Addendum

* p.39 line 19: Sensitivity should be: detection limit.

* p.39 line 22: Should read: From the graph it can be seen that for a proton beam at 3MeV the lowest detectable limit is for elements in the range Z=27 to 30.

* p.39 line 26: Sensitivity should be: detection limit.

* The label on the y axis of Figure 3.7B should read: Elemental detection limit (g/g).

* In the caption to Figure 3.7B: Sensitivity should be: detection limit.

* p.49 line 16: ... elemental yields. Normalization of the data to the backscattered yield from the major elemental components C, N and O, was not considered due to the presence of these elements in the supporting nylon foil and the variation in foil thickness. Integrated charge was not considered a valid normalization factor due to the wide variation found in the thicknesses of the freeze dried fibroblasts.

* p.50 line 12: ... scanned region, and negligible charging is found in targets composed of such thin nylon foils and cells.
Application of a
Scanning Proton Microprobe
as a diagnostic tool and the development
of a high brightness ion source

Thesis submitted for
the degree of
Doctor of Philosophy

by

Garry Lindsay Allan, B.Sc. (Hons.)

School of Physics,
University of Melbourne,
December, 1989.
This thesis concerns both the application and future development of a Scanning Proton Microprobe (SPMP). The work involved the use of a microprobe in a biological project which placed heavy demands on beam brightness, and also a program to investigate and address the demand for brighter microprobe beams. The thesis thus falls naturally into two distinct, though related, sections.

The SPMP has been applied to the study of Menkes' disease, a copper-dependent genetic disorder. The disease is expressed in fibroblast cells, and the SPMP was used to map elemental distributions within both normal and Menkes' fibroblasts. An elevated level of intracellular copper was observed within Menkes' cells enabling individual cells to be identified as normal or Menkes' depending upon the copper content of the cell.

Subcellular structure within fibroblasts was investigated by using the microprobe as a Scanning Transmission Ion Microscope (STIM). It was shown that this technique affords sufficient resolution to image the nuclear membrane and nucleoli. However, at this resolution, insufficient beam current was available to permit elemental distributions to be obtained. The elemental content of subcellular and subnuclear components is of fundamental importance to biochemical processes within the cell and to the expression of Menkes' disease. Hence an increase in the resolution of the SPMP is of major importance provided that the beam current can be maintained at levels acceptable for elemental analysis.

Such a significant improvement in microprobe resolution can only be achieved with a brighter primary beam from the accelerator. This requires a brighter ion source. The performance of the existing RF ion source has been studied on a suitable test-bench, and its brightness measured. The possible use of alternative ion sources offering significant gains in brightness was investigated, and an ion source using the process of field ionization was designed and built.

Field ionization sources use a sharply pointed emitter as the site for ion production. This gives these sources an intrinsically high brightness, but in general
they have not been designed so as to produce currents suitable for use in an electrostatic accelerator. The present field ionization source was optimized to produce a maximum current whilst being sufficiently rugged and compact to withstand use within the accelerator. The beam brightness achieved with this source offered a significant increase in source brightness with sufficient current to provide stable operation of the accelerator. The successful implementation of this source would produce a major improvement in the spatial resolution available for imaging and elemental analysis with the microprobe.

This thesis is less than 100,000 words in length.
Acknowledgements

The work described in this thesis was undertaken at the School of Physics, University of Melbourne with the permission of Dr. E.G. Muirhead and Professor A.G. Klein.

I would like to thank my supervisor Dr. George Legge for his guidance and scientific insight into problems associated with my research work. Also I owe a particular debt of gratitude to Dr. Jim Camakaris for his enthusiastic and knowledgeable assistance with the preparation and interpretation of the biological work presented in this thesis.

In conducting research work within the Melbourne microprobe group I have been fortunate in benefiting from the wide ranging expertise of a number of my colleagues. I would like to thank all members of the microprobe group and in particular Peter O'Brien for his competent and thoughtful assistance in many aspects of computing and microprobe research. I also gratefully acknowledge the inspirational assistance and influence of Zhu Jieqing. Further thanks are due to Dr. Alex Mazzolini for his introduction to microprobe research and Dr. David Jamieson, Richard Brown and Glenn Moloney for their excellent contributions to the software used in this work. I have gained much from expansive discussions with Robert Colman. I thank him for many perceptive insights and his contribution to the environment in which this thesis was written. I would also like to thank Graham Bench for his collaboration with the STIM imaging.

This work has benefited from considerable input from the technical staff of both the School of Physics and the Genetics Department. In particular I would like to thank Neil George for his superlative machining of the ion source, and Roland Szymanski for his proficient maintenance of the accelerator. The preparation of the fibroblast cells was performed by Lefi Paul and Angela Bruzzaniti and I thank them both for their perseverance and constructive suggestions.

I also thank Andrew Morton for his temperate guidance in all aspects of style associated with thesis presentation.
I wish to acknowledge the financial support of the Commonwealth Postgraduate Research Award scheme during my candidature.

Finally, I would like to thank my family and friends for their exceptional support and thoughtfulness during the course of this work.
# Contents

Abstract ........................................................................................................... i  
Acknowledgements ......................................................................................... iii  

1 Introduction ................................................................................................... 1  
  1.1 The Scanning Proton Microprobe ............................................................... 1  
  1.2 Imaging and analysis with a SPMP .............................................................. 4  
  1.3 Motivation .................................................................................................. 7  
  1.4 This work ................................................................................................... 8  

2 The Melbourne Proton Microprobe ............................................................... 10  
  2.1 Introduction ................................................................................................ 10  
  2.2 Microprobe Beamline .................................................................................. 11  
  2.3 Specimen Chamber ...................................................................................... 14  
  2.4 Data acquisition ......................................................................................... 18  
  2.5 Data analysis and presentation ................................................................... 19  

3 Menkes' disease and the analysis of human skin fibroblast cells ............ 21  
  3.1 Introduction ................................................................................................ 21  
  3.2 Menkes' disease ......................................................................................... 22  
  3.3 Fibroblast cells ........................................................................................... 25  
  3.4 Formation of cell culture ........................................................................... 26  
  3.5 Preparation of cells for analysis ................................................................. 27  
    3.5.1 Specimen support ............................................................................... 27  
    3.5.2 Culture conditions ............................................................................ 30  
    3.5.3 Cryogenic preparation ..................................................................... 33  
  3.6 Microprobe analysis ................................................................................... 35
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Fibroblast analysis: results and discussion</td>
<td>43</td>
</tr>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>43</td>
</tr>
<tr>
<td>4.2</td>
<td>Specimen contamination</td>
<td>43</td>
</tr>
<tr>
<td>4.3</td>
<td>Elemental content of normal fibroblasts</td>
<td>46</td>
</tr>
<tr>
<td>4.4</td>
<td>Elemental content of Menkes' fibroblasts</td>
<td>48</td>
</tr>
<tr>
<td>4.4.1</td>
<td>Elemental distribution in a mitotic Menkes' fibroblast</td>
<td>55</td>
</tr>
<tr>
<td>4.5</td>
<td>Comparison of normal and Menkes' fibroblasts</td>
<td>56</td>
</tr>
<tr>
<td>4.6</td>
<td>Diagnosis of Menkes' disease</td>
<td>65</td>
</tr>
<tr>
<td>4.7</td>
<td>STIM imaging of fibroblasts</td>
<td>68</td>
</tr>
<tr>
<td>4.8</td>
<td>Conclusion</td>
<td>70</td>
</tr>
<tr>
<td>5</td>
<td>Ion sources: The present system and alternatives</td>
<td>72</td>
</tr>
<tr>
<td>5.1</td>
<td>Introduction</td>
<td>72</td>
</tr>
<tr>
<td>5.2</td>
<td>Pelletron accelerator and beam brightness</td>
<td>73</td>
</tr>
<tr>
<td>5.3</td>
<td>Radio Frequency ion source test bench</td>
<td>77</td>
</tr>
<tr>
<td>5.4</td>
<td>Performance of the RF ion source</td>
<td>80</td>
</tr>
<tr>
<td>5.5</td>
<td>Alternative proton sources</td>
<td>86</td>
</tr>
<tr>
<td>5.6</td>
<td>Field Ionization</td>
<td>90</td>
</tr>
<tr>
<td>6</td>
<td>Development of a high brightness ion source</td>
<td>96</td>
</tr>
<tr>
<td>6.1</td>
<td>Introduction</td>
<td>96</td>
</tr>
<tr>
<td>6.2</td>
<td>Design of a Field Ionization ion source</td>
<td>96</td>
</tr>
<tr>
<td>6.2.1</td>
<td>Source housing</td>
<td>97</td>
</tr>
<tr>
<td>6.2.2</td>
<td>Internal geometry and components</td>
<td>99</td>
</tr>
<tr>
<td>6.2.3</td>
<td>Source vacuum and gas supply</td>
<td>106</td>
</tr>
<tr>
<td>6.3</td>
<td>Emitter tip construction</td>
<td>111</td>
</tr>
<tr>
<td>6.3.1</td>
<td>Tungsten emitters</td>
<td>111</td>
</tr>
<tr>
<td>6.3.2</td>
<td>Iridium emitters</td>
<td>113</td>
</tr>
<tr>
<td>7</td>
<td>Performance of the Field Ionization ion source</td>
<td>117</td>
</tr>
<tr>
<td>7.1</td>
<td>Introduction</td>
<td>117</td>
</tr>
<tr>
<td>7.2</td>
<td>The FI ion source test bench</td>
<td>117</td>
</tr>
<tr>
<td>7.3</td>
<td>Performance of tungsten emitters</td>
<td>119</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

1.1 The Scanning Proton Microprobe

The Scanning Proton Microprobe (SPMP)\(^1\) has undergone continuous development since the first focused probe was operated by Cookson at Harwell in 1970 (Co 72). The interest in probe development has been maintained because of its unique abilities in elemental microanalysis. These qualities result from the combination of three complementary analytical techniques: Particle Induced X-Ray Emission (PIXE), Rutherford Backscattering Spectrometry (RBS) and Nuclear Reaction Analysis (NRA) in a high resolution probe. The microanalytical attributes of all three vary widely as a function of the element of interest and the target matrix composition. However, in concert they provide an overall technique that is sensitive to all elements, with a bulk detection limit as low as 0.01 ppm (Me 76).

The development of the proton microprobe has followed that of the electron microprobe (EMP) which is now available commercially in a highly evolved state. Figure 1.1A is a schematic of the electron optics used for the formation of the electron probe. The electron source is usually a sharp filament negatively biased to create an accelerating field between the filament and an anode. A lens then focuses the beam diverging from the filament. The electron source and lens constitute the electron gun shown in Figure 1.1A. The image formed by this gun

\(^1\)SPMP refers to focused high energy ion probes as well as those using protons.
Figure 1.1

Comparison of probe formation for A: electron probe and B: scanning proton microprobe

A:

B:
IG: Ion gun (Ion source and lens), A: Accelerator, M: Energy analysing magnet, OD: Object diaphragm, AD: Aperture diaphragm, L: Probe forming lens, SC: Scanning coils or electrostatic deflection plates, S: Specimen
is then demagnified by solenoid condenser and objective lenses. Beam divergence, and hence lens aberrations, is controlled by aperture diaphragms before each of these lenses. The objective lens focuses the image formed by the condenser lens onto the specimen, across which it may be scanned by electromagnetic deflection coils. Careful construction of the EMP has enabled it to achieve spot sizes as small as several nanometers. However the electron beam is extensively scattered by the specimen matrix into a pear-shaped volume (Co 78), resulting in an effective beam width comparable to specimen thickness. This effect restricts high resolution work with electron probes to prepared samples of thin cross section, where, with less material being irradiated, the problems involved in the detection of trace elements are exacerbated. Both the electron probe and the proton probe use K-shell ionization of target atoms and the subsequent production of characteristic X-rays as the major means of collecting elemental information on a target. The efficiency of X-ray production is dependent upon the velocity of the ionizing particle. For both protons and electrons, characteristic X-ray production is a maximum when the particle velocity matches the classical orbital velocity of the K-shell electrons in the target atom. For electron probes a beam energy within the range of 15 → 30keV is generally used, providing an adequate overvoltage for the readily detected characteristic X-rays within the range of 1 → 10keV. Because of the large mass difference between protons and electrons, the analytical proton beam is necessarily far more energetic than the electron beam of equivalent ionization cross section; typically most proton microprobes operate with a beam energy of several MeV.

The elemental sensitivity of the electron probe is limited by the primary bremsstrahlung produced by the decelerating electrons within the target matrix. This reduces the peak to background ratio for characteristic peaks within a spectrum. In contrast, the main source of background for PIXE analysis is secondary electron bremsstrahlung which provides a background two to three orders of magnitude less than the background produced by the electron probe (Gu 77)(Le 79). This affords the proton probe a considerable advantage in sensitivity. By way of comparison, the electron probe, when using energy-dispersive detection, can achieve elemental sensitivity within the range 100 → 1000ppm (dry weight) (Co 78). This sensitivity is significantly bettered by the SPMP, where the charge to
mass ratio of the ions is far less, and consequently the background contribution from primary bremsstrahlung is similarly reduced. The result is an elemental sensitivity as low as 0.1ppm (Jo 76) for PIXE elemental analysis with energy dispersive detection.

The higher energy beam used with a proton probe introduces many difficult technical complexities but gives that instrument a further advantage in both beam penetration and divergence within a specimen. Electrons and protons suffer comparable energy loss when traversing a section of specimen. The proton beam, which is at a far higher energy, is therefore able to penetrate deeper into the target. In addition, due to the heavier mass of the proton, beam scattering within the specimen is considerably less than that encountered with an electron probe. In combination these effects give the proton probe the ability to perform meaningful analysis of thick specimens.

A wide variety of designs has been implemented in the construction of existing proton microprobes. Design criteria have been dictated by the difficulties associated with the formation of focused ion beams of MeV energy. A generalized schematic of the most commonly used layout for high energy proton microprobes is shown in Figure 1.1B. The beam is created in the ion source, focused, and then directed into a high voltage accelerator. The accelerator is followed by a magnetic analyser, which selects the desired beam particles and energy, and provides the feedback necessary for energy stabilization of the accelerator. An object diaphragm is used to select the core of the beam from the accelerator and an aperture diaphragm defines the beam divergence and controls angle dependent aberrations in the probe forming lens. Following the lens a beam deflection unit scans the beam across the specimen, or alternatively, the specimen is scanned under a fixed beam.

Historically, the primary beam for proton microprobes has been supplied by accelerators normally used for nuclear physics experiments at low energies. These accelerators are a logical choice, as their usefulness for pure nuclear physics has diminished, leaving them available for applied nuclear experimentation. However unlike the electron probe, where the entire optical system is designed to achieve a minimum probe size, current proton microprobes rely on accelerators that are not
specifically suited to probe formation (Le 82). As the proton microprobe is further
developed by optimization of the beamline and focusing lens, greater demands will
be placed upon the ion optical performance of the accelerator and ion source.

1.2 Imaging and analysis with a SPMP

The greater sensitivity of the SPMP has enabled it to extend successfully areas of
elemental microanalysis that have previously been serviced by the electron probe,
as well as initiate new fields of research. The SPMP has been most successful
in dealing with specialized problems that fully utilize its unique abilities and as
resolution improvements have been realized the range of applications has increased.

The techniques available for rapid imaging with the SPMP are: 1.) STIM
2.) Secondary electron imaging. The techniques available for elemental analysis
are: 1.) PIXE 2.) RBS 3.) Elastic Recoil Detection Analysis (ERDA) 4.) NRA

The imaging techniques have yet to find wide application. Most specimen
selection and target navigation relies upon optical observation of the target. STIM
is used to image the areal density of a thin target by the collection of transmitted
ions (Ov 83). Both the object and aperture diaphragms must be stopped down
dramatically to reduce the transmitted beam current to a level acceptable for
a particle detector mounted at 0° (less than 1fA). This reduction in diaphragm
size results in a significant reduction in beam spot size to below that available for
elemental analysis; nevertheless image formation is rapid due to the high efficiency
of transmission collection.

STIM has found initial application with biological specimens (Sc 87)(Sc
88)(Ov 88), as the targets used in this work are generally thin and thus inher-
ently suited to transmission imaging. The low currents used with STIM permit
a preliminary non-damaging and rapid scan to be performed prior to elemental
analysis. The resolution available for such scans can be as low as 50nm (Be 89).

Secondary electron imaging forms an image of the surface topology of a
target by modulating the intensity of an oscilloscope screen with the secondary
electrons produced by the beam interaction with the target (Yo 79). The image
also shows contrast that reflects the elemental content of the area being scanned.
The beam current required for effective live secondary electron images is of the order of 100pA and is strongly dependent upon surface conductivity.

Conducting targets, both thick and thin, are readily imaged (Br 85), and, as each incident ion generally produces a secondary electron, the secondary electron yield is high enough to allow rapid image formation at both resolutions better than and beam currents smaller than those pertinent to analytical work. Targets that are poor conductors, such as uncoated biological specimens, are able to be imaged on a storage oscilloscope, but require larger currents (Le 88a). For all targets, image quality relies upon a stable target current, as current fluctuations leads to image degradation caused by variations in the electron yield.

Elemental analysis with a SPMP is most commonly performed using PIXE. This established technique has been successfully applied to analytical problems in a wide variety of fields. This is illustrated by a selection of applications of thick-target PIXE analysis with a SPMP: Geochemical targets to evaluate elemental distribution in mineral grains (Sa 87), Metallurgical targets to measure localization of critical elements in smelter products (Le 88), Thin-film and trace element analysis of semiconductors (Br 85).

The targets used in the examples given above are composed of a non-organic (Z > 9) matrix and so PIXE analysis is complicated by the abundance of low energy X-rays produced by the major matrix constituents. Frequently the elements of interest are heavy (Z > 45) and hence it is desirable to optimize collection of the low energy L series X-rays from these heavy elements. At convenient accelerator energies the L series X-ray production cross sections can be quite small, and peak detection exacting, due to the proliferation of L series lines, the large bremsstrahlung background, and interference from lighter and heavier elements’ X-ray peaks. Often elements only appear in a spectrum as weak L lines. In such cases a high-sensitivity measurement requires a significant amount of charge to be deposited on the target and the abundant X-rays removed by filtering or electronic gating. To obtain this sensitivity while keeping irradiation times to a practical level requires high currents. These are obtained by using large diaphragms which, unfortunately, lead to a loss of resolution. Increased beam intensities are tolerable for these targets as they are generally resilient to ion-induced damage.
Thin-target PIXE analysis with the SPMP has found application in the analysis of the elemental content of biological cells and tissues (Ob 82)(Li 84)(He 86)(Va 85)(Ob 87), and also in the determination of elemental distributions in botanical specimens (Ma 85).

The PIXE spectra from thin targets are generally uncomplicated. However there is a major interest in trace elements in thin biological targets. As the thin-target X-ray yields are low, high target currents are desirable to permit the detection of trace elements within a reasonable time.

Focused ion beams using $^4$He$^+$ and RBS are used routinely where depth resolution and film analysis are required. In a similar manner ERDA has been used for hydrogen profiling (He 86). The Melbourne SPMP has found recent and innovative application in the ion beam analysis of semiconductors. The ability to channel a focused ion beam (In 81) has provided new insights into damage studies in implanted semiconductors (Mc 87) and combined with the depth resolution of RBS offers an accurate measurement of the location of elemental impurities in a host matrix. The changes in RBS yield encountered with a scanning channeled microbeam can be used to image structural variation and imperfections in a crystal lattice (Mc 83). However channeling with a microbeam places a number of stringent ion optical demands on the probe forming system. Channeling requires a convergence half angle of $\alpha < 0.15^\circ$. Target beam current stability must be greater than 30% over a one second time period and sufficient current must be available in the focused beam spot to provide an adequate RBS yield.

Most NRA work with ion microprobes has been performed at Harwell (Co 87). This specialized technique is suited to the analysis of low Z elements in a heavy-element matrix and to the detection of fluorine via the reaction $^{19}$F(p,αγ). The technique has found only limited application with microbeams as most reactions of interest have relatively small cross sections. The low yields thus obtained necessitate that beam target currents be as high as possible.

The examples of imaging and analysis discussed above highlight the need for an optimal compromise between target current and beam resolution in all spheres of SPMP application and reinforce the need for stable target irradiation.
1.3 Motivation

The SPMP has found particular application in biology where elemental information at the cellular and subcellular level is of fundamental importance. The electron probe has been widely applied to the study of macro elements within cells and cell strata (Ha 73), and now the SPMP, with its ability to detect both macro and trace elements in biological systems, is being applied to an increasingly wide range of biological problems. The elemental content of individual cells is of great importance as basic biochemical processes depend upon the presence and transport of both macro and trace elements within cells. This is reflected in the known elemental dependence of a variety of genetic diseases. A particular genetic disorder, Menkes' disease, is known to effect copper metabolism in the human body. The discovery of the link between this disease and a defect in copper uptake and transport, has resulted in an intensive and successful study of Menkes' disease. It has been shown that skin fibroblast cultures from Menkes' patients exhibit intracellular copper levels elevated above that obtained from normal fibroblasts (Go 76). However, direct comparison between the copper levels of individual cells has required an analytical technique capable of resolving single cells whilst providing an elemental sensitivity that is able to detect trace levels of copper within these cells. An electron microprobe has been applied to elemental studies in Menkes' disease but the copper sensitivity was found to be inadequate. The SPMP, with its greater sensitivity, is then a logical choice for single-cell studies involving Menkes' disease.

This work has been motivated by the need to determine the elemental distribution within human skin fibroblasts as an adjunct to the study of Menkes' disease. The SPMP at its existing level of development is able to spatially resolve single cells, but subcellular elemental detail cannot be clearly discerned. As the function of subcellular features is integral to the biological operation of the cell, it is evident that further improvements to the resolution of the SPMP is of major benefit to cellular microanalysis.
1.4 This work

The work presented in this thesis details the Melbourne SPMP, the use of this probe in the elemental microanalysis of single fibroblast cells and the development of an ion source especially suited to a high resolution SPMP.

In Chapter 2 the Melbourne SPMP is described in the configuration used for the collection of all microprobe data presented in this thesis. A wide range of technical and developmental work on the microprobe beamline and specimen chamber has been undertaken during the course of research for this thesis. Also included in Chapter 2 is a description of the techniques of data collection, analysis and presentation used for the work presented in Chapters 3 and 4.

Chapter 3 focuses on the genetic disorder Menkes' disease and its connection to elevated copper levels in cultured fibroblast cells. The technique of culturing both normal and Menkes' fibroblasts on a surface suitable for sensitive microprobe analysis is detailed as are the operational conditions used on the microprobe.

The results of the microprobe analysis of both normal and Menkes' cells are presented in Chapter 4. The sensitivity of this technique highlights the possibility of elemental contamination, which is discussed, as are the refinements necessary to ensure valid elemental results are obtained. The elemental content of both normal and Menkes' cell types are categorized and a comparison formed between the two cell types. The possibility of using this technique to identify both a Menkes' sufferer, and a heterozygote is expounded. Internal structure of the fibroblast is shown in a high resolution STIM image of the cell. Elemental data cannot be obtained with equivalent resolution and the chapter concludes by discussing the present limitations of the microprobe system for cellular and subcellular analysis.

The effective spatial resolution of the Melbourne SPMP for elemental analysis is limited by the accelerator ion source. The existing source has been studied on a suitable test bench. Chapter 5 contains the results of this investigation and a study of possible alternative sources compatible with the existing accelerator and microprobe.

Chapter 6 deals with the development and construction of a Field Ionization (FI) ion source specifically suited to the requirements of a high resolution proton
microprobe. The source fundamentals are derived from the design of traditional field ion microscopes and are adapted and modified to be suitable for use in the harsh environment found within an electrostatic accelerator.

The performance of this source was evaluated on a test bench and the results of these tests are presented in Chapter 7. Steps were taken to ensure that current output was a maximum whilst acceptable levels of current stability were maintained.

Chapter 8 reviews the work covered by this thesis and indicates areas for improvement of the FI ion source as a prelude to its installation in the accelerator.
Chapter 2

The Melbourne Proton Microprobe

2.1 Introduction

The Melbourne Proton Microprobe (MP) initially began operation in 1975. The MP system employed the principle of orthomorphic focusing with a Russian quadruplet of magnetic quadrupole lenses. This technique had first been used on the Harwell microprobe (Co 72), and produces a demagnified image of an object diaphragm in the target plane. Since its inception and during the continuous development that has occurred since 1975, the Melbourne probe has maintained a number of distinctive design features aimed at establishing and enhancing its usefulness as an analytical instrument. The microprobe has retained the magnetic quadrupole quadruplet focusing system (see Figure 2.1).

The microprobe obtains its primary beam from a Pelletron electrostatic accelerator. The cleanliness of the accelerator vacuum is compatible with the vacuum requirements of a sensitive high resolution microprobe, hence the microprobe beamline and specimen chamber are able to be operated at high vacuum. This ensures that target specimens are examined under the ultra-clean conditions necessary for the reliable measurement of trace element concentrations in specimens. The evolution of the MP system has resulted in a probe designed for optimal data collection and sensitivity with a wide range of target specimens.

A significant advance in target handling and data collection was achieved in 1984 with the commissioning of a new specimen chamber (Le 86). The chamber’s design was based on experience gained during the use of the initial microprobe.
Figure 2.1
Schematic diagram of Pelletron Accelerator and MP beamline.

Pelletron Accelerator:

MP beamline:
M: Magnetic beam steerer, N: Monitor Faraday Cup, O: Object diaphragm, P: STIM object strips, Q: Removable Faraday Cup, R: Ion pump (125ls⁻¹), S: Aperture diaphragm strip, T: Anti-scattering collimator, U: Removable Faraday Cup, V: Quadruplet of magnetic quadrupole lenses, W: Si(Li) X-ray detector, X: Magnetic scanning coils, Y: Specimen chamber, Z: Specimen chamber vacuum lock, G1: Beam-line support girder, S1: Girder support block
specimen chamber (Ma 82), and features a rapid change multiple target facility. Target position is now controlled by a highly accurate microprocessor driven X-Y stage, and a high-power optical microscope is available for target observation. The probe has also undergone a continuous improvement in beam spatial resolution, the most significant advance occurring in 1980 with the installation of a new set of quadrupole lenses designed specifically for the MP system (Le 82). This has enabled beam resolutions of 1μm to be achieved routinely.

The data collection technique is central to the effectiveness of the microprobe. The Melbourne SPMP has been used to develop a total quantitative recording and analysis technique (Le 79)(Ob 88). This technique involves the storage of all radiation events in real-time sequence and hence offers the maximum possible versatility as all elemental data is recorded directly onto disk or magnetic tape with a single irradiation. Afterwards, the data can be sorted and analysed with off-line computer facilities. These facilities provide the following four distinct forms that can be used to represent radiation (both X-ray and scattered particle) spatial distributions: 1.) Point map 2.) Contour map 3.) Grey scale mapping 4.) Colour mapping.

This chapter details the Melbourne Proton Microprobe in the configuration in which it was used to perform the microanalytical work presented in this thesis.

2.2 Microprobe Beamline

The MP system is represented schematically in Figure 2.1. The primary beam for this system is supplied by a National Electrostatics Corporation 5U Pelletron accelerator and consists typically of either 3MeV protons or 2MeV He+ ions at currents of the order of 1 → 10μA. This beam is incident upon the Monitor Faraday Cup at the entrance to the microprobe beamline. The cup is used to monitor constantly the beam current entering the microprobe line, and provides the initial major collimation by only accepting the core of the primary beam through a 300μm diameter central hole in its backing plate. An electromagnetic steerer, attached to the microprobe girder, immediately precedes the monitor cup and is used to steer the beam along the central optical axis of the quadrupole lenses. Under normal
operating conditions the monitor cup must dissipate ~ 30W over an area of 3mm² in a high vacuum environment, and so to avoid the possibility of overheating, the cup is made of molybdenum with a water-cooled tantalum backing plate.

Immediately following the monitor cup is a series of circular object diaphragms located in a 50µm thick platinum strip. This strip is sufficiently thick to stop 3MeV protons, and was commercially supplied to satisfy custom design requirements. The four object sizes available on this strip are: 25µm, 37.5µm, 50µm, and 100µm. In addition to these a separate 5µm diaphragm is available. This diaphragm is formed in a 150µm thick platinum disc. The 5µm diameter circular hole is 8µm deep. At this depth the hole becomes conical in cross section and diverges out to a diameter of 200µm on the opposite side of the disc. As 3MeV protons have a range of 26µm in platinum, straggling can occur with this object, producing a beam with a diffuse halo of low energy ions. However the stringent collimation of the microprobe stops any off axis ions and the high magnetic fields in the quadrupole lenses strongly deflect straggled paraxial ions, so that the beam spot in the target plane is a demagnified image of the 5µm section of the diaphragm.

Both the object strip and the 5µm diaphragm disc are positively located in a water-cooled copper block. The cooling is used to prevent distortion of the diaphragms, which can result if heat build up occurs. A micrometer drive, with a positional accuracy of 1µm, is used to move the object diaphragms across the beam. A diaphragm is selected for use when it is placed directly behind the 300µm hole in the monitor Faraday Cup. The beam may then pass through the defining object and down the beamline.

A series of aperture diaphragms ranging in size from 0.25mm to 4mm are located 5m downstream from the object diaphragm, and are used to define the angle of divergence of the beam that will be accepted into the quadrupole lens system (see Figure 2.2). The aperture diaphragm strip was formed by drilling a series of holes in a 100µm thick tantalum plate. This plate is sufficiently thick to stop 3MeV protons, the range of which is 32µm in tantalum.

The desired aperture diaphragm is selected by a mechanical drive system that moves the strip laterally across the beam with a positional accuracy of 0.1mm.
Figure 2.2
Dimensional schematic of the MP beam line. All lengths are in millimetres.
Object Diaphragm  Aperture Diaphragm  Quadrupoles  Image Plane
Immediately following the selected central aperture is an anti-scattering collimator, also made from 100µm thick tantalum sheet, which prevents the transmission of ions through the off axis diaphragms.

Following the example set by Cookson et al. (Co 72), the MP system uses a quadruplet of magnetic quadrupoles to focus the beam. A single quadrupole lens will focus the beam in one plane and defocus the beam in a perpendicular plane, hence two or more quadrupoles are required for a point focus. The quadrupoles are operated as a Russian antisymmetric quadruplet (Dy 65), a configuration which sets alternate lenses with opposite polarity with the first and fourth lenses at equal excitation and the second and third lenses also at equal excitation. This lens configuration forms an orthomorphic and stigmatic point image.

The quadrupoles are located 3.8m downstream from the aperture and were manufactured in the School of Physics workshop (Le 82). Their performance has been subject to extensive study (Ja 85).

Each quadrupole is mounted on a trolley which allows it to be moved parallel to the beam axis along an accurately leveled baseplate. Throughout the duration of the experimental work undertaken for this thesis the quadrupoles were placed together and as close to the target plane as possible. (Minimum working distance: 208mm).

Quadrupole alignment is critical, with off axis displacements causing large steering effects, so each trolley has three separate quadrupole positional adjustment mechanisms. Set screws are used to move the quadrupole horizontally, an eccentric cam placed under the quadrupole moves it vertically and a micrometer is used to rotate accurately the quadrupole about the beam axis. A high level of current stability is necessary in order to minimize excitation aberrations in the quadrupoles. The quadrupoles use a current supply that is stable to better than 10ppm, and is controlled by an accurate resistive control box.

Immediately following the quadrupoles are the electromagnetic scanning coils. There are two sets of coils wound to a saddle-like shape, both sets being 8cm long, while one has 12 field windings, the other 16. Each set of coils consists of a pair of windings with one pair rotated 90° with respect to the other. The windings are bound to a rotatable plastic sleeve that enables scans to be set up at
any angle. The beam is scanned by a triangular current waveform which is applied to the coils.

Due to the importance of trace elements in biological systems and the low cross section for X-ray production, it is often necessary to irradiate the biological specimens for a number of hours in order to obtain suitable statistics for elements of interest. This places demands on the long-term mechanical stability of the microprobe beamline. The beamline has therefore been constructed to a high level of mechanical rigidity and is housed in a temperature controlled laboratory. The beam tubing is firmly mounted on a rigid square section girder which is elevated above the laboratory floor by two adjustable pillars that are bolted to the floor. The mechanical vibration of the beamline has been measured to be less than 1nm over the frequency range of 0.1 to 1000Hz.

2.3 Specimen Chamber

The applicability of the MP system depends greatly upon the design of its specimen chamber. The present specimen chamber (see Figure 2.3) features a vacuum lock and a multiple target facility that permits the user to run on a variety of different targets without venting the chamber to air during target changing. This makes the microprobe analysis more time efficient and enables an ultra high vacuum to be maintained in the specimen chamber.

The materials chosen for the specimen chamber were selected on the basis of their suitability for use in an ultra-high vacuum environment. The chamber body was milled from a solid block of stainless steel and stainless steel components are used wherever possible. With the exception of one Viton O-ring the chamber is sealed by an all metal gasket system and is pumped directly by a 120 ls⁻¹ ion pump. After baking out at 60°C the chamber has a base pressure of less than 1 x 10⁻⁷ mbar.

A vacuum lock is located below the specimen chamber and is pumped by a 125 ls⁻¹ turbomolecular pump. A manually operated target changing mechanism moves a target support block from the vacuum lock to the specimen chamber, where it is held in position by an arm connected to an X-Y stage. A gate valve
Figure 2.3
Schematic diagram of a side view of the specimen chamber.

A: Target holder
B: Microscope eyepiece
C: Prism
D: Turret holding the five internal objective lenses
E: Concave mirror
F: Photometric camera
G: Goniometer
H: Target stage and stepper motors
I: Scanning coils
J: Faraday cup
K: Si(Li) detector
L: Backscattering charge particle detector
M: Electron detector light pipe
N: Electron detector phototube

Top centre is the Goniometer, which is connected to the X-Y stage. To the lower right is the electron detector photomultiplier tube. The beam enters the chamber from the centre right, after passing through the bore of the last magnetic quadrupole. The magnetic deflection scanning coils surround the beam tubing immediately before it enters the chamber. Below this top chamber, and projecting radially outwards are six rotary motion feedthroughs which control the position of detectors and a mirror on a concentric ring system in the base of the top chamber. Beneath the feedthroughs is a mid-chamber gate valve which separates the top chamber from the lower vacuum lock.
separates the vacuum lock from the specimen chamber, and target transfer only occurs when the pressure in the vacuum lock is below $2 \times 10^{-6}$ mbar. The complete change-over time for a target support block, which can hold up to four separate targets, is approximately 10 minutes.

Target position is controlled by a micrometer-driven X-Y stage with each micrometer being turned by a stepper motor. A Signetics 2650 based microprocessor controls the stepper motors to a positional accuracy of 0.5µm over an available range of 1cm.

Thin biological targets can be accurately observed with a high power transmission binocular microscope system. The objectives are encased in stainless steel and placed directly behind the target within the specimen chamber. A prism mounted behind the objectives optically links them to either the oculars or a photometric camera mounted on the top of the chamber. The four internal objectives are mounted on a rotatable turret, which is gear driven by a rotary feedthrough allowing the selection of magnifications ranging from $\times 100$ to $\times 800$. Specimen illumination is achieved by the placement of a microscope lamp outside the chamber, which can illuminate the target area directly through the glass side window, or light, reflected from an internal mirror, can be transmitted through the specimen to the internal objective.

The microscope is used both for specimen observation and to enable observation of the beam spot position and focus on a glass slide. When the proton beam is focused on a transmission target both the objectives and the prism are rotated out of the path of the transmitted beam allowing the beam to be collected by a Faraday Cup (see Figure 2.4).

The proton microprobe makes extensive use of Particle Induced X-ray Emission (PIXE) to determine information on the elemental composition of a target matrix. As there is often a strong interest in elements that are present in trace concentrations, it is necessary to employ an efficient, high resolution X-ray detector. The MP specimen chamber uses a custom designed Lithium drifted Silicon X-ray detector (Si(Li)) supplied by EG & G Ortec Inc. The Si(Li) has an extended snout that allows the 12.5mm$^2$ crystal to be placed as close as possible to the target without interfering with the scanning microbeam. The Si(Li) snout
Figure 2.4
Plan of the MP specimen chamber.

A: Target holder
E: Concave mirror
I: Scanning coils
J: Faraday cup
L: Backscattering charged particle detector
M: Electron detector light pipe
N: Electron detector phototube
O: Forwards scattering charged particle detector
P: External light source
Q: Glass side window
enters the chamber at 45° to the beam path (see Figure 2.3). The detector position can be varied at this angle as the detector is joined to the chamber within extendible bellows and the dewar is mounted on a trolley that runs along a 45° ramp. This permits the detector to be wound in to within 20mm of the target, at which point it subtends a solid angle of 31msr. The detector was supplied with an 8µm thick beryllium window, which is insufficient to stop backscattered protons at the 3MeV bombarding energy typically used on MP. A filter holder has been attached to the detector snout so that additional filters may be added when necessary. For all the 3MeV proton work conducted on MP and included in this thesis an additional beryllium filter of 120µm thickness was placed in front of the detector. This is sufficient to stop backscattered protons at 3MeV, the range of which is 98µm in beryllium. This additional filter greatly attenuates low energy X-rays, so that for PIXE analysis with 3MeV protons the detector is sensitive to elements heavier than and including silicon. The detector has a measured energy resolution of 160eV (FWHM) for the 5.895keV $^{55}$Mn Kα X-rays produced by an $^{55}$Fe source.

Further elemental information can be obtained from the energy distribution of forward and backscattered particles. The backscattered data presented in this thesis were collected with a Tennelec silicon charged particle detector placed at a scattering angle of 150°. The detector had an active area of 50mm², subtended 31.3msr and had an approximate energy resolution of 12keV. All forward scattering data discussed in Appendix 1 were collected with an identical detector placed at 70°.

For high resolution work an accurate knowledge of the relative position of the proton beam and the specimen is of major importance. The internal optical microscope enables the target to be accurately located with respect to the beam position; however it requires a glass slide to be placed in the beam path, then removed while the target is placed in the correct position relative to that of the beam. This is tedious and may require a number of time consuming attempts to align low yield targets. The technique is also inapplicable to stopping targets. In order to circumvent these problems a secondary electron detector was designed and built (see Figure 2.5).
Figure 2.5
Schematic diagram of the secondary electron detection system.

A: Electron collector (Aluminium coated scintillator)

B: Glass light pipe

C: Vacuum feedthrough

D: Optical coupling

E: E.M.I 30mm photomultiplier
The secondary electron detector collects electrons liberated from the surface of the target by the bombarding proton beam. The detector is composed of a collector, lightpipe and photomultiplier.

The collector consists of a 500µm thick disc of plastic scintillator covering the termination of a 14mm diameter glass light pipe, with a layer of aluminium, approximately 0.03µm thick, evaporated onto the scintillator. The aluminium layer extends around to the side of the light pipe, where it makes contact with a stainless steel biasing ring. This ring is connected to a positive polarity high-voltage power supply via a suitable high-voltage vacuum feedthrough. The collector itself is 60mm from the front surface of the target and is raised to a voltage of 6kV. The electric field between the collector and the grounded target attracts secondary electrons produced by the irradiation of the target, and these electrons have sufficient energy when they reach the collector to penetrate the conducting aluminium coating covering the scintillator. Optical pulses produced by the electrons striking the scintillator pass down a pure glass light pipe which leaves the specimen chamber and connects via optical coupler to an E.M.I 30mm photomultiplier tube. This 11 stage tube is used at a maximum gain voltage of −1.5kV, and the output signal is passed through an Ortec 113 preamplifier and an Ortec 410 linear amplifier, before being fed into the Z modulation input of a B.W.D Model 845 storage oscilloscope. The X and Y traces of the oscilloscope are driven by the X and Y scanning outputs respectively, enabling a secondary electron image of the scan region to be displayed on the oscilloscope screen. The use of a storage oscilloscope allows a quality image of the specimen surface to be built up over a short time interval (−10s). The image is produced with the spatial resolution of the proton beam and can be enhanced by the optimization of storage parameters on the oscilloscope. The displayed region corresponds exactly to the area on the target being irradiated by the scanning beam. Navigation on the surface of the target is convenient as a large scan allows observation of a wide area on the target's surface. The target may then be moved around in front of the beam, enabling features of interest to be selected. The scan size may then be reduced to concentrate the beam on the desired area.

The use of the electron detector with fibroblast cells is detailed in Section
2.4 Data acquisition

The flexibility and usefulness of the microprobe is greatly dependent upon a rapid and accurate data acquisition system. The system described was used for all the microprobe data presented in this thesis; however it may also be expanded for additional detectors and current integration (see Appendix 1) (Ma 82). The MP acquisition system is based upon the event by event recording of an event triplet, consisting of a detector energy E and the coincident beam position X and Y. The beam position coordinates are derived from the deflection current in each of the scanning coils at the time of the event.

The MP data acquisition electronics used for this work are represented schematically in Figure 2.6. Acquisition of an event is initiated by radiation entering either the Si(Li) X-ray detector or backscattering detector; this results in current pulses which are integrated by close coupled preamplifiers, to produce voltage pulses that are proportional to the energy of the incident radiation. These pulses are amplified and fed into a multiplexer. A fast coincident timing pulse is generated by a timing single channel analyser (TSCA), and the leading edge of this pulse is used to transmit the appropriate detector signal through the multiplexer, which is run in a two channel state. The multiplexer allows each input channel to be offset in the memory of the PDP 11/40 computer, thereby producing separate spectra for each detector. Typically both detectors will address a 2K region in memory. The multiplexer produces a 'busy' pulse that indicates that the energy ADC (ADC 0) is converting a transmitted analogue signal. This pulse is fed into a gate and delay generator (GDG) and is used to gate both ADC 1 and ADC 2. These two ADCs then sample the voltage across standard one ohm resistors in series with both the X and Y scanning coils respectively, and hence record beam position at the time of the radiation event. The multiplexer 'busy' pulse is also used to gate the energy ADC, (ADC 0), via two GDGs, and another GDG is used on this pulse to produce an enabling pulse on the computer interface after the digital conversion of the event triplet E, X and Y.
Figure 2.6
Schematic diagram of data acquisition electronics.

Charge collection
FC: Shielded Faraday cup, K: Keithley 603 Electrometer, CD: Current digitizer (Ortec 439), C: Counter (Ortec 771)

Detector signals and timing
1.) X-ray Signals
BS: Si(Li) Bias supply (Ortec 459), PA: Preamplifier (Ortec 117B), LA: Linear amplifier (Ortec 452), TSCA: Timing single channel analyser (Canberra 2037)
2.) Particle Detector Signals
PD: Backscattering particle detector (Tennelec), PA: Preamplifier (Ortec 142), LA: Linear amplifier (Ortec 572), TSCA: Timing single channel analyser (Canberra 2037)
3.) General
MUX: Four channel multiplexer (Ry 85), GDG: Gate and delay generator (Ortec 416A), R: Ratemeter (NE), ADC: Analogue to digital converter (Tracor Northern NS623)

Scanning signals
SC: Scan controller, PS: Current power supply, A: Voltage amplifier and line driver
Data acquisition was conducted on an online PDP 11/40 computer with 64K of 16 bit memory. Each ADC writes to one word to record an event, as shown in Figure 2.6.

Beam current is collected by the Faraday Cup and amplified by a Keithley Electrometer. A current digitizer is then used to monitor the charge. A series of scaler counters log the total charge deposited on the target (see Figure 2.6).

2.5 Data analysis and presentation

The MP data acquisition system produces an event by event record on either disk or magnetic tape of all detector energies along with the coincident beam position coordinates. A system has been developed for rapid offline sorting and analysis of the recorded data. The system makes use of the PHYDAS (PHYsics Data Acquisition System) computing facility (Mo 85), which is based around a Data General MV8000 computer. Initially, the data is transferred to disk on the MV8000 computer. This transfer takes place along a custom high speed data link between the MV8000 and an offline PDP 11/40 computer as shown in Figure 2.7. The data is then rapidly sorted into a readily accessible data base by the MV8000 and stored on a work disk.

Upon completion of the sort the fast-link is then reconfigured, so that the offline PDP 11/40 can then be used to analyse and display the data. Software previously developed on the PDP 11/40 can be used for the extraction of energy spectra from selected regions of interest within the scan region (Ma 82). The offline computer uses a high resolution Tektronics storage oscilloscope to display the mapped data. A region of interest is defined by the movement of a point cursor, whose position is controlled by the keyboard. The ability to select a particular area within a scan, and to extract a spectrum from that region is especially powerful when undertaking analysis of cell cultures, where cells grow in close proximity and a single scan may cover a number of cells. The high resolution screen also enables subcellular regions of interest to be selected so that spectra may be extracted from these areas.

The sorted data can also be displayed with a suite of programs which are
Figure 2.7

Schematic of the computer system used for data analysis.
executed on the MV8000 computer. A 2 and 3 dimensional contour mapping program is available which uses drawn contours to join points of constant radiation yield and presents these contours as a surface defined by three mutually orthogonal axes. The X and Y axes correspond to the X and Y scan directions and the Z axis is used to represent the number of events collected within a selected energy range. The resultant maps feature a series of contours representing levels of intensity in radiation yield. The contour data set can be rotated for viewing from any angle, with direct viewing along the Z axis presenting a two dimensional view of the data. The use of contour plotting is illustrated in Figure 4.1B. For these maps the X-ray intensity at a particular point can be determined by counting the number of contours up to the contour level associated with that point. The two dimensional map uses a total of ten contours and the three dimensional map fifty contours, with every fifth contour corresponding to a contour in the two dimensional map and highlighted by an enhanced width. Comparison of the relative yield between separate contour plots can be formed by the normalization of the Z axis of the data set. The program also provides the option of smoothing statistical fluctuations in the data set. The smoothing is performed by convoluting the data with a rotated Gaussian representation of the microprobe beam spot. The examples in Figure 4.1B have been smoothed by this method.

The data set can also be displayed by using a grey scale point map to represent radiation intensity. Figure 4.12B is an example of such a grey scale map which can display up to 32 shades at a resolution of 12 pixels per mm.
Chapter 3

Menkes' disease and the analysis of human skin fibroblast cells

The work presented in the following two chapters was performed in collaboration with Dr. J. Camakaris from the Genetics Department, University of Melbourne, who performed the bulk measurements of Table 3.1 and the work described in Section 3.4.

3.1 Introduction

The important role played by heavy trace elements in a wide range of biochemical processes, has invited considerable research aimed at eliciting a greater understanding of the functions performed by these elements. Clinical studies have provided a background on the physical effects of heavy elements on the human system and technological improvements have enabled links to be defined between heavy elements and known clinical diseases.

In 1961 Menkes et al. (Me 62) described a syndrome associated with infant mortality. It had been known since the early 1950s that copper deficiency posed a potential health risk for infants and in 1972 this syndrome was positively linked to a disturbed copper metabolism by Danks et al. (Da 72). The disease is genetically inherited, and has been labeled Menkes' disease. The known copper link allows Menkes' disease to be used to provide greater insight into fundamental genetics and copper metabolism, hence research into the disease has created considerable
interest (Da 83).

The mutant gene is expressed in cultured cells. Consequently cell cultures have offered a convenient and controlled environment for the study of this disease. Bulk measurement by atomic absorption spectrophotometry has shown that Menkes' fibroblast cultures exhibit elevated intracellular copper concentrations (Go 76). This technique can differentiate between a Menkes' fibroblast culture and a normal control fibroblast by comparing the intracellular copper level. However, fibroblast cultures taken from a female carrier cannot, in most instances, be differentiated from the normal control by this method. In addition, the preparation and analysis time required for a bulk measurement limits the effectiveness of the technique for prenatal diagnosis of a Menkes' fetus, as an amniocentesis can only be performed sixteen weeks after conception and a further period of approximately four weeks is required for a bulk analysis. This length of time is necessary in order to grow sufficient cells for the bulk measurement.

The Melbourne SPMP has sufficiently good spatial resolution to resolve individual fibroblasts, and hence has the potential to differentiate between the two cell types by sampling a small population. Small populations of fibroblasts (~1000 cells) can be grown rapidly in culture over a period of approximately 6 days. Thus if this is combined with efficient microprobe analysis, an alternate, more workable diagnostic test would be available for the detection of a Menkes' fetus. The technique could also be extended to identify female carriers from their expected bimodal distribution of Menkes' and normal fibroblasts.

This chapter presents work performed in developing fibroblast cultures under conditions which allow the cells to be analysed accurately by the SPMP. The chapter also details the method used for the SPMP analysis of fibroblasts.

3.2 Menkes' disease

Within the nucleus of most cells in the human body are 46 chromosomes, which are thread-like structures consisting of DNA and proteins. The chromosomes contain genes which code for the cellular proteins and enzymes and play a critical role in sex determination. In a normal human male, there are 22 pairs of similar homologous
chromosomes, while the 23rd pair is odd as it is formed of dissimilar chromosomes termed X and Y. These are the sex chromosomes. In a human female, they are homologous chromosomes, X and X.

Menkes’ disease was first described by Menkes in 1961 and is generally inherited from a carrier-mother (heterozygote) by her male offspring. Figure 3.1 shows the pattern of inheritance of the mutant gene. The disease can be readily identified in an affected baby by a range of distinct symptoms. The child has unusual ‘steel-wool like’ hair, an abnormal facial appearance and severe neurological abnormalities. The skin is particularly lax and is deficient in pigmentation. Body temperature can be as low as 29°C and mental retardation is apparent. Skeletal defects can be found and bones tend to be prone to fracture. Notably all major arteries are distorted. Death usually results from the rupture of an artery before the time the child reaches 3 years of age.

As with most inherited disorders, Menkes’ disease is rare. In 3 births out of 400, a baby is born with a genetic disease. Menkes’ disease is found in approximately 1 live birth in 50,000 (Da 83). Even though the disease is rare its inheritance pattern means that tests need to be developed to detect affected fetuses and carriers within families (termed target groups) where the mutant gene exists.

Menkes’ disease is termed X-linked as it results from a mutation on the X-chromosome. In a given cell only one X chromosome is active. Therefore the female heterozygote (potential mother of an affected child) will have on average half her cells mutant and the other half normal. The normal cells compensate for deficiencies in the mutant cells, so that the heterozygote remains unaffected by the disease. Hence the disease is referred to as X-linked recessive. The Y-chromosome in a male carrying the mutation does not have a copy of the gene which is affected in Menkes’ disease and so the full effects of the disease are expressed.

Menkes’ disease was identified as being associated with a disturbed copper metabolism by Danks et al. (Da 72). In normal cells excess levels of heavy metals induce the protein metallothionein, which then acts to bind and detoxify these

1Two affected females have been observed (Iw 79)(Fa 83).
Figure 3.1

Sex determination and chromosome inheritance for Menkes' disease. The mutant gene is carried by the female in any pairing. Four possible inherited genes are equally probable, two of which produce normal male and female children. The mutant gene may, however, be passed on to a female heterozygote, who will remain essentially unaffected. It may also be passed to a male offspring who will be fully afflicted by the disease. Although the potential exists for a Menkes' male to pass on the gene, in reality no such child lives long enough to mate. The mutant gene is therefore carried by the heterozygote.
**Sex chromosomes**

- **X**
- **Y**
- **X+X**: Heterozygous Female Carrier
- **X+Y**: Menkes mutant Male
- **XY**: Normal Female
- **XX**: Normal Male

**Four possible pairs of sex chromosomes**

**Female**

- **X+X**

**Male**

- **XY**
  - **X+**: Mutant chromosome

**Cross**

- **X+X** ⊗ **XY**

**Menkes inheritance**

**Sex chromosomes**
metals. It is believed that the mutation in Menkes' cells leads to a defect in intracellular copper transport, which allows copper to be accumulated in a form which induces the overproduction of metallothionein as a secondary effect. This overproduction will cause Menkes' cells to retain copper in a biologically inaccessible form inside the cell. Thus Menkes' patients have been found to exhibit elevated copper levels in the extractive organs (kidneys and duodenum). With the copper supply being impeded at this point diminished copper levels are found in the liver, serum and brain. The tendency of Menkes' cells to accumulate copper is also seen in the elevated copper content of Menkes' cells grown in culture.

It has been shown that a variety of Menkes' cells preferentially accumulate abnormally high amounts of copper (Ca 80) and can be differentiated from normal cells of the same type by comparing the copper level found within the cell. The copper uptake and retention in both normal and Menkes' cells can be enhanced by growing the cells in a copper rich medium. Table 3.1 shows the results of bulk measurements, by atomic absorption, of the copper content of a range of normal and mutant human cells. The results were obtained from measurements made on cell cultures containing approximately $1 \times 10^6$ cells (Ca 80). The table clearly shows that Menkes' disease is consistently expressed as elevated copper levels in the cultured cells. These results however give no specific information on the elemental content or distribution within an individual cell. Such information, particularly that relating to copper, is fundamental to the detection of carriers and an understanding of Menkes' disease.

Fibroblasts derived from skin biopsies were considered the cell line most suited to the investigation of the elemental distribution within Menkes' cells with a SPMP. This choice was based on a number of factors. The fibroblast is a robust and readily grown cell that plates as a monolayer and clearly expresses the Menkes' mutation. Fibroblasts have previously been used for a number of studies involving the uptake and transport of copper in Menkes' cells (Go 76)(Ca 80) and sufficient research on bulk samples has been undertaken to establish expected elemental concentrations (Ca 80).
Table 3.1: Bulk measurement of the copper content (µgCu/10^6 cells) of a variety of human cell lines. Two sets of results taken from (Ca 80) are shown. One set was for the cells cultured in normal growth and maintenance media (FbGM and FbMM, see Section 3.4), the other set was obtained from cell growth in FbGM and a copper rich maintenance medium (FbMMCu+, see Appendix 2). Both sets show the results for normal and mutant Menkes' cell lines and are presented as mean±SD with the number of observations in parentheses.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Normal medium (0.05 → 0.07 µg/ml Cu)</th>
<th>Copper-loaded medium (6 µg/ml Cu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal fibroblasts</td>
<td>0.023±0.013 (12)</td>
<td>0.113±0.074 (12)</td>
</tr>
<tr>
<td>Menkes fibroblasts</td>
<td>0.282±0.091 (6)</td>
<td>0.770±0.156 (6)</td>
</tr>
<tr>
<td>Normal amniotic</td>
<td>0.013±0.014 (9)</td>
<td>0.142±0.070 (8)</td>
</tr>
<tr>
<td>Menkes amniotic</td>
<td>0.278±0.059 (5)</td>
<td>1.147±0.284 (3)</td>
</tr>
<tr>
<td>Normal CLC †</td>
<td>0.002±0.002 (3)</td>
<td>0.024±0.005 (3)</td>
</tr>
<tr>
<td>Menkes CLC †</td>
<td>0.024±0.004 (3)</td>
<td>0.057±0.010 (3)</td>
</tr>
</tbody>
</table>

† CLC: Continuous Lymphoblast Cells

3.3 Fibroblast cells

The fibroblast is a cell engaged in the active synthesis of the components of fibrous tissue. The cells predominate where new connective tissue is being formed, e.g. in the healing of wounds. Fibroblasts are capable of slow ameboid locomotion and their morphology is therefore variable. The morphology of the cells depends on whether they are in vivo or a culture preparation and also upon the available nutrients. Most often healthy fibroblasts in culture are elongated and spindle-shaped. However, they may assume irregular triangular or polygonal shapes. This variation in shape is necessary if the cell is to fulfil its role in connective tissue. Additionally, cell dimensions can vary widely. The width of a characteristic spindle-shaped fibroblast may lie between 15 and 40 µm and the length between 50 and 200 µm.

A fibroblast plated on a surface in culture will be flattened, with a max-
imum thickness of approximately 3µm. The nucleus is ellipsoidal, with a small eccentricity and contains dispersed chromatin and several nucleoli. The nucleus generally has a major axis length of around 30µm. Organelles evident in the cytoplasm include a number of mitochondria. Centrioles are located in close proximity to the nucleus and along with the endoplasmic reticulum provide the main morphological feature in the cytoplasm (Al 83).

Cultured fibroblasts have provided a useful system for a variety of metabolic and biochemical studies (Vi 81) (Wh 82) (Go 76) and as these cells adhere strongly to surfaces used for mounting they are particularly suited to SPMP analysis. The alternative of artificially attaching individual cells to a specimen support is difficult and compromises sensitivity by risking elemental contamination.

3.4 Formation of cell culture

All fibroblast cultures were grown in an identical manner. Normal cells were sourced from disease-free subjects and Menkes' cells were sourced from several male Menkes' patients at the Royal Childrens Hospital, Melbourne. A skin sample, extracted by punch biopsy, was used to form the initial culture. The fibroblasts were grown from this sample in Eagle's basal medium buffered with 20mM N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic acid (Hepes, Calbiochem) and containing 10% fetal calf serum. This growth medium is termed Fibroblast Growth Medium, and is hereafter referred to as FbGM. The cells were grown in culture flasks for typically 5 passages, while stored in an incubator at 37°C. The fibroblasts were detached from the culture flasks with Trypsin-EDTA and stored at liquid nitrogen temperature in a solution of FbGM + 10% Dimethyl sulfoxide (DMSO) until required for sample preparation.

Sample preparation was initiated by thawing the cells and checking cell viability with trypan blue staining. Damaged and dead cells stain with the dye trypan blue due to a breakdown in the integrity of the cell membrane. The cellular suspension was washed several times to remove residual DMSO. The suspension was then placed in a culture flask and incubated. Cell attachment occurred after several hours. The culture was then left undisturbed for 24 hours. Under
favourable growth conditions fibroblasts divide every 24 hours. To ensure sufficient area for cell growth and to circumvent contact inhibition amongst cells, the culture was split 1 in 3 every 3 days at which time the FbGM was renewed.

The cells used for plating were selected between the 7th and 12th mean population doubling in vitro. Cell viability was again checked with trypan blue staining before the cells were deemed suitable for the final stage of specimen preparation.

3.5 Preparation of cells for analysis

3.5.1 Specimen support

Cellular microanalysis presents the major problem of devising a preparative technique which will preserve as accurately as possible the elemental conditions found within the living cell. Cells placed within a high vacuum system must be stabilized. However, the accepted classical techniques of cellular preservation and presentation, staining, fixing and embedding are unacceptable for elemental microanalysis because of the elemental contamination and redistribution that is introduced by these techniques. In addition cellular permeability can be altered by these treatments. This may result in the loss of electrolytes from the cell and the effect may be further exacerbated by chemical dehydration of the cell.

It is obvious that minimal interference is desirable in cell preparation. Microanalyses performed by electron probes have favoured a cryogenic preparation of cells (Ec 74). With such a technique, labile electrolytes are locked into the position in which they are found in the living cell by the rapid freezing of the water within the cell. The sudden increase in the viscosity of intracellular fluids prevents the migration of elements of interest within the cell and retains intracellular fluids within the cellular membrane. The water can then be removed by sublimation under high vacuum, leaving a stable dehydrated specimen. This technique affords minimal chemical interference with the cell (provided that cryoprotectives and artificial nucleating agents are not used) and as such was considered to be the most suitable method of preparing fibroblast cells for analysis by the SPMP.

Specimen mounting is of major importance and several options are avail-
able for the mounting of specimen cells for analysis by the SPMP. Experience in specimen mounting has been accrued from work conducted by electron probes. However the proton probe, with its greater elemental sensitivity, places more stringent requirements upon mounting techniques.

An optimum mounting surface would not interfere with the cell, and allow it to express the form and cellular metabolism that would be present in vivo. In addition the ideal mounting surface would not contribute any measurable background radiation. Such a surface does not exist and compromises must be made. For X-ray elemental analysis, the most appropriate materials for specimen support surfaces are generally of low atomic number, so that background characteristic X-rays from the support surfaces are too low in energy to be efficiently collected by the Si(Li) detector. Such surfaces must be of high purity to avoid the production of contaminant X-rays.

Following the method developed by Hall at Cambridge, Echlin et al. (Ec 73) used thin aluminium-coated nylon foils as the specimen support for the EMP examination of cells and tissue sections. The nylon foil is extremely thin (< 2µm) and hence produces less bremsstrahlung than alternative thick supports such as graphite disks. Nylon foil, without a conductive coating, has been used successfully for the mounting and microanalysis of cells and tissue with the Melbourne SPMP (Ob 87). The foils proved a suitable choice for this work as the surface of the foil is amenable to cell growth, the nylon is acceptable in culture media and, as the foils are optically transparent, specimen visualization is readily performed in both the culture and the specimen chamber. These attributes were used to advantage by growing and analysing the fibroblasts on the same foil surface. This approach served to minimize external interference with the cells, thereby limiting the possibility of introduced contamination and possible morphological disturbances that can effect localized elemental concentrations (Go 81a).

The nylon foils proved to be a suitable support material for the cell culture during both the period of cell growth and target irradiation. However, as this experimental technique requires that the cells be grown on nylon, the preparation of both the nylon and the foils was critical. DuPont Elvamide 8061 nylon resin (in chip form) was dissolved with iso-butyln alcohol at 70°C to form a liquid nylon
Figure 3.2
Nylon foil manufacture.

A:
The Teflon coated dish is filled with filtered deionized water. The target holder blanks are restrained at 45° to the vertical by a stainless steel support block.

B:
The surface of the water is swept by an initial drop of nylon, which is then removed.

C:
Two drops of nylon (liquid at 70°C) are placed on the water surface. The nylon spreads widely forming a thin immiscible layer. The water level is then reduced by a siphon until it is 2mm below the top of the target holders. The nylon skin is then allowed to polymerize on the surface of the water for four minutes.

D:
The remainder of the water is removed and the nylon skin drapes across the target holders.

E:
Excess nylon is melted away by a clean heating iron and the target holders are separated, now with a foil attached.
solution. A thin layer of nylon was then placed across a target holder, as shown in Figure 3.2. Hostafan proved to be the most suitable material for use as a target holder. The inert polyethylene based plastic was compatible with all preparatory techniques used in this work and proved more suitable than alternatives such as Teflon. The Hostafan holders were easily formed and cleaned, had good vacuum properties and allowed the nylon to readily adhere to their surface. In addition, these holders did not produce spurious detectable X-rays from impinging beam halo when used in the SPMP specimen chamber.

The Hostafan target holders were pressed from a 1mm thick sheet of Hostafan, with both the sheet and presstool being cleaned in alcohol prior to use. The rectangular target holders were shaped to fit the target changing mechanism in the microprobe specimen chamber and featured a 7mm hole offset toward one end (see Figure 3.3A). The target holders were thoroughly cleaned in alcohol and deionized water before being sterilized in a high pressure autoclave. These steps were deemed necessary to avoid bacterial contamination of the cell culture.

Ultra-clean conditions are required during foil preparation, as the high level of sensitivity provided by the SPMP makes the technique especially susceptible to contamination (see Section 4.2). In particular a microscopic dust particle or metallic contaminant can invalidate a data set if it is positioned within a localized scan. Users of the MP system have access to a dedicated clean room, especially designed to ensure that target preparation (including foil manufacture) may be undertaken with a minimal risk of contamination. The clean room is pressurized to exclude dust and incoming air is filtered. All microprobe target preparation is conducted within this environment, with the exception of high vacuum freeze-drying which is performed in an adjoining room due to the risk of oil vapour contamination from vacuum pumps (Ha 80). The clean room features two high power (×2000) Olympus light microscopes. Both offer phase contrast microscopy and photomicrography. One of the pair is an inverted microscope and is suited to the in vitro examination of cell cultures.

As the nylon foils are weakly adhesive they are kept vertically in clean

2The effects of stainless-steel target holders are documented in Section 4.2
Figure 3.3

Growth of fibroblast cultures on nylon foil.

A:

Hostafan foil holder. The holder is 15mm x 20mm and readily mounted in the target changing mechanism in the MP specimen chamber. The circular hole is 7mm in diameter.

B:

The foil is held horizontally by a Pactene support bracket. A 30μl droplet of fibroblasts in suspension is then placed centrally on the nylon foil. The droplet contains approximately $3 \times 10^3$ cells and is left to settle under gravity for 1 hour.

C:

The foil, mounted in the support block is then placed within a sealed culture dish. The support block raises the foil above the base of the culture dish and can accommodate two Hostafan foil holders. The culture dish is filled with FbGM and the medium is changed every 3 days.
protective containers within a dry-air desiccator until required for use. Target specimens are stored in a similar manner. The good condition of the nylon foil surface was integral to the successful use of the foils. The fibroblasts adhered preferably to a flat nylon surface. Hence the production of a suitable foil surface demanded a reproducible technique in foil manufacture, and the selection of the most suitable foils. It was found necessary to place rigid constraints upon the allowable polymerization time of the nylon (four minutes, as detailed in Figure 3.2). Further polymerization resulted in a nylon skin with set wrinkles formed from minute disturbances in the water surface. When such a skin was placed across the holder the wrinkles remained, producing surface undulations in the finished nylon foil. Similarly, insufficient polymerization time allows density changes to occur in the film of nylon as it is lowered across the foil holders, again resulting in minute irregularities in the surface of the nylon. Each foil was examined with an optical microscope, and only high quality ripple-free foils were chosen for use.

Typically, acceptable foils were ~ 0.7µm thick. Figures 3.4A and B show the RBS and X-ray spectra obtained when a blank nylon foil was irradiated with 1.8µC of charge from a 3MeV proton beam. It can be seen that within the detectable range of elements, the nylon is composed almost entirely of carbon, nitrogen and oxygen. No statistically significant elemental contamination is observable.

### 3.5.2 Culture conditions

It was necessary to have a reproducible technique for the final preparation of the cell cultures. Such a technique was developed using nylon foils as the specimen support. Cell attachment was assisted by treating the foils with the synthetic polypeptide polylysine (Poly-l-lysine, (Hydrobromide), Sigma Chemical Company, USA) (Ma 74). The foils were dipped into an aqueous solution of polylysine (Concentration 100µg/ml) for 5 minutes then air dried for 20 minutes in a laminar flow hood, to form a molecular monolayer. The highly basic polylysine binds, via charge interaction, the acidic residues of cell coats and results in consistent and vigorous cell growth. Following this treatment the Hostafan holders, with foils attached, were placed in small Petri dishes and supported in a horizontal position by inert and clean Pactene blocks, as shown in Figure 3.3B. In order to
Figure 3.4

A:

RBS spectrum from a blank nylon foil. The foil was irradiated with 1.8\(\mu\)C of 3MeV protons. Beam current was maintained at 1nA. X-ray peaks of no elements heavier than oxygen are present in this spectrum.

B:

X-ray spectrum from a blank nylon foil irradiated under the same conditions as A.
A.

![Graph showing the relationship between backscattered proton energy (MeV) and number of counts. Peaks for C, N, and O are visible.]

B.

![Graph showing the relationship between X-ray energy (eV) and number of counts.]

Number of Counts

BACKSCATTERED PROTON ENERGY (MeV)

X-RAY ENERGY (eV)
eliminate bacterial contamination both the Pactene block and foil were thoroughly sterilized prior to use. The block was initially washed in alcohol and autoclaved; next both the block and foil were U.V. irradiated for 20 minutes. With the nylon foil supported horizontally a 30µl droplet of the plating fibroblasts in suspension was placed centrally on the nylon foil. This droplet contained approximately 3 \times 10^3 cells, considered to be the optimum number for initiating the growth of a fibroblast culture under these conditions. The cells were then left undisturbed, to settle under gravity and attach over a period of 4 hours. The complete incubation was conducted at 37°C.

The culture dish was then filled with FbGM (see Figure 3.3C) and the culture incubated at 37°C for a period of between 24 and 40 hours. This was a critical period during the development of the culture and so the cells were frequently examined with an optical microscope. This was most conveniently performed with an inverted microscope, as the transparent foils were mounted low in the clear Petri dishes. Observations were made for evidence of a developing bacterial contamination, detachment of cells and clumping of cell groups. The occurrence of any of these three events results in an unusable culture. No level of contamination is acceptable; poor cell attachment was indicative of an uneven foil surface; and clumping, which occurred when cell membranes were damaged and adhesive, resulted in localized cellular densities which were excessively high, obscuring the base monolayer of cells and hence rendering that area of the foil unacceptable for analysis. Observations of the culture were also made to monitor the rate of cell division and ultimately cell confluence. A foil completely covered in a monolayer of fibroblasts in intimate contact was defined as being 100% confluent. The cellular density was considered to be optimum when the fibroblasts were between 60% and 70% confluent on the nylon foils. In almost all experimental preparations, two cell cultures (of the same type) were grown in the one Petri dish. Comparisons could then be made between cultures grown under identical conditions.

When the desired foil coverage was reached, the rate of cell growth was significantly reduced by the replacement of the growth medium with a maintenance medium of Eagle's basal medium buffered with 20mM Hepes and containing 5% fetal calf serum. This medium was designated Fibroblast Maintenance Medium.
and is hereafter referred to as FbMM (Ca 80). FbMM contains insufficient serum to promote normal growth of cells and as a result incubation in this medium retarded cell growth rates significantly. The ionic content of the cells was allowed to stabilize by leaving the cells in this medium for between 96 and 120 hours. This procedure was adopted because studies performed by Camakaris et al. (Ca 80), utilizing bulk techniques, have shown that reproducible results are obtained under these conditions, rather than during the use of actively growing cells.

In order to measure accurately the elemental content of a cell, all culture medium from around and on top of the cell must be removed and the cell preserved in as close to its living state as possible. The removal of the medium was achieved by washing the cells, foil and entire holder in a suitable wash solution. The choice of solution was governed by a number of factors: 1.) The cells must undergo as little change as possible during immersion, 2.) The wash solution must not produce elemental contamination on or about the cells, 3.) The copper content of the solution must be known to be negligible, and 4.) The wash solution must not produce a thermal or osmotic shock to the cell population and it must be of a compatible pH.

A wide variety of buffers and wash solutions are used in cellular studies. Few however satisfy the requirements imposed by these experimental conditions. Three solutions were considered: distilled water, isotonic ammonium acetate (pH 6.5) and isotonic Tris(hydroxymethyl)aminomethane (TRIS-HCl, pH 7.5, Sigma Chemical Company, USA). The cell washing period was found to take up to 30 seconds, so an initial test was performed to assess the suitability of each solution. The results of this test are presented in Table 3.2. The cultures, including an inviolate control sample, were observed under a microscope and morphological changes noted. Cell viability was monitored by periodic in situ trypan blue staining.

There is a known connection between morphological change and intracellular elemental disturbance (Mo 78). Obviously evidence of any morphological disturbance resulting from immersion in a wash solution is experimentally unacceptable. Characteristically the normally elongated cells distend and assume a rounded appearance in a hostile environment, with cellular death characterized by cytoplasmic breakdown and nuclear disintegration and dissolution.
Table 3.2

This table shows the reaction of normal fibroblast cultures (S94, passage 9) to immersion in the two possible wash solutions and distilled H₂O. The cultures were visually examined at three time intervals and cell viability was determined by in situ trypan blue staining. An unaffected control sample was maintained for comparison. Any disturbance of cell morphology will have a deleterious effect upon intracellular elemental distributions (Mo 78) and is obviously unacceptable.
### WASH SOLUTION

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Ammonium Acetate</th>
<th>TRIS-HCl</th>
<th>Distilled H₂O</th>
<th>Control Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 min</td>
<td>99% Viable Cells healthy</td>
<td>99% Viable Cells healthy</td>
<td>99% Viable Cells rounded Some elongated</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>99% Viable Cells healthy</td>
</tr>
<tr>
<td>5 min</td>
<td>99% Viable Cells healthy</td>
<td>99% Viable Cells healthy</td>
<td>99% Viable Cells rounded and bloated</td>
<td>99% Viable Cells healthy</td>
</tr>
<tr>
<td>20 min</td>
<td>70% Viable Cells elongated but detaching</td>
<td>90% Viable Cells elongated but detaching</td>
<td>95% Viable Cells bloated and detached Patches of cells only</td>
<td>99% Viable Cells healthy</td>
</tr>
</tbody>
</table>
From Table 3.2 it can be seen that in the presence of either TRIS-HCl or ammonium acetate cell cultures retained 99% cell viability and unchanged morphology for up to 5 minutes. This compared with the effect of distilled water, where, as might be expected, significant morphological changes occurred in under 2 minutes exposure; a result is readily attributable to osmotic shock, and it leaves the cells vulnerable to ionic redistribution. Both TRIS-HCl and ammonium acetate were used as wash solutions. Figures 3.5B and C shows how each foil was rapidly immersed in the washing solutions.

### 3.5.3 Cryogenic preparation

The washed cells must immediately be subjected to cryogenic preparation. A number of cryogens are available; again most of them having been developed as a part of specimen preparation for electron microprobes (Ec 73a). An ideal cryogen will instantaneously immobilize solutes through the entire cell and not introduce any contamination. Available cryogens are, however, non-ideal and it is accepted that some redistribution of soluble elements must occur when freezing (Dö 75). However the chosen cryogen must minimize this effect. Most commonly, liquid nitrogen, iso-pentane and freon are used as cryogens. Liquid nitrogen was chosen for this work because of its low boiling point (77K), absence of contamination and convenience of use.

The cell matrix is composed of heteropolymers with bound H₂O and an amount of unbound H₂O. Cryogenic preparation requires that the H₂O first be frozen, then removed by sublimation under high vacuum. During the freezing of the cell, coexisting solid and liquid phases are present. This permits electrolyte movement toward the liquid phase and hence elemental redistribution within the freezing cell. Therefore, in order to preserve elemental integrity, a high freezing rate is required. A monolayer of fibroblasts on a thin nylon foil is ideally suited to rapid freezing.

Provided that the foil is rapidly plunged into the liquid nitrogen, complete cell freezing will be virtually immediate, as the fibroblasts have an extremely large surface area to volume ratio and a small absolute volume (Va 82). Rapid cooling also minimizes morphological damage due to ice crystallization (Bo 73), an effect
Figure 3.5
The washing and freeze-drying of the cell culture.

A:
The cells were grown on foils in a Petri dish.

B:
First immersion in the primary washing solution. Volume of the buffer was 200 ml. Each foil was immersed once.

C:
Second immersion in the secondary washing solution. Volume of the buffer was 200 ml. Each foil was immersed once. After washing, as much residual buffer solution was removed from the foil and holder as possible. This was done by concussing the holder and absorbing the extraneous fluid.

D:
The foil and holder were rapidly plunged into liquid nitrogen and maintained in the cryogen until thermal equilibrium was attained with the surrounding liquid.

E:
The frozen specimens were then placed in the freeze-drier and it was immediately evacuated. The specimens remained at a vacuum of $10^{-7}$ mbar for 24 hours. An ultra-fine leak valve was then used to bring the foils slowly up to a dry nitrogen atmosphere over a period of 2 hours. This technique was adopted to avoid possible rupturing of the fine foils.
that has been linked to elemental redistribution (Go 81b).

In this work, excess wash solution was removed from the washed foils by concussion and absorption. The foils were then plunged immediately into a liquid nitrogen bath as shown in Figure 3.5D. The entire foil assembly was then retained in the cryogen until thermal equilibrium was established between the foil and the surrounding liquid. Ice recrystallization within frozen biological material is negligible provided that the temperature is held below 143K (Ec 73). Ice recrystallization was avoided with this work by loading the frozen foils directly into a cold aluminium block. The block was precooled to liquid nitrogen temperature and resided in a liquid nitrogen bath. The foil holder maintained good thermal contact with the block and was radiatively cooled from both sides.

The cryogenic preparation was completed by removing the water from the frozen cells by sublimation under high vacuum. The cells, on the support foils, were transported in bulk within the cooled aluminium block, to the freeze-drier. The block was rapidly transferred to the drier, where it was thermally isolated; the drier was then immediately evacuated (see Figure 3.5E). A pressure of $1 \times 10^{-4}$ mbar was obtained after 10 minutes pumpdown and the base pressure of $1 \times 10^{-7}$ mbar was achieved after approximately one hour. The drying rate of the cells is dependent upon the rate of sublimation of ice within the cells, which is a function of the vapour pressure of ice at the specimen temperature and decreases sharply with a decrease in temperature (Bo 69). The drying rate also depends on the specimen size and shape, the relative amount of bound and unbound water and to a lesser extent the pressure. A monolayer of fibroblasts offers a high drying rate for reasons similar to those responsible for the rapid freezing rate. The large surface area to volume ratio and the small absolute volume minimize the resistance to sublimation of water molecules inside the cells. It is known that a monolayer of cells requires only several hours to dry at around 200K (Go 81b). So to ensure that the fibroblasts were completely dried, the entire aluminium block, which was thermally isolated in the vacuum system, was allowed to warm-up to room temperature over a period of 24 to 48 hours. The drier was kept at the base pressure of $1 \times 10^{-7}$ mbar during this time.

At the end of the drying period, a dry nitrogen atmosphere was slowly
introduced into the freeze-drier. The specimens were then removed and placed in a desiccator over silica gel until required for analysis.

3.6 Microprobe analysis

After preparation and freeze-drying, fibroblast cultures were transferred directly from the desiccator to the MP specimen chamber for analysis. Up to four separate nylon foils could be loaded into the specimen chamber at any one time. Each target was then sequentially moved into position as required. Target transfer from the vacuum lock (see Section 2.3) occurred at a pressure of $5 \times 10^{-6}$ mbar or better and target irradiation took place at pressures of less than $5 \times 10^{-7}$ mbar.

The MP system offers an optimum resolution of 50nm (Be 89). This resolution is achieved when the system is operated as a Scanning Transmission Ion Microscope (STIM, see Section 4.7)(Le 88). Under these conditions minimum object and aperture diaphragm sizes are selected, and a pair of micrometer driven slits immediately following the object diaphragm is used to reduce further the effective object size. The current is reduced to approximately 0.1fA for STIM analysis and for the present system the theoretical maximum current at a resolution of 50nm is approximately two orders of magnitude higher. This current is insufficient for meaningful elemental analysis and is limited to this level by ion optical constrictions imposed on the microprobe by the beam available from the accelerator. These restrictions are elucidated and addressed in Chapters 5 to 7.

For a given experimental situation a compromise must be made between available resolution and beam current. A target current of 100pA is considered to be the minimum useful current for PIXE analysis (Le 87) and the MP system presently provides a resolution of 1µm at this current. However, for any particular application, many practical considerations control the choice of beam resolution and current.

A fibroblast cell plated on a thin nylon foil can be considered to be a thin target for protons at MeV energies. Fibroblasts in vivo are generally 3µm in thickness (To 68), but the freeze-drying process is known to cause the cells to flatten substantially. Cell thickness was monitored by examining the energy
distribution of Rutherford backscattered protons. These scattered protons have been deflected from their incident path by elastic Coulomb collisions with the effectively stationary target nuclei. The reduction in energy of the scattered proton depends on the masses of the projectile and target nucleus and the ratio of the incident and scattered projectile energies can be written (Ch 78)

\[ K = \frac{E_s}{E_0} = \left( \frac{m_p \cos \theta + \sqrt{M^2 - m_p^2 \sin^2 \theta}}{M + m_p} \right)^2, \tag{3.1} \]

where \( m_p \): Mass of the incident proton; 
\( M \): Mass of the target atom; 
\( E_0 \): Incident proton energy; 
\( E_s \): Scattered proton energy; 
\( \theta \): Scattering angle in the laboratory reference frame (150° for this work); 
\( K \): Kinematic factor.

Figure 3.6 depicts the situation encountered with RBS analysis of both the nylon foil, and the fibroblast mounted on the nylon foil. Firstly, considering the nylon foil alone, protons scattering through an angle \( \theta \) from the front surface of the nylon foil have a resultant energy \( E_s = K E_0 \), where \( K \) depends upon the mass of the nucleus. The foil composition is C_{12}H_{22}N_2O_2 (Ch 88), and at backward scattering angles the elements readily detected are carbon, nitrogen and oxygen. There is a distinct upper limit to the energy of the backscattered protons from each of these elements, \( K E_0^C \), \( K E_0^N \) and \( K E_0^O \).

As the protons traverse the nylon foil, they lose energy at a rate \( \left( \frac{dE}{dx} \right)^{\text{Foil}} \). For a compound target such as the nylon, Bragg's Rule can be applied to find \( \left( \frac{dE}{dx} \right)^{\text{Foil}} \) as a weighted sum of the stopping powers of each element in the foil. A proton arriving at the back of the foil has an energy \( E \), where

\[ E = E_0 - \int_0^{t_f} \left( \frac{dE}{dx} \right)^{\text{Foil}} \, dx \tag{3.2} \]

and \( t_f \) is the foil thickness.

If this proton is backscattered at the rear of the target, from an atom of element \( i \), with kinematic factor \( K^i \), then immediately after scattering the proton
Figure 3.6

The diagram shows how an idealized Rutherford backscattered energy spectrum is generated from nuclear backscattering off carbon, nitrogen and oxygen within firstly the nylon foil and subsequently the cell and nylon foil. The proton beam is normally incident upon the target at an energy $E_0$ and backscattered particles are collected at $\theta = 150^\circ$ (for all experimental work in this thesis). An experimentally-derived backscattering spectrum from a fibroblast plated on the nylon foil is shown in the lower half of Figure 3.6. The energy widths of the backscattered peaks are increased beyond that obtained from the nylon foil alone (see Figure 3.4A) due to the increased target thickness.
To charged particle detector

Fibroblast Cell

Backscattering Proton Energy

Backscattered Proton Energy (MeV,)

NUMBER OF COUNTS

2.05 2.10 2.15 2.20 2.25 2.30 2.35 2.40

BACKSCATTERED PROTON ENERGY (MeV.)
will have an energy $K'E$ and will lose further energy on its path back through the foil. The backscattered proton will leave the foil with an energy

$$E_i^f = K^i E - \int_{0}^{t_p} \left( \frac{dE}{dx} \right)_{\text{Foil}} dx,$$

(3.3)

where $t_p$ is the path length of the scattered proton ($t_p = \frac{t_f}{\cos \theta}$); $E_i^f$ then defines the lower limit to the energy peak for protons backscattering from atoms of element $i$. The top left of Figure 3.6 shows an idealized backscattering spectrum generated by irradiating the nylon foil with a proton beam of energy $E_o$. The energy width of a RBS peak resulting from element $i$ is $\Delta E^i = K^i E_o - E_i^f$. This can be written

$$\Delta E^i = K^i \int_{0}^{t_f} \left( \frac{dE}{dx} \right)_{\text{Foil}} dx + \int_{0}^{t_p} \left( \frac{dE}{dx} \right)_{\text{Foil}} dx.$$

(3.4)

Assuming that the rate of energy loss is constant (valid as $t_f \leq 1\mu m$), and using its value at the surface, then

$$\Delta E^i = t_f \left( K^i \left( \frac{dE}{dx} \right)_{E_o} + \frac{1}{\cos \theta} \left( \frac{dE}{dx} \right)_{K E_o} \right).$$

(3.5)

The average foil thickness over a scanned region can then be determined from backscattering energy widths, experimental geometry, and energy loss of the projectile in the target of interest. From an average of four nylon foils it was found that $\Delta E^C = 28 \pm 6\text{keV}$ (see Figure 3.4) with detector resolution and straggling amounting to $17\pm2\text{keV}$. From this the average foil thickness was determined to be $\bar{t}_f = 0.66\mu m$ ($\sigma=0.18\mu m$). As the cell is plated directly onto the nylon, the RBS spectra extracted from within the cell boundary give a measure of the combined average thickness of the cell and the foil. The composition of the cell matrix is well approximated by that of skin tissue, the bulk composition of which is known (Th 86), so that the stopping power of the cell can be calculated. The lower part of Figure 3.6 shows a typical backscattering spectrum resulting from the irradiation of a fibroblast on a nylon foil. It can be seen by comparing this figure with Figure 3.4A that the backscattering peaks have broadened. This is due to additional energy loss in the cell. Using a knowledge of the foil thickness, the average fibroblast thickness was found to be $\bar{t}_c = 0.62\mu m$. This value had a standard deviation of $0.18\mu m$ but examples were found as thin as $0.3\mu m$, indicating that the freeze-dried cell was up to an order of magnitude thinner than the cell in vivo.
A proton incident upon this target can interact with inner shell electrons of an atom of a particular element \(X(A,Z)\) and eject one or more of these electrons. The vacancies thus created are filled by outer shell electrons, leading to the emission of a characteristic X-ray (the PIXE process). The distribution of these X-rays is isotropic, and as the protons lose only \(\sim 20\text{keV}\) in traversing the target, beam energy loss is negligible and thin target approximations can be used. The number of a particular characteristic X-ray emitted from the element \(X\) can then be written

\[
n_{X_i} = \sigma_{X_i}^m N_p t_z \frac{N_A \Delta \Omega}{A} \epsilon_d C_{abs}^X
\]

(3.6)

where

- \(i\): Indicates the shell being filled (K, L or M);
- \(N_p\): Number of incident protons;
- \(t_z\): Equivalent thickness of \(X\) in mass per unit area;
- \(N_A\): Avogadro's number;
- \(A\): The atomic mass of element \(X\);
- \(\Delta \Omega\): The solid angle subtended by the effective volume of the X-ray detector crystal;
- \(\epsilon_d\): The X-ray detector efficiency at the energy of the characteristic X-ray;
- \(C_{abs}^X\): The absorption reduction factor for \(X_i\). This factor allows for X-ray attenuation between the target and the detector crystal.

\(\sigma_{X_i}^m\): X-ray emission cross section. This can be written as a product of the ionization cross section \(\sigma_{i}^{\text{ion}}\) and the fluorescence yield \(\omega_{X_i}\),

\[
\sigma_{X_i}^m = \sigma_{i}^{\text{ion}} \omega_{X_i}.
\]

(3.7)

For this work elemental sensitivity is required for both the macro elements and the heavier trace elements present in fibroblasts. This was necessary as the distribution of trace and macro elements in fibroblasts was unknown and sensitivity to any elemental perturbation resulting from Menkes' disease was of major importance. As a result X-ray production and detection efficiency must be optimal within the energy range of the characteristic X-rays produced by the macro and trace elements within fibroblasts. In particular, a high detection sensitivity is required for copper, specifically the Cu \(K_{\alpha}\) X-ray of 8.04keV.
For general PIXE analysis the elemental sensitivity is dependent upon a number of factors: (i) the X-ray background, (ii) interference from other characteristic peaks, (iii) energy resolution of the detector, (iv) count-rate limitations, (v) heating effects, and (vi) charging effects. In the specialized case of microbeam analysis of a thin biological cell, points (iv) and (vi) are inconsequential as extremely low currents are used and the X-ray yields are low.

Figure 3.7A (data points) shows the experimentally determined X-ray cross section for Kapton (C\textsubscript{22}H\textsubscript{10}N\textsubscript{2}O\textsubscript{4}). A hydrocarbon target of this nature provides a good simulation of a biological matrix (Th 86) and offers a good representation of the X-ray background produced during the irradiation of a biological target (Gu 77). It can be seen from this graph that the background cross section diminishes by 10\textsuperscript{3} between 2keV and 6keV and a further 10\textsuperscript{2} between 6keV and 10keV. This background shape is due to a variety of competing effects (see the caption for Figure 3.7A) and favours the detection of biological trace elements whose characteristic X-rays are found at energies greater than 6keV. Figure 3.7A also shows the X-ray detector efficiency curve (solid line). This curve indicates that X-rays below 6keV are heavily attenuated by the Beryllium filter, whereas X-rays between 6 and 20keV are detected with greater than 95% efficiency. These experimental effects result in a calculable sensitivity. Figure 3.7B shows the expected sensitivity of the Melbourne SPMP when 3MeV protons are incident upon a biological matrix. A peak to background ratio of one was used to define the minimum detectable characteristic peak. From the graph it can be seen that a 3MeV proton beam affords the greatest sensitivity for elements within the range Z=27 to 30. This is ideal for the present work as copper has Z=29.

The low background in the region of the Cu \textit{K}\text{a} peak ensures that for a thin biological target the sensitivity is not limited by the peak to background ratio, but rather the counting statistics available within a reasonable time interval.

Given a specified matrix and a nominated element of interest, equation (3.6) can be rewritten

\[ n_{X_i} = \sigma_{X_i}^{em} N_p K_{X_i}^{Matrix} K_d C_{abs} X_i, \]  

(3.8)

where \( K_{X_i}^{Matrix} = \frac{N_{A}}{t} \) is a constant for a given target;
Figure 3.7

A:

The background cross section ($d\sigma/dE_r$, indicated by circles) measured on the MP system, for the irradiation of Kapton with 3MeV protons (data from Gu 77). Contribution to the background cross section is provided by (i) Secondary electrons, (ii) Bound electrons interacting with incident protons, and (iii) Protons interacting with target nuclei. The background cross section is a minimum between 10 and 30keV and is a factor of 10 greater at the energy of the Cu$K\alpha$ X-ray (8.04keV). The background cross section increases with bombarding energy (Fo 74) and with 5MeV protons the background cross section can be expected to be two orders of magnitude higher at 8keV. The same energy scale is used to represent the Si(Li) X-ray detection efficiency (solid line). The efficiency shown is corrected for attenuation due to the 120µm thick Beryllium filter that protects the detector crystal from backscattered particles. The detector efficiency is greater than 95% for X-ray energies between 6keV and 18keV. The detector efficiency was determined from information supplied by the manufacturer (Or 78).

B:

The elemental sensitivity of the MP system, for the detection of K X-rays from elements within a Kapton matrix, irradiated by a 3MeV proton beam (data from Gu 77). The sensitivity limit was defined by a peak to background ratio of one.

C:

The K-shell ionization cross section for copper verses proton energy over the range accessible by the Pelletron accelerator. The cross section was calculated using ECPSSR theory (Energy Coulomb Perturbed Stationary State Relativistic) by Cohen and Harrigan (Co 85).
\[ K_d = \frac{\Delta \Omega}{4\pi} \epsilon_d \] is a constant for a given detector and a specified geometry.

If \( \Delta \Omega \) is made as large as possible then

\[ n_x \propto \sigma_{X_u}^{\text{en}} N_p C_{\text{abs}}^{X_u}. \]  

(3.9)

Within the energy range accessible by the Pelletron accelerator, the X-ray production cross section for copper (\( \sigma_{\text{Cu}}^{\text{en}K_a} \)) is a monotonically increasing function of bombarding proton energy and is shown in Figure 3.7C. Hence the Cu \( K_a \) X-ray yield is maximized by operating the accelerator at the highest practicable energy. However the background cross section increases with energy as does \( C_{\text{abs}}^{X_u} \), as filtering is introduced to protect the X-ray detector from increasingly energetic backscattered particles. Such filtering dramatically attenuates the low energy X-rays from the macro biological elements. The choice of beam energy is also controlled by the fact that beam instability increases at high energies. A suitable compromise between the background cross section, X-ray yield, low energy X-ray attenuation and accelerator performance is achieved by using a 3MeV proton beam. At this energy \( \sigma_{\text{Cu}}^{\text{en}K_a} \) is approximately 50% of the maximum value attainable with this accelerator (see Figure 3.7C), but attenuation of the P \( K_a \) X-ray by protective filtering is six times less than that which would occur with a 5MeV beam.

From equation (3.9), under conditions of optimal beam energy for fibroblast analysis, the number of X-rays collected from a given target will depend directly on \( N_p \), the number of incident protons. As a fibroblast is a very thin target, the X-ray yield is low; hence it is important to maximize \( N_p \) to ensure that a statistically significant number of Cu \( K_a \) X-rays are collected in a reasonable time. To do this requires a compromise between beam current and irradiation time. A beam current of between 500 and 1000pA was deemed suitable for fibroblast analysis. Within this range of beam current the Melbourne SPMP provided a spatial resolution of 2 to 4\( \mu \)m depending on accelerator performance. Higher currents, though providing a more rapid analysis, can only be achieved with inferior resolution. A further increase in beam size is unacceptable for this work as it would prevent the resolution of intercellular separations. With beam currents in
the acceptable range approximately two hours was required to irradiate a fibroblast sufficiently to obtain useful statistics on the trace element X-ray peaks. Longer irradiation times compromised high resolution data due to the deleterious effects of long term instability in the lens, accelerator and target-holding mechanism.

After freeze-drying, the fibroblasts were supported by the nylon foils in a monolayer approximately 60% confluent (some cells were lost during freeze-drying) across a 154mm² area of nylon foil (see Figure 3.8A). This level of coverage left sufficient extracellular space for the resolution of individual cells by the SPMP. Two methods were used to localize the beam scan about selected cells.

The secondary electron detector (see Section 2.3) was initially employed to image the cells. Positive charging of the scan region, due to the non-conducting target material, inhibited secondary electron production. As a result images of the cells were of insufficient quality to be of use in target navigation and cell selection.

The charging problem was solved by coating the foil with a thin evaporated carbon coating. Carbon was chosen to provide a conductive layer in preference to aluminium as it produces a smaller contribution to the bremsstrahlung (Sa 81). The edge of the Hostafan foils, and hence the carbon layer, was electrically connected to the target changing mechanism with colloidal graphite. The specimen could then be earthed or maintained at a bias voltage.

The thin carbon coating prevented charge localization, permitting secondary electron images of fibroblasts to be formed. The secondary electron yield primarily reflects topographical surface features and to a lesser extent variation in the atomic number of the surface elements (Tr 84).

Figure 3.8B shows a secondary electron image obtained from a carbon coated foil. The scan size was 22µm x 62µm and the beam resolution was 3µm. The target was grounded, the electron collector biased to 6.3kV, and the photomultiplier operated at maximum gain (−1.5kV). The 3MeV proton beam was scanned at 75Hz while the storage image was formed. These conditions offered the maximum contrast between the cell and the nylon foil, yet for many fibroblasts the technique was unable to image the thin extremities of the cytoplasm. This proved particularly disadvantageous in regions of high cell density, as the secondary electron image was unable to show the overlapping of cell extremi-
Figure 3.8

A:

A section nylon foil with the plated fibroblasts after freeze-drying. The cell shapes are consistent with that expected of healthy fibroblasts. Cell density is high enough to permit healthy cell growth, yet sufficient extracellular space remains for individual cells to be resolved by the microbeam.

B:

The secondary electron image of a carbon coated fibroblast. The scan size was $22\mu m \times 62\mu m$. The image was formed with a beam current of 1nA and a resolution of 3$\mu m$. The image was built up on the storage oscilloscope over a ten second period.

C:

A freeze-dried normal fibroblast that has been scanned by the microbeam for two hours. A total charge of $4.86\mu C$ was deposited at a current density of $1 \times 10^{-10} A\mu m^{-2}$. The scan size was $40\mu m \times 232\mu m$. Both the foil and cell have been discoloured by the irradiation.
ties. The evaporated carbon layer also introduced elemental contamination that significantly misrepresented the true elemental content of the fibroblasts. This introduced contamination is discussed in Section 4.1.

As effective secondary electron imaging of fibroblasts compromised the elemental integrity of the target, and as this method was only able to offer inexact imaging of thin low-contrast sections of fibroblasts, an alternative, though less convenient, technique was employed for specimen location.

The thin, transparent nature of both the foil and the cells permitted the target to be viewed with the high power internal optical microscope system (see Section 2.3). Reflected light was used to illuminate the foil, highlighting the cells such that cell boundaries were clearly defined as were the nuclei. Such conditions were favourable to navigation across the freeze-dried specimen and facilitated cell selection. Healthy, spindle-shaped fibroblasts were selected for analysis and a rectangular scan of appropriate dimensions and orientation was set up on a glass cover slip, before the cell was placed directly in the beam path. Figure 3.8C is a photomicrograph of a normal fibroblast after it has been scanned by the microbeam. The rectangular scan was $40\mu m \times 232\mu m$ and a total of $4.9\mu C$ of charge was deposited in this region. The beam has discoloured the scanned area but beam damage is negligible (see Appendix 1) and cellular detail can still be discerned in polarized light.
Chapter 4

Fibroblast analysis: results and discussion

4.1 Introduction

This chapter presents the results of the SPMP analysis of both normal and Menkes' fibroblasts.

The high elemental sensitivity and spatial resolution provided by the SPMP leaves the technique vulnerable to the effects of microcontaminants. As outlined in Chapters 2 and 3 the preparative techniques were designed to minimize the introduction of contaminants. Nevertheless elemental contamination was encountered at first, so modifications were made to the preparative techniques to eliminate sources of such introduced contamination. This was possible as the SPMP is readily able to detect contamination by comparing elemental distributions to physical features and identifying non-biological elemental contaminants. Bulk analysis and spot or line-scan microprobe analysis have greater difficulty detecting and identifying contaminants and hence more readily allow low level contamination to go undetected.

4.2 Specimen contamination

There were three stages at which the fibroblast cultures were at risk of contamination. A) Culture preparation, washing and freeze-drying. B) Carbon coating. C) Specimen storage. The sources of contamination are considered at each stage be-
A) Bacterial contamination of the fibroblast cultures was identified by examination with an optical microscope. All cultures were examined prior to washing and any cultures showing evidence of contamination were excluded from the preparation.

Initially fibroblast cultures were washed in either TRIS-HCl or ammonium acetate. After freeze-drying, physical differences were apparent between the cultures subjected to each of the two washing procedures. Fibroblast cultures buffered in TRIS-HCl had small crystalline deposits across the surface of the foil. This deposition was uneven, appeared random and on occasion, covered the cells. The cultures washed with the volatile ammonium acetate buffer showed no visible sign of surface deposition. The cultures were otherwise physically identical with no evidence of morphological disturbance.

Cultures prepared with both types of buffers were then examined with the SPMP. Differences were found in the elemental content of the types of prepared cells. The TRIS-HCl washed cultures showed the presence of a strong chlorine concentration in areas where the crystalline deposits had been seen, indicating that the deposition was a TRIS-HCl salt. Such deposition is unacceptable as it increases the X-ray background, attenuates X-rays from elements of interest and invalidates any measurement of intracellular chlorine. Figure 4.1A shows an X-ray spectrum from the scan of a Menkes' fibroblast cell that had been washed with a TRIS-HCl buffer. The spectrum is dominated by a particularly strong chlorine peak, the tail of which contributes significantly to the bremsstrahlung and sulphur peak areas. Although the other elemental peaks remain unaffected by the chlorine peak, a surface coating of salt crystals will attenuate the yield of low energy X-rays from underlying elements. No accurate measurement of chlorine concentration was possible with this buffer, as shown in Figure 4.1B. The phosphorus map shows the cell outline; in contrast, high chlorine concentration is found around, as well as within, the cell.

Cultures washed with ammonium acetate were free of any discernable contaminants. This was determined by scanning areas on the nylon that were devoid of cells; background in these areas was negligible, indicating that the washing
Figure 4.1

A:

An X-ray spectrum from the scan of a Menkes' fibroblast that has been washed with TRIS-HCl buffer. The irradiating beam was 3 MeV H\(^+\) and a total charge of 9.2\(\mu\)C was deposited over the scan region.

B:

Phosphorus and chlorine maps from the same cell as in A. The scan dimensions were 33\(\mu\)m (X) \(\times\) 100\(\mu\)m (Y). A total of 10 contours are used in the two dimensional maps and 50 contours are used in the three dimensional maps, with each enhanced contour corresponding to a contour in the corresponding two dimensional map.
X-ray Energy (keV)

Number of Counts

X-ray Energy (keV)

PHOSPHORUS

PHOSPHORUS

CHLORINE

CHLORINE

0 2000 4000 6000 8000

X 50

P  S  K  Cl  Fe  Cu  Zn

2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0

20 μm

20 μm

20 μm

20 μm

A
technique was successfully removing the culture medium. In subsequent culture preparation ammonium acetate was employed exclusively as a wash solution.

Initially stainless-steel holders were used as supports for the nylon foil, and these holders were fixed in position in the culture dish with stainless-steel supports. Both of these components were thoroughly cleaned and sterilized before use; yet metallic contamination was evident in the microanalysis of cells grown on these foils. Figure 4.2A shows the X-ray spectrum from the scan of a Menkes' fibroblast grown under these conditions and washed in ammonium acetate. The cells showed no physical reaction to the possible toxic effects of the stainless-steel, but mapping revealed that chromium and iron contamination were evenly distributed throughout the scan region, presumably transported by the culture medium. Consequentially all stainless-steel components were removed from the culture dish, and a Pactene support block and Hostafan foil holder substituted.

The use of glassware in all stages of the nylon foil and culture preparation resulted in some scan areas being contaminated by minute glass fragments. These fragments resulted from the repeated stirring of the nylon during its re-heating prior to use and were not visible in the nylon but were revealed in scans as localized and extremely high concentrations of Si, K, Ti and Zn. Figure 4.2B is an X-ray spectrum from a $32\mu m \times 108\mu m$ scan region about a Menkes' fibroblast that was found to contain a $6\mu m \times 15\mu m$ glass fragment. The silicon contaminant peak from the glass fragment contributes significantly to the area under the phosphorus peak and hence affects phosphorus maps. Contamination of this nature was eliminated by regularly formulating fresh nylon and changing to plastic dishes.

B) As discussed in Section 3.6, a carbon layer evaporated onto the cells was necessary for effective secondary electron imaging. PIXE analysis of the specimens both before and after this evaporation showed that such evaporations tended to add contaminations as well as carbon. Figure 4.2C shows two spectra from the same scan area around a normal fibroblast. The solid-line spectrum is that obtained during the initial irradiation, during which $7.4\mu C$ was deposited. After the carbon layer was added, an identical scan was performed with the same amount of charge deposited on the scan region. The spectrum, indicated by dots in Figure 4.2C, was then collected. It was obvious from this spectrum that Si, S and Fe
Figure 4.2

A:

An X-ray spectrum from the scan of a Menkes’ fibroblast. The cell was grown in a media dish where a stainless-steel support was used to hold the nylon foil and a total charge of 3µC was deposited on the scan region. The presence of the non-biological elements Cr and Ni indicate that the use of stainless-steel in the culture dish has introduced contamination.

B:

An X-ray spectrum from the scan of a Menkes’ fibroblast. A total charge of 4.2µC was deposited on the scan area. Elemental maps revealed that a glass fragment has resulted in Si and Ti contamination.

C:

Two spectra from the scan of a normal fibroblast. Identical irradiation conditions were used for both spectra and a charge of 7.4µC was deposited over the scan region during each irradiation. The solid-line spectrum was obtained during the initial irradiation and the dot spectrum was collected after the foil had been carbon-coated.
contamination had resulted. Mapping these elements revealed that Si and S were evenly distributed across the scan region (previously S had only been found within the cell), an effect consistent with these elements being associated with the carbon layer. In contrast the Fe contamination was found in localized concentrations. The other elements appeared unaffected, but in some coated cells localized copper contamination was found. As this element is of critical importance to this work, all elemental data on carbon-coated cells were discarded.

C) The cells were stored in desiccators over silica-gel and evidence of silicon contamination was apparent in specimens stored for periods greater than three months. This contamination was rendered negligible by minimizing the time between freeze-drying and analysis.

4.3 Elemental content of normal fibroblasts

After refining the preparative techniques to overcome contamination problems, and ensuring that beam induced specimen damage was negligible, a number of normal fibroblasts, from a variety of sources, were examined with the SPMP. This was to establish the elemental yield obtained from a normal fibroblast under the defined experimental conditions. By analysing a number of normal cells in this way a data set could be built up to characterize the elemental content of a normal fibroblast. Examination of Menkes' cells under identical conditions could then be used to reveal elemental perturbations resulting from Menkes' disease.

Few measurements on the bulk elemental content of fibroblasts have been performed. The intracellular potassium concentration in mouse fibroblasts has been measured by Frantz et al. (Fr 81), the calcium content of human fibroblasts was measured by Shapiro and Lam (Sh 82) and an electron microprobe study of the bulk concentrations of Na, Mg, P, S, Cl, K and Ca in human fibroblasts has been performed by Abraham et al. (Ab 85). No trace elemental measurement on fibroblasts has been performed and no maps of the elemental distributions within fibroblasts have been reported.

Figure 4.3A shows an X-ray energy spectrum from a normal fibroblast. The cell was plated on a nylon foil, as described in Chapter 3. The contribution to the
Figure 4.3

A:
An X-ray energy spectrum from a normal fibroblast. The cell was irradiated with a 800pA beam focused to a 3µm spot and scanned over a rectangular area of 32µm (X) by 132µm (Y) at a frequency of 25Hz for 2.5 hours. The cell itself received a charge of 1.5µC.

B:
Energy spectrum of backscattered protons for the same cell and irradiation conditions discussed in A.
spectrum from the foil beneath is negligible as can be seen by comparison with Figure 3.4B. The major intracellular elements (within detection limits) are P, S, Cl, and K. Trace amounts of Fe, Cu and Zn are also present.

The additional elemental information provided by backscattered protons is shown in Figure 4.3B. The major elements in this spectrum, C, N, and O, are present in both the cell and its supporting nylon foil, with a similar backscattering yield from the cell and the nylon. The heavy elements in the backscattered spectrum come exclusively from the cell. Detector resolution and target thickness effects combine to prevent individual peaks from being resolved in this region of the spectrum. The backscattered data does however give a useful measure of the sodium and magnesium content of the cell.

Figure 4.4 shows contour map representations of the elemental distributions within this normal fibroblast cell. These maps were formed from the smoothing of each data point by a rotated Gaussian representation of the beam spot. In these maps, with the exception of the copper map, 50 contours are used to represent the distribution. Each contour then corresponds to 2% of the maximum number of counts for that element with every fifth contour being highlighted by an enhanced width.

The C, N and O maps, which were generated from the backscattering data, reflect the density distribution of the scanned region. These maps have significant backgrounds outside the cell boundary due to the presence of each of these elements in the nylon foil. The sodium distribution, which is also derived from backscattered proton data, is indistinct due to the low counting statistics available for that element.

The phosphorus map was derived from the $K_\alpha$ and $K_\beta$ X-ray peak for that element. As this peak is located on the bremsstrahlung background, approximately 20% of the mapped data result from the background radiation. Hence there is a low level of apparent phosphorus concentration found outside the cell. It was found by physical correlation that the cell nucleus corresponded to the major peak in the phosphorus map. Examination of the C, N and O maps show that the nucleus is a region of greater mass density than the surrounding cytoplasm. However the greater relative height of the phosphorus peak in the nucleus indicates that the
Figure 4.4

Three dimensional elemental maps for the normal human fibroblast discussed in Figure 4.3. The cell nucleus corresponds to the maximum in the phosphorus map. Fifty contours are used to represent each elemental distribution with the exception of the copper map. The copper distribution is normalized to the peak of the phosphorus distribution and the vertical scale of the map has been increased by a factor of 30 to show features. The following table gives the total number of events used in composing each map. This table provides a guide to the statistical significance to be attached to each distribution.

<table>
<thead>
<tr>
<th>Element</th>
<th>Counts in scan region</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>143657</td>
</tr>
<tr>
<td>N</td>
<td>20229</td>
</tr>
<tr>
<td>O</td>
<td>7249</td>
</tr>
<tr>
<td>Na</td>
<td>189</td>
</tr>
<tr>
<td>P</td>
<td>20073</td>
</tr>
<tr>
<td>S</td>
<td>11043</td>
</tr>
<tr>
<td>Cl</td>
<td>50029</td>
</tr>
<tr>
<td>K</td>
<td>75430</td>
</tr>
<tr>
<td>Fe</td>
<td>146</td>
</tr>
<tr>
<td>Cu</td>
<td>22</td>
</tr>
<tr>
<td>Zn</td>
<td>39</td>
</tr>
</tbody>
</table>
nucleus is a phosphorus rich area of the cell. The sulphur, chlorine and potassium maps were obtained in a similar manner to the phosphorus map. The sulphur map shows that this element is far less concentrated in the nucleus than is phosphorus. Sulphur is significantly depleted immediately between the nucleus and the rest of the cell. Chlorine and potassium show similar distributions, with both being more concentrated in the nucleus than is sulphur. The iron, copper and zinc distributions were formed from the Kα peaks for each element. There is little X-ray background for these peaks but, due to the trace level concentrations and the poor statistics available, meaningful interpretation of their spatial distribution was not possible, apart from noting no evidence of strong localizations.

The contour levels can be normalized between maps to enable the comparison of elemental intensity. The peak of the copper map in Figure 4.4 is normalized relative to the number of counts in the peak of the phosphorus map and the vertical (contour) scale of the map has been increased by a factor of 30. Each contour in the copper map is therefore representative of 1/30 of the number of counts symbolized by a single contour in the phosphorus map. Phosphorus has proved to be suitable element for the normalization of the intracellular elemental content of fibroblasts (Ab 85), due to its stability in the presence of induced ionic mobility and its presence in all cellular compartments.

Ionizing and heating effects arising from the irradiating beam can cause specimen damage and result in the movement of elements within the target matrix. These effects were monitored by collecting elemental yield as a function of deposited charge during the irradiation of a number of fibroblasts. In each case there was no significant reduction in the elemental yield over the duration of the scan. The spatial distributions within the scans were also found to be reproducible, with internal elemental features being reproduced when the cells were re-irradiated in an identical manner. These two results are presented in detail in Appendix 1.

4.4 Elemental content of Menkes’ fibroblasts

The Menkes’ fibroblasts were cultured from a Menkes’ disease patient with techniques of cell cultivation and target preparation that were identical to that devel-
oped for the normal fibroblasts. The Menkes' cells were taken from three separate cell lines each sourced from a different patient and the cells exhibited growth rates similar to that of normal fibroblasts. A control sample of normal fibroblasts was cultured alongside each Menkes' culture and identical analytical conditions were maintained with the SPMP to ensure valid comparison between the different cell types.

It is known that a phenotypic feature of Menkes' fibroblasts is elevated concentrations of copper (Go 76)(Ca 80). Hence it was anticipated that increased intracellular copper levels would be found within individual Menkes' fibroblasts. Figure 4.5A shows a typical X-ray spectrum from a Menkes' fibroblast. This cell was irradiated with a charge of 2.1μC, approximately 1.4 times the amount of charge deposited on the normal cell in Figure 4.3. It can be seen that for the Menkes' cell, the major elements are present in the same relative proportion. The same is true of the trace elements with the exception of copper.

The elemental variation between the two cell types was studied by examining the relative normalized elemental yields. Two options were considered in normalizing the data for the comparison of the elemental content of normal and Menkes' cells. It is common in PIXE experiments to normalize the data relative to the bremsstrahlung background (Ue 78). However, for this experimental work a significant component of the bremsstrahlung background is due to the presence of the nylon foil beneath the cell. An uncertainty of 30% was present in the measured value of the foil thickness and the variation in foil thicknesses lay within this range. The actual variation may be less than 30% but could not be measured more accurately due to the limitations of the energy resolution of the RBS measurement. STIM analysis of nominally identical foils, using an alpha particle beam, has recorded an average foil thickness of 0.8±0.1μm, suggesting that the variation in foil thickness is closer to 13% (Ch 89). The cell thickness is comparable to that of the nylon foil, and will contribute a similar fraction to the bremsstrahlung yield. If the elemental yields, which result exclusively from within the cell region, are normalized to the total bremsstrahlung yield, an uncertainty of at least 13% will exist due to the variation in the bremsstrahlung yields from the nylon foil beneath different cells.
Figure 4.5

A:
An X-ray energy spectrum from a Menkes' fibroblast. The cell was irradiated with a 700pA beam focused to a 4µm spot and scanned over a rectangular area of 36µm (Y) by 160µm (X) at a frequency of 25Hz for 2.5 hours. The cell itself received a charge of 2.1µC. Menkes' cell spectra differed from the spectra obtained from normal cells and were identifiable by the ratio of the copper to zinc peak heights. For Menkes' cells the copper peak area was equal to or greater than the area of the zinc peak. For normal cells the copper peak areas were always smaller than the zinc peak areas.

B:
Energy spectrum of backscattered protons for the same cell and irradiation conditions discussed in A. No significant differences were apparent between the backscattering spectra obtained for normal and Menkes' cells.
The diagram shows two X-ray energy spectra. The upper graph, labeled A, displays the number of counts versus X-ray energy (keV) for various elements such as K, Cl, P, S, Fe, Cu, and Zn. The lower graph, labeled B, shows the number of counts versus back scattered proton energy (MeV) for elements C, N, O, Na/Mg, and P. Both graphs are plotted on a logarithmic scale for the number of counts and a linear scale for the energy.
Despite the presence of this large error, a bremsstrahlung normalization was performed to see if any elemental trends were apparent between the normal and Menkes' cells. The bremsstrahlung normalization factor was generated from the 3keV bremsstrahlung yield obtained within a 50eV energy window between the Cl Kβ and the K Kα peaks. This region was chosen as argon is absent from biological targets and for these specimens no other characteristic X-ray peaks are found at the same energy. The width of the energy window was chosen so that there was no contribution from the Gaussian tails of the Cl Kβ and K Kα peaks. The bremsstrahlung X-rays at this energy arise almost entirely from the deceleration of knocked-on secondary electrons within the target matrix (Gu 77). For fibroblasts on nylon foils, the intensity of this radiation is directly proportional to the thickness or density of the scanned region. The results of the bremsstrahlung normalization are shown in Table 4.1 and were obtained for a set of 82 cells consisting of 39 Menkes' cells and 43 normal cells. The Menkes' cells were sourced from three cell lines: Me26α, Me6a-1, and Me39a and the normal cells were sourced from the cell lines S87 and S94. Subgroups of these cells were grown and analysed at different times in separate preparations of FbGM and FbMM.

The ratio of the normalized elemental yields for each group permits a comparison to be made between the elemental content of the two cell types. In order to find the limits for the ratio of the means, the data were log transformed and a two sample t-test was applied to the transformed data to find the limits for the difference between the two log transformed means. The limits for the ratio of the means was then found by raising e to the power of the limits calculated by the t-test. The limits for the Menkes'/Normal ratio define, for the given data set, the range in which the ratio will be found with 95% certainty.

The trend evident from Table 4.1 is that the Menkes' cells contain approximately five times more copper than the normal cells. For the bremsstrahlung normalization this is the only identifiable elemental difference occurring between the two cell groups. The elevated copper level was found in each of the different Menkes' cell lines.

Normalization of the data to phosphorus was considered as the second option. It was noted upon the examination of the elemental maps from all the cells.
Table 4.1: The mean relative elemental yields from normal and Menkes' fibroblasts. Individual yields were normalized to the bremsstrahlung yields, as defined in Section 4.4, before averaging. The data were obtained from 43 normal and 39 Menkes' fibroblasts and the means are shown ± SD. The ratio of the means of the normalized yields are also shown. The range indicated is the two standard deviation limit (i.e. 95% certainty) for the ratio of the Menkes' and normal mean values.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Menkes'</th>
<th>Ratio Menkes'/Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>45.8±13.7</td>
<td>41.4±10.4</td>
<td>0.82→1.02</td>
</tr>
<tr>
<td>S</td>
<td>10.6±5.2</td>
<td>8.3±3.9</td>
<td>0.66→1.00</td>
</tr>
<tr>
<td>Cl</td>
<td>53.1±19.3</td>
<td>57.7±21.8</td>
<td>0.95→1.23</td>
</tr>
<tr>
<td>K</td>
<td>132.2±45.9</td>
<td>138.3±18.6</td>
<td>0.99→1.16</td>
</tr>
<tr>
<td>Fe</td>
<td>0.271±0.289</td>
<td>0.383±0.454</td>
<td>0.75→1.74</td>
</tr>
<tr>
<td>Cu</td>
<td>0.019±0.009</td>
<td>0.101±0.058</td>
<td>4.27→7.10</td>
</tr>
<tr>
<td>Zn</td>
<td>0.118±0.060</td>
<td>0.073±0.031</td>
<td>0.94→0.98</td>
</tr>
</tbody>
</table>

that the phosphorus maps most clearly expressed the shape of the cell, indicating that phosphorus was present throughout the cytoplasm and up to the cell membrane. Phosphorus has proved to be strongly correlated to potassium, sodium and magnesium in fibroblasts, yet remains stable within the cells even when the K/Na ratio is so strongly perturbed that the bulk of the intracellular potassium is replaced by sodium (Ab 85). Phosphorus has also been used to normalize X-ray yields from the electron probe analysis of fibroblasts, where the phosphorus yield was considered to be a measure of the cellular mass under the beam (Ab 85). For the present work phosphorus normalization is the most legitimate elemental normalization and is more attractive than bremsstrahlung normalization as the phosphorus X-rays will come exclusively from within the cell and be independent of foil thickness variation. Table 4.2 shows the elemental X-ray yield, normalized to the phosphorus X-ray yield, for the group of cells used for Table 4.1.

From Table 4.2 it can be seen that, for the macro elements, there are
Table 4.2: The mean relative elemental yields from normal and Menkes' fibroblasts. Individual yields were normalized to the phosphorus yield before averaging and are shown ± SD. The data were obtained from 43 normal cells and 39 Menkes' cells. Also shown is the 95% certainty range for the ratio of the Menkes' to normal mean values.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Menkes'</th>
<th>Ratio&lt;sub&gt;Menkes'/Normal&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>0.232±0.104</td>
<td>0.197±0.055</td>
<td>0.75→1.04</td>
</tr>
<tr>
<td>Cl</td>
<td>1.274±0.642</td>
<td>1.465±0.609</td>
<td>0.99→1.42</td>
</tr>
<tr>
<td>K</td>
<td>3.16±1.67</td>
<td>3.45±0.527</td>
<td>1.03→1.34</td>
</tr>
<tr>
<td>Fe</td>
<td>5.7 x 10⁻³±5.9 x 10⁻³</td>
<td>8.6 x 10⁻³±9.1 x 10⁻³</td>
<td>0.86→1.81</td>
</tr>
<tr>
<td>Cu</td>
<td>4.5 x 10⁻⁴±2.7 x 10⁻⁴</td>
<td>2.5 x 10⁻³±1.1 x 10⁻³</td>
<td>4.56→7.97</td>
</tr>
<tr>
<td>Zn</td>
<td>2.56 x 10⁻³±8.7 x 10⁻⁴</td>
<td>1.84 x 10⁻³±5.9 x 10⁻⁴</td>
<td>0.62→0.85</td>
</tr>
</tbody>
</table>

no identifiable differences in the phosphorus normalized elemental yield, between Menkes' and normal cells. The large variances present in these results, reflect the degree of biological variability observed during single cell analysis. Despite uniform conditions of cell growth, the intracellular content of healthy and morphologically sound cells was observed to vary widely. The inferior counting statistics available for the trace elements accentuated this variation. No difference between normal and Menkes' cells could be identified for iron, but a minor and statistically significant decrease in the zinc content of Menkes' cells was observed. A t-test applied to the difference between the means of the Zn/P ratio for Menkes' and normal cells showed that the probability that the two means were equal was less than 0.004% and that the most likely value for the reduction of zinc in the Menkes' cells was 28%. It is possible that zinc does play a minor and as yet unidentified role in Menkes' disease as both copper and zinc can be bound by metallothionein. The magnitude of the decrease in zinc is subject to statistical variation and it will be necessary to examine a larger number of cells in order to clarify the response of zinc to the genetic perturbation of Menkes' disease.

An approximately sixfold increase in the copper level of Menkes' cells was
Table 4.3: The mean copper elemental ratios for normal and Menkes' fibroblasts. The ratios are shown ± SD. The data was obtained from 43 normal cells and 39 Menkes' cells. The range of the ratio of the means of each ratio for the Menkes' and normal cells are shown in the third column. This range represents the 95% certainty limits for the distribution of the ratio of Menkes' to normal.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Menkes'</th>
<th>Ratio Menkes'/Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu/P</td>
<td>4.5×10⁻⁴±2.7×10⁻⁴</td>
<td>2.5×10⁻³±1.1×10⁻³</td>
<td>4.56→7.96</td>
</tr>
<tr>
<td>Cu/S</td>
<td>2.4×10⁻³±1.8×10⁻³</td>
<td>1.34×10⁻²±7.0×10⁻³</td>
<td>4.90→9.47</td>
</tr>
<tr>
<td>Cu/Cl</td>
<td>3.9×10⁻⁴±2.4×10⁻⁴</td>
<td>1.8×10⁻³±1.0×10⁻³</td>
<td>3.82→6.76</td>
</tr>
<tr>
<td>Cu/K</td>
<td>1.51×10⁻⁴±8.8×10⁻⁵</td>
<td>7.3×10⁻⁴±3.9×10⁻⁴</td>
<td>3.95→6.67</td>
</tr>
<tr>
<td>Cu/Fe</td>
<td>0.133±0.099</td>
<td>0.542±0.370</td>
<td>3.09→7.50</td>
</tr>
<tr>
<td>Cu/Zn</td>
<td>0.197±0.169</td>
<td>1.48±0.88</td>
<td>6.18→11.28</td>
</tr>
</tbody>
</table>

observed. The consistency of this result was investigated by using the multi-elemental sensitivity of the SPMP to examine the differences between normal and Menkes' fibroblasts for copper normalized to each element. The results of this are shown in Table 4.3.

Again Menkes' cells show a five to six fold greater level of copper content, relative to normal cells, when the yields are normalized to each of the macro elements. The values given for the iron and zinc normalizations are consistent with the observed trends but the high variances present for these elements prohibit meaningful interpretation. The high Menkes'/Normal ratio obtained for Cu/Zn normalization is a result of the reduction of zinc in the Menkes' fibroblasts.

The factor of six difference in the copper level between normal and Menkes' fibroblasts was lower than that found using bulk analysis. The bulk results for normal and Menkes' fibroblasts shown in Table 3.1 indicate that approximately a thirteen fold higher level of copper was found in Menkes' cells relative to normal cells when analysed by the atomic absorption technique.

This discrepancy between the SPMP results and the bulk measurement highlights the advantage of the direct analysis technique employed by the SPMP,
where the cell is grown and analysed on the same surface and is only subjected to washing in a volatile buffer before freeze-drying. In contrast to this the bulk measurement began with fibroblast monolayers in 25cm³ flasks. The same growth and maintenance media used for the SPMP study, FbGM and FbMM, were also used for these bulk measurements. The cells were then washed twice in the flasks with calcium- and magnesium-free phosphate-buffered saline (PBS). Following this, cell detachment was induced with versene-trypsin and the cells were again washed twice in PBS, washed once in 0.9% NaCl, and finally resuspended in 0.5ml of 0.9% NaCl. The cells were then collected as a pellet and lysed in either 50 or 100µl of concentrated nitric acid after which the cell lysate was vortexed and 1µl replicates were assayed for copper by an atomic absorption spectrophotometer (Ca 80).

The number of steps necessary for the bulk measurement of intracellular copper leaves the technique vulnerable to the possibility of changes in the copper level. Of major concern is the technique used for cell detachment. The fibroblasts, which are strongly adhesive cells, must be detached from the flasks in order to perform the experiment. Simply scraping the cells from the flasks damages the cells. The only viable alternative was considered to be versene-trypsin, a proteolytic enzyme. Versene-trypsin detaches the cells by breaking up the proteins that bond the cell to the plastic and neighbouring fibroblasts. However evidence has been found from measurements of the ⁶⁴Cu uptake in fibroblasts, that trypsinization accentuates the difference in the copper level between normal and Menkes' cells (Ca 89). Trypsinization affects the cell membrane, and copper associated with the membrane may be lost. This will lower the measurement of the intracellular copper content of normal cells to a greater degree than the Menkes' cells as the copper within Menkes' cells is more strongly bound by metallothionein.

The lower ratio of the intracellular copper level between Menkes’ and normal cells measured by the SPMP supports the proposition that the use of versene-trypsin does affect the measurement of intracellular copper by bulk techniques.

Examination of three dimensional elemental maps for the Menkes' fibroblasts revealed little difference from the maps obtained for the normal cells. Figure 4.6 shows a set of three dimensional elemental maps for the Menkes' cell discussed in Figure 4.5. The highly variable shape of cultured fibroblasts makes compari-
Figure 4.6

Three dimensional elemental maps for the Menkes' fibroblast discussed in Figure 4.5. With the exception of the copper map fifty contours are used to represent each elemental distribution. The copper distribution has been normalized to the peak of the phosphorus distribution and the vertical scale of the map has been increased by a factor of 30. This permits a comparison between the contour levels of the copper distributions for the normal and Menkes' cells shown in Figures 4.4 and 4.6 respectively. The following table gives the number of events used in composing each map.

<table>
<thead>
<tr>
<th>Element</th>
<th>Counts in scan region</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>75913</td>
</tr>
<tr>
<td>N</td>
<td>8854</td>
</tr>
<tr>
<td>O</td>
<td>4080</td>
</tr>
<tr>
<td>Na</td>
<td>124</td>
</tr>
<tr>
<td>P</td>
<td>26266</td>
</tr>
<tr>
<td>S</td>
<td>7951</td>
</tr>
<tr>
<td>Cl</td>
<td>77285</td>
</tr>
<tr>
<td>K</td>
<td>116076</td>
</tr>
<tr>
<td>Fe</td>
<td>330</td>
</tr>
<tr>
<td>Cu</td>
<td>178</td>
</tr>
<tr>
<td>Zn</td>
<td>70</td>
</tr>
</tbody>
</table>
SULPHUR
CHLORINE
POTASSIUM
IRON
COPPER
ZINC
son between the elemental distributions of the two cell types difficult. For all the elements, with the exception of copper, no differences could be seen in the elemental maps, with similar trends for localizations and concentration gradients being shared by the two cell types. In Figure 4.6 the copper map is again normalized to the phosphorus map in the same manner used for the cell in Figure 4.4. A direct comparison between the Cu/P content of the two cells may be performed by comparing the number of contours associated with a given point on either map. The greater relative copper content of the Menkes' cell can then be seen in the relative height (in contours) of the two distributions.

4.4.1 Elemental distribution in a mitotic Menkes' fibroblast

Fibroblasts in culture are formed by mitotic division, that is, cell division giving rise to two daughter cells with the same number of chromosomes as the parent (Ma 74a). Fibroblasts in FBGM have a reasonably high mitotic index, in that a cell will divide on average every 24 hours. It was found that in FbMM cell division still occurred but at a much reduced rate. Hence there existed a finite probability that a cell would be dividing when the culture was freeze-dried and that this cell would be observed and analysed by the SPMP. Out of a total of 198 analysed cells, one cell (a Menkes') appeared to be undergoing mitosis. Visual examination of this cell with the light microscope revealed a binucleated appearance, characteristic of telophase and cytokinesis, the final stages of mitosis and the only stages of the mitotic process identifiable in a freeze-dried cell with the microscope in the specimen chamber. Examination of the elemental distribution within this cell indicates the elemental separations present during cell mitosis.

Figure 4.7 shows the elemental distributions obtained for the dividing fibroblast. The position of the cross-hairs in the phosphorus map correspond to the position of the nuclei in the plated cell. It can be seen from the phosphorus map that these two nuclear regions are associated with local maxima in phosphorus concentration; this effect is also seen for the elements S, Cl and K. The maps for all these elements show a similar form. The phosphorus map indicates that one nuclear region (N1) has a considerably greater mass density than the other (N2), and the iron concentration in N2 is depleted relative to N1. This contrasts with
Figure 4.7

A series of elemental maps of a Menkes' fibroblast undergoing mitosis. The scan size is 44µm x 160µm and a total charge of 10.4µC was deposited on the scan region. The position of the two nuclei may be identified from the two dimensional phosphorus map. In this map the cross-hairs represented by dashed lines intersect at one nucleus (N1), and the cross-hairs represented by solid lines intersect at the other nucleus (N2). Ten contour intervals are used for the two dimensional map and they correspond to the enhanced width contours in the three dimensional map, where a total of fifty contour levels are used. The following table gives the total number of events used in composing each map.

<table>
<thead>
<tr>
<th>Element</th>
<th>Counts in scan region</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>41727</td>
</tr>
<tr>
<td>S</td>
<td>20305</td>
</tr>
<tr>
<td>Cl</td>
<td>40629</td>
</tr>
<tr>
<td>K</td>
<td>142211</td>
</tr>
<tr>
<td>Fe</td>
<td>187</td>
</tr>
<tr>
<td>Cu</td>
<td>180</td>
</tr>
<tr>
<td>Zn</td>
<td>155</td>
</tr>
</tbody>
</table>
the copper map where similar copper concentrations are found in both nuclei. A
greater concentration of zinc is found in N1 than N2.

From both the visual appearance of the cell and the elemental maps it
appears that the cell is in the state of cytokinesis where the cell membrane is
beginning to pinch inward and separate the daughter nuclei and the two halves of
the cytoplasm. The elevated calcium concentration known to be present in earlier
stages of mitosis was not observed.

4.5 Comparison of normal and Menkes’ fibroblasts

The SPMP analysis of the two groups of fibroblasts, normal and Menkes’, pro-
vides a range of elemental information that may be used to classify the cell type.
This elemental information can be used to discriminate between the two fibrob-
last groups, so that a given cell may be identified as normal or Menkes’ by the
examination of its elemental composition.

For a thin fibroblast mounted on a nylon foil, the ratio of X-ray counts
detected for a given pair of elements will be directly proportional to the ratios
of their concentrations per unit volume within the cell. Each cell can then be
characterized from the X-ray data by a range of intracellular ratios (X_i).

The identification of a cell as either normal or Menkes’ from these elemental
ratios invites the use of the statistical technique of discriminate analysis. Discrim-
inate analysis is a multivariate statistical method that forms a linear function of
the available classification variables X_i, such that this linear function provides a
maximal discrimination between the two groups (Ar 87). The technique requires
that an original data set be classified independently of the measured variables X_i;
for this work this necessitates the examination of a wide number of cells that are
known to be normal or known to be Menkes’, and the formation of group data sets
formed by the elemental ratios (X_i) for each cell. These variables are then used
to define a discriminate function Z for p variables

\[ Z = b_0 + b_1X_1 + b_2X_2 + \ldots + b_pX_p. \]  

(4.1)

For this function to discriminate well between the two groups, the coeffi-
cients, b_i, are chosen so as to maximize \( \Delta^2 \), the Mahalanobis’ distance, which is a
measure of separation between the two groups (Ar 87) and is defined:

\[ \Delta^2 = \sum_i b_i (\bar{X}_{Mi} - \bar{X}_{Ni}) \]  

(4.2)

where \( \bar{X}_{Mi} \): Mean of Menkes' variable \( X_i \);
\( \bar{X}_{Ni} \): Mean of Normal variable \( X_i \).

As the SPMP results confirmed that a high intracellular copper concentration is a phenotypic feature of Menkes' fibroblasts, this element was considered to be the most suited for use in a discriminant test. The ratios that offered the best statistics and discrimination were formed between copper and the macro elements. Hence, the form of the discriminate function for fibroblast analysis becomes

\[ Z = b_0 + b_1 \left( \frac{Cu}{P} \right) + b_2 \left( \frac{Cu}{K} \right) + b_3 \left( \frac{Cu}{S} \right) + b_4 \left( \frac{Cu}{Cl} \right) . \]  

(4.3)

The calculated discriminate function allows an allocation rule to be applied, where there is a minimum chance of misclassification of a cell. The cut-off value of the discriminate function for the optimal separation of cell types is:

\[ Z_0 = b_0 + b_1 \left( \frac{Cu}{P} \right) + b_2 \left( \frac{Cu}{K} \right) + b_3 \left( \frac{Cu}{S} \right) + b_4 \left( \frac{Cu}{Cl} \right) . \]  

(4.4)

For a given cell, if \( Z > Z_0 \) then the cell is considered Menkes', and if \( Z < Z_0 \) the cell is considered normal.

This assignment rule for the linear discriminate function assumes multivariate normality of the \( X_i \), equal covariance matrices, uncontaminated samples and correct classification of the original data set (La 75). For this work; care during the preparation of the cell cultures ensured that cross-sample contamination and misclassification did not occur. The intracellular X-ray intensity ratios vary widely, however, as a result of biological variability and generally exhibit nonnormal distributions. In order to stabilize the large variances and produce distributions that well approximate normal distributions, a logarithmic transformation was applied to the cell elemental ratios (the ratios were initially multiplied by \( 1 \times 10^5 \)).
A theoretical concept of a lognormal distribution is presented. The theory and application of these distributions have been thoroughly evaluated (Ai 57) and for each data set univariate normality was tested by applying the Kolmogorov-Smirnov test (Pe 63). This test determines the goodness-of-fit of each data set to a normal distribution. The Kolmogorov-Smirnov Z value was calculated from the largest difference in absolute value between the variable distribution and a normal distribution, and the two tailed probability was calculated to estimate the degree of normality expressed by the distribution (Ko 83). All lognormal distributions used in the analysis satisfied the Kolmogorov-Smirnov normality test; this was necessary as multivariate normality cannot be achieved with one or more nonnormal univariate distributions.

A further test of multivariate normality and equality of covariance matrices was obtained by applying Box's M Test to the covariance matrices generated during the discriminate analysis (No 85). The significance result of the Box's M Test was used along with the Kolmogorov-Smirnov normality tests to ensure that the selected variables satisfied the criteria for use in a linear discriminate function and application of the assignment rule. For small sample sizes, as in this work, it is known that the linear discriminate function can still perform well even if the covariance matrices are not too dissimilar (An 73).

A variety of codes is available for discriminate analysis. The program SPSS* (No 85) was used to calculate the discriminate function coefficients $b_i$.

Three separate data sets were deemed suitable for discriminate analysis. Set 1 comprised a total of 96 cells formed from a group of 39 cells (Group 1: Menkes') and a group of 57 cells (Group 2: Normals). These cells were known to belong to their designated group and were grown on separate foils. Both normal and Menkes' groups were sourced from a variety of cell lines. The Menkes' cells were from the cell lines: Me26a, Me6a-1 and Me39a. The normal cells were from the cell lines: S87, S94 and S97. Subgroups of these cells were grown and analysed at different times in separate preparations of FbGM and FbMM. The elemental results from the analysis of this set of cells were expected to reflect the maximum range encountered under the defined experimental conditions.

Set 2 comprised 43 cells known to be either Menkes' or normal. Each
group was formed from only one cell line. The 21 Menkes' cells were from the line Me39a and the 22 normal cells were from the line S94. All the cells were grown concurrently on four separate foils (two for each cell type), and in media from the same preparations of FbGM and FbMM.

Set 3 was a prepared 50:50 mixture of the two cell groups grown for Set 2. Four foils were seeded with this equal mixture of Menkes' and normal cells under the same culture conditions as given in Chapter 3, but Set 3 contained the results of the analysis of one foil only. This set was prepared concurrently with Set 2 and under identical conditions and contained a total of 36 cells. This preparation enabled the cells in Set 3 to be classified from the results of Set 2. The mixture of cells in Set 3 was chosen in order to reproduce the distribution of normal and Menkes' cells encountered in a heterozygote, where theoretically an even distribution of normal and Menkes' cells is obtained. The randomness of X-chromosome inactivation, however, does affect the cell mixtures obtained by biopsy. This is discussed in Section 4.6.

Consider firstly the analysis of Set 1 and the Cu/P ratios obtained for this set. The Cu/P ratio is a suitable choice for a discriminating elemental ratio due to the stability of phosphorus under conditions of induced ionic mobility (Ab 85), and the presence of this element in all cellular compartments. Figure 4.8A shows the frequency distributions of the log Cu/P ratio in Set 1. The normal cell data is represented by a solid line and the Menkes' cell data by a dashed line. It can be seen that two distinct distributions are apparent, highlighting the considerably higher level of intracellular copper found within Menkes' fibroblasts. The two components of the bimodal distribution share a central region of overlap that indicates the possible misclassification of 20% of the cells in each group if the Cu/P ratio alone is used to discriminate between cell groups. This would result in an unacceptably high misclassification rate for identifying individual cells as normal or Menkes'.

Similar bimodal lognormal distributions with approximately the same degree of overlap were found for plots of Cu/K, Cu/S and Cu/Cl. Table 4.4 shows the means and standard deviations of the transformed classification variables calculated from Set 1.
Figure 4.8

A:

The frequency distribution of log Cu/P for the cells in Set 1. The solid line represents the results for normal cells and the dashed line represents the results for the Menkes' cells. These data are normalized to the same number of cells number in each group.

B:

The frequency histogram of discriminate values (Z) for both the normal (solid line) and Menkes' (dashed line) fibroblasts in Set 1. The discriminate cutoff point is zero and the discriminate function completely separates the two distributions about this point. Again these data are normalized to the same cell number.
Table 4.4: Means and standard deviations of the classification variables used in the discriminant analysis of Set 1.

<table>
<thead>
<tr>
<th>Classification Ratio</th>
<th>Transformed Means and Standard deviations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Cu/P</td>
<td>1.55±0.37</td>
</tr>
<tr>
<td>Cu/K</td>
<td>1.09±0.31</td>
</tr>
<tr>
<td>Cu/S</td>
<td>2.14±0.47</td>
</tr>
<tr>
<td>Cu/Cl</td>
<td>1.46±0.36</td>
</tr>
</tbody>
</table>

A discriminate analysis was performed on Set 1 using Cu/P, Cu/K, Cu/S and Cu/Cl as the classification variables. This was done to investigate the feasibility of separating the groups of Menkes' and normal cells using these elemental ratios. All variables were equally weighted and entered into the analysis simultaneously. The Mahalanobis' distance ($\Delta^2$) was calculated using all the classification variables. The coefficients that maximized $\Delta^2$ are given in Table 4.5.

Table 4.5: Set 1 discrimination coefficients.

<table>
<thead>
<tr>
<th>$b_0$</th>
<th>-5.689</th>
</tr>
</thead>
<tbody>
<tr>
<td>$b_1$</td>
<td>0.943</td>
</tr>
<tr>
<td>$b_2$</td>
<td>1.579</td>
</tr>
<tr>
<td>$b_3$</td>
<td>1.262</td>
</tr>
<tr>
<td>$b_4$</td>
<td>-1.150</td>
</tr>
</tbody>
</table>

The coefficient values are correlated and hence the magnitude of a coefficient does not reflect its contribution to the discriminate function. This contribution may be determined by examining the pooled within-groups correlations between the classification variables and the discriminate function. For Set 1 the descending order of correlation of the classification variables to the discriminate function was Cu/S, Cu/K, Cu/Cl and Cu/P. The coefficient values shown in Table 4.5 were found to produce 100% discrimination (no cells in the data set misclassified) be-
tween the two groups of normal and Menkes' cells about a cut-off point at $Z_0=0$. The distribution of the discriminate function is shown in the frequency histogram of Figure 4.8B. In this figure the discriminate value ($Z$) is calculated for each cell using the transformed classification variables. The results are presented as a histogram, with the number of cells in the Menkes' group normalized to the number of cells in the normal group.

A direct application of the allocation rule to this result would indicate for subsequent cells that, if $Z > 0$ the cell is Menkes', and if $Z < 0$ the cell is normal. However, as the allocation rule has been determined to be the best for the two sample groups that have been used in forming the discriminate function, this rule is falsely optimistic when applied to cells outside these sample groups. Though 100% discrimination was achieved in this two group situation, a discriminate function that offers 50% discrimination is performing no better than chance. In order to test the possibility of misclassification, the data block for Set 1 was randomly split into two parts. One half of the randomized data block was used to derive the discriminate function, and this function was then used to predict the classification of the cells in the other half of the data block. The result of such a test is considered to give a good measure of the actual misclassification rate (No 85). For Set 1 a misclassification rate of 2% was obtained for each of two independent tests with randomized data. In each case the method of analysis was identical to that applied to the original data set.

It was assumed in the following work that a 2% misclassification rate was present when a discriminate function generated from a cell data set was used to discriminate between cells not in the original data set.

Analysis of Set 1 demonstrated that up to 100% discrimination between normal and Menkes' cell groups was possible when the four elemental ratios were used. An identical method of discriminate analysis was then applied to Set 2. The same classification variables were used and the distribution statistics for the transformed values of these variables are shown in Table 4.6.

A new discriminate function was generated from the data in Set 2 specifically to discriminate between cells from the same cell lines and culture conditions used for both Sets 2 and 3. Table 4.7 shows the discriminate function coefficients
Table 4.6: Means and standard deviations of the classification variables used in the discriminant analysis of Set 2.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Transformed Means and Standard deviations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Cu/P</td>
<td>1.76±0.18</td>
</tr>
<tr>
<td>Cu/K</td>
<td>1.20±0.21</td>
</tr>
<tr>
<td>Cu/S</td>
<td>2.51±0.19</td>
</tr>
<tr>
<td>Cu/Cl</td>
<td>1.63±0.18</td>
</tr>
</tbody>
</table>

Table 4.7: Set 2 discrimination coefficients.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>b₀</td>
<td>-13.134</td>
</tr>
<tr>
<td>b₁</td>
<td>3.836</td>
</tr>
<tr>
<td>b₂</td>
<td>0.747</td>
</tr>
<tr>
<td>b₃</td>
<td>2.754</td>
</tr>
<tr>
<td>b₄</td>
<td>-1.896</td>
</tr>
</tbody>
</table>

that resulted in 100% discrimination between the normal and Menkes' cells in Set 2. The frequency histogram of the discriminate scores for Set 2, generated by these discriminate coefficients is shown in Figure 4.9A.

Roughly equal numbers of normal and Menkes' cells were used in Set 2, hence the small group-number normalization factor of 1.1 was used. The classification results for all the cells in Set 2 are shown in Table 4.8. For Set 2 the cell type is known implicitly, however group membership probabilities were calculated from the discriminate score using Bayes' rule. The probability that a cell with a discriminate score of $Z$ belongs to Group $i$ is given by its posterior probability $P(G_i | Z)$. This value was calculated from Bayes' rule (No 85)

$$P(G_i | Z) = \frac{P(Z | G_i)P(G_i)}{\sum_{i=1}^{2} P(Z | G_i)P(G_i)}$$

(4.5)
Figure 4.9

A:

The frequency histogram of discriminate values for both the normal (solid line) and Menkes' (dashed line) fibroblasts in Set 2. The discriminate function coefficients for these cells are shown in Table 4.1. The discriminate cut-off point is zero and the discriminate function completely separates the two distributions about this point. The data for the Menkes' group are normalized to the same cell number as the normal group.

B:

The frequency histogram of discriminate values for both the normal (solid line) and Menkes' (dashed line) fibroblasts in Set 3. The discriminate values were calculated from the coefficients generated by Set 2. These data are not normalized to the same cell number in each group. Both a Menkes' and a normal cell are represented by the histogram value at Z=0. The distribution of Z for the normal cells shows no correlation to the normal cell data obtained for Set 2. The cells measured in Set 3 display a greater range of discriminate values than that obtained for Set 2. In particular three normal cells in Set 3 recorded discriminate values lower than the lowest value in Set 2.
Figure A: Graph showing the relative frequency distribution of discriminant values.

Figure B: Graph showing the frequency distribution of discriminant values.
Table 4.8

The classification table for Set 2. Cells numbered 1 to 22 are known to be normal and cells 23 to 43 are known to be Menkes'. The discriminate cut-off point is zero. The group allocation probabilities were calculated from the discriminate value (No 85) and sum to one as a cell must be a member of one of the two groups. The discriminate function has managed to produce 100% discrimination between the cells in this set, however three normal cells have a probability of greater than 0.1 of being Menkes' and five Menkes’ cells have a probability of greater than 0.1 of being normal.
<table>
<thead>
<tr>
<th>Cell number</th>
<th>Discriminant score</th>
<th>Probability of being a normal cell</th>
<th>Probability of being a Menkes' cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-3.8628</td>
<td>1.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>2</td>
<td>-2.9278</td>
<td>0.9999</td>
<td>0.0011</td>
</tr>
<tr>
<td>3</td>
<td>-2.6550</td>
<td>0.9999</td>
<td>0.0011</td>
</tr>
<tr>
<td>4</td>
<td>-2.6614</td>
<td>0.9998</td>
<td>0.0002</td>
</tr>
<tr>
<td>5</td>
<td>-2.2688</td>
<td>0.9994</td>
<td>0.0006</td>
</tr>
<tr>
<td>6</td>
<td>-2.1339</td>
<td>0.9991</td>
<td>0.0009</td>
</tr>
<tr>
<td>7</td>
<td>-2.0521</td>
<td>0.9988</td>
<td>0.0012</td>
</tr>
<tr>
<td>8</td>
<td>-1.9546</td>
<td>0.9984</td>
<td>0.0016</td>
</tr>
<tr>
<td>9</td>
<td>-1.9477</td>
<td>0.9984</td>
<td>0.0016</td>
</tr>
<tr>
<td>10</td>
<td>-1.7861</td>
<td>0.9972</td>
<td>0.0028</td>
</tr>
<tr>
<td>11</td>
<td>-1.7843</td>
<td>0.9972</td>
<td>0.0028</td>
</tr>
<tr>
<td>12</td>
<td>-1.7822</td>
<td>0.9972</td>
<td>0.0028</td>
</tr>
<tr>
<td>13</td>
<td>-1.7517</td>
<td>0.9969</td>
<td>0.0031</td>
</tr>
<tr>
<td>14</td>
<td>-1.3574</td>
<td>0.9887</td>
<td>0.0113</td>
</tr>
<tr>
<td>15</td>
<td>-1.1725</td>
<td>0.9794</td>
<td>0.0206</td>
</tr>
<tr>
<td>16</td>
<td>-0.9104</td>
<td>0.9525</td>
<td>0.0475</td>
</tr>
<tr>
<td>17</td>
<td>-0.8499</td>
<td>0.9426</td>
<td>0.0574</td>
</tr>
<tr>
<td>18</td>
<td>-0.8277</td>
<td>0.9385</td>
<td>0.0615</td>
</tr>
<tr>
<td>19</td>
<td>-0.7157</td>
<td>0.9135</td>
<td>0.0865</td>
</tr>
<tr>
<td>20</td>
<td>-0.6498</td>
<td>0.8947</td>
<td>0.1053</td>
</tr>
<tr>
<td>21</td>
<td>-0.1292</td>
<td>0.6048</td>
<td>0.3952</td>
</tr>
<tr>
<td>22</td>
<td>-0.0124</td>
<td>0.5102</td>
<td>0.4898</td>
</tr>
<tr>
<td>23</td>
<td>0.1200</td>
<td>0.4025</td>
<td>0.5975</td>
</tr>
<tr>
<td>24</td>
<td>0.1642</td>
<td>0.3680</td>
<td>0.6320</td>
</tr>
<tr>
<td>25</td>
<td>0.4675</td>
<td>0.1776</td>
<td>0.8224</td>
</tr>
<tr>
<td>26</td>
<td>0.5403</td>
<td>0.1444</td>
<td>0.8556</td>
</tr>
<tr>
<td>27</td>
<td>0.6382</td>
<td>0.1089</td>
<td>0.8911</td>
</tr>
<tr>
<td>28</td>
<td>0.8142</td>
<td>0.0041</td>
<td>0.9359</td>
</tr>
<tr>
<td>29</td>
<td>1.0314</td>
<td>0.0324</td>
<td>0.9676</td>
</tr>
<tr>
<td>30</td>
<td>1.1817</td>
<td>0.0200</td>
<td>0.9800</td>
</tr>
<tr>
<td>31</td>
<td>1.3128</td>
<td>0.0131</td>
<td>0.9869</td>
</tr>
<tr>
<td>32</td>
<td>1.5700</td>
<td>0.0057</td>
<td>0.9943</td>
</tr>
<tr>
<td>33</td>
<td>1.6571</td>
<td>0.0042</td>
<td>0.9958</td>
</tr>
<tr>
<td>34</td>
<td>1.6598</td>
<td>0.0042</td>
<td>0.9958</td>
</tr>
<tr>
<td>35</td>
<td>1.6852</td>
<td>0.0039</td>
<td>0.9961</td>
</tr>
<tr>
<td>36</td>
<td>1.8049</td>
<td>0.0026</td>
<td>0.9974</td>
</tr>
<tr>
<td>37</td>
<td>1.9284</td>
<td>0.0017</td>
<td>0.9983</td>
</tr>
<tr>
<td>38</td>
<td>2.3657</td>
<td>0.0004</td>
<td>0.9996</td>
</tr>
<tr>
<td>39</td>
<td>2.4078</td>
<td>0.0004</td>
<td>0.9996</td>
</tr>
<tr>
<td>40</td>
<td>2.7183</td>
<td>0.0001</td>
<td>0.9999</td>
</tr>
<tr>
<td>41</td>
<td>3.3895</td>
<td>0.0000</td>
<td>1.0000</td>
</tr>
<tr>
<td>42</td>
<td>3.4451</td>
<td>0.0000</td>
<td>1.0000</td>
</tr>
<tr>
<td>43</td>
<td>3.6752</td>
<td>0.0000</td>
<td>1.0000</td>
</tr>
</tbody>
</table>
where $P(G_i)$: Prior probability that a cell belongs to Group $i$. For this work the prior probability for each group was assumed to be 0.5.

$P(Z_{G_i})$: Conditional probability of obtaining a score of $Z$ given the Group $i$.

In this two group situation the sum of the posterior probabilities for each group must be one. The posterior probabilities provide a useful guide to the effectiveness of the discriminate function. For Set 2 a total of eight cells lies in a range where the probability of misclassification is greater than 0.1 and three cells (21, 22 and 23) have probabilities of being misclassified approximately equal to or greater than 0.4. For these three cells the discriminate function is performing little better than chance. Cells other than those in this intermediate range between the two groups are well classified by the discriminate function.

The discriminate coefficients for Set 2 (shown in Table 4.6) were then applied to the cells in Set 3, which were from the same cell lines as Set 2 and grown under identical conditions, but each foil had been seeded with a 50:50 mixture of normal and Menkes' cells. The discriminate function was used to label unidentified cells as normal or Menkes' by the use of the allocation rule.

Figure 4.9B shows the frequency histogram of the discriminate scores for Set 3 when the coefficients generated by Set 2 were applied to the elemental ratios for each cell in Set 3. No group-number normalization was applied to these data as group membership was to be determined by the discriminate function. It is immediately obvious from this histogram that the expected 50:50 distribution of normal and Menkes' cells was not obtained. The distribution of cell types after the application of the allocation rule can be seen more clearly in Table 4.9, where the posterior probabilities for group membership are shown.

Absolute application of the allocation rule to the cells in Set 3 divides the set into 26 normal cells (Cell nos. 1→26) and 10 Menkes' cells (Cell nos. 27→36). However the anticipated misclassification rate of 2% must be considered in determining the most likely ratio of the normal to Menkes' cells. This rate of misclassification predicts that one cell in the set will be misclassified. The posterior probabilities for group membership can then be used to identify the cell most likely to be misclassified by the discriminate function. In Set 3 cell 26
Table 4.9

The classification table for Set 3. Cells numbered 1 to 26 were classified as normal. Cells numbered 27 to 36 were classified as Menkes'. Again the discriminate cut-off point is zero. The classification probability provides a guide to the likelihood of a cell belonging to one or other of the specified groups. Three cells classified as being normal have a probability of greater than 0.1 of being Menkes' and two cells classified as being Menkes' have a probability of greater than 0.1 of being normal. The allocation rule, when applied in this situation, has been shown to have a misclassification rate of 2%. Conservatively one of three cells 26, 27 or 28 may be misclassified.
<table>
<thead>
<tr>
<th>Cell number</th>
<th>Discriminant score</th>
<th>Probability of being a normal cell</th>
<th>Probability of being a Menkes' cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-6.5066</td>
<td>1.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>2</td>
<td>-4.5064</td>
<td>1.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>3</td>
<td>-4.2024</td>
<td>1.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>4</td>
<td>-3.7061</td>
<td>1.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>5</td>
<td>-3.2789</td>
<td>1.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>6</td>
<td>-3.2494</td>
<td>1.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>7</td>
<td>-2.8856</td>
<td>0.9999</td>
<td>0.0001</td>
</tr>
<tr>
<td>8</td>
<td>-2.5827</td>
<td>0.9998</td>
<td>0.0002</td>
</tr>
<tr>
<td>9</td>
<td>-2.5678</td>
<td>0.9998</td>
<td>0.0002</td>
</tr>
<tr>
<td>10</td>
<td>-2.5322</td>
<td>0.9998</td>
<td>0.0002</td>
</tr>
<tr>
<td>11</td>
<td>-2.3337</td>
<td>0.9996</td>
<td>0.0004</td>
</tr>
<tr>
<td>12</td>
<td>-2.3056</td>
<td>0.9996</td>
<td>0.0004</td>
</tr>
<tr>
<td>13</td>
<td>-2.1614</td>
<td>0.9993</td>
<td>0.0007</td>
</tr>
<tr>
<td>14</td>
<td>-2.0680</td>
<td>0.9990</td>
<td>0.0010</td>
</tr>
<tr>
<td>15</td>
<td>-1.6960</td>
<td>0.9967</td>
<td>0.0033</td>
</tr>
<tr>
<td>16</td>
<td>-1.6870</td>
<td>0.9966</td>
<td>0.0034</td>
</tr>
<tr>
<td>17</td>
<td>-1.6573</td>
<td>0.9963</td>
<td>0.0037</td>
</tr>
<tr>
<td>18</td>
<td>-1.5567</td>
<td>0.9941</td>
<td>0.0059</td>
</tr>
<tr>
<td>19</td>
<td>-1.4815</td>
<td>0.9933</td>
<td>0.0067</td>
</tr>
<tr>
<td>20</td>
<td>-1.1907</td>
<td>0.9806</td>
<td>0.0194</td>
</tr>
<tr>
<td>21</td>
<td>-1.1648</td>
<td>0.9789</td>
<td>0.0211</td>
</tr>
<tr>
<td>22</td>
<td>-0.8787</td>
<td>0.9475</td>
<td>0.0525</td>
</tr>
<tr>
<td>23</td>
<td>-0.8659</td>
<td>0.9454</td>
<td>0.0546</td>
</tr>
<tr>
<td>24</td>
<td>-0.5847</td>
<td>0.8727</td>
<td>0.1273</td>
</tr>
<tr>
<td>25</td>
<td>-0.4754</td>
<td>0.8272</td>
<td>0.1728</td>
</tr>
<tr>
<td>26</td>
<td>-0.0015</td>
<td>0.5012</td>
<td>0.4988</td>
</tr>
<tr>
<td>27</td>
<td>0.0421</td>
<td>0.4653</td>
<td>0.5347</td>
</tr>
<tr>
<td>28</td>
<td>0.0695</td>
<td>0.4430</td>
<td>0.5570</td>
</tr>
<tr>
<td>29</td>
<td>0.8020</td>
<td>0.0666</td>
<td>0.9334</td>
</tr>
<tr>
<td>30</td>
<td>0.9661</td>
<td>0.0374</td>
<td>0.9626</td>
</tr>
<tr>
<td>31</td>
<td>1.4478</td>
<td>0.0095</td>
<td>0.9905</td>
</tr>
<tr>
<td>32</td>
<td>1.7601</td>
<td>0.0034</td>
<td>0.9966</td>
</tr>
<tr>
<td>33</td>
<td>2.6584</td>
<td>0.0002</td>
<td>0.9998</td>
</tr>
<tr>
<td>34</td>
<td>2.7842</td>
<td>0.0001</td>
<td>0.9999</td>
</tr>
<tr>
<td>35</td>
<td>3.0631</td>
<td>0.0000</td>
<td>1.0000</td>
</tr>
<tr>
<td>36</td>
<td>4.0962</td>
<td>0.0000</td>
<td>1.0000</td>
</tr>
</tbody>
</table>
(classified a normal cell) has the greatest probability of misclassification (0.4988), and hence statistically this cell would be the one most likely to be incorrectly classified. High probabilities of misclassification also exist for cells 27 and 28, both of which have been classified as Menkes' with the respective probabilities of 0.47 and 0.44 of being normal. To account for these high probabilities, in this data set, a conservative misclassification rate of 8% was assumed.

A number of factors may give rise to the unequal division of normal and Menkes' cells in Set 3. The 36 cells sampled were taken from many different sites on the foil surface to avoid selective sampling from an area predominately populated by one group. As a result Set 3 should be representative of the normal-Menkes' distribution across the whole foil. In previous bulk measurements where known normal and Menkes' cells were mixed in equal proportion and grown for several passages, the total copper concentration of the cell population was found to decrease with increasing passage number. This has been speculated to be the result of a growth advantage of the normal cells over the Menkes' cells (Ca 80), but the proportional change in the normal-Menkes' distribution cannot be ascertained by the bulk measurement. Also a large error exists in the seeding levels of the two cell groups. This error may be up to 20%, giving rise to the possibility that the normal cells were seeded with up to a 1.5:1 advantage over the Menkes' cells. The combination of both of these effects offers an explanation for the predominance of normal cells in Set 3. Given the error in seeding, and the error in misclassification (assuming misclassification probabilities <0.1), then the growth advantage of normal cells over Menkes' cells in culture is between 1.2 and 5.2.

The analysis of Set 3 offered the opportunity to directly compare normal and Menkes' fibroblasts by including a cell from each group in a single scan. As the cells can only be identified after the experimental run, it was only by good fortune that, in a scan performed on two cells, one proved to be normal (Cell 20), the other Menkes' (Cell 39). Figure 4.10 shows the three dimensional elemental maps of these cells two cells growing side by side on the foil. It is difficult to present these maps to show the distribution within each cell to full effect, as the cell thicknesses vary significantly. For a given element this results in a difference in the maximum counts found within each cell region of the scan. Thus, in a contour
Figure 4.10

A set of three dimensional elemental maps for cells 20 and 36 from Set 3. The scan size was 40µm × 104µm and a total charge of 6.0µC was deposited on this area over a period of 3.5 hours. The beam current was 650pA and the beam spatial resolution was 4µm.

Different total contour numbers were chosen to highlight the distributions for each element.

Cell 35 (Menkes’) is the cell located on the upper left-hand side of each map. The carbon, nitrogen and oxygen maps indicate that the cell is less dense than Cell 20 the adjacent normal cell. The two cells are separated by 4µm at the point of closest approach. The table below gives the total number of events used in composing each map.

<table>
<thead>
<tr>
<th>Element</th>
<th>Counts in scan region</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>137990</td>
</tr>
<tr>
<td>N</td>
<td>20221</td>
</tr>
<tr>
<td>O</td>
<td>9107</td>
</tr>
<tr>
<td>Na</td>
<td>538</td>
</tr>
<tr>
<td>P</td>
<td>71409</td>
</tr>
<tr>
<td>S</td>
<td>45416</td>
</tr>
<tr>
<td>Brem</td>
<td>2048</td>
</tr>
<tr>
<td>Cl</td>
<td>106981</td>
</tr>
<tr>
<td>K</td>
<td>204532</td>
</tr>
<tr>
<td>Fe</td>
<td>333</td>
</tr>
<tr>
<td>Cu</td>
<td>273</td>
</tr>
<tr>
<td>Zn</td>
<td>204</td>
</tr>
</tbody>
</table>

Table 4.10, on the following page, shows the different elemental yields from each cell. These differences in yield necessitates the use of a range of contour levels for each element. Thirty contours were used to represent the distribution of C, N, O, Na, P, S, Cl, Bremsstrahlung, and K. Twenty contours were used for Cu and fifteen for Fe and Zn.
Table 4.10 Intracellular X-ray and RBS yields for cells 20 and 35. The X-ray data have been normalized to a bremsstrahlung (as defined in Section 4.4) yield of 500. It has been assumed that the foil thickness does not vary over the area of the scan.

<table>
<thead>
<tr>
<th></th>
<th>Normal (Cell 20)</th>
<th>Menkes' (Cell 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area (μm²)</td>
<td>620±50</td>
<td>1240±50</td>
</tr>
<tr>
<td>P</td>
<td>23000±1300</td>
<td>19600±960</td>
</tr>
<tr>
<td>S</td>
<td>8600±700</td>
<td>8500±550</td>
</tr>
<tr>
<td>Cl</td>
<td>36000±2000</td>
<td>30000±1300</td>
</tr>
<tr>
<td>K</td>
<td>87000±4000</td>
<td>80000±3000</td>
</tr>
<tr>
<td>Fe</td>
<td>51±12</td>
<td>73±15</td>
</tr>
<tr>
<td>Cu</td>
<td>22±6</td>
<td>126±14</td>
</tr>
<tr>
<td>Zn</td>
<td>62±12</td>
<td>49±9</td>
</tr>
<tr>
<td>C</td>
<td>27000±1500</td>
<td>32000±1400</td>
</tr>
<tr>
<td>N</td>
<td>3100±250</td>
<td>3800±200</td>
</tr>
<tr>
<td>O</td>
<td>1700±160</td>
<td>2000±140</td>
</tr>
<tr>
<td>Na</td>
<td>140±50</td>
<td>70±32</td>
</tr>
</tbody>
</table>
Table 4.10 Intracellular X-ray and RBS yields for cells 20 and 35. The X-ray data have been normalized to a bremsstrahlung (as defined in Section 4.4) yield of 500. It has been assumed that the foil thickness does not vary over the area of the scan.

<table>
<thead>
<tr>
<th></th>
<th>Normal (Cell 20)</th>
<th>Menkes' (Cell 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area ($\mu m^2$)</td>
<td>620±50</td>
<td>1240±50</td>
</tr>
<tr>
<td>P</td>
<td>23000±1300</td>
<td>19600±960</td>
</tr>
<tr>
<td>S</td>
<td>8600±700</td>
<td>8500±550</td>
</tr>
<tr>
<td>Cl</td>
<td>36000±2000</td>
<td>30000±1300</td>
</tr>
<tr>
<td>K</td>
<td>87000±4000</td>
<td>78000±3000</td>
</tr>
<tr>
<td>Fe</td>
<td>51±12</td>
<td>73±15</td>
</tr>
<tr>
<td>Cu</td>
<td>22±6</td>
<td>126±14</td>
</tr>
<tr>
<td>Zn</td>
<td>62±12</td>
<td>49±9</td>
</tr>
<tr>
<td>C</td>
<td>27000±1500</td>
<td>32000±1400</td>
</tr>
<tr>
<td>N</td>
<td>3100±250</td>
<td>3800±200</td>
</tr>
<tr>
<td>O</td>
<td>1700±160</td>
<td>2000±140</td>
</tr>
<tr>
<td>Na</td>
<td>140±50</td>
<td>70±32</td>
</tr>
</tbody>
</table>
map, a greater proportion of contours are used to display one cell in preference to the other. To overcome this problem a variety of contour levels were used in the maps (see the caption for Figure 4.10). From the backscattering maps it can be seen that Cell 20 (the cell on the upper left-hand side of the maps) is thicker than Cell 35 (Menkes'). As a result the phosphorus map shows a greater P Kα X-ray yield for Cell 20 than for Cell 35. The peak in the phosphorus map for Cell 35 corresponds to the cell nucleus. The position of the nucleus in Cell 20 could not be identified.

For both cells the distribution of the other macro elements is similar to that obtained for phosphorus. Table 4.10 shows the normalized elemental yields obtained for each cell. The table indicates that the elemental yields, with the exception of copper, are similar for each cell. Cell 35 has approximately six times the copper content of Cell 20. This is reflected in the copper map which is dominated by the copper distribution for Cell 35. Relatively far less Cu Kα X-ray yield is obtained from the area within the scan occupied by Cell 20.

4.6 Diagnosis of Menkes' disease

The results presented in Section 4.5 have shown that the SPMP is able to distinguish individual Menkes' fibroblasts from normal fibroblasts in a single culture. This has ramifications in the diagnosis of Menkes' disease.

In most cases a Menkes' heterozygote will be free of the disease symptoms and exhibit normal serum copper levels. However some heterozygotes may show symptoms of Menkes' disease (Ta 81) and the full range of Menkes' symptoms has been seen in two females (Iw 79)(Fa 83). This occurs because random chromosome inactivation in a female begins early in fetal development and follows a binomial distribution. On average heterozygotes will possess a 50:50 mix of normal and mutant cells, but some cell areas will contain more normal cells and others more mutant cells (Ho 80). As the cells form a mosaic a sampling error could occur due to a biopsy being taken from an area containing predominately normal cells. Bulk analysis of this sample will show that the cells have normal copper levels. Alternatively normal cells may have a growth advantage over mutant cells and
whilst growing the large numbers of cells required for bulk analysis the population may be overtaken by normal cells. Clearly there is an advantage in being able to estimate the copper content of individual cells where a small population of cells containing elevated concentrations of copper can be identified. Bulk measurements have found copper levels intermediate between normal and mutant phenotypes in one third of standard fibroblast cultures formed from a single skin biopsy of a heterozygote (Ca 80)(Ho 80). These results, are inconclusive in identifying a potential heterozygote however the SPMP analysis offers the advantage of a rapid and positive identification of a heterozygote, as by analysing individual cells from a small sample only one definite Menkes' cell need be identified to classify the culture as being sourced from a Menkes' heterozygote. This means of identification would diminish the problem of mosaicism of cells in a biopsy from a single site, and if a number of foils are prepared, each sourced from a different biopsy, a SPMP analysis that sampled cells from all the foils would ensure that limitations on heterozygote identification due to cell mosaicism would be overcome.

A guide to the number of cells that needs to be analysed for the diagnosis of a heterozygote can be taken from the results in Set 3. In biological work the significance of most results is expressed with the two standard deviation limits (95%) of uncertainty. Applying this limit to the cells in Set 3 divides the results into the breakdown shown in Table 4.11. If Set 3 is considered representative of

<table>
<thead>
<tr>
<th>No. of cells</th>
<th>Normal</th>
<th>Menkes'</th>
<th>Uncertainty &gt; 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>7</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

a heterozygote culture and a cell is considered an 'identified' Menkes' cell if it is defined with 95% certainty, then it may be shown that 5 cells must be examined in order to classify the culture as one which contains Menkes' cells with greater than 98% certainty. In practice however the analysis of at least 40 cells would be necessary to ensure that the overrepresentation of normal cells, due to their growth advantage, was avoided.
The SPMP also has a potential role in the prenatal diagnosis of Menkes’ disease. As can be seen from Table 3.1 Menkes’ amniotic cell lines also express the copper accumulation phenotype and tests, based on the copper levels in amniotic cells, are available for the intrauterine diagnosis of Menkes’ disease. These tests require the formation of a bulk culture consisting of at least $1 \times 10^6$ cells and the total analysis time is approximately four weeks. Such a lengthy diagnosis period compromises the utility of the technique. The SPMP technique developed for the analysis of fibroblasts could also be applied to amniocytes. Amniocytes are a fibroblast-like cell present in the amnion, the sac of fluid surrounding a fetus. The similarity between fibroblasts and amniocytes extends to their response to culture conditions. Amniocytes can be grown on the nylon foil under the same culture conditions that were used for the fibroblasts. Hence the possibility exists that the technique used for identifying mutant fibroblasts could also be extended to amniocytes (Ca 80)(Ho 80). The small number of cells necessary for SPMP analysis, relative to the requirements of a bulk measurement, permits the growth of a culture in 6 days. Diagnostic time beyond that would only be limited by the SPMP analysis time. This time period would be governed by the number of cells required for diagnosis, which would be dictated by the discriminant scores, and hence group assignment probabilities, of the cells as they were analysed.

For amniocyte diagnosis the culture would consist of either exclusively normal cells or exclusively Menkes’ cells. Set 2 can then be used as a guide to the number of cells that need to be considered for diagnosis. Table 4.12 shows the breakdown of cell identification, with 95% certainty, for the cells known to belong to each group in Set 2.

On the basis of Table 4.12, where a cell can be considered ‘identified’ if it can be defined as being normal or Menkes’ with 95% certainty, it may be shown that only three cells need be examined in order to classify each group as either normal or Menkes’ with 98% certainty. In practice however, the serious clinical implications of the diagnosis would necessitate the examination of at least 20 cells with the possibility of the analysis of a greater number of cells if ambiguous classification probabilities were obtained.
Table 4.12: The number of cells identified with 95% certainty from each of the two groups in Set 2. The number of cells known to belong to each group is shown in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Identified</th>
<th>Uncertainty &gt; 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal(22)</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>Menkes'(20)</td>
<td>15</td>
<td>5</td>
</tr>
</tbody>
</table>

4.7 STIM imaging of fibroblasts

STIM imaging provides the optimum resolution achievable with a SPMP. Fibroblasts were subjected to STIM analysis in order to examine density variation within a cell and establish the cellular features revealed by resolution improvements beyond that presently available for elemental analysis.

The STIM technique has been used with the SPMP to form high resolution images of thin targets (Se 83)(Ov 83). Image contrast with STIM is provided by the energy loss of the transmitted ions. A surface barrier detector placed at 0° is used to determine transmitted particle energy and the exceptionally high collection efficiency of bright field imaging necessitates a low beam intensity on target, which is used to advantage as beam current is attenuated by restricting the microprobe diaphragms, resulting in a minimum beam spot. The stopping power of the biological matrix is approximately ten times greater for alpha particles than protons of equivalent energy, thus the STIM imaging of fibroblasts was performed with alpha particles to obtain superior depth resolution.

The MP system was reconfigured to operate as a STIM by using the smallest available object and aperture diaphragms (5µm and 0.25mm respectively) and a 2MeV 4He⁺ beam. A set of orthogonal, taper-edged microslits was then wound into position directly following the object diaphragm. Judicious adjustment of the microslit position reduced beam current and resulted in an effective object size of ~ 0.2µm (Be 87). Angle dependent lens aberrations were minimized by the selection of the smallest aperture diaphragm and hence the resultant beam spot was limited primarily by chromatic aberration. The transmitted particles

68
Figure 4.11

A:

A SEM image of a freeze-dried fibroblast mounted on a nylon foil. The image was formed with a resolution of 10 nm and the scale is indicated by the horizontal bar which represents a length of 10 μm. The central ellipsoidal region within the cell is the nucleus.

B:

A magnified image of the nucleus shown in A. The scale is shown in the bottom right-hand corner.
were collected by a 50mm\(^2\) silicon surface barrier detector. This detector was placed 24cm downstream from the target and operated with an energy resolution of 25keV. A transmission particle count rate of approximately 1.5kHz was used and this corresponded to a beam current of approximately 0.2fA.

Initially fibroblasts, prepared as detailed in Section 3.5, were imaged with a SEM. These images were formed to reveal, at high resolution, the structure of the prepared cells. Electronmicrographs of the prepared fibroblasts are shown in Figure 4.11. These images show that the surface of the freeze-dried cells had been disrupted during washing, freezing and drying. This disruption possibly resulted from the use of a hypertonic buffer. The SEM is only capable of identifying surface feature, so internal structure within the nuclei is not revealed.

The STIM data collected from the irradiation of a fibroblast are shown in Figure 4.12. Figure 4.12A shows the transmitted particle spectrum. Little structure is apparent in the spectrum, the shoulder occurring at 1860keV representing the lower energy limit for particles traversing the nylon foil only. Figure 4.12B is the STIM image formed from the same data set. The image was produced from events occurring within a selected energy window (1654 \(\rightarrow\) 1971keV) and is presented on a 96 \(\times\) 358 pixel map. The average energy loss of transmitted particles in each pixel was used to construct the image and these points were smoothed by a Gaussian representation of the beam spot in the target plane. The estimated diameter of the beam spot was 0.2\(\mu\)m. The final image intensity is represented from a range of 32 possible shades of grey.

It can be seen from Figure 4.12B that the energy-loss imaging provided by STIM offers sufficient contrast and resolution to clearly highlight the cell outline, nuclear membrane and three possible nucleoli. The image of the nuclear membrane appears to be approximately 1\(\mu\)m thick (in the X-Y plane). This width is at least an order of magnitude greater than the width of the nuclear membrane in vivo and comes about due to an edge effect, with the perimeter of the nuclear membrane wall appearing thicker to the STIM beam. The average energy smoothing then accentuates the apparent width of the membrane. The three nucleoli are between 2 and 3\(\mu\)m in diameter. Previous PIXE analysis had proven to be incapable of discerning the nuclear boundary or subnuclear components.
Figure 4.12

A:

The energy spectrum of transmitted 2 MeV \(^4\)He\(^+\) ions for the STIM analysis of a fibroblast. The target current was estimated to be 0.2fA. The scan region was irradiated for 600 seconds and 516,180 events were collected at a count rate of approximately 1.5kHz.

B:

Mapped data from the data set described in A. The mapped points were from within the energy range 1654 → 1971keV. Beam resolution was estimated to be 0.2\(\mu\)m and the data set was smoothed by a rotated Gaussian of this diameter. This average energy map utilizes a grey scale composed of 32 grey shades. The scan size was 24\(\mu\)m × 90\(\mu\)m and the map is 96 × 358 pixels. Darker regions represent areas of higher density. The nuclear membrane is clearly visible and the regions of higher density within the nucleus are possible nucleoli.
4.8 Conclusion

The SPMP can detect copper in both normal and Menkes' fibroblasts. A fivefold increase in the intracellular copper level was found in the Menkes' fibroblasts. This difference in the copper level is less than that obtained from bulk analysis probably due to the minimal interference necessary for the SPMP measurement. A minor but statistically significant decrease in the zinc content was also found and this has not been previously described. Zinc is known to be bound to metallothionein, a protein over-produced by the Menkes' mutation. It is possible that in mutant cells copper is bound to metallothionein at the expense of zinc. The confirmation of a decrease in the zinc content of Menkes' cells requires the analysis of larger populations of normal and Menkes' cells. At the present time it is not clear whether such a small decrease in zinc may cause any abnormalities other than the copper-related abnormalities described (Da 83).

Using copper-based elemental ratios the SPMP can identify individual mutant fibroblasts in culture. This can be used for the diagnosis of Menkes' disease, although a minimum of twenty cells must be examined by the SPMP to ensure that a valid result is obtained. The time required for diagnosis is then very much limited by the irradiation period for each cell. Under the present microprobe conditions the average period of irradiation necessary for sufficient statistics of the Cu Kα X-ray is between 2 and 3 hours. This results in a diagnosis period that requires up to 60 hours of microprobe beam time. Obviously a reduction in this component of the diagnosis time should be sought.

STIM analysis of the fibroblasts has shown the extra intracellular feature revealed by high resolution imaging. Elemental information at the same resolution would provide fundamental insights into transport mechanisms within the cells. This is of major interest in Menkes' disease where the localization of copper within fibroblasts remains unknown. In particular the following two specific areas of Menkes' research invite further application of a high resolution microprobe: (i) Some evidence has been found from studies with copper toxicity that copper accumulates in the nucleus of cells (Ar 89), and this work has been qualitatively supported by the present work (see Appendix 2). (ii) There also exists a possi-
bility that in Menkes' cells metallothionein polymerizes and accumulates within lysosomes. Lysosomes within fibroblasts are about 2\(\mu\)m or less in diameter and if the metallothionein accumulation is present then the lysosomes would entrap copper. Both of these examples highlight immediate applications available for a high resolution microprobe; however serious attempts at this work must await significant resolution improvements.
Chapter 5

Ion sources: The present system and alternatives

5.1 Introduction

The preceding chapters have shown that at its present level of development, the SPMP is capable of determining elemental distributions within individual biological cells. The existing resolution of several microns provides useful elemental information on large-scale cellular features, but subcellular structures such as mitochondria and other organelles will remain unresolved until the SPMP is capable of submicron resolution with sufficient beam current for elemental analysis.

The STIM analysis of fibroblasts has shown that the spatial resolution of the Melbourne SPMP can be improved by reducing the size of both the object and aperture diaphragms. However, this leads to a reduction in the beam current available for target irradiation. In elemental analysis work with small target currents (<100pA), excessively long irradiation periods are required in order to collect data with satisfactory statistics. Such long irradiation times demand an exceptional degree of mechanical stability if high resolution data is to be obtained, and place unreasonable demands on accelerator usage. For PIXE analysis of biological cells the lowest target current tolerable for routine use is approximately 100pA. Under typical accelerator conditions the Melbourne SPMP will produce a 100pA target current with an object diaphragm size of 25μm and an aperture diaphragm size of 0.5mm. Assuming that the beam has a rotated Gaussian cross
section, the measured FWHM of the beam spot for this configuration is $1 \mu m$. The resolution can be improved for this set of diaphragm sizes by reducing lens aberrations (Ja85) and increasing the demagnification factor. The present microprobe system has been optimized within physical constraints to achieve the maximum demagnification ($\frac{1}{M} \sim 22$) for a Russian antisymmetric quadrupole quadruplet system. Lens aberrations have been, and continue to be, studied extensively (Ja 87) (Mo 87), but the first order limit to the lens performance is close to being reached for the present system. A significant improvement in beam resolution can therefore be achieved only by a large reduction in the object and aperture diaphragms. With the present accelerator this results in a massive reduction in target current, rendering most elemental microanalysis impossible.

This chapter describes the investigation of possible modifications to the Pelletron accelerator to improve its suitability for use with the proton microprobe. At the present time most ion microprobes (the MP system included) are attached to accelerators designed primarily for low energy nuclear physics research (Le 87), and as such, these accelerators are not especially suited to the ion optical demands of a high resolution probe forming system. In order to determine the limitations of the present 5U Pelletron accelerator it is necessary to consider the ion optical parameters relevant to the matching of the accelerator to the microprobe system.

5.2 Pelletron accelerator and beam brightness

A variety of optical elements are required to form the focused microprobe beam. These are represented in Figure 2.1, a schematic of both the 5U Pelletron accelerator and the microprobe beamline. The beam is created in the RF ion source. It is then extracted from the ion source canal region and focused by a four electrostatic element lens (combination Einzel and Immersion lens). Next, the beam is further accelerated by a bias voltage then mass analysed by a Wien filter. This filter selects the desired ion species and provides an energy resolution of 750eV for $H^+$ at 25kV, a typical bias voltage. A 1.16mm diameter aperture at the exit of the Wien filter defines the beam entrance to the accelerator and an electrostatic steerer following the filter is used to direct the beam along the axis of the acceler-
ator. The accelerating column consists of five sections, with each section designed
to provide up to 1MeV acceleration potential. Upon exiting the accelerator, the
beam is collimated by a set of four adjustable object slits before passing through
an energy dispersive dipole magnet. Two image slits following the magnet are used
in combination with the object slits to select the desired ion beam and provide
the energy stabilization of the accelerator. The measured beam energy spread is
700eV at 3.5MeV (Ba 86). The beam from the accelerator then passes through
both the object and aperture diaphragms before being focused by the quadruplet
of magnetic quadrupoles, as described Section 2.2. The focused beam contains
only those ions that have traveled within a paraxial envelope through the system,
off axis trajectories being discarded by the rigid collimation of the probe. The rays
that are important to the formation of the beam spot are those found close to the
optical axis of the system and with small angles of convergence or divergence.

The path of the proton beam through the entire probe-forming system may
be defined relative to a right-handed coordinate system x, y, and z. The optical
axis may be considered to be the z axis. Each particle in the beam may, at any
point in time, be characterized by a conjugate position and momentum coordinate
pair, (x,px), (y,py), and (z,pz). The entire beam can thus be represented by a
six dimensional phase space. Liouville's theorem may then be applied to the
motion of the particles within this phase space. This theorem states that the
density of points in phase space remains constant under the action of conservative
forces. Both stationary electric and magnetic fields provide conservative forces
upon the charged particles moving through the field, and Liouville's theorem holds
independently for each of the two dimensional subspaces (x,px), (y,py), and (z,pz).

For a particle moving along the z axis under the action of a conservative force
the four dimensional phase space (x,px, y, py) may be considered to be a conserved
quantity. The particle trajectory coordinates, (x,x'), where x' is defined as x' =
\arctan(p_y/p_x), are of greater interest than the beam position-momentum coordinates
(x,px). If px is constant, as in an energy normalized system, then the transverse
phase space areas are proportional to the areas xx' and yy'. These areas may
be used to define the energy (E) normalized emittance \( \epsilon_n \) as a beam invariant

74
quantity
\[ \epsilon_{nx} = \pi x' \sqrt{E} \quad \epsilon_{ny} = \pi y' \sqrt{E} \]  

(5.1)

The mean beam brightness, for a beam of current \( I \) may be defined from this (Wa 62)
\[ B_n = \frac{2I}{\epsilon_{nx} \epsilon_{ny}} \]  

(5.2)

and is also conserved in an aberration-free system. The brightness may be defined as the ion current per unit area per unit solid angle (Se 67) and for a source of radius \( r_s \), with small beam half angle \( \alpha \), the normalized brightness can be written
\[ B_n = \frac{I}{\pi^2 r_s^2 \alpha^2 E} \text{ A m}^{-2} \text{ rad}^{-2} \text{ V}^{-1}. \]  

(5.3)

The invariance of normalized beam brightness has significant consequences for the microprobe system. It dictates that the brightness of the focused microbeam is limited by the brightness of the primary beam from the accelerator and ultimately by the brightness of the ion source. In this way the ion source may be considered to be the object of the entire probe forming system.

In an idealized apertured system the radius of the Gaussian image \( r_i \) may be written in terms of the system magnification \( M \) and the half angle of convergence \( \alpha_i \)
\[ r_i = Mr_s = \frac{1}{\pi \alpha_i} \left( \frac{I}{B_n E} \right)^{\frac{1}{2}}. \]  

(5.4)

It is clear from this that, for a given convergence angle and target current, the image radius is limited by the beam brightness. A brighter beam would be able to offer a reduced first order image size for a given current, or greater current for a fixed first order image size.

The Melbourne microprobe system is non-ideal and higher order terms contribute significantly to the final spot size (Ja 85). These terms, which include spherical, chromatic and parasitic aberrations, act to degrade the beam and reduce its brightness. Ion optical studies of the entire probe forming system have been, and continue to be, undertaken at Melbourne (Di 83)(Ja 87)(Mo 87)(Co 89a). The aim of these studies was to optimize the ion optical transport system in the accelerator so that the maximum source brightness would be made available to
the microprobe, and to maximize the demagnification of the microprobe beamline
and reduce higher order aberrations in the quadrupole lenses.

In theory every lens system is ultimately limited by chromatic or spherical
aberration, given that parasitic aberrations are removed by careful construction,
alignment and shielding. The role played by brightness in determining the final
microprobe image size ($r_i$) is best understood by considering two limiting cases.
Consider firstly a chromatic aberration limited beam size. This situation occurs
in STIM analysis (see Section 4.7) where the effective sizes of the object and
aperture diaphragms are made as small as possible. The optimum radius under
these conditions is given by

$$r_i^c = \frac{1}{\sqrt{\pi}} \left( C_c \frac{\Delta E}{E} \right)^{\frac{1}{4}} \left( \frac{I}{B_n E} \right)^{\frac{1}{8}},$$  \hspace{1cm} (5.5)

where $C_c$ : Chromatic aberration coefficient;
$\Delta E$ : Beam energy spread.

From equation (5.5) it can be seen that, for a given beam energy $E$, reducing $C_c$
and $\Delta E$ will have the greatest effect in improving chromatically limited spatial
resolution. As $r_i^c \propto B_n^{-\frac{1}{4}}$, an order of magnitude reduction in $r_i^c$ requires an
improvement in brightness of four orders of magnitude.

The second limiting case of microprobe resolution occurs when the beam
is dominated by spherical aberration. In practice the spatial resolution of the
microprobe beam is limited by spherical aberration when large divergence angles
are used for PIXE analysis. Under these conditions the optimum radius for the
beam spot is

$$r_i^s = \left( \frac{1}{\pi} \right)^{\frac{1}{8}} \left( \frac{16}{27} \right)^{\frac{1}{8}} C_s \left( \frac{I}{B_n E} \right)^{\frac{1}{8}},$$  \hspace{1cm} (5.6)

where $C_s$ : Spherical aberration coefficient.

Equation (5.6) shows that the reduction of $C_s$ has less of an effect upon $r_i^s$
than an increase in beam brightness, and that an improvement in source brightness,
if maintained into the microprobe beamline, will produce a reduction in the beam
spot proportional to $B_n^{-\frac{1}{4}}$. Therefore, to improve spatial resolution by an order of
magnitude an increase in brightness of three orders of magnitude is required.
From both equations (5.5) and (5.6) it can be seen that the beam current $I$ is directly proportional to the brightness under conditions of constant spot size. As was discussed in Section 4.8 improvements in the beam current available for analysis is of significant benefit in enhancing the versatility of the SPMP as a diagnostic instrument. An increase in the beam brightness promises a means for achieving this goal.

It can be seen from the above that along with minimizing lens aberrations and beam energy spread, it is important that higher brightness beams be sought. In response to this a study of the RF ion source was undertaken to establish the brightness available to the microprobe from the existing ion source. In order to conduct these studies a test bench facility was built.

### 5.3 Radio Frequency ion source test bench

During accelerator operation the ion source and its ancillary equipment is inaccessible positioned within the high voltage terminal of the accelerator. Hence, in the absence of a terminal telemetry system (Ho 77), accurate monitoring and adjustment of source parameters is impossible. In addition, modifications to the source would require the interruption of the accelerator applications program as well as the expense and down-time of an accelerator tank opening. In response to these difficulties an ion source test bench facility was constructed to allow an unhindered study of the ion source and its associated equipment. It was necessary on this test bench to duplicate the electrical, ion optical and vacuum conditions encountered within the accelerator terminal, whilst at the same time providing a means of monitoring source and beam parameters.

The existing ion source system uses a modified Ionex 320 RF ion source and this was replicated on the test bench. This commercial source is based on the design of the original Oak Ridge RF source by Moak, Reese and Good (Mo 51). It’s incorporation in the test bench is depicted in Figure 5.1. The source operates by ionizing the source gas ($H_2$ for $H_2^+$ and $H^+$, $^4He$ for $^4He^+$ and $^4He^{++}$) which is admitted via a fine control leak valve into a Pyrex bottle. An RF oscillator, operating at 80MHz with a maximum power output of 80W is inductively coupled
Figure 5.1
A schematic representation of the RF ion source test bench.

A: Insulating supports (Pactene)
B: High voltage deck
C: Bias voltage protection resistor
D: Bias voltage power supply
E: Focus lens power supply
F: Extractor lens power supply
G: Source magnet power supply
H: RF oscillator
I: Oscillator balance meter
J: Oscillator current meter
K: Panel meters for power supply
L: Probe power supply
M: Thermocouple gauge control unit
N: Variac control panel
O: Source gas bottle
P: High precision leak valve
Q: Thermocouple gauge head
R: Ion source probe
S: Source bottle (Pyrex)
T: Source magnet
U: Oscillator couplings
V: Faraday cup
W: Aperture
X: Scanning arm
Y: Cold cathode vacuum gauge
to the source bottle and excites a plasma. Free electrons within the plasma have a mean free path of $\sim 1\text{cm}$ and are accelerated by the RF field to produce ion pairs by collisions with the neutral gas molecules. An axial D.C. magnetic field is used both to shape the plasma and to dampen plasma oscillations. Extraction of the ions is accomplished by the application of a positive voltage of several kV (Probe voltage) between an anode at one end of the Pyrex bottle, and the source base. As the plasma is a good conductor it is essentially at the anode potential. Ions are extracted from the plasma via a 1mm diameter aluminium canal sheathed in a sapphire insulating sleeve. The end of the sleeve projects into the source. At the end of this sleeve is formed a concave plasma boundary which serves to focus the ions into the canal. In operation the source pressure within the Pyrex bottle is approximately $5 \times 10^{-3} \text{ mbar}$ and for $\text{H}_2$ gas a beam consisting of 85% protons is produced (Or 74). The general characteristics of RF ion sources have been studied extensively (Eu 54)(Co 62)(Ga 62)(Be 69)(Ca 69)(Ne 75). From this work it has been found that source characteristics depend greatly upon the design of the source and the various source parameters, and so direct extrapolation of source characteristics from well studied RF source designs is not possible.

Within the accelerator a preinjection bias voltage is applied to the ion source base and is used to accelerate the beam to an energy that enables it to be successfully matched to the entrance of the accelerating column. This bias voltage may be as high as +40kV and hence the test bench was constructed to isolate electrically the source, lens elements and power supplies. This isolation was achieved by suspending all components which were required to be maintained at the bias voltage, on an insulated platform. Source parameters were then controlled via Perspex insulating rods (see Figures 5.3A and B).

Immediately following the ion source is the first element of the four element electrostatic lens. This first element is shaped to follow closely the exit canal of the ion source, and then expands in diameter to 3.5cm, the internal diameter of the three other identical cylindrical lens elements. The first and third lens elements are maintained at an identical voltage ($0 \rightarrow -16\text{kV}$ relative to the bias voltage) by the Extractor power supply (see Figure 5.1) and the second element is controlled by a Focus power supply (also $0 \rightarrow -16\text{kV}$ relative to the bias voltage). The final
Figure 5.2

Block diagram of electrical circuitry for the ion source, electrostatic lens and bias power supply as configured on the test bench.

V: Variac autotransformer

F1: Fuse

R1: 120 MΩ

R2: 120 MΩ

R3: 35 MΩ

The power supplies and layout shown here, with the exception of the diagnostic instrumentation, are identical to that used in the Pelletron accelerator. The voltage supplies are not stabilized against load. This is not important for the lens element supplies which need only electrostatic. The probe and bias supplies must be capable of supplying beam current loads. These supplies are rated considerably in excess of any expected current loading; however at currents within 30% of the maximum load the output voltages of these supplies were found to vary by up to 5%, in response to current loading.

The bias voltage was measured by a calibrated electrostatic voltmeter which had an accuracy of 3%. Other voltages were measured with calibrated voltage-divider circuitry permitting the probe, extractor and focus voltages to be measured to within ±2.5%. The 240V line was stabilized and all circuitry was brought up to normal operating temperature before measurements were taken.
Figure 5.3

A:
Photograph of the RF ion source test bench in operation. The wire mesh screen was used to attenuate RF interference to the measurement equipment.

B:
Photograph of the high voltage deck and source control rods.
lens element is maintained at ground potential relative to the bias voltage. The voltages applied to all the electrostatic lens elements were measured as shown in Figure 5.2.

On the test bench the ion source/lens combination was attached to a stainless steel beam monitoring chamber, which was evacuated by both turbo molecular and ion pumps (see Figure 5.1). A base vacuum of less than $1 \times 10^{-7}$ mbar was attainable in this chamber. All ion source parameters were monitored by calibrated gauges, as shown in Figures 5.1, 5.2 and 5.3. The voltage supplies and ion source equipment duplicated the equivalent components used in the Pelletron and considerable care was taken to ensure that stray RF fields did not interfere with the measurement equipment. This involved the shielding of all cables and the use of RF filters.

Effective long-term use of the microprobe necessitates a stable beam from the ion source. For the RF ion source beam stability is directly related to the plasma stability, hence plasma conditions were monitored by the measurement of all the source variables: oscillator-plate current and balance, probe voltage and current, magnet current and gas pressure. The gas pressure was monitored indirectly by two gauges, a thermocouple gauge in the gas supply line and a cold cathode gauge in the beam monitoring chamber. Gas pressure and throughput were controlled by a high precision leak valve. The gas flow was calibrated as a function of valve position and gas bottle pressure (see Figure 5.4A). The source magnet position was made variable along the central axis of the source and the axial magnetic field was measured as a function of magnet current (see Figure 5.4B). The oscillator was inductively coupled to the plasma via two metallic slip rings which fitted around the source bottle. Both the separation of the rings and their position along the length of the bottle could be varied (see Section 5.4). The oscillator power output was not externally controllable but was dependent on plasma loading. In order to monitor the power output from the oscillator the plate current was measured and the oscillator power output was calibrated as a function of plate current. The calibration graph is shown in Figure 5.4C. The oscillator power available to this source was the maximum tolerable for the given source dimensions and materials. Higher levels of RF power risked damage to the
Figure 5.4

A:
Calibration graph for the mechanical gas metering and shut-off valve. The source gas was ultra-high purity H₂ and was fed to the source from a 500cm³ gas bottle pressurized to 40 bar.

B:
Magnet field as a function of current for the axial ion source magnet. The magnetic is used to intensify the discharge in the region of the extraction canal. The axial magnetic field results in electron trajectories that avoid the glass walls so that each electron is used more efficiently for ionization.

C:
RF power as a function of the oscillator plate current. The oscillator frequency was set at 80MHz and the power output was not externally controllable. During source operation the oscillator balance and plate currents were monitored.
source bottle (Or 74).

The aperture at the rear of the Wien filter was duplicated in the beam monitoring chamber, as it serves as a defining object for the accelerator optics. Two types of aperture were used. One aperture was located in a quartz block which was coated with an evaporated copper layer to prevent charge accumulation which could steer the beam. The beam was readily focused onto this aperture as scintillation was visible through the viewing port in the beam monitoring chamber. Another aperture, of identical dimensions and constructed of aluminium, was placed in position during quantitative measurements. Following the aperture was a linear scanning arm, used to measure the beam profile and divergence angle. This device consisted of a fine stainless steel wire 450µm in diameter which was stepped across the beam path 6cm below the aperture. This method of beam profile measurement has been commonly used in emittance and brightness studies (Ch 67)(La 79)(Ha 80)(Br 80). For this work the beam current incident upon the wire was recorded as a function of wire position. The resultant angular distribution was a convolution of the beam profile and that of the wire as it intercepted the beam. An example of the beam profile, as measured in the plane of the scanning arm (see Figure 5.1) is shown in Figure 5.8A. The total beam current transmitted through the aperture was recorded by a Faraday Cup in the base of the beam monitoring chamber. A negatively biased ring preceding the entrance to the Cup was used to retain secondary electrons. The total brightness of the beam was then able to be calculated from a knowledge of the diameter of the aperture, the divergence angle of the beam and the total beam current.

5.4 Performance of the RF ion source

Implementation of the RF ion source on the test bench revealed the interdependence of source and lens parameters. Due to the absence of diagnostic instrumentation within the accelerator the extent of these interdependencies were hitherto unknown and severely hindered attempts to quantitatively investigate source performance as a function of the source and lens parameters.

The output of an RF ion source is directly related to the plasma condi-
tions. There are four variables which affect the character of the discharge and the resultant ion beam: gas pressure, RF field (and its magnitude and coupling to the plasma), axial magnetic field, and probe voltage. These source parameters are not at all independent. For instance, both the gas pressure and the magnetic field will affect the RF fields by influencing the electrical properties of the plasma which in turn affect the RF coupling. Also, if the probe voltage is not stabilized, variation in the resistive load of the plasma, which may be caused by a change in any of the plasma parameters, will affect the probe voltage. There are other similar interdependencies, such as the self-pumping phenomenon of RF ion sources (Or 74) which can change the state of the plasma and have a major effect upon the quality and magnitude of the extracted beam. In addition, the lens voltages and the bias voltage were found to be interdependent, the former being indirectly affected by the latter. The bias voltage power supply was not stabilized and hence the bias voltage changed in response to the load, which was the beam current. This further complicated the interrelationship between parameters as the beam current changed in response to the plasma parameters. As a consequence of these complex relationships it was not possible to quantitatively examine the source characteristics as a function of only one of the control variables. However, it was possible to note general patterns in the source and extracted beam characteristics and from these to identify the source conditions best suited to the ion optical requirements of the microprobe.

Initial work was directed at ascertaining the source conditions that offered the maximum beam stability. Beam instability occurs when the plasma changes its density distribution about the entrance to the extraction canal. Consequently the stability of the beam was controlled by the parameters that influenced the plasma, specifically: probe voltage, magnetic field, gas pressure and oscillator power. The beam stability was monitored in two ways. The beam was focused onto the aperture in the quartz block; the beam position, and hence spatial stability, could then be observed, and simultaneously, the transmitted current measured as a function of time, indicating the beam's current stability. It was found that the beam stability depended greatly upon the coupling of the oscillator to the plasma. The position of this coupling was variable and maximum stability was attained
with the inductive clips placed near the base of the ion source bottle (Al 80). With the clips in this position the plasma was concentrated around the entrance to the extraction canal thereby maximizing $\rho_i$, the ion density, in this region. The clips were placed close together to minimize the steering of the extracted beam when the oscillator load altered due to plasma perturbations resulting from a change in probe voltage, gas pressure or magnetic field.

For a given combination of plasma control parameters there existed an optimum gas pressure which maximized the beam current and provided the most stable current output. This pressure varied between 0.04 and 0.2 mbar (as measured by the thermocouple gauge). Figure 5.5A shows a typical response of beam current to gas pressure. It can be seen from this curve that the oscillator power output changes with the gas load and that the maximum beam current was obtained at the maximum oscillator power. This effect has also been observed with other RF ion sources (Ga 62); however, solely increasing the oscillator power does not necessarily increase the beam current as excessive power can cause the plasma sheath to bulge into the extraction region. This acts to restrict the fraction of emitted ions able to pass through the canal (Ne 75). Gas pressures higher than 0.2 mbar gave greater probe currents but reduced the beam current due to defocusing in the canal and an increased rate of ion recombination in the cathode dark space.

The use of the magnet intensified the discharge about the canal region and this was reflected in greater probe currents. Figure 5.5B shows the probe current as a function of magnetic field for several probe voltages. When the magnetic field was increased the plasma load on the oscillator also increased. This was not found to occur when the magnetic field was held constant and the probe voltage changed (as shown in Figure 5.6A) despite the increase in probe current obtained for increasing probe voltages.

It was found that probe voltages in excess of 2.5kV induced uncontrolled oscillation in the plasma density distribution. These oscillations were unable to be predictably dampened by the axial magnetic field or adjustments to the gas pressure, and resulted in beam steering and current fluctuations. Despite offering the potential of greater current outputs, source operation at probe voltages greater
Figure 5.5

A:
Variation of beam current as a function of gas pressure as measured by the thermocouple gauge. This curve was obtained with no magnetic field and a probe voltage of 0.5kV. Indicated beside the curve is the oscillator power (in Watts). The plasma appeared visibly unstable at pressures less than $3.8 \times 10^{-2}$ mbar and greater than 0.9 mbar.

B:
Probe current as a function of magnetic field for three different probe voltages. The oscillator power is indicated alongside each curve and the source pressure was $6.8 \times 10^{-2}$ mbar. The magnetic field intensified the discharge but, because of defocusing of the beam in the extraction canal, this didn't necessarily correspond to an increase in beam current.
Figure 5.6

A:

Probe current as a function of probe voltage for three magnetic field values. For B=0 the gas pressure was $6.8 \times 10^{-2}$ mbar and the oscillator power was 46W. Predictable plasma conditions were obtained for these values with the probe current having an almost linear response to probe voltage. For B=60 Gauss the gas pressure was 0.075mbar and the oscillator power was 52W. The plasma conditions were less predictable with a probe voltage of 2kV causing the plasma to change state significantly. This is reflected in a rapid rise in the probe current at 2kV. For B=240 Gauss the gas pressure was 0.09mbar and the oscillator power was 60W. For this high value of magnetic field the plasma was obviously unstable and oscillated between modes of excitation at probe voltages greater than 2kV.

B:

Beam current as a function of probe voltage for the plasma conditions given in Figure 5.6A. For the low magnetic field values (B=0, 60 Gauss) the beam current shows a saturation with probe voltage, as is typically found with RF ion sources (Ga 62). The beam current output for the high magnetic field value (B=240 Gauss) shows the effect of high magnetic fields upon beam extraction. For this high magnetic field value the shape of the plasma sheath is highly sensitive to the probe voltage and at probe voltages greater than 1kV the beam is defocused in the extraction canal.
than 2.5kV was considered too unstable to be a viable regime for the microprobe ion source.

The greater plasma excitation and oscillator power obtained at high magnetic fields did not produce correspondingly higher beam currents, as the high magnetic fields ($B > 120$ Gauss) and high oscillator output combined to change the shape of the plasma sheath in such a way as to defocus the beam in the extraction canal. This reduced the transmission of ions from the plasma to the lens system. Figure 5.6B shows the total beam current as a function of probe voltage for the three values of the magnetic field. It can be seen from this graph that the magnetic field can grossly affect the beam current. The use of the magnet increased $\rho_i$ and permitted higher beam currents to be extracted, but the beam current was unpredictable in its response to probe voltage. The most stable conditions were obtained for zero magnetic field. However, this limited the beam current which could be extracted from the source.

In order to determine whether the extracted beam was space-charge limited, a measurement was made of the maximized beam current as a function of probe voltage. The scope of this measurement was restricted by the fact that the oscillator power was not a free, adjustable parameter. It would have been preferable to optimize all four source parameters, including oscillator power. However, as the magnetic field was strongly coupled to the oscillator power both of these parameters were excluded from the optimization. Figure 5.7 shows the plots of $(I_{\text{max}})^{\frac{3}{2}}$ (maximum extracted current) versus probe voltage for two different oscillator coupling positions. Line 2 in Figure 5.7 was obtained for the source with the oscillator coupling set in the manner recommended by the manufacturer, the source performance was improved by modifying the positioning of the oscillator coupling (Al 80) and line 2 was recorded for the source operating in the modified configuration. Both of these results show that the beam is space-charge limited for probe voltages greater than 1.5kV. Under 1.5kV the beam was not space-charge limited. This effect has been observed previously (Ga 62) and presumably is due to a poorly defined plasma boundary in this voltage range under conditions of zero magnetic field.

Due to the many interrelated source and lens parameters it was not possible
Figure 5.7

Two plots shown to reflect the trend of maximized beam current as a function of probe voltage. The magnetic field was zero for both data sets. The $(I_{\text{max}})^{\frac{3}{2}}$ is shown multiplied by $10^4$ and is derived from the current value in $\mu$A. The Child-Langmuir equation (equation 5.11) predicts that for a space charge limited beam $I \propto V_{\text{probe}}^{\frac{3}{2}}$. Both data sets 1 and 2 indicate that for probe settings greater than 1.5kV the beam is space charge limited. Significant deviations from linearity (diminished current yield) were recorded for probe voltages less than 1.5kV suggesting that, under conditions of zero magnetic field, these lower probe voltages failed to shape the plasma sheath for optimum beam extraction. Set 2 was obtained with the RF induction clips positioned as instructed by the manufacturer. The clips were relocated to maximize the plasma density about the extraction canal and minimize plasma instabilities (Al 80). Set 1 was recorded with the clips in the optimized position. The oscillator power output for set 1 was 46W. For set 2 it was 44W. The values alongside the data points in set 2 are the optimum gas pressures $\times 10^{-2}$mbar. For set 1 the maximum current was obtained at $6.8 \times 10^{-2}$mbar for each data point.
to measure brightness as a function of each of the individual parameters. Instead the source was operated in the same manner in which it was used in the accelerator. That is, the optimal beam current was obtained by judicious adjustment of the source and lens parameters: probe voltage, magnet, gas pressure, bias voltage, extractor voltage and focus voltage. Due to the ionization of residual gas the beam path was visible in the monitoring chamber. Hence, by observing the beam it was possible to reduce the beam divergence, whilst maintaining the beam current at its maximum value. The reduction in beam divergence was brought about by optimizing the three interrelated voltages: bias voltage, extractor voltage and focus voltage. The optimized beam was then scanned by the linear scanning arm. Figure 5.8A shows a typical beam profile recorded 6cm downstream from the aperture. The profile is roughly Gaussian in shape, but the distribution shown is considerably more peaked than the actual beam density distribution due to the greater beam cross section sampled by the wire when it was on the beam axis. The distributions were used to define the beam's outer limits so that the beam brightness, at 100% beam intensity, could be calculated. This level of intensity, and all subsequent beam intensity levels, refer to the beam intensity after the Wien filter aperture.

A range of source parameter settings were investigated in order to achieve the brightest possible beam from the source. A maximum brightness of \( B_n = 6.7 \pm 2.0 \) A m\(^{-2}\) rad\(^{-2}\) V\(^{-1}\) was obtained for the source settings shown in the caption for Figure 5.8B, at a gas pressure of \( 7.5 \times 10^{-2} \) mbar. The gas pressure was varied about this value and the response of beam brightness recorded. The variation of brightness as a function of gas pressure, for these optimum settings, is shown in Figure 5.8B. The average value for the optimized beam brightness was \( B_n = 4.9 \pm 1.5 \) A m\(^{-2}\) rad\(^{-2}\) V\(^{-1}\) and this value was taken to be representative of the beam brightness at 100% intensity available to the accelerator from the ion source-lens combination.

The inner core of the beam from the RF ion source can be expected to be brighter than the brightness obtained at 100% intensity (Re 88). For a beam current of 1.5nA, corresponding to approximately 0.006% beam intensity, a beam brightness of 51 A m\(^{-2}\) rad\(^{-2}\) V\(^{-1}\) has been measured in the microprobe line. This was achieved under conditions of optimal source brightness, and is two orders of
Figure 5.8

A:
Beam profile as measured by the linear scanning arm for the following source conditions. The wire was moved in 250μm increments and the current values given were recorded from the beam incident on the wire. The beam brightness measured for the source under these conditions was $B_n = 4.4 \pm 1.3 \text{ A m}^{-2} \text{ rad}^{-2} \text{ V}^{-1}$.

<table>
<thead>
<tr>
<th>Probe Voltage (kV)</th>
<th>Probe Current (mA)</th>
<th>Magnetic Field (Gauss)</th>
<th>Gas Pressure (mbar)</th>
<th>Osc. Power (W)</th>
<th>Bias Voltage (kV)</th>
<th>Ext. Voltage (kV)</th>
<th>Focus Voltage (kV)</th>
<th>Beam Current (μA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4</td>
<td>2.4</td>
<td>.100</td>
<td>0.075</td>
<td>46</td>
<td>18.0</td>
<td>15.5</td>
<td>15.5</td>
<td>28</td>
</tr>
</tbody>
</table>

B:
Beam brightness as a function of gas pressure for the RF ion source. The brightness values were obtained for the following ion source and lens parameter settings.

<table>
<thead>
<tr>
<th>Probe Voltage (kV)</th>
<th>Probe Current (mA)</th>
<th>Magnetic Field (Gauss)</th>
<th>Osc. Power (W)</th>
<th>Bias Voltage (kV)</th>
<th>Ext. Voltage (kV)</th>
<th>Focus Voltage (kV)</th>
<th>Beam Current (μA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4</td>
<td>2.4</td>
<td>120</td>
<td>52</td>
<td>9.75</td>
<td>7.0</td>
<td>14.0</td>
<td>8.5</td>
</tr>
</tbody>
</table>
magnitude higher than the usual level of brightness of the primary beam.

An understanding of limitations inherent in the design of the RF ion source, and in fact any other plasma-type source, can be gained by considering the theory of beam extraction from a plasma. The source current density $J_s$ may be defined in terms of the source current $I_s$ and area of emission $A_s$,

$$J_s = \frac{I_s}{A_s}.$$  \hspace{1cm} (5.7)

Equation (5.3) can then be rewritten for a beam accelerated axially by a voltage $V_a$:

$$B_n = \frac{J_s}{\pi \alpha^2 V_a}.$$ \hspace{1cm} (5.8)

For small $\alpha$ this can be written

$$B_n = \frac{J_s}{\pi V_a} \left( \frac{V_a}{V_r} \right) = \frac{J_s}{\pi V_r},$$ \hspace{1cm} (5.9)

where $V_r$ is the voltage corresponding to the radial emission energy $eV_r$. For a plasma-based source the source current density is given by the ion diffusion current density from a region of zero field in the plasma into the accelerating field. The current density, derived from Langmuir's theory of probe extraction, is given by

$$J_s = \rho_i e \left( \frac{kT_i}{2\pi m_i} \right)^{\frac{1}{2}}$$ \hspace{1cm} (5.10)

where $\rho_i$ : Ion density;

$m_i$ : Ion mass;

$T_i$ : Ion temperature.

In the extraction region of the RF ion source, between the sapphire sleeve and the canal, the current density is limited by space-charge and hence may be expressed by the Child-Langmuir equation:

$$J_s = \left( \frac{4}{9} \right) e \left( \frac{2e}{m_i} \right)^{\frac{1}{2}} \frac{V_a^{\frac{3}{2}}}{d^2}$$ \hspace{1cm} (5.11)

where $V_a$ : Is the potential between the plasma and the canal;

$d$ : Acceleration distance for the ions extracted from the plasma. For the RF ion source $d = 5$ mm.
The theoretical brightness is then

\[ B_n = \left( \frac{4}{9\pi} \right) \varepsilon_0 \left( \frac{2e}{m_i} \right)^{\frac{1}{2}} \frac{V_a^{\frac{3}{2}}}{d^2V_r}. \]  

(5.12)

The radial emission energy may be estimated from the Maxwellian distribution of ion energies, \( \varepsilon V_r = \frac{3}{2} kT_i \), where \( T_i \) is the plasma temperature. For the RF ion source, under stable conditions, at a probe voltage of 2.5kV and an ion temperature of 1500K, the brightness of an extracted proton beam is estimated to be \( B_n \sim 450 \text{ A m}^{-2}\text{rad}^{-2}\text{V}^{-1} \).

This value is an order of magnitude higher than the maximum measured in the microprobe beamline for its RF ion source. This difference occurs because, in practice, beam degradation results from ion losses and charge exchange events in the extraction region. Also, the experimental value for the brightness, being measured after the lens and accelerator system, will be subject to aberrations (Co 82). The derivation of the theoretical value for the beam brightness from the plasma source highlights limitations in achieving a higher brightness. With this source there is little scope for increasing \( V_a \) as plasma instabilities arise for probe voltages greater than 2.5kV. Also decreasing \( d \) introduces the risk of electrical breakdown across the sapphire sleeve and \( V_r \) remains limited by the discharge temperature.

In view of the inherent limitations in beam stability, reproducibility and brightness encountered with the RF ion source, an investigation of alternative ion sources was undertaken.

### 5.5 Alternative proton sources

In considering alternative ion sources, more suited to a SPMP than the RF ion source, it is of interest to review the evolution of the Scanning Transmission Electron Microscope (STEM). A significant improvement in the resolution of the STEM was brought about by the advent of the Field Emission (FE) electron source. The high brightness offered by this source permitted the STEM to approach its theoretical limits of resolution, achieving probe sizes of a few angströms with currents of the order of a nanoampere (Cr 71). The FE electron gun was first introduced by
Crewe et al. (Cr 68) and incorporated special electrodes designed by Butler (Bu 66). The electron source was derived from the FE microscope and offered a source brightness as high as $B = 10^8$ A m$^{-2}$ rad$^{-2}$ V$^{-1}$ (Cr 68)(Re 85). This level of brightness is four orders of magnitude greater than that available from thermionic electron sources and results from a small effective source size of $\sim 10$nm, compared with the effective source size for thermionic emitters of $\sim 50\mu$m. The FE source is extremely sensitive to vacuum conditions and suffers from short-term current fluctuations of the order of 2 to 5%. This, along with its limited total current output, restricts the useful range of probe sizes over which the FE source is superior to the thermionic source. In general the FE source gives a much greater depth of focus and is used for probe sizes less than 300Å. The FE source is of less use for scanning electron microscopy where electron scattering in solid specimens limits the resolution to $\sim 10$nm and thermionic sources are able to satisfy brightness requirements.

The optical requirements of the STIM were seen as being analogous to those of the STEM by Levi-Setti who proposed and built an ion probe with a field ionization (FI) ion source (Le 74). This source is based on the FI microscope and uses a single point emitter which acts as a small effective source ($\sim 1$nm) to produce a low current ($\sim 1$nA), high brightness beam from the ionization of gas atoms in a high electric field. This source is suited to a STIM where high resolution is sought with only small target currents ($< 1$pA). However, the SPMP requires target currents of at least 100pA and due to this requirement initial consideration was given to conventional commercial ion sources as potential replacements for the RF source.

A number of criteria must be satisfied by any potential replacement ion source: 1) The source must be able to produce a proton beam and, preferably, an alpha particle beam as well. These light ions are necessary for effective PIXE and RBS analysis at MeV energies. 2) The source must be able to operate within the accelerator terminal (see Figure 2.1). This imposes a number of restrictions. The space available in this region is limited, the power available is restricted to 1000W and the source must be able to operate without frequent attention. 3) The gas load of the source must be able to be pumped by the existing accelerator.
vacuum pumping system. 4) Source beam energy spread must be low (< 70eV). 5) Sufficient current must be produced to operate the accelerator voltage stabilization system. This necessitates an ion current of at least 1nA. 6) Sufficient gains in beam brightness and stability must be offered by an alternative to make development work worthwhile.

These criteria significantly restrict the number of sources that are viable replacements for the RF ion source. Table 5.1 shows a listing of ion source types that satisfy points 1) – 4) above, along with their performance characteristics most relevant to their choice as a replacement ion source. Several sources have been excluded from this table as they are not relevant to the present study, yet may ultimately be used in accelerators attached to microprobes. The first of these, the Capillaritron source (Ma 81), is a constrained plasma-type source, which uses a discharge maintained at the pointed end of a capillary, through which the source gas flows. Due to its small source size of 25µm and its low beam divergence of 0.035 – 0.140rad, this source can offer a beam brightness of up to 100 A m⁻² rad⁻² V⁻¹. Unfortunately the Capillaritron suffers from an exceptionally wide beam energy spread and produces negligible amounts of H⁺ (Go 82). Also excluded from the table is a particularly bright single point heavy ion source, the liquid metal ion source (LMIS). This source has been demonstrated to have a brightness of $B_n = 1 \times 10^5$ A m⁻² rad⁻² V⁻¹ (Kr 75), a current output of up to 10µA and source lifetimes which depend on the metal used but can be as long as 1000h. A wide range of heavy metallic ions can be produced by this source (Cl 78), but these ions are of little use for PIXE, RBS, or NRA analysis techniques. Lithium has also been used in a LMIS (Cl 78a) and development work has begun on adapting a LMIS to an accelerator with the ultimate aim of using a Li⁺ beam for SPMP analysis (Re 87)(Re 88a). This light ion is the best option for the application of a LMIS in a high energy SPMP as lithium can be effectively used for RBS, NRA and some applications of PIXE analysis. It is estimated that the source radius of the LMIS may be as low as 25nm (Kr 75). A source of this size is much less than the aberrations present in conventional lens systems and recent attempts at adapting a LMIS for accelerator use have found that the intrinsic source brightness may be readily lost (Re 88).
Table 5.1

Several ion source types and their performance parameters most relevant to microprobe beam formation are shown. The current values given refer to the H+ beam component unless stated otherwise. The sources listed are gaseous sources capable of proton production and with operating pressures and gas loads compatible with the Pelletron accelerator. Sources with high power requirements (>1000W), unnecessarily high current output and large beam energy spreads (>70eV) were not considered for inclusion.

Accurate comparison between the sources is difficult as source performance data is frequently obtained by different techniques. Brightness values are often quoted at unspecified beam intensities; the values given assume greater than 90% beam intensity and may only be considered to be a general guide to the performance levels attained by each source type.

Notes to Table 5.1
1: Manufacturers specification.
2: The ion source presently used in the Pelletron.
3: These data are derived from general results in the literature. The beam currents assume 85% production efficiency for H+. The brightness values were calculated assuming an angular spread of 0.12rad half angle and a beam energy of 5keV.
4: Duoplasmatron design most readily adapted to the Pelletron, can be used with the existing ion source lens.
5: These data are derived from general results in the literature. The beam currents assume a 60% production efficiency for H+. The brightness values were calculated assuming an angular spread of 0.14rad half angle and a beam energy of 30keV.
6: The brightness and current values given here are for He+ ions.
7: These data are derived from general results in the literature for low current PIG sources. A 75% production efficiency for H+ is assumed. The brightness values were calculated assuming an angular spread of 0.14rad half angle and a beam energy of 10keV.
8: The energy spread value is for electric fields less than $2 \times 10^{10}$ Vm$^{-1}$. 
<table>
<thead>
<tr>
<th>Ion source type</th>
<th>Max Current ( \times 10^{-3} ) A</th>
<th>Brightness ( B_n ) ( \text{A m}^{-2} \text{rad}^{-2} \text{V}^{-1} )</th>
<th>Energy Spread (eV)</th>
<th>Ref.</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF Ionex 320</td>
<td>0.85</td>
<td>8</td>
<td>( \sim 65 )</td>
<td>Or 74</td>
<td>1</td>
</tr>
<tr>
<td>RF Ionex 320 modified</td>
<td>0.5 ( (\text{H}^+, \text{H}_2^+ )</td>
<td>5</td>
<td></td>
<td>Al 80</td>
<td>2</td>
</tr>
<tr>
<td>RF(^a)</td>
<td>2</td>
<td></td>
<td></td>
<td>Re 88</td>
<td></td>
</tr>
<tr>
<td>RF(^b)</td>
<td>0.8</td>
<td></td>
<td></td>
<td>La 79</td>
<td></td>
</tr>
<tr>
<td>RF(^c)</td>
<td>0.1→8.5</td>
<td>0.13→13</td>
<td>30→500</td>
<td>Wi 73</td>
<td>3</td>
</tr>
<tr>
<td>Duoplasmatron Ionex 350B</td>
<td>6</td>
<td>2</td>
<td>15</td>
<td>Or 74</td>
<td>4</td>
</tr>
<tr>
<td>Duoplasmatron(^a)</td>
<td>0.6→60</td>
<td>0.2→17</td>
<td>10</td>
<td>Wi 73</td>
<td>5</td>
</tr>
<tr>
<td>PIG Axial Extraction</td>
<td>0.2 ( (\text{He}^+ )</td>
<td>1.3</td>
<td></td>
<td>Bi 75</td>
<td>6</td>
</tr>
<tr>
<td>PIG(^a)</td>
<td>1 ( (\text{He}^+ )</td>
<td>0.13</td>
<td></td>
<td>Ba 76</td>
<td>6</td>
</tr>
<tr>
<td>PIG(^b)</td>
<td>0.01→0.8</td>
<td>0.5→5×10^{-3}</td>
<td>50</td>
<td>Wi 73</td>
<td>7</td>
</tr>
<tr>
<td>Field Ionization</td>
<td>( 10^{-5} \rightarrow 10^{-4} ) ( (\text{H}^+, \text{H}_2^+ )</td>
<td>( \sim 10^8 )</td>
<td></td>
<td>Or 77</td>
<td>8</td>
</tr>
</tbody>
</table>
Of the sources that are shown in Table 5.1, the RF, Duoplasmatron and PIG sources are all plasma based. These sources are designed for use in an accelerator and are readily able to supply sufficient current for stable operation of the Pelletron. An expression for the brightness of these plasma sources can be found by combining equations (5.9) and (5.10);

\[ B_n = \frac{\rho_i e (kT_i)^{1/2}}{\pi V_r (2\pi m_i)^{1/2}}. \]  

(5.13)

If it is assumed that \( eV_r \), the radial emission energy of the thermally emitted ions, takes its most probable value, then

\[ B_n = \frac{\rho_i e^2}{\pi (2\pi m_i kT_i)^{1/2}}. \]  

(5.14)

From equation (5.14) it can be seen that in order to obtain a beam of high brightness from a plasma source, a low temperature plasma of high ion density is required. The brightness of plasma sources has been limited by attainable ion densities and temperatures (Wo 77) and the fundamental limitation of the Child-Langmuir equation. As a result the brightness values for the plasma sources in Table 5.1 are similar and are of the order of 10 A m\(^{-2}\) rad\(^{-2}\) V\(^{-1}\). The energy spread inherent in the beams extracted from plasma sources results from a number of factors: variations in the potential where the ions are created, thermal energy spread (\( \Delta E \sim 0.4eV \)), charge exchange in the extraction region during initial ion acceleration, and, for sources forming a plasma sheath, the ions having an energy spread representative of the sheath potential (10 – 20eV) (Wi 73). The combination of these effects results in the plasma-type sources having a lower limit to their energy spread of 10eV.

The brightness limitation inherent in the plasma sources is overcome by the FI source shown at the bottom of Table 5.1. This source achieves exceptional brightness from its small virtual source size in the same manner as the FE electron source, and due to the greater mass of the ions, the virtual source size is even smaller for FI. Also, space-charge limitations are not encountered with this source due to the exceedingly high electric field at the emitter. Dyke and Dolan (Dy 56) have shown that space-charge effects occur in FE at \( J_e \sim 10^{12} \) A m\(^{-2}\). For protons this limit will be lower by the ratio of the square root of the proton and electron
mass, hence for protons formed above a FI emitter the space charge limit becomes \( J_p \sim 10^{10} \text{ A m}^{-2} \). This current density is never attained in practice even above localized emission sites where \( J_{p \text{max}} \sim 10^7 \text{ A m}^{-2} \). The energy spread inherent in a FI beam is a function of the electric field and gas pressure. A careful choice of ionization parameters can ensure that the beam energy spread is less than that attainable with a plasma source. However, several deficiencies inhibit the use of the FI source with a SPMP. The source has a small current output which is only just sufficient to stabilize the Pelletron accelerator, the beam is a mixture of H\(^+\) and H\(_2^+\) and, unlike the other sources listed in Table 5.1, the FI source has not been employed in a high-voltage accelerator and is unavailable commercially.

From a consideration of equations (5.5) and (5.6) it is clear that if an order of magnitude improvement in SPMP resolution is to achieved by an increase in beam brightness alone, then an improvement in source brightness of at least three orders of magnitude will be necessary. This fact favours the development of a FI source as it is the only source with sufficient intrinsic brightness to make the necessary development work worthwhile.

Consequentially, consideration was given to the process of FI, the ultimate aim being the development of a FI source suitable for use in the Pelletron accelerator.

5.6 Field Ionization

The process of FI was first demonstrated experimentally by Müller (Mü 51) and was a logical extension of his pioneering work in FE microscopy. Müller's initial work in FI microscopy involved the imaging of the crystallographic structure of metallic field emitters by the introduction of an imaging gas (H\(_2\) or He) into the high field region surrounding the positively biased emitter. Gas atoms and molecules attracted to the tip by polarization forces may then be ionized by the quantum mechanical tunneling of electrons from the atom or molecule into the tip. Such tunneling is energetically possible as the electric field modifies the electron's potential energy well. The resultant positive ion is then accelerated away by the high electric field. FI is a complex phenomenon and it has been necessary to
introduce approximations in explaining the processes involved in the production of a FI beam (Go 61) (Mü 69) (Hr 68) (Va 70) (Iw 75).

FI of an atom or molecule may occur in electric fields greater than $1 \times 10^{10}$ Vm$^{-1}$. These fields can be produced by an emitter of submicron radius biased to several kilovolts. For an atom in the vicinity of the tip there exists a critical distance $x_c$, which is the distance of closest approach for ionization. This distance is given to good a approximation by

$$x_c = \frac{(V_i - \phi)}{F}, \quad (5.15)$$

where $V_i$: Ionization potential of the atom;

$\phi$: Work function of the emitter metal;

$F$: Electric field.

The probability of ionization is greatest just before $x_c$ and ionization cannot occur at distances less than $x_c$. For a hydrogen atom on tungsten or iridium $x_c \approx 0.4$ nm at $F = 2.3 \times 10^{10}$ Vm$^{-1}$.

The field at the surface of the emitter may be well approximated by

$$F_o = \frac{V_o}{k r_t}, \quad (5.16)$$

where $V_o$: The applied voltage;

$r_t$: Tip radius;

$k$: A constant which depends on the geometry of the tip and cathode as well as the shape of the emitter endform. For slender etched tips $k$ is usually in the range 5 - 8 (Sa 77).

A surface field of $2.3 \times 10^{10}$ Vm$^{-1}$ can then be achieved for a 0.1μm radius emitter with an applied voltage of 11.5kV.

The ion current from the ionization zone around the tip depends on the supply of gas molecules to the ionization region and the ionization probability. Both of these ionization variables are complex functions of the tip and gas temperatures, the electric field, as well as the polarization energy of the molecules (Mü 69). Approximations to the FI current are best understood by considering two limiting cases evident in the FI current-voltage characteristic. Figure 5.9 shows
Figure 5.9
A pair of FI characteristic curves, derived from data presented by Levi-Setti (Le 80). Both curves are for the total beam current measured from the FI of H₂. The data have been transposed to tip radii of 0.1µm and a gas pressure of 1 × 10⁻³ mbar. The dotted curve is for FI at 294K, the dashed curve is for FI at 78K. Two distinctive gradient regimes are present in the characteristics. Regime 1 is the low field domain, where the ionization probability for a gas molecule entering the tip region is small. The ion current increases very sharply in this regime, typically as $F^n$, with $n$ between 20 and 40. This strong dependence results from the rapidly increasing ionization probability as a function of $F$. A relatively slow increase in ion current with $F$ is seen in Regime 2. In this regime the ionization probability approaches one. The current increase in Regime 2 results from the supply of gas molecules to the ionization zone increasing with $F$.

The inflection point in the FI characteristics is defined as the intersection point of the two gradient lines in each regime. This is shown for the characteristic at 294K. The inflection point occurs at a set value of $F_0$ for a given gas, gas temperature and emitter material. For the FI of H₂ on Ir at room temperature the inflection point was taken to be at $F_0 = 2.3 \times 10^{10}$ V m⁻¹. Knowledge of the field at the inflection point and the emitter-cathode geometry permits the emitter voltage to be calibrated against $F_0$. 
APPLIED VOLTAGE (kV.)

BEAM CURRENT (pA.)

78K

294K

1

2
two typical FI current-voltage characteristics for H₂. In each characteristic two gradient regimes are apparent on either side of a point of inflection. At any field value the FI current is controlled by the gas supply function \( Z \) and the ionization probability \( p_i \). For low fields \( F < 2.1 \times 10^{10} \text{ Vm}^{-1} \) for H₂, \( p_i \) is a complex function of \( F \), gas temperature, tip material and shape (Mü 69). The current in this regime is limited by the gas supply function and the mean lifetime for ionization of molecules near the critical distance. These are both strong functions of the electric field and as a result the ion current increases rapidly with electric field in this regime.

The high field regime yields the largest FI current and so, as the present application requires the highest possible current, is of greatest interest. For high fields \( F > 2.5 \times 10^{10} \text{ Vm}^{-1} \) \( p_i \to 1 \), and the FI current is given by

\[
I = eZ .
\] (5.17)

An expression for \( Z \) in the high field regime may be derived from gas-kinetic theory. Under conditions of zero field the rate at which molecules at a temperature \( T \) and pressure \( P \) approach an area \( A_o \) is given by

\[
Z_o = \frac{A_o P}{(2\pi mkT)^{\frac{1}{2}}} ,
\] (5.18)

where \( m \) : Mass of the molecule;
\( k \) : Boltzmann's constant.

The electric field enhances the effective cross sectional area of the tip by a factor \( \sigma \), which results from the action of polarization forces. This enhancement factor may be as high as 10 – 100 in the high fields used in FI. The tip endform may be approximated by a hemispherical termination and the supply function can then be written

\[
Z = \frac{2\pi r^2 \sigma P}{(2\pi mkT)^{\frac{1}{2}}} .
\] (5.19)

Southon (So 63) has calculated \( \sigma \) for a spherical emitter to be

\[
\sigma \approx \left( \frac{\pi \alpha F_o^2}{2kT} \right)^{\frac{1}{2}},
\] (5.20)
where \( \alpha \) is the polarizability of the gas, and the permanent dipole moment is assumed negligible.

The ion current in the high field regime may then be approximated by

\[
I \approx \frac{e2\pi r_i^2 P}{(2\pi mkT)^\frac{1}{2}} \left( \frac{\pi \alpha F_0^2}{2kT} \right)^\frac{1}{2}
\]

(5.21)

This simplified expression does not account for the enhancement of the FI current due to the gas supply from the emitter shank (So 63), but it does illustrate the dependence of ion current on a number of source variables. Equation (5.21) predicts that the ion current will be linearly proportional to gas pressure and inversely proportional to temperature, indicating the advantage gained by operating the source at low temperatures and with as high a pressure as possible without inducing ion-neutral beam energy broadening. The increase in FI current available at 78K is shown in Figure 5.10A. These data have been presented by Müller and Tsong (Mü 69) and show that for low fields emitter tip cooling is essential. In the high field domain the relative current advantage of a cooled emitter is much less and the ratio approaches the theoretical temperature dependence predicted by equation (5.21).

Equation (5.21) also predicts that for a given electric field the current can be expected to vary as \( r_i^2 \). In fact it has been found by Southon and Brandon that the current varies as \( r_i^n \) where \( n \) is between 2.5 and 3.0 (So 63a). Levi-Setti (Le 80) has used this relation to deduce the following empirical expression for the comparison of the brightness of two FI sources (\( B_{n1} \) and \( B_{n2} \)), operating at the same field, but with different emitter radii (\( r_1 \) and \( r_2 \)) and applied voltages (\( V_1 \) and \( V_2 \)):

\[
\frac{B_{n1}}{B_{n2}} = \frac{V_2}{V_1} \left( \frac{r_1}{r_2} \right)^{\frac{n}{2}-1}
\]

(5.22)

From both equation (5.21) and (5.22) it is obvious for the present application, where both maximum current and brightness are sought, that the largest possible emitter radii should be used. As \( F \propto r_i^{-1} \) any increase in tip radius must be matched by a proportional increase in the applied voltage and/or a reduction in the emitter-cathode separation in order to maintain source operation at the desired field. In practice the upper limit to the source radius is set by the breakdown
Figure 5.10

A:

A plot of the hydrogen FI current at 78K divided by the current at 300K. These experimental data were obtained by Müller and Tsong (Mü 69) and highlight the advantage provided by the greater thermal accommodation of the molecules at low temperatures. This advantage is less than a factor of ten for field values greater than $2.5 \times 10^{10} \text{ Vm}^{-1}$.

B:

A plot of the fraction of the total ion yield for each of $H^+$, $H_2^+$ and $H_3^+$ during the FI of $H_2$. The plot is derived from data obtained by Clements and Müller (Cl 62). The relative ion yields given here were found to be independent of pressure from $6.5 \times 10^{-4}$ mbar to $1 \times 10^{-1}$ mbar, and temperature from 20K to 300K. The relative abundance at the field evaporation limit ($F \approx 5 \times 10^{10} \text{ Vm}^{-1}$) was said to favour the atomic ion by a factor of ten.
A

FRACTION OF TOTAL ION YIELD

1.00

0.80

0.60

0.40

0.20

0

H₂⁺

H⁺

H₃⁺

B

ELECTRIC FIELD (x 10¹⁰ Vm⁻¹)

FRACTION OF TOTAL ION YIELD

1.00

0.80

0.60

0.40

0.20

0

1.60 1.80 2.00 2.20 2.40 2.60 2.80

ELECTRIC FIELD (x 10¹⁰ Vm⁻¹)
properties of the emitter-cathode gap and for most practical work \( r_t \) has an upper bound of 0.2\( \mu \text{m} \) (see Section 6.2.2).

The FI of \( \text{H}_2 \) results in the production of \( \text{H}^+ \), \( \text{H}_2^+ \) and \( \text{H}_3^+ \) ions. The relative abundance of these ions is a strong function of electric field and has been investigated by Clements and Müller (Cl 62). Their data are shown in Figure 5.10B. It can be seen from this figure that FI in the low field regime favours the production of the \( \text{H}_2^+ \) parent ion and as the field is increased above \( 1.8 \times 10^{10} \text{ Vm}^{-1} \) the beam is a mixture of the three ion species. At fields above \( 2.25 \times 10^{10} \text{ Vm}^{-1} \) \( \text{H}^+ \) production is favoured reinforcing the point that for this application of FI, fields greater than \( 2.2 \times 10^{10} \text{ Vm}^{-1} \) will be necessary.

The FI process has an intrinsic energy spread, as the ionization of gas molecules can occur over a range of distances beyond the critical distance \( x_c \). The energy spread will be smallest in the regime where ionization occurs in a narrow region about \( x_c \). For fields around \( 2.1 \times 10^{10} \text{ Vm}^{-1} \) the energy spread of the \( \text{H}^+ \) and \( \text{H}_2^+ \) beam is approximately 4eV (Ts 64). However, in the high field regime the energy spread increases rapidly due to the effects of field-induced disassociation of the \( \text{H}_2 \) molecules (Ha 74) and a secondary structure resulting from enhanced ionization at distances from the emitter where field-induced resonance states exist (Ja 67). These effects combine to set a lower limit to the energy spread in the FI beam. Additional energy spread can result from pressure broadening and power supply instability.

The energy distribution of \( \text{H}^+ \) ions from the FI of \( \text{H}_2 \) has been carefully measured by Hanson (Ha 75). He found that for fields less than \( 2.4 \times 10^{10} \text{ Vm}^{-1} \) the distribution was dominated by a central peak resulting from the disassociation of vibrationally excited \( \text{H}_2 \) and that the width of this peak was insensitive to gas temperature. The FWHM of the dominate peak was approximately 2.1eV at \( 2.2 \times 10^{10} \text{ Vm}^{-1} \), 4.3eV at \( 2.6 \times 10^{10} \text{ Vm}^{-1} \) and 5.7eV at \( 2.8 \times 10^{10} \text{ Vm}^{-1} \). The full width of the energy distribution includes a contribution from resonance states that dramatically increases with field and temperature. The \( \text{H}^+ \) beam full energy spread at 21K was measured by Hanson to be 15eV at \( 2.2 \times 10^{10} \text{ Vm}^{-1} \), 30eV at \( 2.6 \times 10^{10} \text{ Vm}^{-1} \) and over 40eV at \( 2.8 \times 10^{10} \text{ Vm}^{-1} \). This rapid increase in energy spread as a function of electric field places an upper limit on the field that may
be used with a FI source as this intrinsic energy spread gives rise to intolerably large chromatic aberration in the ion source lens at high fields. The effect of the energy spread of a FI beam upon the ion optical performance of an electrostatic ion source lens is discussed in a thesis by Colman (Co 89a).

From the H⁺ yield given in Figure 5.10B and knowledge of the energy spread of the H⁺ beam component, it was decided that FI within the field range of $2.2 \times 10^{10} < F < 2.6 \times 10^{10} \text{Vm}^{-1}$ would be most suited to this application of a FI source.
Chapter 6

Development of a high brightness ion source

6.1 Introduction

The process of field ionization (FI) is particularly suited to the formation of ion beams for microprobe use. However, no proton source using this process is available commercially. The existing experimental FI sources have been built for mass spectrometry studies of organic compounds (Be 77) or designed as low current sources suitable for microfabrication and imaging purposes (Or 75)(Sc 77)(Ha 79)(Le 80). As none of these sources could be contemplated for use in a high voltage accelerator it was considered necessary to design and build an ion source that utilized the process of FI. It was necessary that this source provide a beam brightness far superior to that available from the existing RF ion source and be suitable for use in the Pelletron accelerator. This latter requirement placed a number of constraints upon the design of the source. The source must be compatible with the accelerator's vacuum system, supply a beam suitable for injection into the accelerator and be both rugged and compact. This chapter describes a FI ion source that was consequently designed and built.

6.2 Design of a Field Ionization ion source

FI can be induced in a gaseous atmosphere with the conceptually simple arrangement of a fine metallic tip at high positive potential with respect to an adjacent
cathode. However there exist many technical difficulties in attempting to successfully utilize the ion optical advantages offered by a submicron emission site. The present FI source was designed as a prototype, to be immediately evaluated on a test bench (see Section 7.2) and ultimately installed in the Pelletron accelerator. The use of this source required a short focus low aberration lens immediately following the emitter. The source was designed both to accommodate such a lens and to allow diagnosis of ion source performance. The materials used in the construction of the source were non magnetic and compatible with ultra-high vacuum.

6.2.1 Source housing

The source housing was required to provide a solid basis for, and allow the accurate alignment of, the internal ion source components. In order to minimize alignment problems the source housing was constructed about a central geometric axis which coincided with the optical axis of the emitter-lens system. The housing was turned from a solid cylinder of austenitic grade 316 stainless steel. Figures 6.1 and 6.2 are diagrams of the source and Figure 6.3 is a photograph of the source. The end-flange size was dictated by the size of the flange on the accelerator column to which the source was to be mated. To ensure that the ion source could be accurately aligned to any following component the source flange was keyed in three positions.

The housing was also required to be rigid and accurately affixed to the flange. Rigidity was achieved by using a wall thickness of 7mm and having the top of the source housing 12.7mm thick and integral with the source walls. The source housing and mounting flange were machined to an interference fit (±3µm) and welded together by the Tungsten Inert Gas (TIG) technique at the lowest possible temperature to avoid distortion.

A symmetric set of four 70mm ports was placed in the source wall for both control of apertures and use as general feedthroughs. A 33mm port was also installed to accommodate a triaxial feedthrough, and a set of three high-voltage electrical feedthroughs was placed asymmetrically in the housing wall (see Figure 6.2). These feedthroughs were constructed from Macor machinable glass ceramic (Co 80) and were designed to transmit the high voltages necessary for a source lens. The ceramic insulator used with the feedthroughs was convoluted to
Figure 6.1

The field ionization ion source. The shaded areas indicate the use of Macor machinable ceramic. All other components have been made from stainless steel, unless indicated.

A: High voltage feedthrough cover (Pactene)
B: High voltage lead from power supply
C: Spring-loaded feedthrough contact
D: Emitter-holder
E: Gas supply line
F: Thermocouple gauge
G: Emitter-holder flange
H: Emitter-holder alignment spigot
I: Aluminium gasket
J: Plenum chamber
K: Lens cradle
L: Linear motion drive
M: Scanning aperture plate
N: Source housing
O: Faraday cup
P: Faraday cup shield
Q: Electron suppression ring
R: Insulating block
S: Faraday cup output line
T: Braided copper shield
U: Mounting flange: 208mm dia.
V: Rotatable 70mm conflat flange
W: Emitter filament
X: Stainless steel cathode cap
Y: Plenum chamber collar
Z: Indium gasket
Figure 6.2

Plan of the field ionization ion source.

A: Thermocouple gauge unit
B: Thermocouple gauge mounting block
C: Emitter-holder flange
D: Emitter high voltage feedthrough (Macor ceramic)
E: Emitter feedthrough cover (Pactene)
F: High voltage feedthrough (Macor ceramic)
G: Triaxial feedthrough
H: Linear motion drive
Figure 6.3

Photograph of the FI ion source test bench. The turbo molecular pump is not shown in this photograph. The source itself is centrally located in the photograph. The high voltage lead enters the source from the top and is surrounded by a white Pactene cover. The gas inlet line also enters the source from the top, and slightly to the right of the high voltage line. The thermocouple gauge head is located on the gas inlet line and the cold-cathode gauge head may be seen on the right-hand side of the source housing. The linear-motion drive for the control of the scanning apertures is present on the left of the housing. The triaxial feedthrough for current measurements, is found on the lower right-hand side of the source. The lead from this feedthrough can be seen connected to an electrometer in the lower left.
inhibit surface tracking, and bonded to the housing wall with an ultra-high vacuum adhesive (Varian Torrseal). The smallest possible surface area of this adhesive was exposed to the vacuum and the feedthrough was designed to minimize retention of trapped gases. With suitable end caps the feedthroughs were able to withstand 40kV without surface tracking or insulator breakdown.

The FI emitter was mounted in an emitter-holder assembly that located on the top of the ion source housing (see Figures 6.1, 6.2 and 6.4). As the prototype source was required to be rugged, uncomplicated and compact, the initial emitter-holder was made without provision for emitter cooling. The alignment of the emitter-holder is critical. This assembly is required to locate accurately the emitter on the central geometric axis, to provide a feedthrough for the emitter voltage, and supply the source gas to the emitter region. A tapered spigot on the top of the source housing supports and locates with a tight fit, the stainless steel flange on the emitter-holder assembly. The spigot and the mating female flange were machined to a high-tolerance slip fit, so as to provide an absolute location of the emitter assembly relative to the source housing. Tilt of the emitter assembly relative to the source housing is less than 1 mrad, and the two components share the same axis to an accuracy of better than ±3µm. The vacuum seal is provided by an aluminium gasket that is compressed uniformly to a thickness of 500µm when the emitter-holder assembly is tightened onto the spigot.

The electrical feedthrough for the emitter was designed to tolerate voltages in excess of 40kV. The feedthrough insulator was formed from a single rod of Macor machinable ceramic and was turned to a tight fit in the emitter-holder flange. Macor glass ceramic was used exclusively within the source as an electrical insulator. The ceramic is an excellent electrical insulator having a volume resistivity of greater than $10^{14} \Omega \text{cm}$ and a nominal D.C. breakdown voltage of 120kVmm$^{-1}$ (Co 80). It has a machining tolerance of 13µm and can be polished to a smoothness of 0.5µm AA (Gr 77). The ceramic may be baked-out and maintained at 800°C and its coefficient of thermal expansion is similar to that of stainless steel ($K_{T,\text{Exp.}}^{\text{Macor}} = 9.4 \times 10^{-6} \frac{\text{mm}}{\text{mm} \cdot \text{C}}, K_{T,\text{Exp.}}^{\text{Steel}} = 10.4 \times 10^{-6} \frac{\text{mm}}{\text{mm} \cdot \text{C}}$). The ceramic also has excellent vacuum properties with an outgassing rate less than that of polished stainless steel ($K_{1}^{\text{Macor}} = 15.4 \times 10^{-10} \text{mbar l s}^{-1} \text{cm}^{-2}$, $K_{1}^{\text{Steel}} = 22.6 \times 10^{-10} \text{mbar l s}^{-1} \text{cm}^{-2}$).
Figure 6.4

A:

A photograph of the emitter-holder flange, plenum chamber and cathode cap. The flange is bolted to the source with eight studs and located by the alignment spigot found on the inner perimeter of the stud holes. The stainless steel and ceramic construction of the plenum chamber can be seen as can the location flange for the cathode cap.

B:

A photograph of the central region of the plenum chamber. An emitter can be seen on the emitter-holder. The clamp which holds the filament in the locating grooves has been removed. An indium gasket is shown on the locating rim for the cathode cap. It is used in this position to form a gas tight seal between the plenum chamber and the vacuum system.
where $K_1$ is the outgassing rate after 1 hr (El 76)), and compares favourably with alternative insulating materials such as polyethylene ($K_1^{Poly} = 30.6 \times 10^{-10}$ mbar l s$^{-1}$ cm$^{-2}$) or Boron nitride ($K_1^{BNitr} = 4.1 \times 10^{-6}$ mm mm$^{-2}$ C$^{-1}$).

A single 1.5mm diameter stainless-steel rod was used as the conductor for the emitter feedthrough and it formed a T-connection with the emitter-filament location bar. The emitter filament is inserted into two 250µm diameter holes machined in this bar, with positive location of the filament being achieved by location grooves milled in the ceramic holder. A ceramic clamp-block holds the filament correctly aligned in the holder.

### 6.2.2 Internal geometry and components

The ion source was required to produce the maximum possible current from a FI emitter. An approximation for the current output from a FI emitter is given by equation (5.21) This equation predicts that current output in the high field regime is linearly dependent upon the gas pressure in the region of the tip. Differential pumping was used in this source to maximize gas pressure near the tip, yet permit high vacuum conditions to be maintained downstream. Figures 6.1, 6.4A and B show the Plenum chamber used to form the high pressure region within the source. This chamber was required to contain a gas pressure of up to 10mbar and accurately locate a cathode 1.3mm from the emitter and on the central axis of the source. This chamber was also required to be detachable to enable both access to the emitter and replacement of the cathode.

The base of the Plenum chamber was made of Macor ceramic and sealed, with an indium gasket, onto the emitter-holder flange. A rim turned in the ceramic locates the chamber, with a tight fit, onto the flange. A stainless steel collar was bonded to the ceramic base and aligned with another locating rim. A range of cathode caps can be placed in position on the collar. The caps are located by a tight fit on their outer rim and an indium gasket is used for a gas tight seal. The use of Macor ceramic in the construction of the Plenum chamber results in the cathode being electrically isolated from the source housing. Hence the cathode can be biased or used for current measurements.

Gas is fed into the Plenum chamber via a 2mm hole through the emitter-
holder flange (see Figure 6.1). A matching 2mm hole is placed in the ceramic base of the Plenum chamber, along with a central 10mm hole through which the emitter-holder assembly protrudes. To ensure that the cathode was located with the maximum possible accuracy, the entire ceramic base of the Plenum chamber was turned from a solid rod of ceramic.

The dimensions of the emitter-holder assembly and the Plenum chamber-cathode cap combination were dictated by the high voltage required for FI with a large radius emitter \( (r_t \sim 0.2\mu m) \). Upper limits to this applied voltage are given in Table 6.1. Table 6.1 shows the electric fields achieved at the surface of the emitter as a function of the applied voltage, tip radius and emitter-cathode separation. The field values were calculated by Colman (Co 89) using a charge simulation method (Ka 82) that modeled the emitter tip and the planar cathode. The cathode was assumed to have a 2mm diameter aperture on the central axis of the emitter. The boundary conditions used in the model matched the geometrical configuration within the Plenum chamber. Table 6.1 indicates that voltages in

<table>
<thead>
<tr>
<th>Tip radius ( r_t(\mu m) )</th>
<th>Emitter-cathode separation: ( d_o ) (mm)</th>
<th>Field at tip: ( F_o \times 10^{10} ) Vm(^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( V_o ): 10kV 15kV 20kV 30kV</td>
</tr>
<tr>
<td>0.1</td>
<td>1</td>
<td>2.88 4.32 5.75</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.67 4.01 5.34</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.59 3.88 5.17</td>
</tr>
<tr>
<td>0.15</td>
<td>1</td>
<td>1.99 2.99 3.98 5.98</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.84 2.77 3.69 5.54</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.79 2.69 3.58 5.38</td>
</tr>
<tr>
<td>0.2</td>
<td>1</td>
<td>1.54 2.31 3.07 4.62</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.42 2.13 2.84 4.26</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.38 2.07 2.76 4.14</td>
</tr>
</tbody>
</table>
excess of 20kV are required to explore source performance in the high field regime 
\( F_0 > 2.2 \times 10^{10} \text{ Vm}^{-1} \). Sufficient freedom was required in the design of the 
ion source to allow electric fields to be as high as \( 4 \times 10^{10} \text{ Vm}^{-1} \) for tips with 
termination radii as large as 0.2\( \mu \)m. Hence the source was required to operate 
at emitter voltages of up to 30kV. Voltages of this magnitude readily give rise to 
problems of spark-over or tracking in a partial or complete vacuum system.

Consider initially the breakdown conditions within the Plenum chamber. 
This is a region of partial vacuum where there is the risk of breakdown occurring 
between the emitter and cathode. Pachen's Law may be used to predict the break-
down voltage between two planar electrodes as a function of separation, pressure 
and gas species (Br 69), but this law does not accurately predict the breakdown 
conditions encountered experimentally for the geometry used in the Plenum cham-
ber. Six factors can be considered to control the breakdown properties of this gap: 
(i) Gas species, pressure and temperature, (ii) Electrode geometry, (iii) Electrode 
separation, (iv) Electrode material, (v) Electrode surface preparation, and (vi) 
External electrical circuitry.

Steps were taken to ensure that the highest possible breakdown voltage \( V_b \) 
was achievable. Although the only gas used was \( \text{H}_2 \) at 293K, a wide range of gas 
pressures was tried, as was a variety of gap distances. The cathode was made from 
304 grade stainless steel, a suitable choice due to its excellent vacuum properties 
and its established resistance to breakdown (La 81). The cathode surface was 
prepared with mechanical polishing. A fine grade diamond paste was used to 
polish the surface; then it was degreased and cleaned in an ultrasonic bath. The 
external circuit affects the response of the initial arc during breakdown. The use of 
a \( 10^{10} \Omega \) protection resistor (see Section 7.2) ensured that the response time for the 
dissipation of capacitive energy was greater than 10\( \mu \)s, which is the characteristic 
time constant associated with the current growth during the initiation of an arc 
(La 81).

It is known that for small gaps, of the order of 6mm or less, the highest 
breakdown voltage for a constant gap at a pressure of \( 10^{-4} \text{ mbar} \), is given by the 
extreme geometry of an anode point opposite a plane (Al 68). This approximates 
the emitter-cathode geometry in the Plenum chamber (except for the presence
Table 6.2: The breakdown voltage between the emitter and cathode.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Tip radius ( r_t (\mu m) )</th>
<th>Emitter-cathode separation: ( d_o ) (mm)</th>
<th>Gas Pressure ( \text{mbar} )</th>
<th>Breakdown voltage ( V_b ) (kV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.15</td>
<td>7.0</td>
<td>( 2.4 \times 10^{-5} )</td>
<td>16.5</td>
</tr>
<tr>
<td>B</td>
<td>0.10</td>
<td>0.2</td>
<td>( 6.6 \times 10^{-4} )</td>
<td>16.0</td>
</tr>
<tr>
<td>C</td>
<td>0.75</td>
<td>7.0</td>
<td>( 1.3 \times 10^{-4} )</td>
<td>16.0</td>
</tr>
<tr>
<td>D</td>
<td>0.25</td>
<td>9.0</td>
<td>( 7.0 \times 10^{-4} )</td>
<td>19.0</td>
</tr>
<tr>
<td>E</td>
<td>0.15</td>
<td>7.0</td>
<td>( 1.3 \times 10^{-2} )</td>
<td>20.0</td>
</tr>
<tr>
<td>F</td>
<td>0.30</td>
<td>4.8</td>
<td>( 1.4 \times 10^{-4} )</td>
<td>20.0</td>
</tr>
<tr>
<td>G</td>
<td>0.30</td>
<td>5.0</td>
<td>( 1.3 \times 10^{-4} )</td>
<td>22.0</td>
</tr>
<tr>
<td>H</td>
<td>0.10</td>
<td>0.2</td>
<td>( 6.7 \times 10^{-3} )</td>
<td>24.0</td>
</tr>
<tr>
<td>I</td>
<td>0.15</td>
<td>0.95</td>
<td>( 6.7 \times 10^{-3} )</td>
<td>24.0</td>
</tr>
<tr>
<td>J</td>
<td>0.15</td>
<td>4.5</td>
<td>( 1.3 \times 10^{-3} )</td>
<td>26.0</td>
</tr>
</tbody>
</table>

of the differential pumping aperture), indicating that the FI geometry favours a high breakdown voltage between the emitter and the cathode. Because theoretical predictions of breakdown voltages in gaseous atmospheres are unreliable (Al 68), it was decided, for this ion source, to experimentally determine the \( V_b \) for a number of emitter-cathode separations at a variety of pressures.

Table 6.2 shows the result of measurements made to identify the onset of electrical breakdown, via the formation of a gaseous discharge, between the emitter and the cathode with the emitter centrally placed opposite the 2.3mm diameter differential pumping aperture. It was noted that surface conditioning played a significant role in raising the breakdown voltage. Measurements A, B and C resulted from directly raising the emitter voltage at a rate of 200 \( \text{Vs}^{-1} \). Under the conditions shown for A, breakdown occurred at only 16.5kV. When the same geometry was conditioned, by raising the voltage at 5 \( \text{Vs}^{-1} \) with 1800 second pauses at each 1kV increment above 10kV, the breakdown voltage was significantly increased and much higher gas pressures were tolerated. This is demonstrated by the comparison between measurements A and E, where the only difference was
that Measurement E was taken with a conditioned gap. Aside from A, B and C, all measurements were taken after the gap had been conditioned.

Varying the emitter-cathode separation did not have a significant effect on the breakdown voltage. This result was expected (Al 68), and high breakdown voltages were still achieved with small values of \( d_o \) (the emitter-cathode separation) at high Plenum chamber gas pressures (measurements H and I). The presence of the aperture causes the pressure in the region of the tip to be reduced for these measurements (see Section 7.4.1). The breakdown voltage bore no identifiable relationship to \( F_o \), and for values of \( d_o \) less than 2mm (the diameter of the differential pumping aperture) the onset of breakdown appeared to be a voltage-related phenomenon.

The breakdown properties of the vacuum found outside the differential pumping aperture are much more readily understood. In this region the gas pressure was always less than \( 10^{-4} \) mbar during source operation (see Section 6.2.4). This pressure is below the gas discharge domain and breakdown is independent of the pressure or nature of the residual gas (Al 68). Under these conditions the mean free path of molecules is large in comparison to the ion source dimensions, and a charged particle drawn from one electrode to another is unlikely to collide with a residual gas molecule and sustain a breakdown path. This results in the breakdown properties of a given high vacuum gap being controlled by factors (ii) to (vi) mentioned above. The ion source was designed to accommodate, in this high vacuum region directly following the emitter, a three element asymmetric electrostatic lens, which has the bulk of its central electrode concentrated near the first electrode. It has been shown repeatedly in calculations and experiments performed during field emission electron gun design, that this lens structure offers optimal lens characteristics for a single point source (Or 79)(Ri 78). For a lens to be used in this type of application, the aberration coefficients must be minimized to maintain as much of the intrinsic brightness of the source as possible. In the case of an electrostatic lens of this nature both \( C_s \) and \( C_e \) can be reduced for a given lens design by scaling down the lens dimensions. Hence it was desirable to minimize the working distance, electrode size and electrode separation. This scale reduction is limited however by a number of practical considerations. Primarily,
the lens elements require sufficient separation to avoid spark-over during operation. The breakdown voltage ($V_b$) for planar electrodes, in vacuum and separated by 2mm or greater, is given by

$$V_b = Kd^\alpha,$$  \hspace{1cm} (6.1)

where $K \sim 40\text{kVmm}^{-\alpha}$, $\alpha \sim 0.4$ and $d$ is the separation (La 81). This indicates that if a 2mm separation is allowed between lens elements, the vacuum gap would be able to withstand a potential of 53kV. For focusing of the ion beam two voltage modes are available. A decelerating mode initially decelerates the beam in the first gap and accelerates it in the second. This mode gives strong focusing for central electrode voltages that are only $\frac{1}{19}$ the emitter voltage. For this work a lens used in the 'decel' mode would require a maximum electrode potential of 3kV. An alternative accelerating mode may be used, where the beam is initially accelerated in the first lens gap then decelerated in the second. It has been shown that in this mode for working distances less than 2mm the central electrode must be opposite in polarity to, and typically a factor of seven greater than, the emitter voltage. This mode is desirable in that it results in low aberrations because the beam diameter remains small as it passes through the lens. However, if the source is operated in the high voltage regime, the lens voltages necessary to focus the beam would be in excess of 100kV. It was considered impractical to design a lens system capable of focusing the beam in the 'accel' mode. Lens dimensions were thus governed by the voltage requirements of the 'decel' mode. The voltage limitation would then be the convoluted surface distance across the insulating ceramic between electrodes. A lens cradle was designed to support the three element electrostatic lens in a cylinder of ceramic 50mm high and 77mm in diameter. This lens support system has proven to be satisfactory at the voltages necessary for focusing a beam in the 'decel' mode (Co 89a).

The lens cradle was suspended from the roof of the source housing and is shown in Figure 6.1. The primary role of this cradle was to support and locate the ion source lens. Obviously, to avoid the introduction of misalignment aberrations in the lens system the cradle must be located about the central optical axis. The cradle itself was turned from a solid cylinder of grade 316 stainless steel and was
located by an interference fit into a turned groove in the top of the source housing. A 12mm wide rim on the top of the cradle prevented tilting of the cradle relative to the source housing. The cradle was placed in position using an alignment jig and a press; although its design permitted its removal, the cradle remained in position throughout this experimental work. A number of holes was drilled in the wall of the lens cradle to enable effective pumping of the cathode region and to allow for electrical feedthroughs to the lens to be inserted.

The secondary role of the lens cradle was to provide a suitable support for beam monitoring equipment during the development of the ion source. Each of a pair of 1.4mm wide slots was milled on opposing sides of the cradle in such a location that, with the cradle in position, the slots were 1.67mm and 7.20mm from the cathode. A pair of aperture strips, located by the slots in the cradle, were then able to be driven across the beam by linear motion drives (see Figures 6.1 and 7.1). Accurate positioning of the aperture strips was of critical importance. The strips were made of stainless steel, and the edges of each strip were rounded to fit the milled slot. Each strip was matched to its corresponding slot by the abrasive reduction of the strip width. The strips then made a tight fit in the slots and carried centrally placed diaphragms across the beam. Each strip contained three diaphragms. The strip closest to the emitter had diaphragm sizes of 0.455mm, 2.290mm and 4.020mm (±1μm). The second aperture strip contained diaphragms 2.840, 6.030 and 9.565mm in diameter. The thickness of each diaphragm was 0.2mm and their positioning was such that the beam could not reach the Faraday Cup by passing through two diaphragms on the same strip.

The base of the lens cradle also served as a support for a Faraday Cup (as shown in Figure 6.1). This Cup was guarded by an electron suppression ring, biased to -300V. This voltage was calculated to be sufficient to prevent the most energetic secondary electrons escaping from the Cup which was shielded from stray fields by a secondary stainless steel cup. The current carrying wire from this Faraday Cup was surrounded by ceramic beads which were used to support a sheath of braided copper wire. This output was then fed via a triaxial feedthrough and a short (0.3m) triaxial lead to a Keithley 603 electrometer (see Figure 7.1).

An accurate knowledge of the internal dimensions of the source was neces-
sary for beam analysis. Prior to the final cleaning of components, all fixed internal source dimensions were measured to an accuracy of 12µm and demountable components involved in beam analysis were measured to an accuracy of 1µm.

6.2.3 Source vacuum and gas supply

For stable FI, ultra-high vacuums are required prior to the introduction of the source gas. One aim of the source design was to achieve the highest possible vacuum in the region of the emitter. The source was built to operate on the FI ion source test bench which was able to be evacuated to less than $5 \times 10^{-8}$ mbar (see Section 7.2). All measurements of the source vacuum were performed whilst the source was attached to the test bench.

In designing the source consideration was given to the choice of materials in order to minimize possible outgassing (desorption) rates. Both stainless steel and Macor ceramic (the major source constituents) have the lowest available outgassing rates for materials of their type (see Section 6.2.2)(El 76). The outgassing rate strongly depends upon the state of the surface. All components used in the ion source and exposed to the vacuum were mechanically polished and degreased. The degreasing involved repeated washing with a degreasing solvent and a detergent, followed by a number of rinses in deionized water and an oven bake-out at 120°C. It has been acknowledged by Routh that such treatment, if undertaken carefully, lowers the surface outgassing rates (Ro 76) and for stainless steel a reduction by a factor of 7.7 in the outgassing rate is achieved (El 76).

The theory of outgassing has been derived by Dayton (Da 63), and the outgassing rate $K_h$ may be written

$$K_h = K_u + K_1 t_h^{-\gamma},$$

(6.2)

where $t_h$ is the pump-down time, $\gamma$ is a surface dependent constant (after a few minutes of initial pumpdown) and $K_u$ is the limiting outgassing rate (as $t_h \to \infty$). The desorption rate is endothermic with both $K_u$ and $K_1$ being proportional to temperature. The evacuation of the ion source began with pumping at room temperature ($P_{t=1hr} \sim 1 \times 10^{-6}$ mbar) to remove physically absorbed water vapour, followed by a bake-out of the entire source and test bench to accelerate desorption.
Within the source metallic gaskets were used for all eight vacuum to air seals. Five of these seals were copper, which has an outgassing rate approximately twice that of stainless steel ($K_{i}^{\text{Copper}} = 4.65 \times 10^{-10} \text{ mbar l s}^{-1} \text{ cm}^{-2}$). Aluminium wire was used for the other seals on the gas input line, between the emitter-holder flange and the source housing and as the gasket between the source and the test bench. Aluminium was used for these seals as it has a outgassing rate lower than alternatives such as gold wire ($K_{i}^{\text{Aluminium}} = 82 \times 10^{-10} \text{ mbar l s}^{-1} \text{ cm}^{-2}$ c.f $K_{i}^{\text{Gold}} = 2101 \times 10^{-10} \text{ mbar l s}^{-1} \text{ cm}^{-2}$), and is less prone to work-hardening which can result in damage to the stainless steel sealing surfaces. Two indium wire gas seals were used within the source. These seals were between the Plenum chamber and the emitter-holder flange (ceramic to metal, as indium wets the ceramic) as well as between the cathode cap and the Plenum chamber. The use of indium seals limited the bake-out temperature of the ion source to 100°C as the melting point of indium is 156°C. Electrical heating tapes were used to bake the entire source (including gas inlet line and test bench vacuum system). A baking cycle was followed to accelerate pump-down.

The source was designed to minimize the possibility of virtual leaks which can occur in the molecular domain when trapped gases must be pumped through small conductances. Virtual leaks can add significantly to pump-down time and limit the effective ultimate vacuum of a system. Every effort was made to expose to the pumping system areas that could potentially trap gas. All bolts used in the source were milled axially such that a 1mm wide slot ran the length of the bolt. This provided sufficient conductance for the effective pumping of threads. In addition, where possible, tapped threads were exposed to the vacuum system by milled slots. The lens cradle was perforated by many openings to enable the effective pumping of the cathode aperture, and alignment surfaces were surrounded by pumping holes.

The vacuum was monitored by three gauges in the source. A thermocouple gauge (calibrated to H$_2$) measured gas pressure in the inlet line (see Figure 6.1). This gauge was sensitive over the range $10^{-3}$ mbar to 3 mbar to an accuracy of 3%. A cold cathode gauge was attached to the source housing on one of the available
70mm flanges. This gauge was used for the measurement of gas pressure outside the differential pumping aperture and provided an accuracy of ± 2% F.S.D over a range from $1 \times 10^{-3}$ mbar to $1 \times 10^{-7}$ mbar. Also attached to one of the 70mm flanges was a Balzers quadrupole mass spectrometer. This spectrometer was used for partial pressure analysis in the vacuum system and was able to detect partial pressures down to $1 \times 10^{-10}$ mbar. The gauge also gave an integrated total pressure measurement and was able to operate at pressures as high as $1 \times 10^{-3}$ mbar. It was used for the measurement of the mass composition of the source gas and residual vacuum.

Figures 6.5 A and B show the residual gas spectra for the test bench vacuum and the test bench-ion source vacuum. The ultimate pressure in each of these two cases is limited by mass 28 (Essentially N$_2$ as the CO$_2$ contribution is negligible). The presence of masses 28 and 32 in the ratio of 4:1 is characteristic of an air leak (Le 82b). The N$_2$:O$_2$ ratio for A is 4.8 and for B it is 4.6, thus suggesting that the ultimate vacuum was limited by an air leak. However all detectable leaks, to a sensitivity of $1 \times 10^{-10}$ mbar He, had been eliminated. Hence, it was deduced that the ultimate pressure was limited by outgassing within the source. The large H$_2$ peak is characteristic of a UHV stainless steel system pumped by a turbo-molecular pump, as the pump has a low efficiency for H$_2$ (see Section 7.2) and hydrogen diffuses from the chamber walls at low pressures. The presence of H$_2$O along with the protracted outgassing of N$_2$ and O$_2$ indicates that the bake out temperature was not high enough and that a longer pump-down was required. These two factors cannot be changed with the present construction as the bake out is limited to 100°C as explained and longer pump-down times inhibit the usefulness of this source as a diagnostic instrument.

Ultra high purity (99.999%) hydrogen was used as the source gas (Supplier: C.I.G.). This gas was supplied directly to the source via a gas line that had initially been baked and pumped to high vacuum. Figures 6.5 C, D and E show the mass spectra obtained when this gas was introduced into the source at increasingly high pressure. The passage of the H$_2$ gas down the evacuated gas line results in a number of contaminants being admitted into the vacuum system. Figure 6.5 C shows the gas composition at a partial pressure of $1 \times 10^{-5}$ mbar H$_2$. Relative
The gas composition for the test bench and ion source vacuum system.

A:

The residual gas spectrum for the test bench vacuum system only (see Section 7.2). This vacuum was achieved after a bake out and five days of pumping. The heating tapes were turned off for this measurement.

B:

The residual gas spectrum for the ion source and test bench vacuum system. The same pumping conditions were used as in A. The major component is mass 28 ($N_2 + CO$). Some residual $H_2O$ is present. A far higher bake out temperature ($\sim 300^\circ C$) and longer pumping time is required to remove these components.

C:

Gas spectrum obtained when $H_2$ is introduced at a partial pressure of $1 \times 10^{-5} \text{ mbar}$. Some $Ar$ and $CO_2$ is now detectable. The levels of $H_2O$, $N_2$ and $O_2$ are increased.

D:

The $H_2$ partial pressure has been increased by a factor of 3.8. No change is seen in the partial pressure of the other gases.

E:

The $H_2$ gas pressure has been increased by a factor of 11 above that presented in C. The $Ar$ and $CO_2$ partial pressures remain unchanged. The $H_2O$ partial pressure has increased by a factor of 5, $N_2$ by 2.1 and $O_2$ by 5.1.
to the residual vacuum the following contaminant species have been introduced: 
$\text{H}_2\text{O}$, $\text{N}_2$, $\text{O}_2$, $\text{Ar}$ and $\text{CO}_2$. These gases have come from the gas inlet line and have been desorbed by the passage of the $\text{H}_2$ gas. The hydrogen gas pressure was then increased through the range of pressures used during the source operation to monitor the level of contaminants present during source operation. Especially significant is $\text{H}_2\text{O}$, the molecule responsible for tip reactive gas etching (see Sections 7.3 and 7.4). From Figures 6.5 D and E, it can be seen that the levels of Ar and $\text{CO}_2$ were independent of the amount of $\text{H}_2$ or its rate of flow into the chamber. The partial pressures of $\text{H}_2\text{O}$, $\text{N}_2$ and $\text{O}_2$ increased with a greater input of $\text{H}_2$ but at a rate less than the rate of change of the $\text{H}_2$ partial pressure. These gases were possibly being liberated from surfaces within the source which could not be effectively baked. The partial pressure of $\text{O}_2$ showed a disproportionate increase at the highest gas pressure.

In order to understand the source performance, the gas supply to the FI emitter must be known. From equations (5.19) and (5.20) an expression for the supply of neutral gas molecules to the high field region of a spherical emitter can be obtained

$$Z = A \frac{P F_0}{2kT} \left(\frac{\alpha}{m}\right)^{\frac{1}{2}}.$$  \hspace{1cm} (6.3)

$A$ is the area of the high field emission, $P$ is the gas pressure in the region of the tip, $k$ Boltzmann's constant, $T$ the gas temperature, $F_0$ the field at the emitter surface, $\alpha$ the gas polarizability, and $m$ the atomic mass of the gas molecule. It can be seen from this that for a given tip radius, temperature and electric field, the gas pressure linearly controls the gas supply to the emitter.

Gas pressure in the source was measured at two points. The thermocouple gauge measures the gas pressure just prior to the Plenum chamber and the cold cathode or mass spectrometer ionization gauges measures the gas pressure outside the differential pumping aperture. These two gas pressures, along with a knowledge of the physical characteristics of the gas system, were used to calculate the gas pressure in the Plenum chamber and provide an estimate of the gas pressure at the emitter.

Gas throughput was controlled by the same high precision leak valve that
was used with the RF ion source (see Section 5.3). The gas was delivered to this valve at a fixed pressure of 4 bar.

Gas dynamical equations were considered in four sections. The first section (S1) was from the thermocouple gauge through its mounting block to the emitter-holder flange. This section was considered to be a tube of diameter 3.2mm. The second section (S2) was the gas line through the emitter-holder flange, and was treated as a tube of diameter 1.6mm. The Plenum chamber was the third section (S3) and assumed to be a tube of 31.75mm diameter. The differential pumping aperture was considered the fourth section (S4). Each of these sections may be seen in Figure 6.1.

The gas transport phenomenon depends on the pressure. Three distinctive domains exist: the viscous, intermediate and molecular domains. They are defined for a particle gas at a given temperature by the product of tube diameter and gas pressure. During source operation the gas pressure was never high enough to induce viscous flow in any of the four sections. Most often the source was operated in the intermediate pressure domain for S1, S2 and S3. S4 remained in the molecular domain.

In order to calculate the pressure in each section, the conductance (C) for the relevant flow regime must be calculated. The throughput (QS₁) of a section S₁ (I=1,2,3 or 4) is related to the conductance Cₛ₁ and pressure differential across the section ΔPₛ₁:

\[ Q_{S₁} = C_{S₁}ΔP_{S₁} \]  \hspace{1cm} (6.4)

For steady state gas flow conditions \( Q_{S₁} = Q_{S₂} = Q_{S₃} = Q_{S₄} \). Hence by calculating the conductance for each section from its physical geometry and knowing the pressure boundary conditions measured by the thermocouple gauge and the cold cathode/mass spectrometer, the pressure in each section may be found. In this way the gas pressure in the Plenum chamber was calculated. It was assumed that the gas pressure in the region of the tip was equal to the Plenum chamber pressure, as the emitter was placed back from the cathode a distance equal to or greater than the differential pumping aperture diameter.
6.3 Emitter tip construction

A variety of materials have been used to form emitters for both the FIM and FI ion sources (Hr 68)(Le 80). All of these materials are highly refractory metals and have been chosen for their abilities to withstand the stress produced by the high electric fields essential for field ionization. Owing to the need in the present work to field ionize hydrogen, the emitter had to be capable of withstanding electric fields as high as $4 \times 10^{10}$ Vm$^{-1}$. Emitter materials suitable for this work needed a field evaporation limit close to or above this value. Table 6.3 lists possible emitter materials and their field evaporation limits. The factors controlling the choice of the emitter materials are: ease of etching, crystal structure of drawn or formed wire, and resilience to reactive gas etching.

### Table 6.3: This table shows a selection of metals with the highest evaporation fields (Mü 69). These fields are approximate; the actual evaporation field depends on tip endform and temperature.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Evaporation Field $\times 10^{10}$ Vm$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>5.7</td>
</tr>
<tr>
<td>Ir</td>
<td>5.0</td>
</tr>
<tr>
<td>Re</td>
<td>4.8</td>
</tr>
<tr>
<td>Pt</td>
<td>4.75</td>
</tr>
<tr>
<td>Mo</td>
<td>4.5</td>
</tr>
</tbody>
</table>

6.3.1 Tungsten emitters

Tungsten has been a favoured material for use as an emitter since the evolution of the field emission microscope (Mü 37). It has the dual virtues of being easily etched to a submicron termination radius and having a high field evaporation limit. The ion source was initially operated with tungsten FI emitters. Due to the extensive use of this metal for both FE and FI emitters, the many tungsten etching techniques have been well documented (Go 61)(Mü 69)(Hr 68). The tungsten
emitters used in this work were fabricated by an electrolytical etching technique that was initially used for the formation of tungsten FE emitters (Dy 52). A schematic of the etching technique is shown in Figure 6.6.

The emitter was shaped from a section of 125µm diameter polycrystalline tungsten wire that was spot welded to the apex of a filament of 250µm diameter polycrystalline tungsten wire (see Figure 6.7A). The emitter wire was then partially immersed in a solution of 1N NaOH maintained at room temperature. The etchant was held in a clear glass beaker, so that the etching could be observed with a microscope. A low D.C. voltage was then applied between the emitter and a Ni electrode in the etching bath. The etching current was monitored by a milliammeter in series with the emitter.

The tungsten wire is etched preferentially at the surface of the etchant. This results from a convective flow within the etchant that is set up by the motion of the higher density etchant that contains reaction products from the wire surface. Fresh etchant is supplied to the top of the wire, so it etches at a faster rate than the lower section, forming a waist with an even taper.

The final tips were required to have a termination radius of approximately 0.1µm and to have a smooth and even taper. Parameters controlling the etching conditions were varied in order to achieve reproducible high quality results. The final etching technique, described in the following text, was generally successful for 125µm diameter wire and produced an etched tip in approximately 8 minutes.

Initially surface protrusions on both the emitter and hairpin filament were removed by rapid (~ 20s) preliminary etching of the entire assembly. This full immersion etching eliminates spurious off-axis emission sites.

It was found that the length of the emitter taper was dependent upon the concentration of the etchant, and was reduced with etchants of high concentration (etchants with concentrations as high as 4N were used). The 1N NaOH solution was chosen for use as it produced suitably formed emitters, with tapers approximately 0.75mm in length in less than 10 minutes of etching.

The depth of immersion of the wire proved to be a critical factor in controlling the quality of the resultant emitter. As the etching proceeds the length of the wire in the etchant is formed into a waist with an attached section of etched
Figure 6.6

Schematic of the tungsten etching technique. Etching conditions were varied to achieve the desired tip radius and taper length (details in text). The support block was attached to a micromanipulator to offer control over the immersion depth and the beaker volume was 500cm$^3$ to ensure an adequate supply of fresh etchant.
Figure 6.7

A:

Photograph of the mounting of an emitter. The 125μm diameter emitter wire is spot welded to a hairpin (radius 2.5mm), formed from 250μm diameter tungsten wire. Both the emitter and hairpin were located in a jig to maintain alignment during the welding.

B:

SEM micrograph of an emitter which exhibits an even taper free of micro-protrusions.

C:

SEM micrograph of the termination of a tungsten emitter. The bar associated with the micrograph indicates a length of 10μm.
wire. The waist eventually erodes to the point where it can no longer support the weight of the wire suspended beneath it and so fractures. The emitter termination is then rapidly smoothed by the continuing etch. The fracture point depends on the length of the discarded section, and so its position varies with the initial immersion depth. It was found necessary to immerse the emitter blank into the etchant to a depth of 3mm prior to commencing the etch to ensure that sufficient taper was produced in the finished emitter.

An applied voltage of 12V D.C. was chosen as it gave an effective etching rate without excessive gas production, which is liable to disturb the surface of the etchant and affect the evenness of the taper of the emitter. With this applied voltage the etching current initially was approximately 6mA. As the etching progressed the surface area of the emitter was reduced and a gradual reduction in the etching current resulted. After approximately three minutes the waist in the wire was reduced until the break-off occurred. This event was signaled by a rapid fall off in the etching current (usually dropping from 3mA to 1µA). The circuit was then immediately broken by the use of the tapping switch. If there was a delay in removing the voltage from the tip then significant rounding of the endform occurred. In general it was necessary to remove the voltage within 0.5 seconds of the drop-off occurring.

After formation the emitters were examined with either a SEM or an optical microscope. Ill-formed or blunt emitters were rejected. A satisfactory emitter is shown in Figures 6.7B and 6.7C. Figure 6.7B is a SEM micrograph of the end of the emitter showing the even taper produced in the etch. Figure 6.7C is a micrograph of a tungsten emitter tip with a termination radius of 0.1µm.

6.3.2 Iridium emitters

Iridium has also been used as an emitter in the FIM (Mü 69) and some FI sources (Or 75)(Al 88). Its use has been restricted however due to the difficulties encountered in etching an iridium wire blank to a suitable emitter with a submicron termination radius. Iridium was considered for use as an emitter material because of its known resistance to reactive gas etching within the source (Or 75) and its ability to tolerate the necessary high electric fields. Polycrystalline iridium wire of 150µm
diameter was used for the tip formation (Supplier: Goodfellows, UK), and this wire was spot welded to a formed tungsten wire filament. Initial work was conducted with ammonium carbonate \((\text{NH}_4)_2\text{CO}_3\) as an etchant. The etching geometry used was the same as that shown in Figure 6.6, and both A.C. and D.C. voltages up to 15V were used. With this technique tip formation was hindered greatly by the production of gas from the wire endform. The rising gas protected the upper section of the wire from attack by the etchant and prevented the formation of the waist necessary for the fabrication of an emitter. Despite etchant currents as high as 15mA, as both a saturated and unsaturated solution \((\text{NH}_4)_2\text{CO}_3\) proved a weak etchant for iridium and required etching times of over one hour to reduce the wire length by 8mm.

Subsequent iridium etching was performed with sodium hyperchlorite (NaClO) as an etchant. Solutions of NaClO ranging from 5% to 50% proved to be a strong etchant of iridium. Again direct immersion of iridium wire resulted in preferential etching and gas production from the wire endform. The etching results were similar to that obtained for \((\text{NH}_4)_2\text{CO}_3\), but were achieved in 1/20 of the time when a 10% solution of NaClO was used.

In order to force the formation of a waist in the wire, a 20% dilute solution of NaClO etchant was floated on liquid CCl₄. The iridium wire then penetrated completely through the thin (2→2.5mm) layer of etchant and into the inert CCl₄. A platinum electrode was then suspended in the etchant and 5V A.C. was applied between the emitter and the electrode. This technique was successful in limiting the electrochemical attack to a 3mm section of the wire. Etching currents as high as 18mA were employed and typically etching would be completed and the wire severed in 90 seconds. This method did not however offer a reliable method of producing suitable endforms. Surface tension effects inhibited control over the etching, making it very difficult to produce the desired conical taper, and bubbles adhering to the lower end of the wire, which was submerged in CCl₄, tended to bend the wire just prior to drop-off, resulting in a twisted endform. Despite the rapid removal of the applied voltage at the instant of drop-off, tip terminations produced by this technique were greater than 1µm in radius and hence unacceptable.
Following the suggestion of Orloff (Or 84) a second method was used to etch a waist in the iridium wire. This method involved the use of an extremely small platinum electrode that contained a drop of etchant, held in position by surface tension. In order to replenish the rapidly exhausted etchant and sweep away reaction products, a drip-feed system was added. This method is depicted in Figure 6.8. Using this technique a variety of etching conditions were investigated before a successful and reliable method was developed.

Prior to commencing the etch, the entire emitter-filament structure was pre-etched in a bath of 5% NaClO for 10 seconds. This cleaned the wire surfaces and removed microprotrusions.

The actual etching was initiated by aggressively etching the fresh wire with a high concentration solution. Using the micromanipulator control, the wire was extended through the electrode 1.8mm. A drip-feed of 50% NaClO was introduced and a voltage of 10V A.C. applied (both square and sine waves were applied with identical results). The initial etch rate was high, drawing a current of 200mA, and etching over a 2.5mm length of the wire. A photograph of the etching under these conditions is shown in Figure 6.9A. These etching conditions were maintained for approximately 40 minutes by which time a 70µm diameter waist was etched in the wire over a 1mm length (see Figure 6.9B.). Further etching required continuous observation and extreme care. The loop was moved 0.4mm down the wire and the solution changed to 5% NaClO. Etching continued for 20 minutes at a current of 120mA, and the waist was reduced to 10µm diameter. The voltage was then lowered to 5V and etching continued at a current of 60 mA. When the minimum diameter of the waist had been reduced to 2µm, the entire emitter-electrode arrangement was lowered into a beaker of 5% NaClO. It was necessary to perform this action with great care, to avoid mechanically stressing the thinned iridium. The voltage was further reduced to 2V A.C. and the tapping switch was used to carefully control the etching rate. Under these conditions the waist was etched away over a 10 minute period. The voltage was immediately withdrawn when the end-section of the wire fell away.

This method, though time consuming, consistently produced satisfactory iridium emitters. The emitter tapers were clean and free of pitting and protrusions.
Figure 6.8  
Schematic of the iridium etching technique. Etchant is introduced into the platinum loop from a drip-feed source. Each drip flushes out contaminated etchant and re-establishes the etching process. A micromanipulator is used to accurately position the electrode loop and a tapping switch is used to control the etch just prior to the drop-off.
The Micromanipulator is used to control the position of the Pt loop.
Figure 6.9

A:
Close-up of the iridium etching technique. The electrode loop has an internal diameter of 0.5mm and the volume of each droplet of etchant is $8\mu l$. In the upper left is the capillary tube for the drip-feed system which supplied fresh etchant at the rate of one drop every two seconds. In this photograph the etching rate is high and the etchant is discoloured by gas bubbles and reaction byproducts.

B:
Partially etched iridium wire. The length of wire located within the droplet region can be seen etched away. This result was obtained after 1.5 hours.

C:
Termination of an iridium tip. The taper is clean and free of microprotrusions. The radius of this tip is 0.1$\mu$m.
(see Figure 6.9C.), and tip radii were less than 0.3µm.
Chapter 7

Performance of the Field Ionization ion source

7.1 Introduction

This chapter presents the results of operational tests for the FI ion source described in Chapter 6. The source was mounted on a test bench adapted from the RF test bench discussed in Section 5.3. Initially the source was operated with tungsten emitters. However, emitter degradation was found to occur after an unacceptably short period of operation. This problem was overcome by the use of iridium emitters. It was then possible to measure the characteristics of the source, and to evaluate its usefulness as a high brightness source, suitable for microprobe application.

7.2 The FI ion source test bench

The test bench detailed in Section 5.3 was modified to accommodate the FI ion source. All RF ion source equipment was removed. As beam measurements were to be conducted inside the source, the glass viewing port at the front of the monitoring chamber was not required, and so the turbo molecular pump was relocated to this port. This gave the pump unimpeded access to the monitoring chamber. The monitoring chamber was then pumped at 170 l/s for N₂ at pressures less than 1 × 10⁻³ mbar and H₂ was pumped at 110 l/s at pressures less than 5 × 10⁻⁴ mbar. Under these conditions the chamber was consistently able to be pumped to
below $5 \times 10^{-8}$ mbar in less than 12 hours. The relevant gas spectrum is shown in Figure 6.5A. The vibration coupling of the turbo pump to the ion source chamber was found to be negligible, with the upper limit to the vibration amplitude along the X, Y and Z axes being less than 0.02µm. This was in agreement with the manufacturers claims (Ba 80). The pumping provided by the turbo molecular pump was augmented as required by the ion pump attached to the bottom of the monitoring chamber (as shown in Figure 5.1). The source gas was stored in a 2.8m³ bottle and supplied to the precision leak valve via an ultra-clean regulator (Supplier: C.I.G.).

Effective use of a FI source requires that the energy spread of the ion beam be minimized. This necessitates the use of a high stability power supply for the emitter voltage. A Bertan 612A power supply was used in this role. The supply provided a $0 \rightarrow 50$kV voltage range and a stability of 10ppm. This stability was achieved at constant room temperature by using a highly regulated line voltage from a Stabilac supply. The Bertan supply was capable of a current output of up to 300µA, an overload protection circuit limiting the supply output to this maximum current. The supply was remotely controlled by resistive programming and both power supply voltage and current outputs were monitored directly from the supply to an accuracy of ±10V and ±10µA respectively.

The emitter tip is susceptible to damage caused by an uncontrolled spark-over between the emitter and the cathode. Such an event can draw up to 300µA from the supply, and dissipates sufficient energy to damage catastrophically the emitter by melting and distorting the endform. These discharges were quenched by a $10^{10}$Ω protection resistor placed in series with the tip. This is shown in Figure 7.1. A low resistance ($R_{int} = 1000Ω$) electrometer was also placed in the voltage supply line. The electrometer provided a true measure of the total current to a sensitivity of 0.1nA and allowed the emitter voltage to be calculated accurately. The total current measured in this way did not provide an accurate measure of the beam current produced by the source. The total current included earth leakage current and a contribution due to backstreaming electrons from secondary electron production at the cathode (see discussion in Section 7.4.1). The high voltage lead was initially shielded by an earthed sheath; however it was found that this
Figure 7.1

A schematic of the FI ion source and its associated instrumentation. The shaded region indicates the ceramic emitter-holder. The protection resistor ($10^{10} \Omega$) and the electrometer in the high voltage line were both electrically floated on a platform insulated by Pactene rods and designed to minimize leakage currents. The electrometer scale was able to be remotely changed whilst the unit was at high voltage.

A pair of scanning apertures, were driven by precision linear motion drives mounted in the 70mm ports located in the source-housing wall.

A Keithley 603 electrometer was used to measure the beam current incident in the Faraday Cup. This electrometer permitted beam currents to be measured with a sensitivity of $1 \times 10^{-13} \text{A}$. 
contributed significantly to the leakage current. The sheath was removed and at
8kV the total leakage current in the high voltage line dropped by a factor of ten
to 120pA. A calibration graph was formed for the earth leakage as a function of
line voltage, and this graph was used to correct the total current measurements,
so that the total emitter current could be established.

All current measurements, apart from the total current measurement in
the high voltage line, were performed with Keithley 603 electrometers. Careful
shielding of the Faraday Cup and its output line permitted beam currents to be
measured to a sensitivity of $1 \times 10^{-13}$ A, with the electrometer providing a current
accuracy of ±2% FSD.

The effective lower limit at which FI currents can be measured is determined
by background ionization events resulting from the presence of a high voltage
applied to the emitter filament. In order to gain knowledge of these residual
ion currents, the source was operated without an emitter, but with the support
filament in position. This hairpin filament was described in Section 6.3.1.

At ultra-high vacuum ($P = 5 \times 10^{-8}$ mbar), no ion current was recorded
in the Faraday Cup, whilst voltages of up to 30kV were applied to the filament.
This situation changed markedly when the source gas was introduced. Figure
7.2A shows the residual positive ion currents measured in the Faraday Cup, as a
function of the applied voltage for a variety of source pressures. These currents,
it was deduced, resulted from non beam ionization, as the currents remained the
same even when the aperture strip was wound into a position which blocked off any
direct path between the cathode aperture and the Faraday Cup. The ionization
current was found to increase with gas pressure but not applied voltage. It was
assumed during FI current measurements that the residual ionization current was
present, and the value of that current was taken as the limitation to current
sensitivity for a given gas pressure.

7.3 Performance of tungsten emitters

The ion source was initially operated with tungsten FI emitters. These were con-
structed by the method outlined in Section 6.3.1. A limitation was immediately
Figure 7.2

A:

The residual ionization current impinging on the Faraday Cup during FI current measurements. The three curves are for different plenum chamber pressures. A: $2.5 \times 10^{-2}$ mbar, B: $1.5 \times 10^{-2}$ mbar, C: $1.7 \times 10^{-3}$ mbar. No current was measured in a vacuum of $5 \times 10^{-8}$ mbar.

B:

The FI current-voltage characteristics for a tungsten emitter at 293K. The emitter radius was 0.25µm and the emitter cathode separation 3.4mm. A gas pressure of $4.3 \times 10^{-4}$ mbar was used and the beam half angle was 279 mrad.
apparent when the tungsten emitters were used. An obvious current fall-off occurred during source operation. Under conditions of constant voltage and gas pressure, the ion current was observed to decrease at rates up to 1% of the initial current every 10 seconds.

From equation (5.21), it can be seen that theory predicts that the FI ion current is a function of the two interdependent variables $F$ and $r_t$. The decrease in FI current could only have resulted from a change in the tip structure. This change came about from the modification of the tip endform due to reactive gas etching. This field induced etching occurs due to the presence of nitrogen and water molecules in the vicinity of the tip (Mü 69)(Or 75). These two molecules, unlike low ionization potential molecules, are able to approach the tip in the low field region on the emitter shank and chemically react with the substrate atoms; this results in the formation of surface complexes, which are field evaporated at field strengths lower than that necessary for field evaporation of pure tungsten. This emitter degradation results in an increase in tip radius, which for a given tip voltage, reduces the electric field at the surface of the emitter ($F_o$), which in turn decreases the ionization probability and ion current despite an increase in the emission surface area. This form of tip etching cannot be controlled and was ultimately found to render the emitter unusable.

Nevertheless, it was possible to investigate source characteristics with tungsten emitters. Figure 7.2B shows the current-voltage characteristics obtained with a tungsten emitter. Two distinct gradient regimes can be seen, these regimes occurring on either side of the characteristic FI inflection point (or knee), which is found at $F = 2.3 \times 10^{10}$ Vm$^{-1}$. The relatively large tip radius of 0.25 µm required the application of a voltage of 21kV in order to achieve this field value. Due to emitter degradation, reproducibility of these characteristics was poor with an accelerated rate of emitter degradation being observed at high voltages.

The rate of emitter degradation as a function of time was monitored by sampling the beam current every 0.5 seconds. It is known that even under optimum conditions short term current fluctuations occur during FI of a gas (Or 75) (Be 77), with low frequency fluctuations affecting FIM images (Mü 69). These fluctuations result from the random activation and deactivation of emission sites due to the
presence of absorbates on the tip. The effect is independent of gas pressure and temperature (Or 75), depends very strongly on the applied electric field and is largest at the point of inflection. This indicates that, in order to reduce the statistical significance of the current fluctuations, large tip radii and the high current regime should be favored.

Figure 7.3A shows the short term current fluctuations recorded for a tungsten emitter. The beam was sampled at a frequency of 2Hz and this limited the sensitivity of the measurement. The fluctuations in FI beams have been frequency-analysed by Orloff and Swanson (Or 77), and their work found almost all of the frequency spectrum to be less than 10Hz, with the dominant frequency component being less than 2Hz. This indicates that Figure 7.3A should provide a reasonable representation of the short term beam fluctuation. The fluctuations range over ±2% of the beam current, and during the displayed time interval there was no net decrease in the current.

Long term emitter degradation was of far greater significance. Figure 7.3B shows the result of tungsten emitter degradation over 2000 seconds. The field induced etching of the tip can be seen to limit dramatically the effective lifetime of the emitter. The current remained within a range of ±25% of the initial current value for 1000 seconds, thereafter it decreased linearly at the rate of 0.06pAs⁻¹ until the current was 12% of the initial current value after 2000 seconds. Attempts were then made to reform the emitter. The option of thermally heating the emitter to promote the formation of a hemispherical emitter from the surface migration of the tungsten atoms was not available with this source. As an alternative, field evaporation of the emitter was attempted. This was conducted in a vacuum and resulted in field enhancement at protruding lattice sites; preferential field evaporation occurred in these areas and the tip was smoothed. However as such smoothing takes place the overall tip radius increases as the endform moves up the conical shank of the emitter. For this work field evaporation of the tungsten emitters stabilized the current output for short periods of time (up to 100 sec) but failed to rejuvenate the tips and often produced tips with radii greater than 0.5µm, which were unusable because of the excessive emitter voltage then required.
Figure 7.3

A:

Short term current stability for a tungsten FI emitter. The emitter tip radius was 0.3µm and the emitter-cathode separation 3.9mm. A gas pressure of $1 \times 10^{-4}$ mbar was used at 293K. The emitter voltage was 24kV and the data was collected via a computer-driven voltage sampling circuit, that monitored the voltage output of the electrometer, at 0.5 second intervals.

B:

Current stability for a tungsten emitter over a longer time period. The emitter radius was 0.25µm and the emitter-cathode separation 3.4mm. A gas pressure of $2 \times 10^{-4}$ mbar was used at 293K. The applied voltage was 17.6kV.
7.4 Performance of iridium emitters

Iridium has been established as an emitter material which particularly resists reactive gas etching (Or 75). Hence due to the severe tip degradation that occurred with tungsten emitters, polycrystalline iridium emitters were used in the source.

It was immediately obvious that these emitters were capable of stable operation in the ion source vacuum system. Figure 7.4A compares the stability of an iridium and a tungsten emitter. The iridium stability test was conducted at a gas pressure of $4.3 \times 10^{-3}$ mbar and an applied emitter voltage of 12kV, which corresponded to an electric field of $2.6 \times 10^{10}$ Vm$^{-1}$. The emitter had been operated for a total of 46 hours prior to the stability test. The emitter had a varied history. Operational time was divided between stability measurements at various gas pressure and voltage values, current-voltage characteristic measurements and angular distribution measurements. Electric fields of up to $4.5 \times 10^{10}$ Vm$^{-1}$ had been used in that time but no evidence of emitter-cathode sparkover had been observed. The emitter had not been field evaporated.

Immediately prior to the stability test, a current-voltage characteristic was measured for the source. This is characteristic 1 shown in Figure 7.4B. The position of the FI inflection point for this characteristic confirmed that the emitter radius was still 0.1µm after 46 hours of operation. The emitter voltage was reduced to 12kV from 19kV at the completion of the characteristic measurement and the source conditions were held constant for 9.5 hours. During this time the beam current was found to fluctuate by ±3% about the average current value of 1520pA, indicating that the iridium emitters offered a substantial improvement in emitter stability over the tungsten emitters. Following the stability run, the emitter characteristic was remeasured, the result being shown as characteristic 2 in Figure 7.4B.

Little difference was found between the two characteristics. This difference is illustrated in Figure 7.5A, which shows the ratios of the currents (Char. 1/Char. 2) at given emitter voltages. The different sizes of the error bars reflect the accuracy of the electrometer on different scales. If the characteristics before and after the stability test were identical, then the ratio plot would be found along the
Figure 7.4

A: The top graph shows a comparison of current stability between an iridium emitter and the tungsten emitter shown in Figure 7.3B. The lower graph is a linear representation of the current stability test for the iridium FI emitter. The emitter tip radius was $\sim 0.1 \mu m$ and the emitter-cathode separation 4.8mm. A gas pressure of $4.3 \times 10^{-3}$ mbar was used at 293K. The emitter voltage was maintained at 12kV. The current stability remained within $\pm 3\%$ of the average current of 1520pA for the duration of the test.

B: Current-voltage characteristics for the emitter described in A. Characteristic 1 (circles) was measured before the stability test shown in A, and characteristic 2 (squares) was measured after the stability test. Identical source conditions were maintained during the stability test and the measurement of both characteristics.
Figure 7.5

A:

The ratio of the current values for the two characteristic curves shown in Figure 7.4B.

B:

The ratio of current values for successive characteristic curves under the emitter conditions given in Figure 7.4A. Data set 1 (represented by data points with error bars) is for the initial cycling of the emitter voltage, Data set 2 is depicted by a dashed line joining each data point, and was collected at the same voltage intervals as Data set 1. Data set 2 represents the second cycling of the emitter voltage. For clarity no error bars are drawn for Data set 2; the error bars for those data closely resemble those of Data set 1. The characteristics for each cycle are very similar to Figure 7.4B.

C:

Beam current as a function of gas pressure for an iridium emitter. The applied voltage was 12kV, which corresponded to an electric field of $2.5 \times 10^{10}$ Vm$^{-1}$ at the surface of the emitter. The beam half angle was 223mrad and the emitter-cathode separation was 4.8mm.
dotted line. A significant deviation was observed for voltages less than 10.5kV, indicating the greater amount of current available at low fields after the stability test. This can be compared with the effect observed, under the same conditions, when the emitter voltage is taken up to 15kV and back to zero twice in succession, without the effects of an intervening stability test. Such a cycle is shown in Figure 7.5B. Data set 1 in this figure shows the ratio of currents between two characteristic curves. The ratio was formed by dividing the current obtained as the voltage was increased towards its maximum of 15kV (Current up), by the current obtained as the voltage was decreased (Current down). The current-voltage characteristics were mapped out twice in succession by this method, the second result being shown by Data set 2. The two graphs show that a high level of reproducibility is obtained during the voltage cycles and as the two graphs show a similar form, they indicate that a similar degree of minor tip modification is occurring during the voltage cycles. Only current values between 9 and 9.5kV show true deviation from the preceding half cycle, within the limits of experimental accuracy. By comparison, Figure 7.5A shows that the emitter has been reformed during the stability test in such a way as to increase the current yield at low voltages. This effect could be the result of the bombardment of negative ions, which can produce small zones of high electric field.

7.4.1 Current output of iridium emitters

Given that this FI source was to operate with hydrogen gas at 293K and that a beam of sufficient brightness, angular intensity and total current is required to form a focused beam suitable for injection into the Pelletron accelerator, the available source variables were investigated with the initial aim of maximizing beam current. From equation (5.21), it can be seen that the beam current is expected to be linearly dependent upon gas pressure in the supply-limited regime for tip fields greater than $2.3 \times 10^{10}$ Vm$^{-1}$. Figure 7.5C shows the beam current produced by an iridium emitter ($r_t \sim 0.1\mu$m) as a function of the gas pressure in the region of the emitter. For this work, the emitter was operating in the high field regime and Figure 7.5C reflects the expected linear response to gas pressure. The gas pressure in the region of the tip is dependent upon the emitter-cathode
separation. The differential pumping aperture was either 1.3 or 2.3mm in diameter and the flow through this aperture produced a gas pressure profile into the Plenum chamber. This region of lower gas pressure was assumed to extend a distance into the Plenum chamber comparable to the pumping aperture diameter and so the emitter-cathode separation was chosen to avoid this low pressure region.

Due to the linear dependence of the FI current upon gas pressure it is desirable to operate the source at the highest gas pressure possible without causing the deleterious effects of emitter-cathode sparkover. The maximum pressure which permitted effective FI source operation for emitter-cathode separations of greater than 2.3mm was $7 \times 10^{-3}$ mbar. Figure 7.6A shows the effect of a fatal sparkover on an emitter. The dotted curve was measured after the sparkover. Although the emitter was destroyed, a small number of ionization sites were still present as the measured current was greater than the residual ion current shown in Figure 7.2A.

Increasing the gas pressure also increases the energy spread ($\Delta E$) of the FI beam. The magnitude of $\Delta E$ affects the effective brightness of the source as it determines the chromatic aberration produced by the source lens. As it was desirable to minimize the pressure broadening of the ion beam energy distribution, the gas pressure was limited to ensure that the mean free path of the ions was an order of magnitude greater than the emitter-cathode distance ($d_o$). For $d_o = 2.3$mm, this corresponded to a maximum pressure of $\sim 7 \times 10^{-3}$ mbar.

As shown in Figure 7.4B the current-voltage characteristics of a given tip varied with use due to surface modification effects. Also, different emitters exhibited contrasting emission characteristics and hence the current-voltage characteristics varied from tip to tip. This is highlighted by two examples in Figure 7.6B. The solid line is a characteristic obtained for a 0.1$\mu$m radius tip at a gas pressure of $1.6 \times 10^{-3}$ mbar. The dashed line was recorded for a 0.15$\mu$m tip under the same conditions. The shape of the inflection point is different for each emitter, with the larger radius emitter consistently producing the greater current.

As the cathode was able to be electrically isolated, the cathode current was measured as a function of emitter voltage. Similarly the placement of an electrometer in series with the tip permitted measurement of the total emitter current. Both of these currents were investigated in conjunction with the measurement of
Figure 7.6

A:

The effect of a severe sparkover on emitter characteristics. The solid line was measured for an \( \sim 0.1\mu m \) radius emitter immediately prior to a sparkover at 19kV and \( 5 \times 10^{-3}\) mbar of gas pressure. The emitter-cathode separation was 4.8mm. After the sparkover the endform had evaporated and the emitter was terminated by a flat conical cross section 2µm in diameter. The characteristic measured under these conditions is shown by the dotted line. The electric field values only apply to the undamaged emitter.

B:

Beam current as a function of the electric field at the surface of the emitter (\( F_o \)). The solid line was recorded for an emitter with \( r_t \sim 0.1\mu m \) and the dashed line was from an emitter with \( r_t \sim 0.15\mu m \). In each case the gas pressure was \( 1.6 \times 10^{-3}\) mbar.

C:

A comparison between the beam current (solid line) and the cathode current (dashed line), as measured by an electrometer between the cathode and ground. The scale for the cathode current is on the right hand vertical axis. The emitter radius was \( 0.06\mu m \) and the gas pressure was \( 5 \times 10^{-3}\) mbar.

D:

A comparison between the beam current (solid line) and the total current (dashed line), as measured by the electrometer in series with the emitter. The scale for the total current is on the right hand vertical axis. The emitter radius was \( 0.1\mu m \) and the gas pressure was \( 5 \times 10^{-3}\) mbar.
Figure A: Beam current as a function of applied voltage and electric field.

Figure B: Beam current at different applied voltages and electric fields.

Figure C: Comparison of cathode current and total current with applied voltage.

Legend:
- Solid line: Beam current
- Dashed line: Cathode current
- Dotted line: Total current
beam current. This was done in order to compare results with those presented by other workers, where the quoted FI current is derived from a meter in series with the emitter (Or 77)(Le 80). Figure 7.6C shows both the beam and cathode current characteristics, recorded simultaneously. The cathode current consisted of the beam current that was not transmitted through the differential pumping aperture and a secondary electron current which resulted from the ion beam striking the grounded cathode. As can be seen from Figure 7.6C, the cathode current does not exhibit the strong inflection point found in the beam current data. The ratio of the cathode current to beam current is least at the beam current inflection point (6.6kV, see Table 7.1), perhaps reflecting a greater transmission of the ion beam at this field value. Either side of the inflection point the cathode current increases much more rapidly than the beam current, as shown in Table 7.1. From this it appears that the cathode current is not simply proportional to the beam current and it cannot be used to give a reliable measurement of beam current variation.

Table 7.1: Current ratios from Figures 7.6C and 7.6D. The values given are for the upper and lower limits to the emitter voltage. The intermediate value is for the ratio obtained at the beam current inflection point.

<table>
<thead>
<tr>
<th>Figure 7.6C</th>
<th>Cathode current</th>
<th>Beam current</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emitter voltage (kV.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td>6.6</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>Figure 7.6D</td>
<td>Total current</td>
<td>Beam current</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>10.7</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>13.1</td>
<td>6.0</td>
<td></td>
</tr>
</tbody>
</table>

Figure 7.6D shows a pair of beam and total emitter current characteristics. The total current shown here has been corrected for leakage effects. These correc-
tions were less than 8% for each voltage value. The total current, shown by the dashed line and represented on the right hand scale in Figure 7.6D, resulted from the total ion current produced by the emitter, with a significant component due to negative ions and backstreaming electrons. The electrons resulted from secondary production at the cathode and an aperture that restricted the accepted beam half angle ($\alpha_s$) to 489 mrad. It can be seen from this figure and Table 7.1 that the measured total current exceeds the beam current by up to an order of magnitude. This is considerably in excess of estimates of the secondary electron contribution in other systems (Or 77). The total emitter current does not follow the shape of the beam current characteristic below an applied voltage of 11kV and so, for this source, cannot be used to monitor the beam current produced by the source.

The beam current produced by the FI of $\text{H}_2$ is composed of $\text{H}^+$, $\text{H}_2^+$ and $\text{H}_3^+$. Only the $\text{H}^+$ component is of use for the microprobe beam; the other components can be discarded by the accelerator's spectroscopic dipole magnet (see Figure 2.1). The relative abundance of each ion species depends on the ionization field and the protrusion of atomic sites on the emitter surface. The relative abundance of the $\text{H}^+$ ion is critical to the final application of this source to the SPMP. Clements and Müller measured the relative ion abundance obtained with the FI of $\text{H}_2$ as a function of electric field (Cl 62). Their results are presented in Figure 5.10B and show that the $\text{H}^+$ ion is favoured in the high field regime of $F_0 > 2.3 \times 10^{10} \text{V m}^{-1}$, whereas $\text{H}_2^+$ is the dominant ion at lower fields. At a field close to the field evaporation limit for iridium ($F_0 = 5 \times 10^{10} \text{V m}^{-1}$), the $\text{H}^+$ ion is even more strongly favoured with 90% of the beam $\text{H}^+$. The relative ion abundances obtained by Clements and Müller have been used to derive the expected composition of the ion beam from this source. Figure 7.7 shows a measured characteristic (solid line) for beam current obtained with the use of $\text{H}_2$ in this FI source. The figure also shows the anticipated ion currents produced during the source operation. It can be seen from this figure that the use of fields greater than $2.3 \times 10^{10} \text{V m}^{-1}$ is desirable in order to take advantage of the predominance of the $\text{H}^+$ ion in the high field domain. In contrast to this, the yield of $\text{H}^+$ ions is unacceptably small during source operation at fields of less than $2.0 \times 10^{10} \text{V m}^{-1}$.

In order to operate the Pelletron accelerator a beam of at least 1nA is
Figure 7.7

The expected composition of the FI beam. The solid line indicates the total measured beam current as a function of electric field for an iridium emitter of 0.1μm radius, operating at a gas pressure of $5.7 \times 10^{-3}$mbar. The acceptance half angle of the beam was 234mrad. The broken lines represent the ion components of the beam. The data for the relative ion abundance were taken from (Cl 62).
required from the ion source. Most of this beam is used for current feedback in the energy stabilization system that monitors the beam on the object and image slits that are located before and after the dipole magnet (Figure 2.1). In order to determine the upper limit to the total ion current available from this source, the ion source parameters were optimized. Figure 7.8A shows the beam current-voltage characteristic for the source conditions that resulted in a maximum current output. The gas pressure in this example was limited to $5 \times 10^{-3}$ mbar, due to the onset of a discharge in the gas. This was registered on the electrometer in the high voltage line as current fluctuations of the order of 100 nA, above the line current of 400 nA. The beam current at this point was 21 nA. The protection resistor suppressed these discharges by lowering the emitter voltage by approximately 1 kV in response to each current fluctuation. While in this way damage to the emitter was avoided, it was not considered prudent to operate the emitter under such conditions for a long time period.

Figure 7.8B shows the result of a current stability test for the emitter discussed in Figure 7.8A. The current output was a maximum, but the emitter voltage had been reduced by 400 V to circumvent breakdown problems. Prior to the test the emitter had been operated under varying conditions for 18 hours. From Figure 7.8B it can be seen that even at the extremely high current output, current stability is good. Some evidence of emitter degradation can be deduced from a straight line fit to the linear data set, which indicates a current fall-off at the rate of 0.029 pAs$^{-1}$. This damage was possibly the result of undetected breakdown events.

### 7.4.2 Beam angular distribution

The beam angular distribution has a critical influence on the focusing of the ion beam. Hence before the source could be used with a lens the beam angular distribution was investigated. This was achieved for a variety of source conditions by using the set of defining apertures and scanning slits discussed in Chapter 6.

The shape of the angular distribution of a FI current has been theoretically predicted to be completely determined by the geometry of the emitter (Go 77) and it has been well established experimentally that the crystallographic orientation of
Figure 7.8

A:

The FI beam current-voltage characteristic that was obtained at the optimum settings for the ion source parameters. A maximum current of 21.6nA was obtained at a voltage of 20.4kV. The gas pressure in the region of the emitter was $5 \times 10^{-3}$ mbar. The emitter radius was $\sim 0.1\mu$m and the emitter-cathode separation was 2.2mm. The half angle of divergence of the beam was 489mrad.

B:

Current stability of the emitter described in A, whilst operating at a high current output. The gas pressure was $5 \times 10^{-3}$ mbar and the applied voltage 20kV.
A

APPLIED VOLTAGE (kV.)

BEAM CURRENT (pA.)

0 5000 10000 15000 20000

TIME (sec.)

BEAM CURRENT (nA)
the emitter governs the shape and extent of the distribution (Or 75)(Or 78a)(Le 80).

The anisotropy of the distribution is primarily due to the presence of high field sites on the crystal structure which regionally increase the gas supply and ionization probability (Mü 69). A number of workers have taken advantage of this by selecting the crystallographic structure of the emitter such that the FI current is confined to narrow emission about the emitter axis (Or 77)(Ha 79)(Le 80). For this work, however, polycrystalline emitters were used. These emitters were formed from drawn iridium wire which has a preferred grain orientation of (111) and (100).

The angular distribution of the beam current for these emitters was measured with a series of small aperture diaphragms which were scanned across the beam and offered an angular resolution of 55mrad (see Figure 7.1). Beam transmitted through these diaphragms was collected in the Faraday Cup and the transmitted current values were corrected for the effect of finite thickness of the diaphragms.

Figure 7.9A shows the beam angular distribution obtained for a polycrystalline emitter at 16.5kV. The full width of the beam subtended a half angle of 500mrad, with the limits being determined by emission sites on the shank of the emitter. The roughly Gaussian shape of this angular distribution is typical for polycrystalline emitters, with 76% of the beam found within a cone of half angle 300mrad. Though this value is typical of that obtained with polycrystalline emitters it is surpassed by cooled (77K), annealed single crystal iridium which can offer an emission half angle of 130mrad (Or 78a), and specialized tip construction techniques with tungsten emitters have yielded sites with extremely confined emission half angles of 8.7mrad (Bö 88). Figures 7.9A and 7.9B show the effect of reducing emitter voltage, upon the angular distribution. As $F_0$ is reduced (from $2.3 \rightarrow 2.13 \rightarrow 2.02 \rightarrow 1.9 \times 10^{10}$ Vm$^{-1}$) the emission pattern changes in a manner that is difficult to predict. Also as the voltage is reduced the distributions indicate that off axis emission sites continue to contribute significantly to the ion current. This is expected with polycrystalline iridium where preferentially the larger (111) and (100) planes are exposed perpendicular to the central emission axis. Higher
Figure 7.9

A:

Comparison between angular distributions obtained for a polycrystalline iridium emitter. The solid line indicates the angular distribution from the emitter at a voltage of 16.5kV, which corresponded to an electric field of $2.9 \times 10^{10}$ Vm$^{-1}$. The gas pressure was $5 \times 10^{-3}$ mbar. Under these conditions a total current of 1460pA was accepted into a half angle of 420mrad. The dashed line denotes the angular distribution obtained when the emitter voltage was lowered 900V and the same gas pressure maintained. At this voltage 800pA was accepted into 420mrad.

B:

Beam angular distributions for the same conditions as in Figure 7.8A. The voltage is lowered to 14.8kV, represented by the solid line, with 290pA into $\alpha_s = 420$ mrad. Finally the angular distribution for a voltage of 13.8kV is shown. The total beam current at this voltage was 87pA.
fields are present on surrounding planes; hence emission sites on the central region of the tip pass into the low-field high-gradient regime on the characteristic curve before the off axis sites. Current output from central axis emission sites then decreases more readily with voltage than the current output from off axis sites.

For the same emitter, the response of the angular distributions to changes in gas pressure is shown in Figure 7.10A and 7.10B. The results for four different gas pressures are presented at two different emitter voltages. The shape of these distributions reflects the emission characteristics of the tip as a function of gas pressure. It can be seen from these figures that the distribution shapes are generally similar for a given gas pressure and hence insensitive to the change in $F_o (2.3 \rightarrow 2.02 \times 10^{10} \text{Vm}^{-1})$. This has already been seen in the two solid line curves in Figures 7.9A and 7.9B. The change in gas pressure produces a marked alteration in the emission properties of the tip, with lower pressures resulting in a larger off axis emission.

The current peaks of these distributions (i.e. the current accepted along the optical axis by the 55mrad scanning system) for gas pressures $1 \rightarrow 3$ are in direct proportion (within 10%) to the linear gas pressure response predicted from Figure 7.5C. Gas pressure 4 is in a region of non-linear response (see Figure 7.5C) and the measured current at 14.8kV is 25% less than the predicted current.

The beam current angular intensity is of major interest to beam formation with a FI source. This is due to the fact that the virtual emission size of the source is of the order of $1 \times 10^{-3} \mu \text{m}$ (Al 88), and in order to effectively focus the maximum current from such an intrinsically bright source a high angular current density is desired. The angular distribution of the ion beam has been used to calculate the angular density assuming an annular emission area (Co 89a). Figure 7.11A shows both the differential and average current angular density as a function of beam half angle for the source conditions described in Figure 7.8A. The density distribution is not strongly peaked as it reflects the broad divergence of the FI beam from a polycrystalline emitter. It can be seen from Figure 7.11A that the maximum current angular density achieved with this source was $0.15 \mu \text{Asr}^{-1}$. This compares with angular intensities of up to $1 \mu \text{Asr}^{-1}$ for (110) oriented iridium tips at 78K (Or 79). Still higher values of angular intensity have been achieved by
Figure 7.10

A:

Beam angular distributions as a function of gas pressure for the polycrystalline emitter used for the data sets in Figure 7.8. The angular distributions were taken at an emitter voltage of 16.5kV. The following gas pressures were used: 1: \(5 \times 10^{-3}\text{mbar}, 2: 4 \times 10^{-3}\text{mbar}, 3: 2 \times 10^{-3}\text{mbar}, 4: 8 \times 10^{-4}\text{mbar}\).

B:

Beam angular distributions as a function of gas pressure for the same emitter as in A. The emitter voltage was 14.8kV. Nos. 1 → 4 denote the same gas pressures as in A.
Figure 7.11

A:  
Beam angular current density as a function of angle for the source conditions given in Figure 7.8A. The solid line is the differential current density \( \frac{dI}{d\theta} \) and the dashed line is the average angular current density \( \frac{I}{\theta} \).

B:  
Total beam current as a function of beam half angle for the source conditions described in Figure 7.8B.
cooling single crystal tungsten emitters to less than 20K. For this work highly confined beams of \(5 \rightarrow 10 \mu \text{Asr}^{-1}\) have been regularly measured (Ha 79)(Le 85). Recent experimental results with local protrusions on field emitters have lead to predictions of hydrogen angular current densities of \(\frac{dI}{d\alpha} = 50 \mu \text{Asr}^{-1}\) (Jo 88).

The average angular intensity can be used to calculate the total beam current as a function of beam half angle (Co 89a). The distribution of beam current as a function of half angle for the source conditions described in Figure 7.8A are shown in Figure 7.11B.

### 7.5 Brightness of iridium emitters

The beam brightness of a FI source operating at a known potential \(V_s\), may be calculated from a knowledge of the beam current \((I_s)\), emission area and angular distribution as indicated by equation (7.1),

\[
B_n = \frac{I_s}{\pi^2 r_s^2 \alpha_s^2 V_s}, \tag{7.1}
\]

where \(r_s\) is the effective source radius and \(\alpha_s\) the half angle of emission.

All ions in a FI source are created at the emitter, which is of submicron dimension. The source, however, may be considered to be virtual and located at the centre of the hemispherical termination of the tip with a finite size due primarily to the transverse thermal velocity component of the ions. The virtual size of an emitter is effectively equivalent to the surface resolution in a field ion microscope. Gomer has derived an expression for the resolution of a field ion microscope, considering the thermal transverse momentum of the ions and the conservation of angular momentum (Go 61). He gives

\[
r_s = 4\beta r_t \left(\frac{kT}{eV_s}\right)^{\frac{1}{2}} \tag{7.2}
\]

as a measure of virtual source radius at gas temperature \(T\), where \(\beta\) is a constant resulting from field deformation due to the presence of the emitter shank. For this work \(\beta\) was taken to be 1.5. Equation (7.2) does not apply for small angles of emission \(\delta \alpha\), where the virtual source is found between the centre of the emitter...
termination and its surface (Wi 73)(Co 89a). Then for $\delta \alpha < 10\text{mrad}$,

$$r_s \approx \frac{1}{2} r_i \delta \alpha .$$  \hspace{1cm} (7.3)

For the emitter described in Figure 7.8A, at large emission angles $0.8 < r_s < 1\text{nm}$. The small value of the virtual source size gives the FI source its intrinsically high brightness. Beam brightness has been calculated for the H$^+$ beam component from a knowledge of the current-voltage characteristics, angular distribution and virtual source size.

Figure 7.12A shows the H$^+$ beam brightness (solid line) and the H$^+$ beam current (dashed line) for emitter conditions described in Figure 7.8A. The absence of published values for the relative ion abundances in the ion beam at fields above $2.8 \times 10^{10}\text{Vm}^{-1}$ limits the expected values of H$^+$ beam current and brightness that can be shown in Figure 7.12A. However an upper limit to the H$^+$ beam brightness can be defined by the total beam brightness which is represented by the dotted line in Figure 7.12A. Typically the source produced a H$^+$ beam brightness of greater than $10^5\text{A m}^{-2}\text{rad}^{-2}\text{V}^{-1}$ for $F_0 > 2.3 \times 10^{10}\text{Vm}^{-1}$. This is illustrated in Figure 7.12B. Here a different FI emitter was used in the source and the emission half angle was reduced to half that used for the emitter in Figure 7.12A. As a result a more intense region of the beam angular distribution was selected, and a marginally greater effective brightness was obtained for this emitter.

Table 7.2 shows the emission characteristics of the source at maximum beam current. The values given are for the total FI beam. From consideration of these

<table>
<thead>
<tr>
<th>Voltage ((\text{kV}))</th>
<th>$r_i$ ((\mu\text{m}))</th>
<th>$F \times 10^{10}\text{Vm}^{-1}$</th>
<th>Press. ((\text{mbar}))</th>
<th>Current ((\text{nA}))</th>
<th>$\alpha$ ((\text{mrad}))</th>
<th>$\frac{dI}{dn}$ ((\mu\text{A sr}^{-1}))</th>
<th>$B_n$ (\text{A m}^{-2}\text{rad}^{-2}\text{V}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.4</td>
<td>0.1</td>
<td>4.1</td>
<td>$5 \times 10^{-3}$</td>
<td>21.6</td>
<td>489</td>
<td>0.155</td>
<td>$10^6$</td>
</tr>
</tbody>
</table>
Figure 7.12

A:

Brightness ($B_n$) of the H$^+$ component of the beam, as a function of electric field, for the source conditions given in Figure 7.8A (solid line). Also shown on the same plot and referencing the right-hand vertical axis is the expected proton current yield as a function of electric field (dashed line). The brightness of the total ion beam is shown by the dotted line.

B:

Brightness (solid line) and current (dashed line) for the H$^+$ component of the FI beam for a different emitter from that used in Figure 7.12A. The beam current for this data was limited to a divergence angle of 234mrad and the emitter radius was 0.1µm. The gas pressure in the region of the emitter was $5 \times 10^{-3}$ mbar.
application and that it offers an increase in brightness of the order of $10^5$ above that obtained from the RF ion source.

### 7.6 Current state of progress with hydrogen FI sources

It is prudent at this point to consider the performance attained by other FI sources. Table 7.3 shows a comparison between the results obtained with the present source and published values of the emission characteristics for other hydrogen FI sources. The values given in this table have been derived from publications where the intrinsic source brightness has either been measured or estimated. All the values given in this table refer to a FI beam containing both $\text{H}^+$ and $\text{H}_2^+$, and with the exception of Source 1, the listed values of beam brightness can only be considered to be a guide to the level of brightness achieved by each source.

Source 1 in Table 7.3 is the present FI source, designed for in-terminal accelerator use. The source current and brightness are shown at the limits of the viable range of the electric field (see both Sections 5.6 and 7.7). The angular intensity value is the maximum obtained for this source. This value was achieved at a higher electric field but is shown to permit comparison with the maximum angular intensity achieved by other sources. The source parameters associated with this maximum in angular intensity are given in Table 7.2. The first half of the entry for Source 2 shows the performance of a field ion microscope operating at room temperature. These data were included to present a comparison between the present source and another operating at the same field and temperature. It can be seen from the table that the differential pumping and high gas pressure used in the present source results in a far greater current than that produced by the FI microscope. The increase in current exceeds that expected from the gas pressure alone by a factor of 1.3. This difference is most likely due to the greater emission half angle used for Source 1.

The second half of the entry for Source 2 shows the effect of reducing the emitter temperature by a factor of 3.8. These values were also obtained from a field ion microscope and can be compared to the values given at room temperature. A significant increase in current is obtained for the cooled emitter. At a field of 2.6
Table 7.3

This table compares the output obtained from the present source (labeled FI Source 1), with the values published for other FI sources or FI microscopes. All the values given are for the FI of hydrogen. The emitter radius for Source 1 was 0.1µm, and the data shown for Source 2 have been transposed from current-voltage characteristics of several field ion microscopes and normalized to emitter radii of 0.1µm using equation (5.22). The emitter radii for Sources 3 and 4 were not published. Source 5 is for FI above a microprotrusion on a FI emitter, the estimated radius of this protrusion being 10nm. Direct comparison between the sources is difficult as published source currents are often the total emitter current and may include a significant secondary electron component. Also, publications frequently give only inexplicit information on the source emission characteristics. This table can be considered to be, at best, a rough guide to the relative performance of the different sources. The values of the intrinsic brightness and current listed for the present source are given at the limits of the viable operational range of electric field values for this source. The total beam current and total beam brightness are presented to permit comparison with the other sources. Source 2 is a listing of the performance of several field ion microscopes as compiled by Levi-Setti (Le 80). The original references are given. Source 3 is a FI source attached to a SIM operated by Orloff and Swanson. Source 4 is a hydrogen FI source intended for use in ion beam lithography. The entire source and lens system is cooled to cryogenic temperatures. The enhanced gas supply thus obtained results in a particularly high beam angular intensity. The values given for Source 5 are the upper limit of the estimated emission characteristics of a microprotrusion on the surface of a FI emitter. These sites promise excellent emission characteristics with small divergence angles and high currents.
<table>
<thead>
<tr>
<th>Field Ionization Source</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>( F \times 10^{10} \text{Vm}^{-1} )</td>
<td>2.2</td>
<td>2.6</td>
<td>2.2</td>
<td>2.6</td>
<td>( \sim 2.2 )</td>
</tr>
<tr>
<td>( \alpha ) (mrad)</td>
<td>489</td>
<td>300</td>
<td>300</td>
<td>( \sim 150 )</td>
<td>( \sim 150 )</td>
</tr>
<tr>
<td>( \frac{dI}{dt_{\text{max}}} ) (( \mu \text{Asr}^{-1} ))</td>
<td>0.15</td>
<td>not given</td>
<td>not given</td>
<td>0.5</td>
<td>10–60</td>
</tr>
<tr>
<td>Pressure (mbar)</td>
<td>5( \times 10^{-3} )</td>
<td>8( \times 10^{-4} )</td>
<td>8( \times 10^{-4} )</td>
<td>1.5( \times 10^{-2} )</td>
<td>10(^{-4} )</td>
</tr>
<tr>
<td>Temp. (K)</td>
<td>294</td>
<td>294</td>
<td>78</td>
<td>77</td>
<td>10</td>
</tr>
<tr>
<td>Current (pA)</td>
<td>920</td>
<td>4600</td>
<td>13</td>
<td>550</td>
<td>2000</td>
</tr>
<tr>
<td>Brightness ( B_n ) (Am(^{-2})rad(^{-2})V(^{-1}))</td>
<td>5( \times 10^4 )</td>
<td>2( \times 10^5 )</td>
<td>not given</td>
<td>3( \times 10^6 )</td>
<td>1\times10(^7)</td>
</tr>
<tr>
<td>Reference</td>
<td>Al 88</td>
<td>Ts 66</td>
<td>Tu 67</td>
<td>Or 77</td>
<td>Le 85</td>
</tr>
</tbody>
</table>
the cooled emitter produces approximately 7 times the current of the field ion microscope at room temperature. This is consistent with the current gain predicted in Figure 5.10A. The current produced by the present source (Source 1) is still greater however by virtue of its higher operational pressure and its larger beam divergence. Due to assumptions made in the calculation of the beam brightness for Source 2 (Le 80), only a rough comparison can be made with the brightness measured for the present source. Theory predicts however that in the high field regime at least a factor of 14 improvement in brightness should be realised by cooling the emitter to 78K. This improvement comes about from the increase in current and the reduction in the virtual emission area given by equation (7.2).

The values shown for Source 3 were obtained from a FI source operated by Orloff and Swanson (Or 77). This source, which was attached to a SIM, used a polycrystalline iridium emitter and achieved a particularly high gas pressure. The current value recorded for this source was measured by a meter in series with the emitter. As has been shown by the present work, such a method of current measurement is unreliable as it contains a large and unpredictable secondary electron component, as well as a further contribution from leakage currents. Because of this the values presented for Source 3 are falsely optimistic. Nevertheless they can be expected to reflect the trend towards the greater current output available with a high pressure source and a cooled emitter.

Source 4 is an advanced hydrogen FI source intended for application in ion beam lithography. The design of this source takes advantage of the enhanced supply of molecules to the ionization zone due to the surface diffusion of physisorbed molecules on a cryogenically cooled tip. Cooling the tip to 10K is technically complex, however substantial gains in the current angular intensity and brightness have been achieved with this source, which also uses an oriented single crystal emitter. The source has only been operated at low fields to minimize the beam energy spread. Hence the selected ion species is $\text{H}_2^+$. Source 5 show the projected values for the use of $\text{H}_2$ in a source which employs microprotrusions on a FI emitter as the sites for ionization. These modified tips yield beams of low divergence and, as the tips are cooled to less than 20K, the angular intensity is particularly high.
All of the sources discussed above, with the exception of Source 1, cannot be considered for use in an electrostatic accelerator; this is due primarily to the requirements of their emitter cooling systems. Additionally the sources have not been designed with the intention of producing a H+ beam. Although at its existing level of development Source 1 cannot match the claimed performance of Sources 3 to 5, the listed values for Source 1 are accurately representative of its emission characteristics and at its present level of development the source produces sufficient current and brightness to be viable for installation in the accelerator.

7.7 Application and further development of the FI source

As was discussed in Section 5.6, the range of electric fields which can be considered for practicable operation of this source lies between $2.2 \times 10^{10}$ Vm$^{-1}$ and $2.6 \times 10^{10}$ Vm$^{-1}$. From Figure 7.12A, for an emission half angle of 489mrad, the proton current available from the source in this field range is between 250pA and 3nA. A field of $2.35 \times 10^{10}$ Vm$^{-1}$ is necessary in order to produce a current of 1nA. A current of this magnitude is necessary, with the existing detector geometry, for the PIXE analysis of thin biological targets, if trace elements are to be measured. Transmission of this current to the target depends critically on the lens and accelerator system. The brightness of the source at a H+ current of 1nA is $5.5 \times 10^{4}$ A m$^{-2}$ rad$^{-2}$ V$^{-1}$. Equations (5.5) and (5.6) can then be used to provide a guide to the resolution improvement offered by this source under ideal ion optical conditions where the transmission of this brightness and current to the microprobe beam line is permitted. The brightness of the RF ion source was measured to be 5 A m$^{-2}$ rad$^{-2}$ V$^{-1}$ (see Section 5.4), and this is exceeded by four orders of magnitude by the FI source when producing 1nA of current. From equation (5.5) it can be seen that this improvement in brightness would correspond to an order of magnitude reduction in the size of a chromatically limited beam spot. From equation (5.6) it may be deduced that a spherical aberration-limited beam could expect a resolution improvement of a factor of 30. These values represent the upper limit to the attainable resolution improvements with this source and may well be impractical due to the highly divergent beam considered above.
It is obvious that the effective use of this source would require the following lens to have an extremely large acceptance angle and very low aberration coefficients. In order to consider PIXE analysis with a less divergent beam it is of interest to examine the source conditions necessary for a beam current of 100pA, the generally accepted lower limit for PIXE analysis. At a field of $2.6 \times 10^{10}$ Vm$^{-1}$ a half angle of 25mrad must be accepted by the ion source lens in order to transmit a 100pA proton beam. Protons following more divergent trajectories could be accepted and focused by the lens, but excluded from the microprobe by its rigid collimation. This divergent beam current (up to 3nA is available if a divergence of 489mrad is tolerable) could then be used for accelerator stabilization.

With the lenses presently developed for use with virtual point sources, beam divergence is limited to about 10mrad so that spherical and chromatic aberrations do not greatly exceed the first order spot size (Le 80). From Section 4.7 it can be recalled that STIM analysis requires a beam current of less than 1fA. For this source, with an angular current density of 150nA sr$^{-1}$, produced at a field of $2.6 \times 10^{10}$ Vm$^{-1}$, sufficient current for STIM analysis is obtained at a divergence angle of approximately 0.01mrad. Hence existing source lens designs could be used with this source for STIM, but a specialized lens would be required in order that the source be acceptable for PIXE analysis. As a result a concurrent program in lens development has been undertaken in order to investigate the possibility of producing a strongly magnifying electrostatic lens with low aberrations. This lens has been designed to have the largest possible acceptance and is the subject of a thesis by Colman (Co 89a). An understanding of the upper limit to the performance of such a lens can be gained by considering the chromatic aberration present in the lens when used with a FI source. The contribution to the effective source radius due to chromatic aberration in the electrostatic lens is given by

$$
\Delta r_{ca} = \frac{1}{2} \alpha_s C_{ca} \frac{\Delta V}{V_s},
$$

where $\alpha_s$ : Half angle of divergence of beam from the emitter;
$C_{ca}$ : Axial chromatic aberration coefficient of the lens;
$V_s$ : Emitter potential;
$\Delta V$ : Beam energy spread.
For an electrostatic lens the theoretical limit to the chromatic aberration coefficient is \( 2f \), where \( f \) is the focal length of the lens. If a working distance of 1mm can be achieved with this lens then it may optimistically be expected that \( f \approx 3\) mm. For the conditions given above for the production of 100pA proton beam, from a 0.1\( \mu \)m radius emitter, an applied voltage of 10kV is necessary and the energy width of the dominate \( \text{H}^+ \) peak is 4.3eV. Substitution of the numerical values into equation (7.4) results in \( \Delta r_{ca} = 0.03\mu \text{m} \). The contribution to the effective source radius due to spherical aberration will possibly dominate for large \( \alpha \); but it is far more difficult to predict and requires the full higher order treatment of Colman (Co 89a). If the effective source size is limited purely by chromatic aberration, then using equation (7.1) it can be shown that it should be possible to approach a transmitted brightness of \( 2 \times 10^3 \text{ A m}^{-2} \text{ rad}^{-2} \text{ V}^{-1} \) in the post lens section. This is an improvement of three orders of magnitude above the brightness attained by the RF source/lens combination. A further improvement in the transmitted brightness could be expected with the use of larger radius emitter tips, where the component of effective source radius due to the lens aberrations is a smaller fraction of the emission area. The ion optics of the accelerator have also been studied as part of the same body of work (Co 89a). However, in order to determine absolutely whether or not the FI source produces sufficient brightness and current for a SPMP ion source it will be necessary to install the source and lens within the accelerator and measure the aberrations and loss of brightness due to the accelerator. At its present level of development this FI source, with its accompanying lens, would be suitable for such tests.

A number of concessions were made in the construction of the FI ion source in order to permit its operation within an accelerator. Because of this the FI source has been limited in its capabilities as it has foregone several design options in order to be compact and rugged. As a result improvements to the existing source design are possible in order to realize the ultimate potential of the FI source. These improvements, some of which require a considerable degree of technical input, define the course for future work on this source.

Importantly the source has no precise control over the spatial relationship between the emitter and the following lens. Although the source was designed to
align the emitter and lens with the maximum possible precision, inevitably some misalignments will be present. These will introduce misalignment aberrations and cause the beam to be steered. When the source is finally used in the accelerator it will be necessary to control accurately, in three dimensions, the position of the emitter. This could be done by a remotely controlled XYZ stage which would permit the adjustment of the relative positioning of the tip and lens during an alignment test. Also, the source does not use a cooled emitter and, thus far, has only been used with polycrystalline emitters. A cooled emitter and gas supply, though less essential for a high current output in the high field regime, is desirable for a number of reasons. As shown in Table 7.3 thermal accommodation of the gas molecules in the region of the tip enhances beam current, reduces the resonant state contribution to the beam energy spread and reduces the size of the virtual source. Cooling the gas and the emitter of a FI source to temperatures as low as 6.5K has been achieved (Le 85) but to implement such an arrangement within the accelerator would be a significant technical project. Oriented single crystal tips could be used on the present source at its existing level of development and should offer an increase in the beam intensity by a factor of three. If an oriented tip is used in conjunction with an emitter cooled to cryogenic temperatures then at least an order of magnitude improvement in the beam angular intensity could be expected. There also exists the possibility of using advanced tip fabrication techniques to increase source performance. As was discussed in Section 7.6, a technique has recently been developed which uses ion bombardment as a means of fabricating microprotrusions on the surface of FI emitters (Bö 88). These sites of highly localised emission have proved to be both bright and stable (Bö 88a), and promise angular intensities of up to $40 \mu A \text{sr}^{-1}$ with the FI of $H_2$ at temperatures below 20K (Jo 88). The projected output from a source using an emitter of this type is given by the data for Source 5 in Table 7.3. The greater angular intensities offered by these more sophisticated designs would permit a significant reduction in all angle dependent aberrations. For this source, lens aberrations are critical as the exceptional brightness of the FI source results from its submicron emission area and this brightness can readily be lost due to the effects of aberrations in the source lens and accelerator.
Consideration must be given to parasitic aberrations which can also threaten beam quality. Due to the nature of the charging and power-supply system used with the Pelletron accelerator, both the column and the high-voltage terminal region are subject to vibration. These vibrations can be as large as 30µm in the plane of the Wien filter aperture and have the potential to degrade significantly a FI beam. The problems associated with mechanical and voltage instabilities in a high voltage accelerator have been addressed by the Cambridge high voltage electron microscope group, which has employed a voltage-doubling charging system to achieve a terminal potential of 600kV stabilized to 1 part in 10^6 (Ni 78). The entire electron optical column is air suspended and mechanically isolated. It may ultimately be necessary to consider a similar system for the SPMP in order to gain the full benefits of a FI source.
Chapter 8

Conclusion

The SPMP is now well established as a versatile analytical instrument. Since its initial development the SPMP has been employed in an increasingly broad range of fields. This expansion in the range of the SPMP applications has been due to a number of factors, one of the most important being the continuous improvement in the spatial resolution of the instrument. During the biological analysis component of the work described in this thesis the SPMP was applied at its existing level of resolution with sufficient beam current for practicable analysis of thin biological specimens. Concurrent with this analytical work, steps were taken to initiate an improvement in the SPMP ion optical system which would permit an increase in resolution whilst maintaining the beam current at the level necessary for effective microanalysis of thin biological specimens.

The work described in the first half of this thesis has shown that the present level of SPMP resolution is sufficient to perform elemental microanalysis of cultured mammalian cells. The elemental sensitivity of a SPMP is at least two orders of magnitude greater than that of an electron probe so stringent constraints were placed upon the preparative techniques used during culture formation. It was demonstrated that healthy fibroblast cultures could be grown at high confluence on nylon foils which, due to the absence of characteristic X-ray peaks, were a suitable specimen support for X-ray microanalysis. As the cells were grown and then analysed on the same surface, interference with the living cell was minimized.

The successful growth and SPMP analysis of normal fibroblasts led to a study of mutant fibroblasts. These mutant Menkes' fibroblast cells expressed an
elevated intracellular copper content and could be differentiated from normal fibroblasts on the basis of the copper content of the cell. This permitted individual cells to be identified as either normal or Menkes' upon the application of a discriminate function. This suggests that the technique may be applied to the diagnosis of cell cultures based on the cell types identified by the SPMP.

A limitation in the time period required for SPMP analysis was encountered, however. Larger beam currents would reduce irradiation time, but, as intercellular space is limited in the high confluence cultures needed for satisfactory cell growth, a reduction in resolution could not be tolerated.

This problem was addressed by undertaking an investigation of the brightness of the beam available to the SPMP. The brightness of the existing RF ion source was measured on a dedicated test bench and a study of alternative sources conducted. The FI ion source offered the promise of an improvement in beam brightness sufficient to bring about worthwhile gains in SPMP resolution. Though it was known that FI sources with cooled emitters were able to satisfy the current requirements of a SPMP, their complexity and spatial requirements prevented them from being seriously contemplated as the initial replacement for the RF ion source. It was not known if a FI ion source operating at room temperature would be capable of providing sufficient current for a SPMP. In order to determine whether or not a source of this nature was a viable alternative ion source, a room temperature FI source was designed and built. Its performance was evaluated on a suitable test bench and the source was found to offer sufficient beam current to operate the Pelletron accelerator and a beam brightness $10^5$ times greater than that attainable with the RF ion source.

The FI source in its present state, should be able to utilize existing lens designs to produce an order of magnitude improvement in STIM resolution, provided that aberrations due to the accelerator are negligible. With the present source, the higher currents necessary for PIXE analysis can only be obtained with a strongly divergent beam. Under these conditions an advancement in lens design will be necessary to ensure that lens aberrations do not degrade the intrinsic source brightness. In addition a more sophisticated ion source may have to be built.

If the advent of brighter ion sources permits the SPMP resolution to be
improved without a decrease in target current, then concerns will arise due to the possible beam damage effects that may occur in biological specimens. Ionization and thermal damage occurs during the irradiation of the biological matrix. The relationship between the specimen-beam interaction and the target damage mechanism is complex (Ch 89), however it is known that ionization damage in particular will become more severe if the same irradiation dose is applied to a smaller area. The alternative of using high brightness to achieve higher beam current at the same resolution would decrease irradiation time but increase thermal damage. As shown by the STIM analysis of fibroblasts, increased beam resolution reveals additional cellular features, but the biological matrix may be so damaged by the heating and ionizing effects of a high intensity beam that the specimen is destroyed as it is analysed. Elemental losses and large scale ion displacements would then render measurements of the elemental distributions unreliable. General current density limits have been established for biological analysis (Se 83b) and in Appendix 1 it is shown that for the special case of thin target analysis of fibroblasts these limits may be exceeded by a factor of ten without introducing any discernible beam damage effects. Thus, in order to use successfully higher resolution beams for elemental analysis of biological specimens it will be necessary to further, and more accurately, evaluate the acceptable limits for current densities, and to consider the options of specimen cooling and reduced radiation doses in order to minimize thermal damage effects. Obviously reduced radiation doses diminish the advantages gained by employing a brighter beam, suggesting that more efficient X-ray and RBS collection systems will also be required. Such systems are becoming available. This work was performed with an X-ray detector of 12.5mm² active area. The same resolution is now achieved with X-ray detectors of 80mm² active area.

The combination of more efficient radiation detection systems and the brighter beams provided by a FI source give promise of an increased range of applications of the SPMP in the biological sciences. It may be expected that such an advanced microprobe will play a major role in the rapid diagnosis of elementally-dependent diseases.
Appendix 1

Beam-induced specimen damage in irradiated fibroblasts

Beam-induced specimen damage has been observed during the X-ray microanalysis of biological tissue and cells, with both the electron probe and the SPMP (Ha 74)(Ma 82)(Sl 85). Ionizing and heating effects arising from the irradiating beam can result in specimen mass loss, elemental migration and structural damage. These effects severely inhibit the successful measurement of elemental localization within a specimen. As this work is concerned with the correlation of elemental distribution to morphological features, it was considered necessary to monitor any possible beam-induced specimen damage that occurred under the chosen experimental conditions.

Several criteria have been developed as a guide to the safe limits of current densities for negligible specimen damage and mass loss in biological tissue (Se 83). For 3MeV proton irradiation the suggested upper limit to the acceptable current density is $10^{-11} \text{ A} \mu\text{m}^{-2}$. This limit is equivalent to 100pA in the 3µm diameter beam spot generally used for fibroblast analysis. For these studies higher current densities were desirable in order to minimize irradiation time, and so, since the suggested limit is general and strongly target dependent, higher target current densities were used for the fibroblast analysis, while at the same time specimen damage effects were monitored. Typically the MP system is capable of supplying a current density of $1 \times 10^{-10} \text{ A} \mu\text{m}^{-2}$ in the 3µm beam spot and a proton fluence of approximately $10^{18} \text{ cm}^{-2}$ is necessary for trace elemental analysis of fibroblasts. This level of proton fluence has resulted in morphological damage and mass loss with white blood cells (Sl 85) and the current density exceeds the recommended safe value by a factor of ten.

As shown in Figure 3.8C the beam discolours the scan region during analysis. Irradiation with 3MeV protons at charge densities of up to 1nCµm$^{-2}$ failed
to obscure the cells and complete cellular detail could be revealed with an optical microscope using polarized light. Alpha particles at an energy of 2MeV and a charge density of 1nCµm⁻² produced considerably greater discolouration of the scanned region. Such conditions rendered all cellular features indiscernible.

Elemental losses were recorded as a function of deposited charge by modifying the data acquisition circuit shown in Figure 2.6 (Ma 82). Output from the current digitizer in the charge collection branch of the circuit was used to flip an unused bit in the X word upon the occurrence of a charge pulse. This then placed a record of charge increments on the data collection tape. The data could then be sorted to order radiation events as a function of collected charge. Using this technique a number of fibroblasts were investigated under normal running conditions (3MeV H⁺ beam i= 700pA t= 4000s). A current density of 1 × 10⁻¹⁰Aµm⁻² was used and a total charge of 2.6µC was deposited on each scan region. The variation in the elemental yield per unit charge was consistent within the different data sets for each cell. Figure A1.1 shows typical elemental yields as a function of charge for fibroblasts. Each charge increment is 3.25nC and was deposited in approximately 5 seconds. It can be seen from Figure A1.1 that the elemental yield remains constant for all elements except hydrogen. Rapid initial hydrogen loss from biological tissue has been observed with an unscanned 3MeV proton microbeam operating at a current density of 1 × 10⁻¹¹Aµm⁻² (Ma 82). No hydrogen loss was apparent when this same beam was scanned, although this may be because the charge per unit area was four orders of magnitude less than for the unscanned data. For the fibroblast work, the current density used was a factor of ten higher than for the unscanned data mentioned above. Hence the observation of a gradual decrease in the hydrogen yield per unit charge for this work is not unexpected. During the 4000s irradiation period a 30% reduction in the hydrogen yield was observed. Hydrogen loss is the least critical of all possible elemental losses in the fibroblast matrix. Its loss is more readily tolerated than that of heavier and more experimentally pertinent elements as its absence from the cell matrix has a minimal effect on X-ray and RBS yields. As the hydrogen loss was only 30% it was considered that the current densities used for the fibroblast analyses were acceptable. Further research is presently being undertaken to interpret theoretically elemental losses.
Figure A1.1

Elemental yield as a function of irradiating charge for fibroblasts. The hydrogen yield is from forward scattered particles. Carbon, nitrogen and oxygen yields are from backscattered particles. The other elemental data are from PIXE X-rays. The only appreciable elemental loss occurs for hydrogen, where, after the deposition of 2.6μC of charge, there is a 30% reduction in the yield per unit charge.
in organic targets (Ch 89).

The reproducibility of the SPMP analysis of fibroblasts was checked to ensure that damage effects at typical current densities did not result in unacceptable target deterioration during the irradiation period. Reproducibility was tested by subjecting a single cell to two identical irradiations at a current density of $1 \times 10^{-10} \text{A}\mu\text{m}^{-2}$. The results are shown in Figure A1.2 and Table A1.1. Excellent reproducibility was obtained, with the distributions and counting statistics produced by the second irradiation corresponding within statistical variation to that of the first irradiation, indicating that little if any elemental loss or migration resulted from the first irradiation.

Overall, the SPMP examination of fibroblasts at proton current densities up to $1 \times 10^{-10} \text{A}\mu\text{m}^{-2}$ is acceptable. The scan region is discoloured by the irradiation; however morphological detail remains visible and elemental distributions and yields, with the exception of the hydrogen yield, remain unaffected.
Figure A1.2

These elemental maps show the reproducibility of the SPMP analytical technique. A single fibroblast was irradiated with a 3MeV proton beam at a current of 700pA for 100 minutes. The beam spot was 3µm in diameter and a total charge of 4.2µC was deposited over the 24µm x 104µm scan region during this period. The same irradiation conditions were then repeated, with an identical amount of charge being placed on the cell. Data set A shows the elemental distributions obtained in the initial irradiation and B the distributions obtained in the second irradiation. It can be seen by comparing the data sets that a high level of reproducibility exists for all the elements. Any variations are within statistical fluctuations.

A fold in the nylon (and hence increased thickness) is responsible for the elevated yield of carbon, nitrogen and oxygen in the top left-hand corner of the maps.
Table A1.1 Comparison of the X-ray and backscattered yield from each of two separate irradiations of the normal fibroblast shown in Figure A1.2. The values given are peak areas with the background subtracted. The errors are one standard deviation of the peak area and are calculated from peak and background areas.

<table>
<thead>
<tr>
<th>Element</th>
<th>Data set A</th>
<th>Data set B</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>25795±287</td>
<td>25531±280</td>
</tr>
<tr>
<td>S</td>
<td>8840±256</td>
<td>8892±255</td>
</tr>
<tr>
<td>Cl</td>
<td>41489±301</td>
<td>41149±304</td>
</tr>
<tr>
<td>Brem</td>
<td>566±24</td>
<td>581±24</td>
</tr>
<tr>
<td>K</td>
<td>90037±321</td>
<td>89858±322</td>
</tr>
<tr>
<td>Fe</td>
<td>644±27</td>
<td>627±26</td>
</tr>
<tr>
<td>Cu</td>
<td>7±5</td>
<td>8±4</td>
</tr>
<tr>
<td>Zn</td>
<td>51±8</td>
<td>59±9</td>
</tr>
<tr>
<td>C</td>
<td>63387±251</td>
<td>63301±252</td>
</tr>
<tr>
<td>N</td>
<td>9267±96</td>
<td>9349±97</td>
</tr>
<tr>
<td>O</td>
<td>3763±61</td>
<td>3793±62</td>
</tr>
<tr>
<td>Na</td>
<td>109±10</td>
<td>116±11</td>
</tr>
<tr>
<td>Charge (μC)</td>
<td>4.20±0.01</td>
<td>4.20±0.01</td>
</tr>
</tbody>
</table>
Appendix 2

The elemental content of Menkes’ fibroblasts grown in copper-rich media

It is known that both normal and Menkes’ fibroblasts accumulate abnormally high levels of intracellular copper when grown in a culture medium that has an artificially high copper content (Ca 80). The SPMP was used to analyse the elemental content of individual fibroblasts grown in a manner identical to that detailed in Chapter 3 but placed in a maintenance medium of FbMM which contained 6µg/ml of copper. Hereafter this medium is termed FbMMCu+. The copper content of normal FbMM is between 0.05 and 0.07µg/ml.

Previous measurements of the intracellular copper content of fibroblasts grown in FbMMCu+ were performed by atomic absorption on bulk samples containing greater than $1 \times 10^6$ cells (see Section 3.2). For the bulk analysis, cell preparation involved washing with phosphate-buffered saline (PBS), cell detachment was induced with versene-trypsin and further washing occurred with both PBS and 0.9% isotonic NaCl before the cells were resuspended in a 0.9% solution of NaCl. Cell viability was then assessed by a number of means. The cells were subjected to a trypan blue exclusion test. In this test the dead cells are readily identified as they stain blue due to the breakdown of membrane integrity. Observation was also made of cell morphology, with unhealthy cells being identified by a granular appearance and a rounded shape resulting from a breakdown of the membrane. Only solutions containing an indicated 85% or greater number of viable cells were accepted for further analysis.

The toxic effect of elevated copper levels on fibroblasts has been well documented (Ca 80); from these results it was found that Menkes’ cells were more vulnerable to the toxic effects of copper and that, for both Menkes’ and normal cell types, the fraction of dead cells in a culture increased steadily with time. For Menkes’ cells grown in FbMM with an added level of 8.7µg/ml of copper, the cell
death rate over the period of exposure used for this work was approximately 22% cell deaths/day. Furthermore 6.9µg/ml of added copper was sufficient for a total of 50% Menkes' cell death after 6 days exposure. Though in both cases the level of added copper was greater than the 6µg/ml used in this work it illustrates the vulnerability of the cells to copper toxicity. It has been noted (Ca 80) that the observed copper toxicity is a function of culture conditions and that cell vulnerability increases with a decrease in the following: calf serum concentration, pH, and cell confluency.

The Menkes' cells cultured for SPMP analysis were incubated in FbMMCu⁺ for 3 days. During that time cell morphology was examined. A rounded cell appearance or a granular structure, or both, was considered to be indicative of cytotoxicity and the corresponding culture was discarded. Only cultures satisfying the criteria for SPMP analysis as defined in Section 3.5.2 were accepted for further analysis.

The cells examined by the SPMP were all identifiably healthy fibroblasts as determined by visual examination of the freeze-dried culture and the anticipated increase in intracellular copper was obtained. However, an unexpected and significant percentage of cells in the culture were affected by the high copper level. The intracellular contents of certain cells in the cultures reflected elemental abnormalities consistent with the breakdown of the cellular membrane, despite having passed the accepted tests of cell integrity and retaining the characteristic shape of a healthy fibroblast. A failed membrane leads to an uncontrolled flux of ions across the cell boundary, with the cell both taking up and losing ion species. The result of this interchange with the culture medium is a large disturbance in the macro and trace elemental content of the cell. For Menkes' cells grown in FbMMCu⁺, 30% of the cells were identified as having notably abnormal intracellular elemental contents, consistent with the failure of the cell membrane. This differs from the results obtained for Menkes' cells grown in FbMM, where all the cells exhibited elemental contents consistent with the average for that cell type and any deviations shown were explicable within the limits of biological variability.

Elemental abnormalities were identified by examining the ratio of each elemental yield to the 3keV bremsstrahlung count. The bremsstrahlung value resulted
Table A2.1: Comparison between intracellular and extracellular elemental contents for mammalian cells (Al 83).

<table>
<thead>
<tr>
<th>Elemental Component</th>
<th>Intracellular Concentration (mM)</th>
<th>Extracellular Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>5–15</td>
<td>145</td>
</tr>
<tr>
<td>K⁺</td>
<td>140</td>
<td>5</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>30</td>
<td>142</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1–2</td>
<td>2.5–5</td>
</tr>
</tbody>
</table>

from summing beneath the spectra over a 50eV window between the chlorine Kα and the potassium Kα peaks. The reasons for the choice of this region as a measure of bremsstrahlung are discussed in Section 4.4.

Figure A2.1 depicts the extent of elemental abnormalities encountered in the dying cells. In the plots shown in Figure A2.1, Ratio 1 is used to represent the average value for the Menkes’ cells that were grown in FbMMCu⁺ and considered to be examples of healthy fibroblasts. The variation in this ratio results from the different batches of culture media used in preparation (this effect only occurs for the macro elements) as well as normal biological variability. Two points associated with Ratio 1 are used to denote the maximum and minimum values produced by elementally sound cells. Ratios 2 to 4 are used to show the extent of elemental deviation found within three otherwise healthy cells. In most cases it can be seen that the abnormal cells record elemental contents that are well beyond the limits recorded for the healthy cells and up to twelve times higher (the Fe/Brem. ratio for cell 3, Ratio 4). None of these results were due to contamination.

From Figure A2.1 it can be seen that the elementally abnormal cells have an elevated phosphorus content. This is due to the uncontrolled uptake of phosphorus from the culture medium. A similar result is seen for other elements: sulphur, iron, copper and zinc. In contrast to this, the cells are potassium deficient. This result is best understood in conjunction with Table A.2.1.

The live cell uses an active ‘pump’ mechanism to maintain the appropriate
Figure A2.1

A series of ratio plots illustrating the elemental abnormalities encountered when analysing fibroblasts with a failed membrane. These results are for Menkes' cells grown in FbMMCu⁺. Ratio 1 is the average value obtained for Menkes' cells under these culture conditions. The two points associated with Ratio 1 indicate the maximum and minimum values for that ratio. Minimum values lie outside the range of the graph for P/Brem., Cu/Brem. and Zn/Brem. Ratios 2, 3 and 4 each represent values obtained for particular cells that were considered abnormal. These values were not used in determining the averages recorded for Ratio 1.
stable ionic balance between the cell and the surrounding tissue fluid. Table A2.1 lists the intracellular and extracellular concentrations of a variety of ions found in mammalian cells. It can be seen from this table that the live cell maintains a very large potassium gradient across the cell membrane, specifically retaining a high concentration of potassium within the cell. It would be expected that a dead cell would be unable to maintain the potassium gradient and hence would be subject to an efflux of potassium, as was observed in these results. Some qualitative evidence was also seen that these cells had an increased level of intracellular calcium, however this was not quantitatively substantiated due to the interference between the potassium and calcium peaks. Such a result could be anticipated from Table A2.1, which indicates that a minor influx of calcium would result from a membrane failure.

The presence of these abnormal cells has consequences for the bulk analysis of fibroblasts grown in media with artificially high copper levels. Such cells would remain undetected by the bulk technique and, given the high percentage of abnormal cells (30%), could significantly affect the bulk result.

Table A2.2: Comparison between the copper level of Menkes' cells grown in FbMM and Menkes' cells grown in FbMMCu⁺.

<table>
<thead>
<tr>
<th>SPMP</th>
<th>Atomic Absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Cu/P FbMMCu⁺: 2.2 ± 0.4</td>
<td>Absolute Cu FbMMCu⁺: 2.73 ± 1.43</td>
</tr>
<tr>
<td>Average Cu/P FbMM</td>
<td>Absolute Cu FbMM</td>
</tr>
</tbody>
</table>

A comparison was made between the copper level of Menkes' cells grown in FbMM and Menkes' cells grown in FbMMCu⁺. Only those cells grown in FbMMCu⁺ and considered elementally sound were used for this comparison. The use of the copper-loaded medium resulted in a change in the intracellular Cu/P ratio. Table A2.2 shows a comparison between this value and the change in the absolute intracellular copper found by bulk analysis (Ca 80). It appears that approximately a threefold increase in intracellular copper is found when the cells are grown in FbMMCu⁺. The agreement between the bulk result and the SPMP
value is not surprising as in this instance the results are independent of any effects due to the use of trypsinization in the bulk measurement (see discussion Section 4.4).

As Menkes' cells grown in FbMMCu+ accumulate copper to a much greater extent than Menkes' fibroblasts grown in FbMM (Ca 80), copper maps generated from the SPMP analysis of these cells have superior statistics and can be used as a guide to the localization of copper in Menkes' fibroblasts. Figure A2.2 is a series of elemental maps from a Menkes' fibroblast grown in FbMMCu+. The position of the cell nucleus was located by visual examination of the cell both before and after SPMP analysis. On the 2D maps the coordinates of the nucleus are indicated by the intersection of the two cross-hairs found on each map. These can be correlated to the 3D maps. The position of the nucleus on the 3D maps may be found by intersecting the cross-hairs, with the X cross-hair running parallel to the Y axis and the Y cross-hair running parallel to the X axis. A line rising vertically from this intersection point will cross the contour surface at the point corresponding to the nucleus.

From Figure A2.2 it can be seen that the nucleus corresponds to a region of maximum phosphorus yield. This result was consistently observed in the fibroblasts and came about due to the greater mass density and phosphorus concentration found within the nucleus. An adjacent phosphorus peak, of almost the same concentration, was found 7.5µm from the nucleus. The sulphur distribution also peaks in the nucleus and has a secondary peak 7.5µm away. The sulphur map also shows the characteristic even distribution of this element through the cytoplasm to the membrane. The chlorine map shows a distinctive peak for this element in the nucleus, as does the potassium map. The potassium map shows evidence of a secondary peak 7.5µm from the nucleus, although relative to the nucleus this region is significantly depleted in potassium. Also potassium is not as evenly concentrated throughout the cell as is sulphur.

The copper distribution shows two peaks. One peak, the broader of the two, corresponds to the position of the nucleus, indicating a definite copper localization in this area. The second peak is found 7.5µm away in the same position as the secondary peaks identified for the major elements. These localizations were not
Figure A2.2

The elemental distribution for a Menkes' fibroblast grown in FbMMCu+ (see text). The scan size is $21\mu m \times 112\mu m$ and a beam spot of $4\mu m$ was used at a current of $1.2nA$. A total charge of $5.2\mu C$ was placed over the area of the cell. The data has been plotted on a $10 \times 55$ grid and has been smoothed with a rotated Gaussian representation of the beam spot. A total of 550 copper X-rays were recorded over the scan area, with maximum peak height of 9 counts. The following table gives the number of events used in composing each map.

<table>
<thead>
<tr>
<th>Element</th>
<th>Counts in scan region</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>39407</td>
</tr>
<tr>
<td>S</td>
<td>13047</td>
</tr>
<tr>
<td>Cl</td>
<td>61054</td>
</tr>
<tr>
<td>K</td>
<td>76097</td>
</tr>
<tr>
<td>Fe</td>
<td>411</td>
</tr>
<tr>
<td>Cu</td>
<td>550</td>
</tr>
<tr>
<td>Zn</td>
<td>60</td>
</tr>
</tbody>
</table>
statistical in origin as they could be reproduced from a split data set.

Despite the good statistics produced by a Menkes' fibroblast grown in FbMMCu+, it is impossible to identify specific localizations of copper within the cell as the localizations observed with this work may possibly be due to the effects of density variation within the cell. It would appear however that the copper concentration found within the nucleus exceeds the concentration expected from a uniform intracellular copper density, and that localizations may occur within the cell in regions other than the nucleus.

The observation that Menkes' cells may accumulate copper in the nucleus is supported by Argarwal (Ar 89), who reports that copper accumulation does occur in the nucleus and that this accumulation has a deleterious effect upon DNA.
References


Al 80  Allan, G.L., Fourth year report, School of Physics, University of Melbourne (1980), Unpublished.


<table>
<thead>
<tr>
<th>Reference</th>
<th>Author(s)</th>
<th>Title and Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba 80</td>
<td>Balzers</td>
<td>(1980), Description, design fundamentals, examples of application, performance data from Pfeiffer-Turbo molecular pumps, 11.</td>
</tr>
<tr>
<td>Ba 85</td>
<td>Balzers</td>
<td>(1985), Partial pressure measurement in vacuum technology, Balzers AG, Balzers, Liechtenstein, 1.</td>
</tr>
<tr>
<td>Ba 86</td>
<td>Baker</td>
<td>D., Fourth year report, School of Physics, University of Melbourne (1986), Unpublished.</td>
</tr>
<tr>
<td>Be 87</td>
<td>Bench, G.</td>
<td>Fifth year report, School of Physics, University of Melbourne (1987), Unpublished.</td>
</tr>
</tbody>
</table>


Ch 88  Cholewa, M., Private communication (1988).


Co 82  Colman, R.A., Fourth year report, School of Physics, University of Melbourne (1982), Unpublished.


Da 72  Danks, D.M., Campbell, P.E., Stevens, B.J., Mayne, V., and Cartwright, E., (1972), Pediatrics, 50, 188.


Dix, M., Fourth year report, School of Physics, University of Melbourne (1983), Unpublished.


Gu 77  Guy, J., Fourth Year Report, School of Physics, University of Melbourne (1977), Unpublished.


Ho 77 Horrabin, C.W., (1977), A digital data telemetry system for terminal to ground communication in a 10MV Van de Graaf, Presented at the 1977 Particle Accelerator Conference, Chicago.
Iw 75 Iwasaki, H., and Nakamura, S., (1975), Surf. Sci., 52, 588.
Iw 79 Iwakawa, Y., Niwa, T., and Tomita, M., (1979), Brain Dev. (Tokyo), 11, 260.
Ja 85 Jamieson, D.N., Ph.D Thesis, School of Physics, University of Melbourne (1985), Unpublished.


Le 82b  Leybold-Heraeus, (1982), Vacuum Technology its foundations formulae and tables, Leybold-Heraeus GMBH.


Ma 82  Mazzolini, A.P., Ph.D Thesis, School of Physics, University of Melbourne (1982), Unpublished.


<table>
<thead>
<tr>
<th>Ref</th>
<th>Author(s)</th>
<th>Title and Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Or 74</td>
<td>Ortec, (1974),</td>
<td>Ion sources and systems.</td>
</tr>
<tr>
<td>Or 78</td>
<td>Ortec, (1978),</td>
<td>Operating and service manual for Si(Li) X-ray detector, Model No. 78005-04555.</td>
</tr>
</tbody>
</table>


Pe 63  Pearson, E.S., (1963), Biometrika, 50, 315.


163
Sa 87  Samuel, A.E., Fourth Year Report, School of Physics, University of Melbourne (1987), Unpublished.


So 63a  Southon, M.J., and Brandon, D.G., (1963), Phil. Mag., 8, 579.


Author/s:
Allan, Garry Lindsay

Title:
Application of a scanning proton microprobe as a diagnostic tool and the development of a high brightness ion source

Date:
1989

Persistent Link:
http://hdl.handle.net/11343/40767

File Description:
Application of a scanning proton microprobe as a diagnostic tool and the development of a high brightness ion source