Microscopy Tomography (STIMT). This technique enables investigation of three-dimensional structures with resolution down to 100 nm. This work is presented in ***paper No. IX***.
- Development of a facility for detection of impurities in silicon (***paper No. X***). In the future a series on new analytical techniques need to be developed for the characterisation of low level impurities in semiconductor materials including silicon. This work is an excellent example of research and development in this direction.
- A concept for the development of a single ion hit facility (SIHF) is presented in ***paper No. XI***. At the time of this publication only three groups around the globe had been involved in this area of research. And currently, it is considered as one of the fastest growing areas using ion and X-ray microbeams.
- Development of high efficiency detector for MeV ions (***paper No. XII***). This work initially started as a joke. And eventually, led to the discovery of new materials that could be used as highly efficient detectors for ionising radiation in the future.

2. NUCLEAR MICROPROBES

There are about 60 ion microprobe facilities around the world, and usually they are associated with low energy electrostatic accelerators, and operated by teams widely involved in interdisciplinary topics. The high diversity of ion beam analysis methods and their application in many disciplines has been represented already in nine international conferences [1-9] called International Conference on Nuclear Microprobe Technology and Applications (ICNMTA). Breese *et al* edited a book [10] related to the topic of nuclear microprobes. Most of the existing microprobe systems operate with spatial resolution between 1 to 10 μm. However, a few systems achieved a very high resolution down to 50 nm [11-13].

2.1 Nuclear microprobe at the University of Melbourne

The nuclear microprobe systems have constantly been under development [14]. During the time that the author was part of the Micro Analytical Research Centre (MARC) at the University of Melbourne we were using a compact "Russian" quadrupole focusing system

[15]. Since then additional focusing systems have been developed by MARC including split "Russian" quadruplet and high current focusing systems. However, the best ever spatial resolution of 50 nm was achieved [11] on the old nuclear microprobe with the use of that compact Russian quadruplet focusing system.

2.2 Nuclear microprobe at the Institute of Nuclear Physics

The split "Russian" quadruplet systems have been successfully installed at the University of Leipzig [16], Germany and the Henryk Niewodniczanski Institute of Nuclear Physics PAN in Krakow, Poland [17]. While the microprobe system in Leipzig has a typical length of ~9 m, the system in Krakow is the shortest microprobe in the world with a total length of 2.3 m only. The author has been participating in the development and application of both those systems.

3. ANALYTICAL TECHNIQUES

Numerous analytical techniques have been developed in conjunction with the application of nuclear microprobes. The reader is referred to publications [1-10] for details. In this overview, the author will describe in some detail all of the techniques applied in the attached 12 publications.

3.1 Proton Induced X-ray Emission (PIXE)

Particle-Induced X-ray Emission (PIXE) is one of the most powerful methods for elemental analysis using ions. It is based on the excitation of the inner electron shells of the atom by impinging ions, mostly protons. The de-excitation of the atom occurs through the emission of an X-ray, whose energy is characteristic of the atom in question. Most often a Si(Li) detector is employed to detect characteristic X-rays. PIXE can be used for the efficient analysis of elements heavier than aluminium. The literature on PIXE and its applications is overwhelmingly extensive, but the reader is referred to the handbook by the founder of PIXE, Sven Johansson and his co-author John Campbell [18]. The PIXE technique in combination with high spatial resolution of 1 μm offers a very powerful tool for mapping different elements inside individual cells. Table 1 compares a minimum detection limit (MDL) expressed as parts-per-million (ppm) for selected microanalytical techniques and selected elements where the sensitivity is maximum. From table 1, which should be treated as a guide only, it is clear that SEM will not offer sufficient sensitivity

Table 1. Comparison of MDL between different micro-analytical techniques.

Analytical technique	Minimum detection limit (MDL) ppm
SEM (Scanning Electron Microscopy) [19]	10^3
PIXE (Proton Induced X-ray Emission)[18]	1
LAMMA (Laser Microprobe Mass Analysis) [20]	1
SRIXE (Synchrotron Radiation Induced X-ray Emission) [22]	10^{-3}

when concentrations of elements at the few ppm level are presented in the samples. The SRIXE technique based on the synchrotron radiation based X-ray microprobe will offer a sensitivity a factor of about 10^3 better than the PIXE and LAMMA techniques and it has been used lately by the author and collaborators [21]. Detail discussion of the MDL and its dependence from atomic number (Z) is performed in [22].

Investigations of uptake of different chemical compounds by single cells have become a very popular topic and have been performed with different microanalytical techniques using electron, laser, ion and X-ray beams. With the advent of nuclear microprobes with submicron resolution it has been possible to investigate distribution of different elements inside individual cell with sensitivity of a few ppm. Examples of the power of the PIXE technique are shown in the following paragraphs.

3.1.1 Characterisation of anti-AIDS drugs inside cells

Introduction: In this work (***papers I & II***) we documented for the very first time the possibility of detecting newly developed anti-AIDS drugs inside individual cells. The drugs had been developed by the CSIRO laboratory in Australia. Those drugs had been tested for their activity against the HIV virus in a continuous human T-lymphocyte line (MT2) and in peripheral blood lymphocytes (PBLs). These tests were performed at the Fairfield Hospital in Melbourne, Australia. A series of selected drugs were introduced to cells. The concentration and spatial distribution of heavy metals (e.g. cobalt, tungsten) present in the drugs were measured with the use of nuclear microprobe. During these measurements which were performed over a five year period, the beam resolution of about 1 μm were applied and MDL of about 1 ppm was achieved for most elements (e.g. cobalt, tungsten) of interest.

Anti-HIV drug: The drug under investigation in this study is one of a large number of polyanions (heteropolytungstates) that have been tested for their ability to inhibit the replication of HIV in continuous human T-lymhocyte line (MT2 cells) and in peripheral blood lymhocytes (PBL cells) from healthy blood donors. The aim of this study was therefore to establish a method for determining the quantitative distribution of metals in cells treated with a drug at non-toxic levels, and in particular, to establish whether the compound $K_{10}[Co_4(H_2O)_2(PW_9O_{34})_2]\cdot nH_2O$ (n is ca. 25) can enter PBL cells and whether if so, it remains intact within the cell? The subtoxic concentration of drugs varies, generally from about 5 to 200 μg of drug per mL of culture medium. Subtoxic is here defined as those concentrations of the drug below which drug-related morphological changes (observed by light microscopy) and cell death (estimated by counting viable cells in a presence of vital stain) do not occur. By toxic mean when more than 10% of cells are killed in the presence of drug and in the absence of virus.

Sample preparation: Prior to analysis, the PBLs were incubated in cell culture medium (RPMI-1640 containing 10% foetal calf serum and supplemented with interleukin 2) containing subtoxic concentration of 50 μg/mL of drug. Drugs are added 24 hours in advance to give them maximal effect. After 24 hours incubation, cells were harvested by centrifugation at 1500 rpm for 10 min, then resuspended in a small volume of 0.154 M ammonium acetate. For analysis cells from suspension were spotted onto the 1 μm thick nylon foil and immediately snap frozen in isopentane cooled down to near freezing point by liquid nitrogen. The samples were transferred at liquid nitrogen temperature to a vacuum chamber and dried at a pressure of 10^{-6} torr as the cells slowly warm up to ambient temperature over a period of about 12 hours. Typical viable lymphocytes (MT2 and PBL) are about 10 μm in diameter and after plating and freeze drying are about 12 μm in diameter and less than 1 μm in thickness.

Experiment: The freeze dried cells were transferred to the specimen chamber of the nuclear microprobe and individually positioned and scanned with a 3 MeV proton beam of about 30 pA and 1 μm resolution. X-rays emitted by the cell were collected with a Si(Li) energy dispersive detector. In order to minimize thermal damage to the cells, the beam is scanned and all data saved on the computer. Although, as with electron probe irradiation, specimen shrinkage and elemental losses (generally only of light elements including hydrogen and oxygen) from ionization of the specimen. Losses of elements of significance were negligible under the proton beam. Shrinkage was usually smaller than 2% as measured by the scanning transmission ion microscopy (STIM) technique.

Results & discussion: The heavy elements characteristic for this drug ($K_{10}[Co_4(H_2O)_2(PW_9O_{34})_2]\cdot nH_2O$) are clearly measurable above the very low background characteristic of the PIXE spectrum. We produced the elemental maps for all elements present in the spectrum including phosphorus, cobalt and tungsten. The average elemental concentration were obtained from the areas under the appropriate peaks in PIXE spectra. The atomic ratio of two elements (W : Co) in the cell is 12.2 and within

experimental error similar to that in the drug, viz. 14.0. This, together with the observation that the tungsten and cobalt constituents were found in the same areas of cell, suggest that the drug remains intact within the cell. The low antiviral activity of the drug tested in PBLs, compared to MT2 cells, does not therefore appear to be due to drug instability or inability to penetrate the cell membrane.

3.1.2 Chromium permeability studies: PIXE analysis of single V79 cells

Introduction: In the 1930's it had been discovered that chromium complexes were causing serious health problem. For example Cr(VI) complexes were recognized as human carcinogens, and Cr(V) complexes were established as potential carcinogens. For almost ten years we had been investigating the role of Cr, its permeability, cytotoxicity and genotoxicity in collaboration with the School of Chemistry at the University of Sydney. We have shown that micro-PIXE, with its approximate 1 ppm sensitivity for Cr and 1 μm spatial resolution, is ideally suited for mapping Cr inside individual cells. We performed a detailed study with different compounds of Cr(III), Cr(V) and Cr(VI) – ***papers III & IV***.

Cr compounds: Investigations of the permeability of Cr into mammalian cells are important for understanding the role of Cr in the area of nutrition, cancer and toxicity. Intracellular Cr has been quantified using a multitude of techniques including radioactive tracer analysis [23], atomic absorption spectroscopy (AAS) [24], and gas-liquid chromatography (GLC) [25]. The cellular uptake of Cr is dependent on the chemical and physical state of Cr, the extracellular concentration, and exposure period.

Sample preparation: In case of Cr studies inside V79 (individual cultured mammalian V79 Chinese hamster lung cells) cells were seeded at a density of $5x10^5$ cells per tissue culture dish and growth medium (GM) of 5 mL overnight. The GM was removed, and new medium (5 mL) was added before the addition of freshly prepared Cr solutions (0.5 μmol of Cr, 0.1 mL). Control cells were prepared in an identical manner, except that 0.1 mL of water was substituted for the Cr solution. Following the treatment period of 4 hours, the GM was removed and the cells were washed thoroughly with PBS solution (pH 7.4, Oxoid) to remove residual medium. The cells were trypsinised and centrifuged at 245 g for 10 min, the supernatant was removed, and the cells were resuspended in a solution of ammonium acetate (200 mM) and frozen immediately in liquid Freon cooled with liquid nitrogen. The cells were frozen onto the 1 μm thick nylon foil and freeze dried in vacuum. After freeze drying the cells were transferred for the PIXE analysis.

Results & discussion: Table 2 shows the amount of intracellular Cr resulting from exposure of cells Cr compound for 4 hours. We had assumed that the mass of the cell is 4 ng. Spatial elemental mapping indicated that Cr was distributed uniformly through the cell. This strongly suggest that it is present in both the cytoplasm and nucleus. If a Cr complex enters the cell during mitosis it would not have to pass through two membranes

Table 2 Calculated masses of Cr present in V79 cells treated with different Cr compounds

Treatment (4 hours)	Mass of intracellular Cr (fg)
Control	10 ± 6
Cr(III)	22 ± 7
Cr(V)	43 ± 3
Cr(VI)	229 ± 35

to interact with DNA. In fact Cr(VI) or Cr(V) complex would probably be more potent during this phase as the time for intracellular reduction to Cr(III) would be lower. The investigation of the Cr oxidation state inside individual cells was reported in chapter 3.2.

3.2 X-ray Absorption Near Edge Spectroscopy (XANES)

Introduction: The advent of a very broad-band highly focused intense photon beams from synchrotrons has revolutionized the application of photon spectroscopy [26]. In the X-ray Absorption Near Edge Spectroscopy (XANES) technique the X-rays can excite inner-shell electrons from the absorbing atoms with consequent sharp steps in the absorption cross section as the X-ray energy is increased through an inner-shell ionization threshold.

We were able to perform for the first time the XANES analysis of Cr oxidation state inside individual cells as shown in ***paper No. V***. The work was performed with the use of glass capillary that enabled us to focus the beam to about a 20 μm diameter spot. The combination of the glass capillary focusing and XANES system at Brookhaven National Laboratory in the USA was essential for the success of this work.

Additionally to the PIXE technique, the author also used X-ray absorption near edge spectroscopy (XANES) [27] with a focused beam to characterise the Cr oxidation state inside individual cells. The XANES technique involves the use of a monochromator crystal situated between the source of X-rays and the sample to provide monochromatic X-rays of narrow bandwidth (a few eV). It permits the selective fluorescence of individual elemental emission lines, with the added feature that ionisation state and bonding information are also available.

Focusing system: A new type of lens for focusing synchrotron-produced X-rays is the paraboloidally tapered monocapillary optics. These devices [28] have demonstrated the ability to produce a focal spot of 20 μm diameter with an intensity gain of 120 with respect to incident beam at 8 keV using the X26A beamline at the NSLS at Brookhaven.

Results and discussion: No Cr K-edge XANES edges were observed above the background noise for control cells or those that were treated with 0.5 μmol/dish with Cr(III) complexes. For the cells treated with Cr(VI) complexes it is clear that all Cr(VI) (>90%) had been reduced to Cr(III) as evidenced by the shift in the edge and the disappearance of the pre-edge peak typical to Cr(VI).

4. BEAM DAMAGE and its CONSEQUENCES

Current densities up to 150 pA/μm^2 can be achieved with a beam of protons or heavier ions in a microprobe. Such currents are necessary for large current techniques including PIXE and Rutherford Back-Scattering (RBS). At such conditions, the sample usually located in a vacuum will experience an increase in temperature, elemental loss and severe deformation (***papers No. VI & VII***). The author with co-workers devoted a large amount of time and energy in order to investigate these processes and their influence on the final analysis of the sample.

It has been shown that great care needs to be exercised in order to minimise the elevation of temperature and sample deformation. In order to obtain meaningful quantitative data for elemental analysis on biological specimens we were forced to reduce the beam current from 120 pA to less than 30 pA. To compensate for a factor of 4 reduction in the current, we increased the solid angle by a factor of 12 of our X-ray detector for the PIXE technique.

Table 2 shows a rough estimation of energy deposition for different analytical techniques for detecting 10^3 ppm (parts per million) of the same element in a biological cell contained in 10x10 μm^2 scanned area. In table 2, I have compared energy deposition for some of the techniques that enable at least 1 μm^2 of spatial resolution. For the estimate of energy deposition we used an organic sample with ~1.2 g/cm^3 density and a thickness of 1 μm. The value of energy deposited is an estimate only.

Table 2. Comparison between different micro-analytical techniques.

Analytical technique	Energy deposited J/g
SEM (Scanning Electron Microscopy) [19]	10^{10}
PIXE (Proton Induced X-ray Emission)[18]	10^{8}
LAMMA (Laser Microprobe Mass Analysis) [20]	10^{9}
SRIXE (Synchrotron Radiation Induced X-ray Emission) [22]	10^{5}

The process of damage is specific for different micro-analytical techniques due to the different interaction of radiation with matter. According to Kirz [29] a dose of 10 J/g suffices to kill the most resistant living cell and a dose of 10^4 J/g will cause severe structural rearrangements in most organic materials. Dose is only one of many parameters that need to be considered. The nature and extent of damage is also affected by the composition and structure of the absorbing material.

5. DEVELOPMENT and APPLICATIONS of NEW ANALITYCAL TECHNIQUES

Analytical techniques are essential in the formation and analysis of different materials including cells and semiconductors. In the following chapters of this thesis the author will describe the development of several new techniques.

5.1 Scanning Transmission Ion Microscopy (STIM)

In scanning transmission ion microscopy (STIM) [30] the ions are transmitted through the sample and the loss of their energy is measured as shown in figure 1. The magnitude of the energy loss depends on the thickness, density and mass of the sample. Thereby various characteristics of the sample can be investigated. For example, the mass thickness and basic structure can be characterised. Thus STIM can be used both as an imaging technique and to measure the sample thickness or areal density. With STIM measurement the particle detector is placed behind the sample on the beam axis. As such very low currents of few fA could be applied. There are several methods to evaluate STIM data [11]. The STIM technique and its modifications had been applied in ***papers No. VI, VII, VIII and IX***.

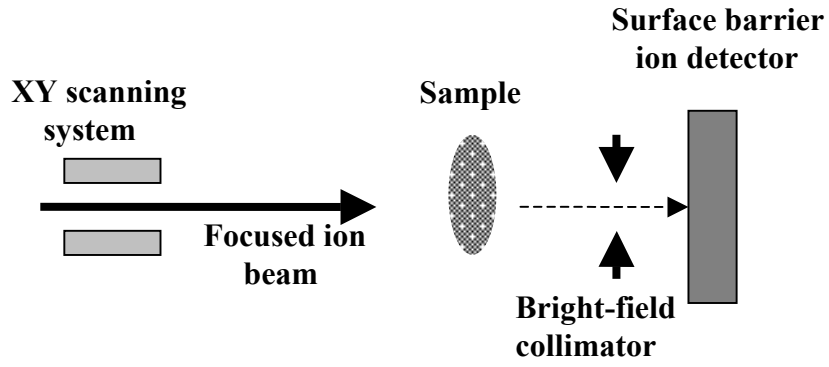

Figure 1 Schematic diagram of the scanning transmission ion microscopy (STIM) set-up with the focused ion microprobe. The focused ion beam is scanned over the sample in X and Y directions. The bright-field (open in the middle – 0-11 mrad) or dark-field (close in the middle – 11-22 mrad) collimators could be used to limit the acceptance angle for ions passing through the sample. The energy of ions passing through the sample is measured with the surface barrier detector.

5.2 Channelling Scanning Transmission Ion Microscopy (CSTIM)

Introduction: The nuclear microprobe, as developed by Cookson *et al.* [15], has typical spatial resolution of about 1 μm for PIXE and several microns for the Rutherford Back-Scattering (RBS) technique because larger currents are required. In ***paper No. VIII*** the author with collaborators introduces a new low current (typically less than 1 fA) and high resolution (down to 50 nm) technique called channelling scanning transmission ion microscopy (CSTIM). This technique is ideally suited for investigation of crystals which are highly sensitive to radiation damage.

The use of Rutherford backscattering (RBS) and nuclear reaction analysis (NRA) is based on well known energy losses of MeV ions in materials [31,32]. However, , in the cases of RBS, NRA or other ion beam techniques combined with channelling in crystalline materials, for crystal quality analysis or impurity lattice location, the channelling energy losses should be used. The channelling energy losses or functions of particle charge, mass and medium, as in amorphous material, but they depend as well on channelling direction and the transverse energy E_T of the ion in the channel. Appleton at al. [33] performed systematic studies on the energy loss of protons channelled through Si and Ge thin single crystals.

The principle of the CSTIM: The CSTIM technique is based on the detection of changes in an ion's energy loss and in the direction in a transmitting sample, due to the presence of crystal defects like interstitial atoms, vacancies and complexes of simple defects up to dislocation lines and loops. The presence of such defects in a crystal structure affects the motion of channelled particles by direct scattering, dechanneling or changing the transverse energy E_T of channelled particles. Wielunski et al. [34,35] showed how such channel deformations leads to dechanneling and direct scattering.

Experiment: The sample for this experiment (attached paper No 8) was a 50 μm thick silicon wafer which had its <111> axis at 30° to the normal. For CSTIM experiments, the 3.9 MeV proton beam was focused to a spot of less than 200 nm. And beam current of 0.2 fA was used. Transmitted protons were detected at 0° by a surface barrier detector with energy resolution of 15 keV full width at half maximum (FWHM). Collimators in front of the detector limited the acceptance angle to 0-11, 0-23 and 11-23 mrad. Another surface barrier detector (SBD) at 135° had an acceptance half angle of 14 mrad. The beam current used with this detector was 1 nA with a spot of 15x21 μm^2. Channeling and nonchanneling spectra were measured with each detector. The sample was damaged with a large current after deposition of 5.8 μC of beam charge in the channelling direction.

Results and conclusions: The CSTIM technique was used to map the damaged area. In this case the beam was scanned over 50x50 μm^2. The nonchanneling peak occurs at an energy loss of 950 keV and is consistent with the silicon thickness of 54±5 μm according to tabulated stopping powers. The proportion of low energy loss particles is highest for the bright field 0-11 mrad collimator and the lowest for the dark field 12-23 mrad collimator. The extreme sensitivity of the CSTIM technique allowed us for mapping of the damage area. The results are shown in the attached paper No. 8. No other known technique is sensitive enough to perform such analysis.

5.3 Scanning Transmission Ion Microscopy Tomography (STIMT)

Introduction: In 1979 Cormac and Hounsfield shared a Nobel Prize in Physiology & Medicine for the development of computer assisted tomography (CAT). Since then the field has exploded and many new developments have been reported [36]. Following this trend we developed STIM Tomography (STIMT) with resolution down to 370 nm as shown in ***paper No. IX***. And this resolution was improved later to below 100 nm [38] making it one of the highest resolution tomography techniques.

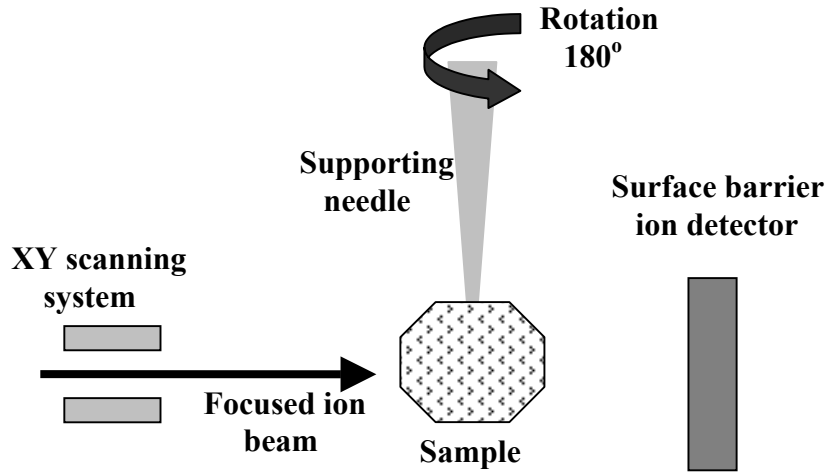

Figure 2 Schematic diagram of the scanning transmission ion microscopy tomography (STIMT) system with the focused ion microprobe. The focused ion beam is scanned over the sample in X and Y directions until single projection data file is collected. After this the sample is rotated and the next projection data is collected until a 180° rotation is made. The energy of ions passing through the sample is measured with the surface barrier detector.

The technique of quantitatively determining variations in the density of matter from the energy losses of individual ions in a focused MeV microbeam in conjunction with computed tomography is called ion computed microtomography [37] or STIM tomography [38]. STIM tomography (STIMT) provides mapping of material density in three dimensions which can give an exceptional view of a specimen shape and internal structure or uniformity. A schematic diagram of the system is shown in figure 2.

Experiment: In our first demonstration of the STIMT technique we used the suspended double-barrelled pyrex micropipette. A straight section of the pipette was selected for the cat scan. For the STIMT technique, each projection was made by magnetically scanning the beam of 3 MeV proton across the sample. The sample was rotated 2° between each projection. The scan was spread over 931x200 channels and covered 340x8 μm^2 during the 180° rotation. The beam of 3 MeV protons provided sufficient energy loss to give good energy contrast at submicron resolution. The beam was focused to a spot of about 200 nm diameter and was continuously scanned over the specimen in random pattern. A silicon surface barrier detector with 15 keV energy resolution was placed 24 cm behind the sample. The solid angle of the detector was 22 mrad.

Results and conclusions: The first 90 projections were analysed with the program SEMPER [39] and the image reconstructed using filtered backprojection algorithm. The estimated spatial resolution after reconstruction was about 310 nm. The calculated from reconstructed data the density of the pipette was about 2.2 g/cm^3 which compares well with the density of pyrex which is 2.23 g/cm^3 as calculated using TRIM [40] code. We demonstrated for the very first time a submicron resolution with a three dimensional STIMT technique. This resolution was later improved to below 100 nm [41] which is the best for the STIMT technique until recently.

5.4 Time-of-flight Heavy Ion Backscattering Spectrometry (TOF HIBS)

The work presented in ***paper No. X*** is an example of a quick response from the scientific community to a growing demand from the semiconductor industry for the development of a highly sensitive analytical tool for detecting impurities in silicon. The present sizes of the smallest features in computer chips are down to 100 nm and still being reduced. To be able to further miniaturize, the semiconductor industry needs to develop silicon wafers with a level of impurities below 10^7 atoms/cm^2 [42]. To perform elemental analysis at or

near surface with such high sensitivity, new techniques are being developed. The time-of-flight heavy ion backscattering spectrometry (TOF-HIBS) technique has been proposed by Doyle *et al.* [43]. The sensitivity of the system in this configuration ranges from ~4x10^{10} atoms/cm^2 for Fe to ~1x10^9 atoms/cm^2 for Au.

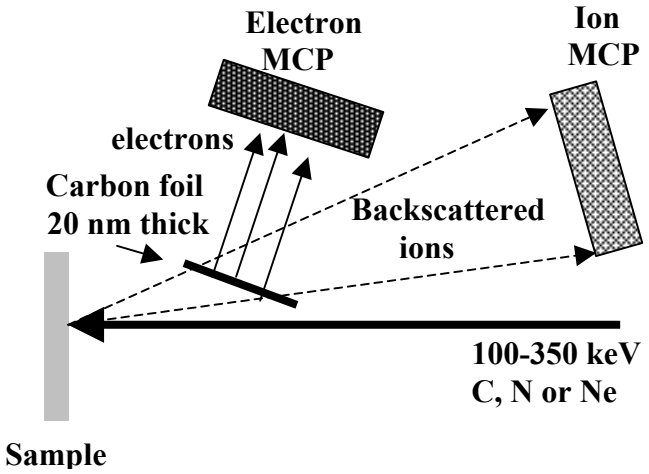

Figure 3 Diagram of the time-of-flight HIBS (TOF HIBS) system at Sandia National Laboratory [43]. The TOF system consists of two event timing detectrors. As backscattered ions pass through the 20 nm thick C foil they produce secondary electrons which produce the START pulse in the electron MCP. The ions are stopped in the ion MCP giving STOP pulse.

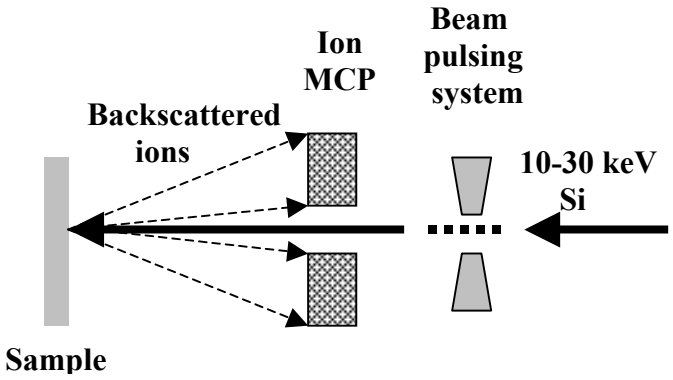

Figure 4 Diagram of the time-of-flight HIBS (TOF HIBS) system at Brookhaven National Laboratory[paper No 10]. The TOF system consists of one timing ion MCP detector which produces the STOP signal from backscattered ions. As primary ions pass through the fast beam pulsing system thick the START signal is produced.

However, in Sandia's experimental setup (figure 3) there is a thin (~20 nm thick) carbon foil in the flight path of backstattered ions. The primary beam energy could not be reduced any further because such a thick carbon foil would stop ions scattered from the measured sample.

In our setup (figure 4) we proposed to use a pulsed ion beam which eliminated the need for a carbon foil to produce the start signal for TOF measurements. Also by using a heavier silicon beam we achieved two improvements: (a) elimination of the scattering of primary beam from the silicon substrate and (b) increase in the cross section for the scattering. The sensitivity of our system in this configuration ~1x10^9 atoms/cm^2 for Au on silicon surface. Which is comparable with the Sandia's system. By decreasing the pulse width for the beam pulsing system from 200 ns to about 1 ns it will be possible to

reduce the sensitivity even further. Since the ***paper No. X*** on our work was published similar systems have been developed around the globe.

6. Single Ion Hit Facility (SIHF)

In recent years [44] a number of groups in Europe, USA and Asia have started to develop, or to plan, installations for the irradiation of individual cells and/or their components to enable the study of certain radiobiological processes in ways that are inaccessible with conventional broad field exposures. In ***paper No. XI*** the author with co-workers presented a design for a system in Melbourne, Australia. While this system was never realised in Australia due to the lack of funding, the author, had been participating in similar projects at GSI, Darmstadt, Germany [45] and in Krakow, Poland.

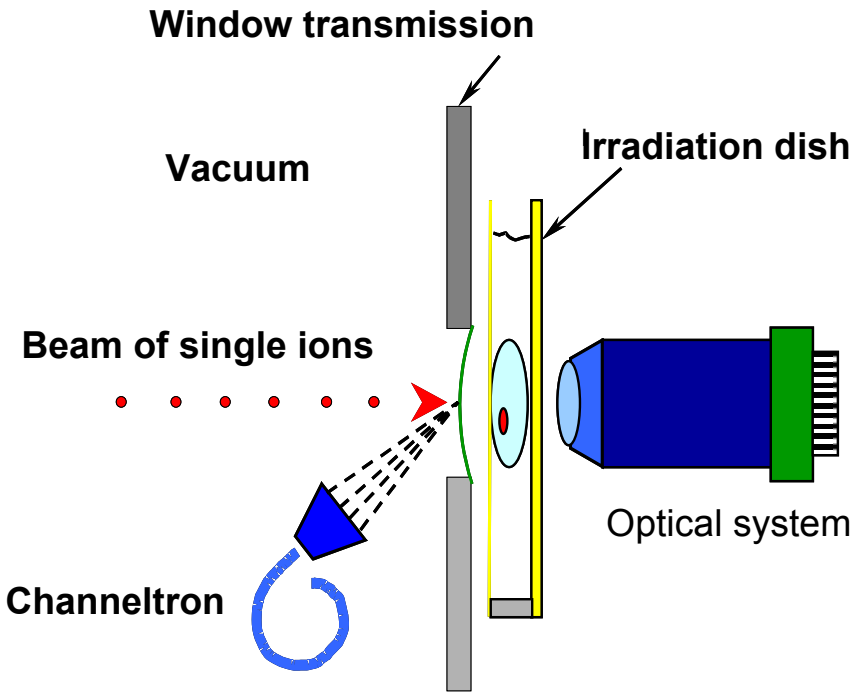

Figure 5 A schematic diagram for the horizontal single ion hit facility (SIHF). Once the single ion is detected and the cell irradiated the beam is switched off. Cells are grown and attached on the thin foil in the vertical "irradiation dish" system which keeps them in the wet conditions. Cells recognition and positioning is performed with an optical system connected with the high sensitivity CCD camera. The process of the cell recognition and positioning, single ion detection and beam switching is controlled by the computer.

A schematic diagram of the SIHF is presented in figure 5. In order to construct the SIHF there are several difficulties on the way:

1. Beam position: the microbeam should be focused to the spot and the position of the spot should be monitored with a very high accuracy.
2. Irradiation dish: to keep cells alive we need to develop a vertical irradiation "wet-cell" system which enables cells attachment and growth in the culture medium..
3. Beam scattering: to limit the scattering of the beam in the vacuum window and in the atmosphere the windows should be extremely thin and the dish positioned as close as possible to the window.
4. Single ion detection: to allow single ion counting, the ion detection should be performed with efficiency as close to 100% as possible.
5. Cell positioning and recognition: in order to irradiate large number of cells in the short time the cell recognition and positioning system should be fully automated and the whole process controlled by the computer.

6. Energy transfer to the cell: a detail modelling should be applied in order to estimate the precise linear energy transfer (LET) to the irradiated cell.

After years of work on heavy ion microlithography [46] it was clear that focusing ion microprobe is a very promising instrument to shoot single ions into single living cells with sub-micron precision. Since 1998 the author has been strongly involved in the development of the SIHF at GSI [47, 48]. Figure 6B shows the first successful use of the SIHF at GSI with 4.8 MeV/nucleon carbon ions.

Using confocal microscopy (figure 6) on immunofluorescence-stained cells, we have investigated at GSI, Darmstadt, Germany [49] the response of CDKN1A (p21), one of the key proteins involved in the DNA damage response pathway, after irradiation with random (figure 6A and focused (figure 6B) beams. Each traversal of an accelerated ion lead to the formation of a single, bright focus of the CDKN1A protein in the nuclei of normal human fibroblast (AG01522B) within 2 min after irradiation at 4 ^{o}C. The particle-induced CDKN1A foci persist for several hours until they become diffuse and vanish.

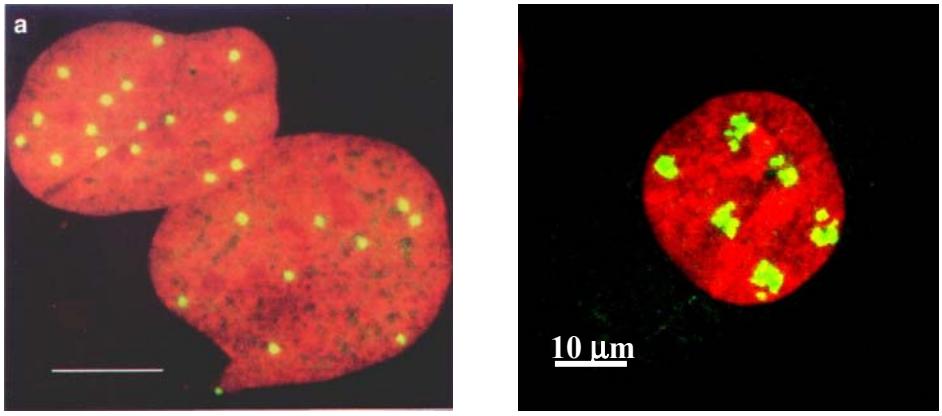

Figure 6 Typical image of the CDKN1A (p21 protein) signal of a cell nucleus after ions irradiation. Figure A: shows [49] irradiation with random beam irradiation of lead ions with 3.1 MeV/nucleon. Figure B: shows irradiation with focused (down to 300 nm) carbon ions with 4.8 MeV/nucleon with single ion hit facility (SIHF). In figure B 15 carbon ions were delivered in each about 1 μm diameter spot inside cell nuclei. Green fluorescence: CDKN1A; red fluorescence: DNA. Bar = 10 μm for both pictures.

7. DEVELOPMENT and APPLICATIONS of DIAMOND DETECTOR

Detectors used for detecting X-rays, ions and electrons play an important role in market of analytical instruments (e.g. SIMS, MALDI-TOF) and in science. Several companies have been delivering detectors and components for these markets. In the last 50 years, the methods for the detection and measurement of ionizing radiation (e.g. x-rays, ions and electrons) have undergone significant evolution [50]. The old detectors are being replaced by new generation detectors. And these new detectors are more frequently based on newly developed materials and technologies (e.g. diamond, high purity germanium, Peltier cooled devices, etc…). Figure 7 show the schematic diagram of the detector (accompanied with a channeltron) and the data recorded from it.

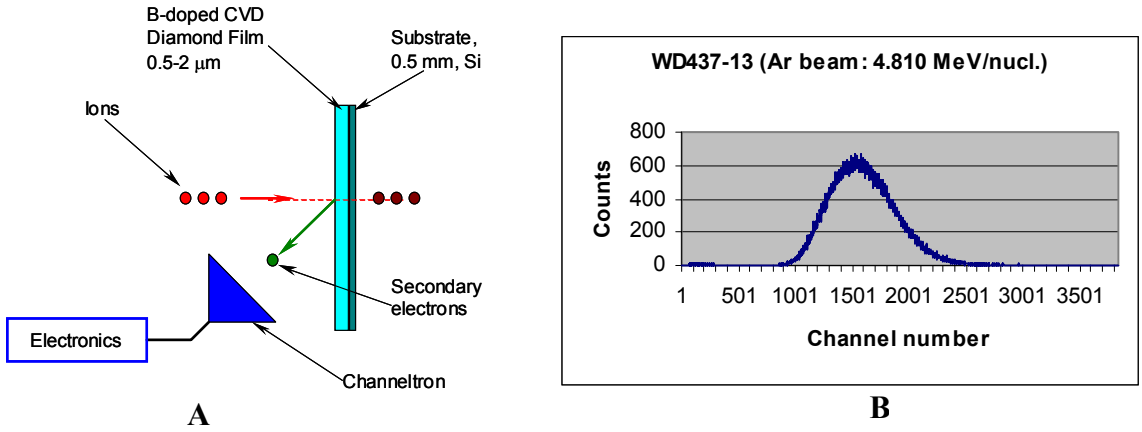

Figure 7 **A:** Schematic figure of the detector unit. Secondary electrons generated by ions from the B-doped CVD diamond film are detected by the electron multiplier (channeltron) detector. **B:** Spectrum recorded by the channeltron from one of the best boron-doped CVD diamond received from the CSEM company in Switzerland. It demonstrates a clear distinction between signal background (single electron background) on the left of the spectrum and peak (true hit peak) on the right.

It should be kept in mind that the channeltron detector at 2.4 KV bias is not strictly linear. As shown in figure 7B an event is classified as a true hit if it is above a threshold set in the valley between the single electron background and the true hit peak. We have produced 10x10 hit (figure 8) single hit pattern in glass using a focused microbeam in vacuum with a diamond detector in front of the glass sample. Figure 8 shows one perfect

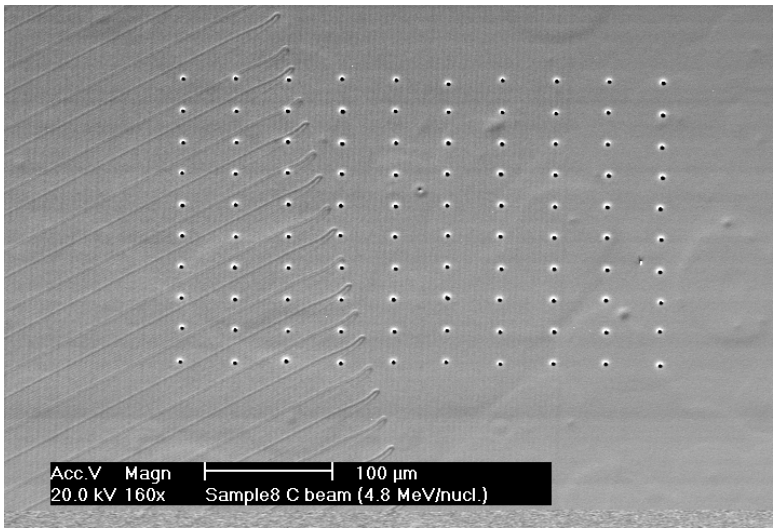

Figure 8 Pattern of 10x10 single carbon ion (4.8 MeV/nucleon) hits on glass [47]. To improve the detection of hits a thin diamond detector was placed in front of the glass sample. The hit positions have been made visible by etching the glass sample in 5% HF for about 1 min. Distance between etch pits 40 μm.

pattern. Out of 20 less perfect ones. This method was used as independent verification of the diamond detector efficiency.

In ***paper No. XII*** the author (with co-workers) has shown the possibility of using thin Boron-doped CVD (chemical vapour deposition) diamond films for the development of a highly efficient detector for ions. Similar developments led by the author at GSI enabled his group to develop a commercial detector for ions by using Boron-doped CVD diamond

[51, 52] as a secondary electron emitter. This line of research has been continued by the author in Singapore.

8. SUMMARY

The development of new analytical techniques is an essential part of our everyday life and is dictated by strong progress in modern science and technology. Both these areas require more precise information about materials and processes involved. Due to these requirements we have been observing a rapid growth in the development of techniques that require both a high spatial resolution and high sensitivity. Modern analytical techniques provide an important interface between science and applications.

The works presented in this habilitation thesis span the period of almost 20 years. During this time the author has been leading the development and applications of several new analytical and micro analytical techniques which have been documented in this thesis. This development has required development of ideas, strong leadership, organisational skills, organisation of funds and groups to carry out the necessary work.

In chapter 3 (supported by ***papers No. I-V***) the use of the PIXE and XANES techniques described an investigation of permeability for selected elements inside cells. It was important to develop new protocols for sample preparation and analysis and a large number of cells were necessary in order to obtain meaningful data. This development was closely associated with work presented in chapter 4 (***papers VI & VII***) where the role of sample damage under the MeV ion beam bombardment was investigated. At that time we were the leading group in the world to perform such studies.

Chapter 5 (***papers No. VII-X***) describes development of new analytical techniques and its possible applications. Development of the SIHF has been probably the most demanding and difficult project and was described in chapter 6 (***paper No. XI***) and it was closely related with development of a diamond detector described in chapter 7 (***paper No. XII***).

A great part of these works were performed by the author at the Micro Analytical Research Centre (MARC) in the School of Physics at the University of Melbourne in Australia. However, some works were performed at GSI in Germany and BNL in USA.

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