AN INTRODUCTION TO ELECTRON MICROSCOPY AND X-RAY MICROANALYSIS

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CONTENTS

1. RELATIVE SCALES.

In the world around us we are used to only seeing objects with the naked eye. Measurements are made usually using metres and millimetres and for longer distances, kilometres. These units form part of the SI system of units. Each unit is related to the other units by 1000 - i.e. 1 kilometre is 1000 meters.

The high magnifications achievable with electron microscopes necessitate using units suitable for smaller measurements. Units used are micro- and nanometer.

A micron is one thousandth of a millimeter, and similarly a nanometer is one thousandth of a micron. It is usual to use the exponent notation when relating these dimensions to meters.

The relationship of the various units and their abbreviations are:

1 millimeter (mm)	$= 1 \times 10^{-3}$	meter
1 micron (µm)	$= 1 \times 10^{-6}$	meter
1 nanometer (nm)	$= 1 \times 10^{-9}$	meter

Historically the Angstrom Unit (Å) was used. 1Å is the same as 1×10^{-10} meter, and nanometer is 10 Å.

2. RESOLVING POWER AND WAVELENGTHS.

The resolving power of a microscope governs the point at which two near features are resolved by the viewer. The eye can usually resolve two points separated by a distance that can be seen by the human eye.

Given a fixed aperture it can be seen that the limit of resolution is proportional to the wavelength of the image forming radiation. For an optical microscope ultraviolet illumination can be used to improve the resolution. Electrons, which have a much shorter wavelength than light, are the extreme example of the use of short wavelengths to improve resolution.

3. HISTORY OF ELECTRON MICROSCOPES.

3.1. Electron microscope development.

In 1897 J.J.Thomson published the results of his theoretical and experimental studies in quantitative aspects of cathode rays, demonstrating that they were composed of streams of negatively charged particles which he named "electrons".

During the following quarter of a century further research into electrons included a number of developments which were to lead to the construction of the first electron beam machines. Key events included Wehnelt's 1903 demonstration that an electron beam could be concentrated by means of an electrostatic field, which established the working principle of the electron gun. De Broglie's demonstrated in 1924, that an electron could be regarded as a wave, which initiated research into the electron's waveform possibilities and Busch’s paper in 1926 on the theory of electromagnetic lenses.

By 1930 a group of German scientists had constructed the first working transmission electron microscope.

The development of the machines during the pre-war period was slowed by a number of technical difficulties which remained to be solved. Obtaining the stable power supplies needed for accurate control of the accelerating voltage and the lens currents was virtually impossible prior to the wartime development of power stabilisers. Avoidance and control of lens aberrations was problematic, as was the production and maintenance of high vacuum inside the microscope column. By the end of the 1940's these problems had been researched and solved, and an important new development was underway.

3.2. Electron probe microanalysis.

In 1948 Raymond Castaing embarked on a Ph.D project at the University of Paris, the aim of which was to develop a practical method of electron probe microanalysis by combining an electron microscope and an X-ray spectrometer. The principle of using characteristic X-rays for chemical analysis had been promulgated by Moseley in 1913 and put into practice with the design and construction of X-ray spectrometers during the inter-war years.

In 1947 Hillier had patented the principle of combining spectroscopy and electron microscopes: in 1950, Castaing arrived at a practical method of achieving this and demonstrated that the method was capable of discerning compositional differences in specimens.

The Cameca Company manufactured the first commercially produced microscope/ spectrometer combination in France in 1958.
At the same time, research into the possibility of obtaining larger specimen areas for microanalysis led to the building of the first scanning electron microscope in 1960.
Further research, carried out in the engineering laboratories at Cambridge University, resulted in the production of the first commercial SEMs by the Cambridge Instrument Company on 1964.

4. MICROSCOPE TYPES.

In our quest to see the unseen, we have built not only better but different types of microscopes. Essentially these fall into one of two major categories:

Transmitting - Energy passed through the specimen differentially refracted and absorbed.
Scanning - Probe forming, energy scanned over the surface. Image built up point by point.

Transmitting:

1.) Transmitting Light Microscope (TLM) - Visible spectrum or selected wavelengths thereof passed through the specimen and gathered. Best resolution = 200 nanometers.
2.) Transmission Electron Microscope (TEM) - Electron beam passed through the specimen and gathered. Differs from TLM principally in its source of illumination. Best resolution = 0.5 nanometers.

Scanning: Although microscopes, these do not use a lens system to produce the final magnified image.

Scanning Electron Microscope (SEM) - Electron beam passed over the surface of the specimen and causes energy changes in the surface layer. These changes are detected and analysed to give an image of the specimen. Yields information only from the surface or near-surface of the specimen. Has an advantage over TEM by having a huge depth of field and gives very pleasing picture. Appears as three dimensional but true 3-D can only be attained by using two pictures taken at different angles. Best resolution = 10 nanometers.

Light and electron microscopes operate on similar principles. They both magnify objects such that the human eye can see detail that would otherwise be invisible. Both types of microscope have a source of illumination, a system of lenses to focus and direct the illumination and a method of viewing the image.

Within the light microscope the sample is illuminated using visible light, and the light is focussed and directed using a system of glass lenses. The final image is viewed through an eyepiece. Light microscopes may be referred to as simple or compound types. A simple type uses a single lens to magnify the object, whereas a compound microscope uses more lenses to produce a more highly magnified image in stages.

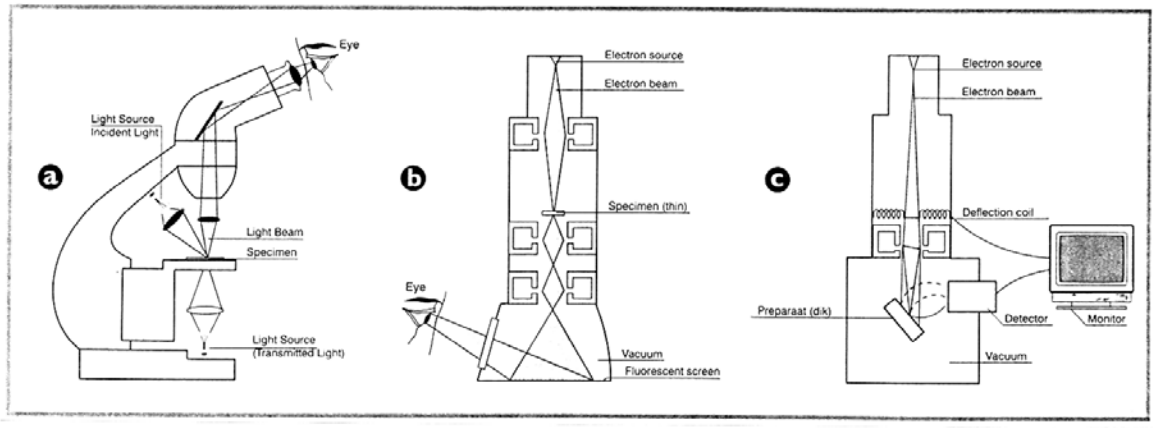
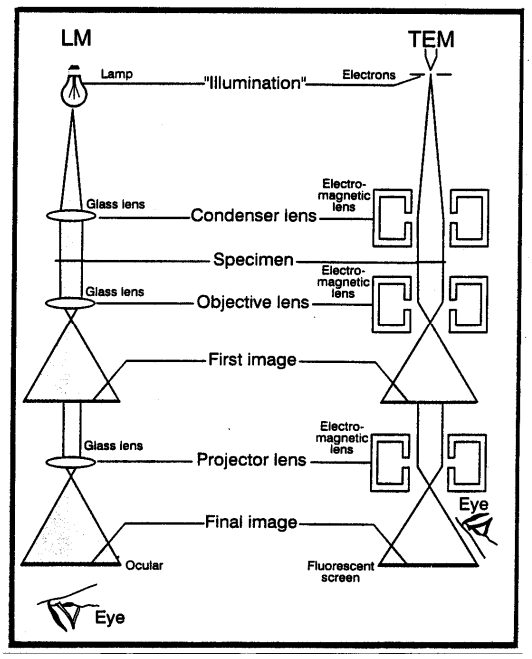


FIG.1a). Light microscope, **b).** TEM, **c).** SEM.

An electron microscope uses an electron gun to provide the illumination, and the electron beam is focussed and directed using electromagnetic lenses. The sample is viewed either on a TV type screen in the case of the scanning electron microscope, or on a phosphor screen in the transmission electron microscope.

4.1 Transmission electron microscope.

The transmission electron microscope allows the investigation of the internal microstructure of samples, provided that they are thin enough to transmit electrons.



The source of illumination in a TEM is contained in the electron gun, situated at the top of the column. Electrons travel at high speed down the column, and are focussed onto the specimen using a combination of magnetic lenses. The microscope can be operated in a number of modes, both image and diffraction, and can be fitted with a variety of detectors, making it a versatile and powerful analytical tool for microstructural investigation at high spatial resolution.

In image mode, the objective lens produces an image of the internal structure of the specimen, which is then projected and magnified, using a combination of projector and intermediate lens, onto the fluorescent screen at the base of the column.

Operation of the TEM in diffraction mode allows crystallographic information, from the sample, to be obtained.

FIG. 2. Ray paths of light in a LM and electrons in a TEM.

4.2 Scanning electron microscope.

The scanning electron microscope (SEM) is commonly used to examine the microstructure of bulk specimens.

It is an electron-optical instrument, which uses a source of electrons to illuminate the specimen. With the new generation of SEM's, the ESEM or Environmental Scanning Electron Microscope, wet, dirty, oily, outgassing and samples as hot as 1 500°C can be viewed.

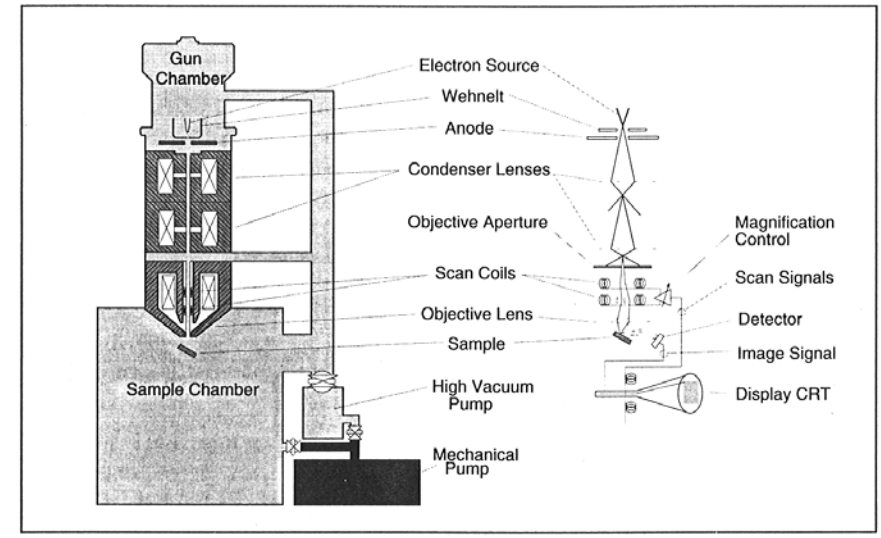


FIG. 3. The functioning of a SEM

These electrons are accelerated down the column and pass through a combination of electromagnetic lenses and apertures to form a fine probe at the surface of the specimen in the chamber area. Both the column and the chamber are held under vacuum to avoid high voltage discharge and scattering of the electrons along their path by residual gas atoms. The beam may be scanned in a rectangular raster across the surface of the specimen by means of a series of scan coils situated above the objective lens. A variety of signals are produced as a result of the interaction of the beam with the specimen, which may be collected by appropriate detectors.

This information can be accessed and displayed in a variety of forms, one of which is an image. The output of the various detectors such as backscattered and secondary electron detectors are used to modulate the brightness of a cathode ray tube (CRT). The raster of the electron beam on the specimen is synchronous with that of the CRT so that information from the sample is built up as a two dimensional image. The SEM is a valuable imaging tool which allows very fine detail to be resolved and, unlike an optical microscope, offers a large depth of field. In addition, it may be combined with appropriate detectors, to serve as a powerful analytical tool.
There are various ways in which to operate the scanning electron microscope, depending on the information required. If high depth of field images are required then a small convergence angle should be used, so that different heights on an irregular surface are all in focus. This can be achieved by using a small objective aperture or a long working distance. However, if X-ray microanalysis is to be performed, probe currents of at least 10^{-10} amps should be used for EDS and at least 10^{-8} amps for WDS.

A higher current will reduce noise in the image. However, the probe diameter increases when the lenses are adjusted to give higher current, so spatial resolution in the electron image is compromised. Thus, there is a trade off between good count rate and low noise images and the ability to see very fine specimen detail. If high-resolution secondary electron images are required, then a small probe size and short working distance should be used. This can be achieved by using both a strong condenser and objective lens setting. This will, however, limit the current in the probe, so the images may appear noisy.

4.3 Environmental scanning electron microscope ESEM.

As mentioned above, samples for conventional SEM generally have to be clean, dry, vacuum compatible and electrically conductive. In recent years the Environmental Scanning Electron Microscope (ESEM) has been developed to provide a unique solution for problematic samples. Examples of specimens which pose problems are wool or cotton tissue, cosmetics, fats and emulsions
(e.g. margarine). Attempts to view a specimen containing volatile components
by placing it in an environmental chamber (see box O) isolated from
the main column by one or more differential pumping apertures used to be hampered by the lack of a suitable electron detector which can work in the atmosphere of the chamber. The Gaseous Secondary Electron Detector makes use of cascade amplification not only to enhance the secondary electron signal but also to produce positive ions which are attracted by negative charge on the insulated specimen surface and effectively suppress charging artefacts.

Environmental Chamber
The pressure-temperature phase diagram for H2O indicates that true "wet"
conditions only exist at pressures of at least 600 Pa at 0°C (environmental microscopists usually refer to 4.6 Torr = 4.6 mm of mercury). In the range 650 to 1300 Pa (5 –10 Torr) therefore the specimen may be observed whilst at
equilibrium with water. Ice cream in the Cryo-SEM: the amount of emulsion in relation to air and water (ice) is an important parameter to study.

5. ELECTRON GUN.

The purpose of the electron gun is to provide a stable source of electrons. There are several types of electron gun routinely used in EMs which vary in their design and emission characteristics. The most commonly used type of electron gun is the conventional triode electron gun, which is made up of three components which are kept under vacuum in the gun chamber.

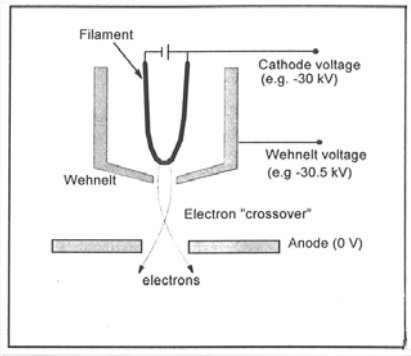


FIG. 4. The high voltages used in the electron gun

The filament or cathode is the source of electrons and is held at negative potential relative to earth potential. A typical tungsten filament is made from a bent piece of wire typically 100µm in diameter. A current is applied to the filament to heat the wire to typically 2700 K, at which point, electrons are emitted from the filament, by a process called thermionic emission. In order for the electrons in the filament to escape from the material, they require sufficient energy to overcome the work-function energy of the material. This energy is

provided by the heat supplied by the filament heating current. The lifetime of the filament will depend on the temperature to which the filament rises. As the temperature increases, so the lifetime may decrease. A good vacuum in the gun area is essential to prevent erosion of the filament by ion bombardment from the gases present in surrounding area.

The "Wehnelt" or grid which is held at a few hundred volts relative to the cathode. The strength of this voltage affects both the form of electrostatic field between the grid and the filament for a given filament current applied. The form of the electrostatic field focuses the electrons between the grid cap and the anode.

The anode is positioned at the base of the gun chamber and held at earth potential, therefore, the electrons are accelerated from the high negative potential of the filament towards the anode. The hole in the anode allows a proportion of the electrons to shoot down the column through a combination of lenses and apertures to the specimen.

6. ELECTROMAGNETIC LENSES.

In optical microscopes, the ability to focus light is achieved by using glass lenses. Electron microscopes use electrons as the source of illumination and the ability to focus electrons in the microscope is achieved using electron lenses. These may be either electrostatic or electromagnetic. Electron lenses are all subject to aberrations but less so in the case of electromagnetic than in electrostatic lenses. The main role of electromagnetic lenses in electron optical columns is to de-magnify the source of electrons to form a much smaller diameter probe incident on the sample.

The lens strength can be varied by adjusting the amount of current, which flows through the windings around the iron core of the magnet. There are two main lenses used in a SEM: the condenser and the objective. The condenser affects the number of electrons in the beam for a given objective aperture size, and the objective lens focuses electrons on to the specimen at the working distance.

7. WORKING DISTANCE.

The working distance is defined as the distance between the lower pole piece of the objective lens and the position of the specimen at which the electrons are focussed onto the specimen. The strength of the objective lens essentially changes the distance between the pole piece and the plane onto which the electrons are focussed. The probe can, therefore, be focussed at different working distances to cope with different specimen heights. The specimen height can be adjusted, but for X-ray microanalysis, there is a recommended working distance for optimum X-ray acquisition, which is specific to the geometry of the detector mount on the SEM chamber.

8. MAGNIFICATION.

The magnification of an electron image is defined as the ratio of the length of one line of the electron beam on the monitor to the width of the area scanned on the specimen.
An increase in magnification can be achieved by reducing the width of the area scanned on the specimen, since the width of the CRT is fixed. In this way magnifications of up to 300,000 can be achieved, provided all the conditions necessary for the best spatial resolution have been optimised. Above these magnifications, no greater detail is observed and greater magnification is often referred to as "empty magnification".

Theoretically, the spatial resolution of the SEM in secondary electron image mode is, ultimately, determined by the size of the scanning spot. This is influenced by electron optical parameters such as working distance, lens settings and electron gun brightness and also by electronic noise in the X and Y deflection currents.

In practice there are a number of other factors that will influence the spatial resolution practically attainable from the SEM in imaging mode. These include accelerating voltage, the state of the specimen surface, detector position and set up and external mechanical vibrations. Spatial resolutions of a nanometer may be obtained with all these conditions optimised.

9. DEPTH OF FIELD.

Depth of field characterises the extent to which image resolution degrades with distance above or below the plain of best focus. With greater depth of field a microscope can better image three-dimensional samples. In the SEM, then depth of field is much greater than for optical microscopes.

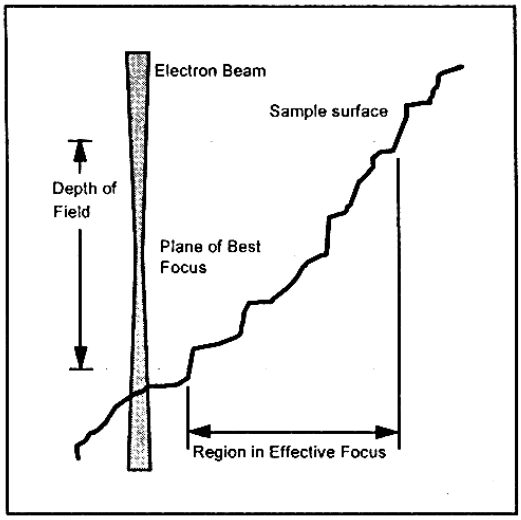


FIG. 5. Depth of field in the SEM.

10. **TYPES OF SIGNAL.**

11. The electron beam interacts with the near surface region of a sample to a depth of approximately a few microns, depending on both the accelerating voltage and the density of the material. Secondary electrons have the smallest volume, followed by backscattered electrons and X-rays. This interaction volume ultimately determines both the spatial resolution and depth from which analysis can be achieved.

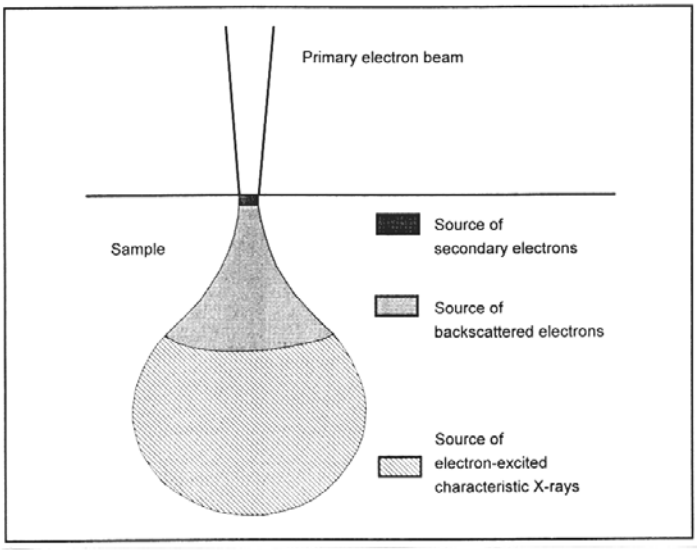


FIG. 6. Each type of signal originates within a specific volume of interaction

Numerous signals are produced as a result of this interaction, which may be detected, by appropriate detectors, to provide information about the specimen.
These signals include low energy secondary emission, Auger electron generation, characteristic X-ray emission, bremstrahlung, backscattered electron emission and cathodoluminescence.

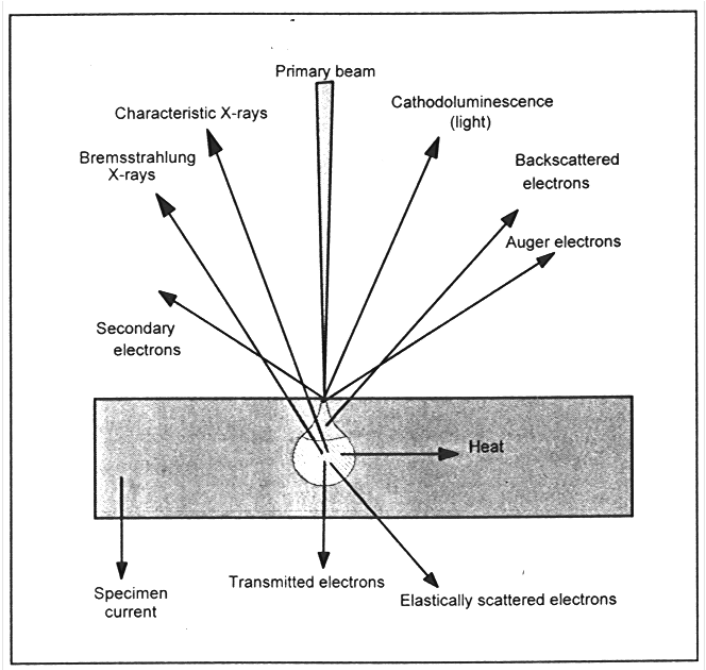


FIG. 7. The interactions of the beam electrons and the sample atoms generate a variety of signals.

10.1 Auger electrons.

The bombardment of the specimen by energetic electrons results in ionised atoms to a depth, depending on accelerating voltage and density of the material, but typically of the order of

1µm. An ionised atom can emit a characteristic X-ray or the energy released as electron fills the initial vacancy may eject another electron from the atom in a "radiationless" transition called the Auger effect.

If an inner K shell electron is ejected and an electron from the L shell fills this vacancy, releases energy and ejects an Auger electron from the L shell, the Auger transition is termed a KLL transition. Measurement of the energy of the characteristic Auger electrons forms the basis of Auger spectroscopy. The energies of the Auger electron peaks allow all elements, with the exception of hydrogen and helium, to be identified, since a minimum of three electrons are required for the emission process.

Auger spectroscopy is a surface sensitive technique, since Auger electrons generated deeper than the top surface layers, will lose their energy signature as they travel out of the specimen. Therefore, the detected signal comprises of electrons generated from only the first few monolayers of the sample - those that have sufficient energy to escape.

10.2 Secondary electrons.

Inelastic scattering of an energetic electron with outer valence electrons leads to the emission of secondary electrons that are characterised by having a kinetic energy less than 50eV. These electrons may be subject to additional scattering events through which energy is lost and therefore only electrons which have sufficient energy to overcome the surface barrier energy and escape the material and contribute to the detected signal; these are electrons at the specimen surface.
Secondary electron emission is one of the most common signals used for imaging in the SEM, since most of the signal is confined to a region close to the incident beam, and gives a high-resolution image. Secondaries are also emitted when backscattered electrons exit the sample, often some distance away from the beam spot.

10.3 Backscattered electrons.

A significant number of the incident electrons which strike a bulk specimen are re-emitted through the surface of the material. These electrons are known as backscattered electrons, which have undergone large angle elastic scattering events in the material, enabling then to approach the surface with sufficient energy to escape. The scattering intensity is related to the atomic number of the atom, the higher the atomic number of the material involved, the higher the backscattering coefficient, and the higher the yield.

This dependence of the backscatter yield with atomic number, forms the basis for differentiating between different phases, thus providing an ideal starting point to guide further microanalysis.

Backscattered electrons give useful information about the composition and surface topography of the specimen.

10.4 Characteristic X-rays.

The interaction of a high-energy electron with an atom may result in the ejection of an electron from an inner atomic shell. This leaves the atom in an ionised or excited state with a vacancy in this shell. De-excitation can occur by an electron from an outer shell filling the

vacancy. The change in energy is determined by the electronic structure of the atom that is unique to the element.

11. SAMPLE CHARGING.

11.1 Charge build up.

As a result of the interaction of the electron beam with the specimen, a number of interactions take place, both elastic and inelastic. This injection of charge produces a number of secondary electrons, and some of the original charge can be re-emitted in the form of backscattered electrons. However, not all the incident charge is re-emitted via these two routes.

11.2 Image distortion.

Charging can distort secondary electron images and affect analytical information from the specimen. The collection of secondary electrons by the detector is influenced by the strength of the field associated with the Faraday cage on the EHT detector, and also the local specimen field.

In some cases, the degree of charging is such that the beam can be deflected from its original position, causing streaks in the image to appear, and in other cases, images can become distorted. The degree of charging and, hence, the effect this has on the image, depends critically on the rate at which the beam is scanned across the specimen.

11.3 Methods of charge reduction.

11.3.1 Coating.

If charging is severe, which is the case for most everyday materials, such as minerals, ceramics, fibres, glasses and biological materials, the specimen generally needs to be coated with a conducting layer, which creates a path along which the excess charge, caused by the beam, can flow to ground.

In addition to insulating materials, many metals and semiconductors are also prone to the build up of insulating oxide layers on the surface. Coating is generally sufficient to prevent the images of such materials being affected.

11.3.2 Specimen tilt.

The yield of secondary electrons is also a function of the specimen tilt.
As the specimen is tilted away from normal incidence, the secondary electron yield increases and so reduces charging of the sample.

11.3.3 Probe current.

Lowering the probe current at the specimen can reduce the effect of charging. This allows the injected charge into the specimen to leak away. The effectiveness of this charge leakage is material dependant.

11.3.4 Scan rate

Charging of the specimen can be reduced by scanning the beam as quickly as possible over the specimen, and building up the image frame by frame, rather than acquiring the image with a long dwell time scan.

11.3.5 Accelerating voltage.

The total electron emission depends on the primary electron energy. Adjustments of this energy will dictate whether the specimen surface will charge negative or positive.
The charging can be reduced by lowering the accelerating voltage, since the secondary electron coefficient generally rises as the beam energy is reduced, whilst the backscattered yield is not a strong function of beam energy.

11.3.6 Choice of detector.

Secondary electron images are extremely sensitive to charging, due to the strength of the bias on the secondary electron detector, and the typically low energies of secondary electrons (<50eV). Whilst secondary electron images are sensitive to low levels of charging, the backscattered component is less so, because of the greater energy of these electrons.
A surface potential of a few volts will not have a substantial effect on the trajectory of a backscattered electron, whose energy could be as great as the incident beam energy.

12. DETECTORS.

Two types of detector are commonly used to detect these electrons: a scintillator type detector (SE), or a solid state type (BSE).

12.1 Secondary electron detector.

The standard detector used in the scanning electron microscope is a combined secondary and backscattered detector, known as the Everhart-Thornley detector.
The detector consists of a Faraday cage, a scintillator, light pipe and photomultiplier tube. Electrons, incident on the scintillator, produce photons, which are then "guided" down the light pipe, via total internal reflection, to the photomultiplier. At the photomultiplier, the signal falls
on the first photocathode, where it is turned back into an electron current. The electrons are accelerated onto successive electrodes, finally producing a cascade of electrons.
The detector is usually operated in two modes. The most common of these is with a positive bias of around 500V on the Faraday cage. The effect of this is to deflect the trajectories of the secondary electrons, emitted from the sample, into the detector.

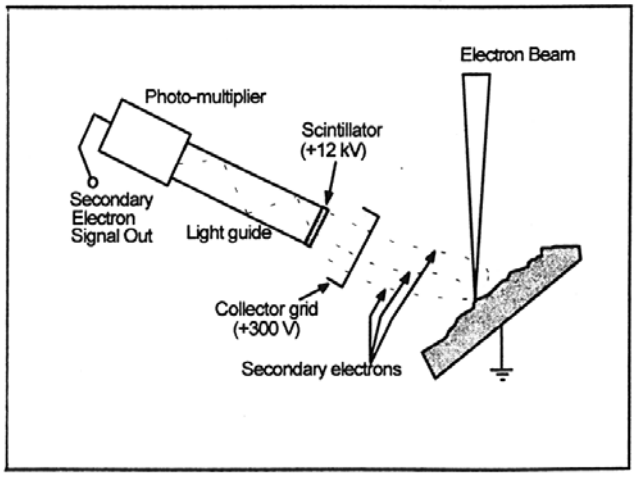


FIG. 8. The Everhart-Thornley detector.

The bias will also accelerate the electrons onto the scintillator. Electrons can be collected from the sample, even if there is not a direct line sight from the specimen to the detector. The collection efficiency of the detector approaches 100% on a flat surface. Backscattered electrons, which have a direct line of sight to the detector, will also be detected and contribute to the signal observed. The positive bias will have little to no effect on these electrons.

With the bias turned off, only those secondary electrons with a direct line of sight to the detector, will be detected, along with the backscattered electrons mentioned above.

12.2 Backscatter detector.

The solid state backscattered detector is mounted close to the specimen, under the final lens and is usually split into four quadrants. The detector will have a hole in the middle for the beam to pass through. The detector is slim enough to be usually left in place, and also to permit simultaneous x-ray microanalysis.

13. SCAN COILS AND RASTERING.

The deflection coils or scan coils are situated above the objective lens in the column and may be used to raster the electron beam over a rectangular area on the surface of the specimen. This raster mode may be used to generate information from more than one location of the beam, in order to build up an image using the emitted signals from the sample

Other such deflection coils in the SEM are the gun shift and tilt coils and the image shifting coils, which are used to align the gun and move the image respectively. The scanning action is achieved by applying current to each of the horizontal and vertical scan coils, so that the probe is deflected to cover a rectangular area on the specimen.
As a result of the interaction of the beam with the specimen, a variety of signals are produced, which may be collected by appropriate detectors.

Such signals are detected end amplified and used to modulate the brightness of the electron beam in the cathode ray tube (CRT). The raster of the electron beam on the specimen is synchronous with that of the electron beam in the CRT.
In this the way, variations in the signal emitted over the scanned area are displayed as variations in brightness on the CRT, and hence, the appropriate information can be displayed in image form. Since the area scanned on the CRT is much bigger than the area scanned on the sample, magnification is achieved.

14. MICROANALYSIS.

As the name suggests, this refers to the analysis of a sample on a microscopic scale, resulting in structural, compositional and chemical information about the sample.
There exists a whole host of analytical detectors that exploit the many signals that may be generated within the sample. X-ray microanalysis specifically gives information about the elemental composition of the specimen, in terms of both quantity and distribution.

14.1 X-ray generation.

As a result of the inelastic scattering of the incident electrons with matter, two main processes can produce X-rays: characteristic X-ray emission and Continuum X-ray emission or bremsstrahlung.

The interaction of a high-energy electron with an atom may result in the ejection of an electron from an inner atomic shell. This leaves the atom in an ionised or excited state, with a vacancy in this shell. De-excitation can occur by an electron from an outer shell filling the vacancy. The change in energy is determined by the electronic structure of the atom that is unique to the element.

This "characteristic" energy can be released from the atom in two ways. One is the emission of an X-ray photon with a characteristic energy specific to that transition and hence, to the element. Detection of such X-rays gives information about the elemental composition of the specimen, in terms of both quantity and distribution. The second way is releasing so called Auger electrons.

The continuum X-ray spectrum or bremsstrahlung (literally translated as braking radiation) is generated, in addition to characteristic X-ray emission, when electrons interact with matter. The emission of these X-ray photons is associated with the deceleration of the atom core. Since the energy lost by the electron can range anywhere between zero up to the value of the incident electron energy, a continuous spectrum of X-ray energies is produced.

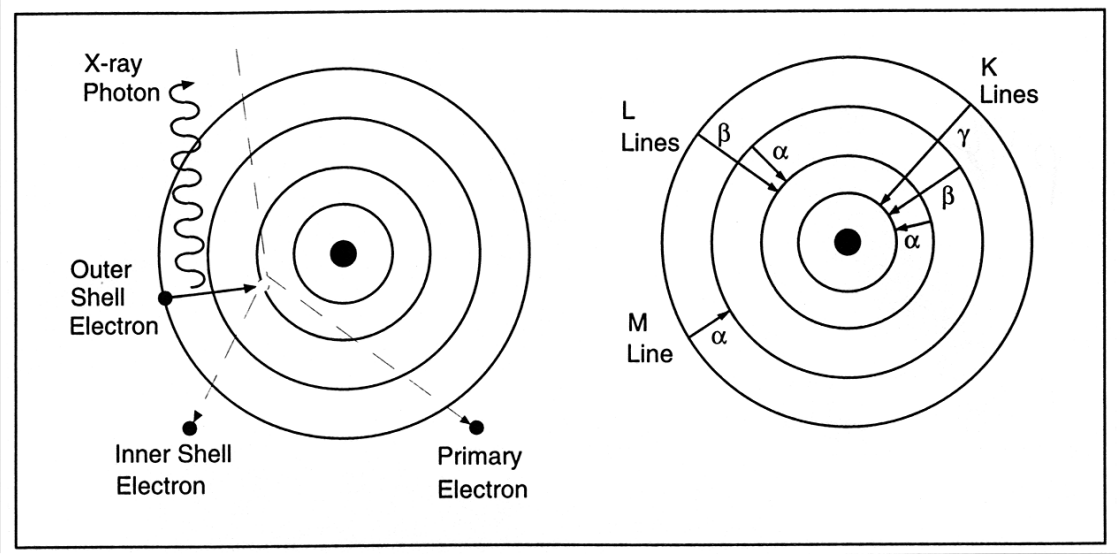
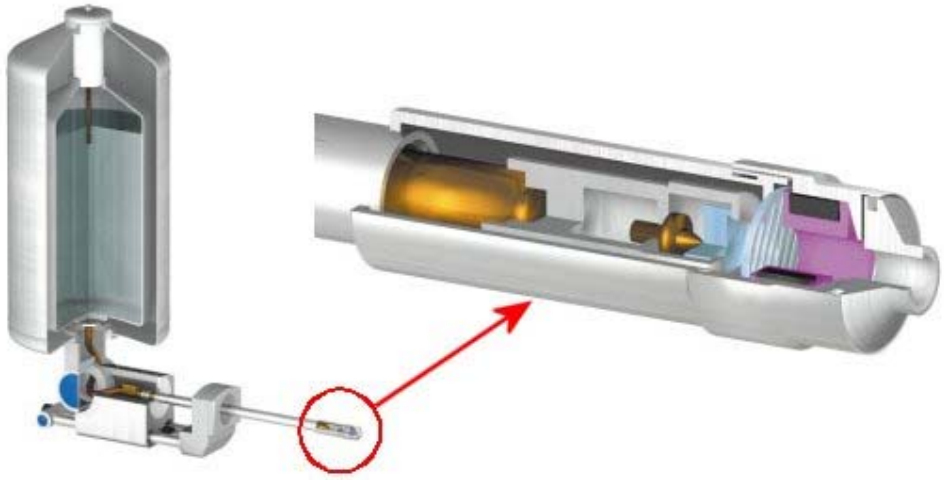


FIG. 9. . X-ray generation.

An X-ray spectrometer collects the characteristic X- rays. The spectrometer counts and sorts the X- rays, usually on the basis of energy (Energy Dispersive Spectrometry – EDS). The resulting spectrum plots number of X-rays, on the vertical axis, versus energy, on the horizontal axis. Peaks on the spectrum correspond to the elements present in the sample.

The EDS Detector



How the EDS Detector Works

The EDS detector converts the energy of each individual X-ray into a voltage signal of proportional size. This is achieved through a three stage process. Firstly the X-ray is converted into a charge by the ionization of atoms in the semiconductor crystal. Secondly this charge is converted into the voltage signal by the FET preamplifier. Finally the voltage signal is input into the pulse processor for measurement. The output from the preamplifier is a voltage 'ramp' where each X-ray appears as a voltage step on the ramp.

EDS detectors are designed to convert the X-ray energy into the voltage signal as accurately as possible. At the same time electronic noise must be minimized to allow detection of the lowest X-ray energies.

How the crystal converts X-ray energy into charge

When an incident X-ray strikes the detector crystal its energy is absorbed by a series of ionizations within the semiconductor to create a number of electron-hole pairs. The electrons are raised into the conduction band of the semiconductor and are free to move within the crystal lattice. When an electron is raised into the conduction band it leaves behind a 'hole', which behaves like a free positive charge within the crystal. A high bias voltage, applied between electrical contacts on the front face and back of the crystal, then sweeps the electrons and holes to these opposite electrodes, producing a charge signal, the size of which is directly proportional to the energy of the incident X-ray.

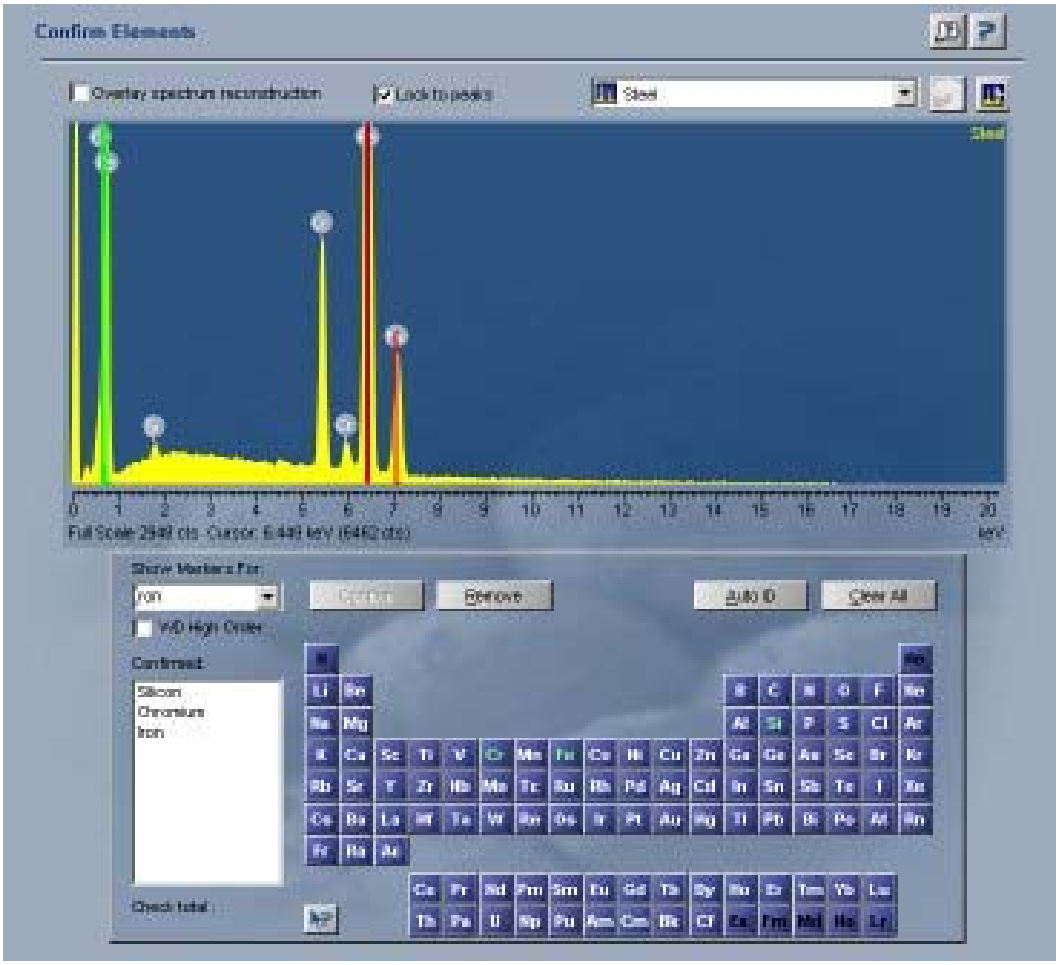


FIG.10. An EDS spectrum displays peaks at energies characteristic of elements present in the sample.

14.2 Qualitative analysis.

Qualitative analysis identifies the elements present in the analysed volume of a specimen, i.e. it answers the question: "what is there?"
An X-ray spectrum is recorded, over a range of energy, within which, relevant lines may be present. The lines, and therefore, the elements, are identified by reference to tables or databases.

14.3 Quantitave analysis.

Quantitave analysis determines how much of a particular element is present in the analysed volume of a specimen, i.e. it answers the question: "How much is there?" or "What is the composition?"
Intensities of X-ray lines from the specimen, are compared with those from standards of known composition. Corrections are made for background and instrumental effects. The composition of the analysed volume is then calculated by applying "matrix corrections", which take into account various factors governing the relationship between the measured intensity and composition. It is important that the volume being analysed is homogeneous, and is representative of the specimen. The sample must be flat and perfectly polished. Polished lines, steps formed by strong chemical etching or electric polishing lead to intensity

variations caused by differences in X-ray absorption due to the topography rather than to the chemical nature of the specimen.
After polishing the sample must be cleaned by washing in water or alcohol and drying in hot air. The use of an ultrasonic cleaner is recommended.

14.4 Spectrum processing

Spectrum processing :
Processing option : All elements analyzed (Normalised)
Number of iterations = 4

Standard :
C CaCO3 1-Jun-1999 12:00 AM
O SiO2 1-Jun-1999 12:00 AM
S FeS2 1-Jun-1999 12:00 AM

Element	Weight%	Weight% Sigma	Atomic%
C K	44.79	0.30	55.82
O K	39.30	0.29	36.76
S K	15.91	0.18	7.43
Totals	100.00		

Processing option : Oxygen by stoichiometry (Normalised)
Number of ions calculation based on 8.00 anions per formula
Number of iterations = 4

Element	Weight%	Weight% Sigma	Atomic%	Compd%	Formula	Number of ions
C K	21.01	0.18	27.34	76.96	CO2	3.21
S K	9.23	0.10	4.50	23.04	SO3	0.53
O	69.77	0.20	68.17			8.00
Totals	100.00					
					Cation sum	3.74

FIG. 11. Quantification results with Wt% (weight %), At% (atomic %),

14.4.5 Spectral artefacts.

Escape peaks- These peaks are caused by the fluorescence of X-rays in the detector. The effects of escape peaks is usually small.
Sum peaks- When two X-rays enter the detector nearly simultaneously in less than the resolving time, their energy will be summed. This occurs at high count rates (± 10000 CPS).

14.5 Standardless analysis.

The same basic approach can be applied without measuring standards. The standards are pure elements, but we can calculate the relative pure element intensities from theory easily with a small computer. So the measured pure element intensities can be replaced with calculated relative pure element intensities. Standardless analysis is as accurate as the ability to calculate pure intensities and as the assumption that the sum of all the concentrations is known.

14.6 Standards analysis.

Standards are materials which are used to relate the intensity of a peak in a spectrum to the concentration of that element in the specimen. They are materials in which the concentration of all the elements are accurately known.
Standards may be pure elements or compounds which contain a known concentration of the element. In order to make a direct comparison between intensity and concentration, a standard specimen is referred to.
Once this is known, this ratio can be used to determine the concentration of that element in an unknown specimen. All the intensities are assumed to have been corrected for background. These corrections account for differences in X-ray absorption, atomic number and the degree fluorescence in the specimen matrix.
The ratio between the apparent concentration and the concentration is a measure of the true concentration is a measure of the ZAF correction, which needs to be included in the calculation. The need for the correction is minimised if the composition of the standard is as similar as possible to the composition of the unknown specimen. Since these matrix corrections can be calculated with only a certain degree of accuracy, the choice of standard material is very important if the quantification is to be as accurate as possible.

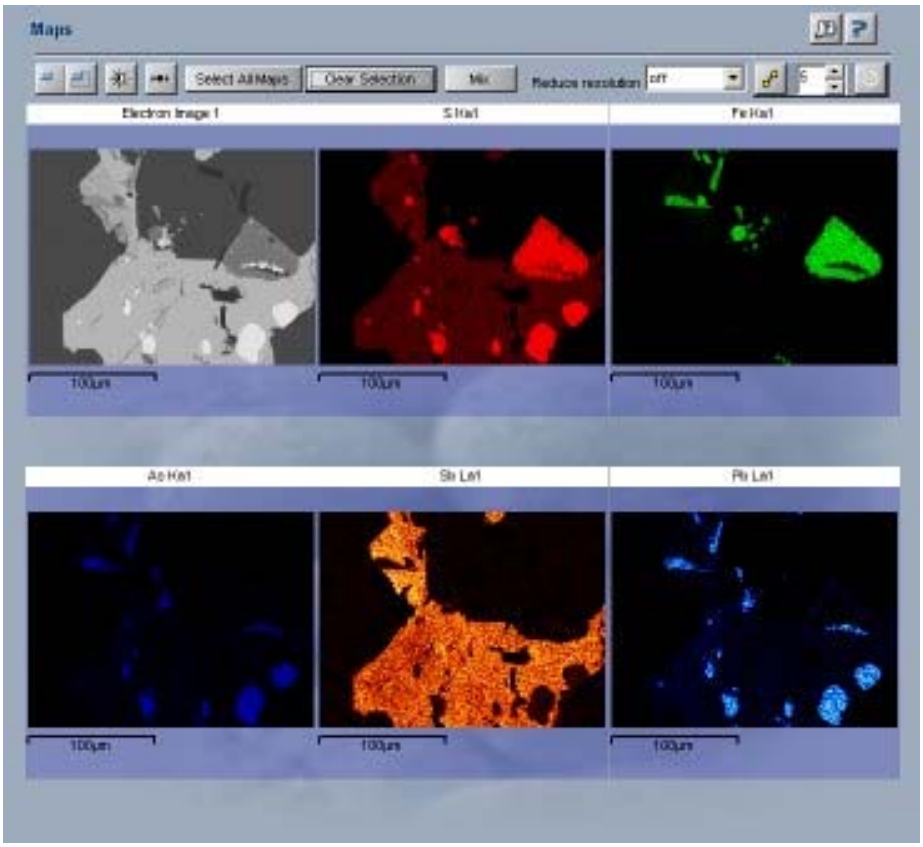
For all these methods the set up is done through a dialog box in the software. If a standard has been measured, ie, a spectrum has been obtained, then the concentrations are entered into the box and RZAF is used to calculate pure element intensities.

When standards are used, the SAME kV and beam current must be used for both the standard and the sample. This is also applicable when comparing different samples in a project.

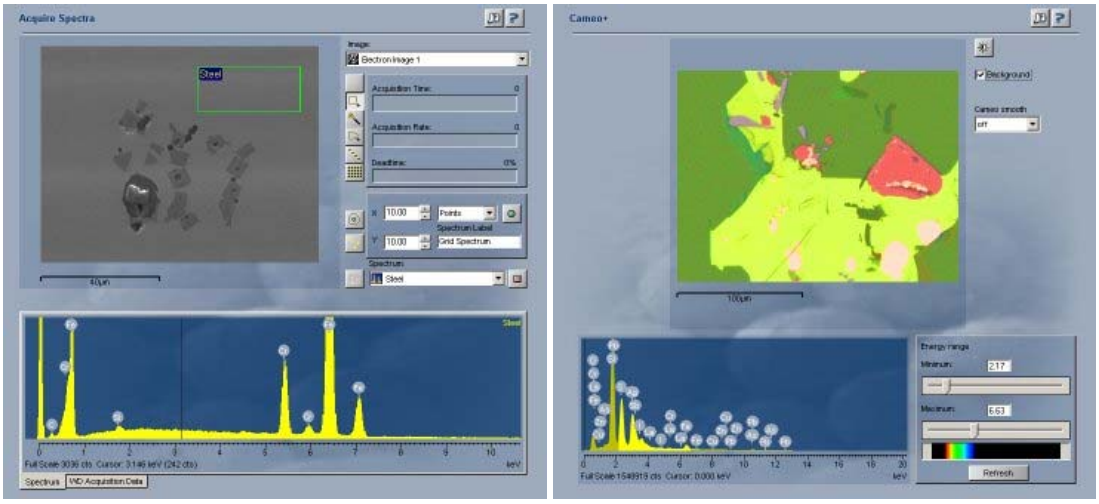
14.7 X-ray mapping

The distribution of elements over a particular area of the sample can be viewed by the acquisition of element specific maps. X-ray mapping utilises the X-ray signal from a specified energy range in the X-ray spectrum in order to show this elemental distribution. There are several modes of mapping, qualitative digital mapping and quantitative digital mapping.

Qualitative mapping gives information on the X-ray intensity distribution over the required field of view. The grey scale value for a given pixel in any digital map corresponds simply to the number of X-rays which enter the X-ray detector within the energy range and, therefore, gives an idea of the elements. Quantitative mapping involves full spectrum processing, including escape peak removal, background subtraction, followed by matrix corrections for each pixel within the image.



14.8 Point and I D **Mapping + Cameo +**



By using point and ID, multiple spot or area analysis can be done on one sample and presnent the results in an Excel format.

15. ACCELERATING VOLTAGE FOR ANALYSIS AND OVERVOLTAGE.

Two physical phenomena come into play when thinking of the choice of voltage. First, the incoming electron must be higher in energy than the critical excitation energy of the element to be excited. If not the atom will not be excited. As the energy of the electron exceeds KeV, more efficient excitation of the element occurs, and we say we have more “overvoltage”. As the “overvoltage” increases, the X-rays are produced deeper in the sample. At some point the

depth become so great that the absorption of the X-rays leaving the sample dominates over the increases in the number of X-rays produced.
The "overvoltage" is define as:

U= kV/KeV

It will be different for each element in the sample. The rule of thumb for the selection of accelerating voltage is that U should be >2, but <20 for general work. For quantitative work, U should be >2 but < 10.

16. SURFACE TOPOGRAPHY.

16.1. Effects of topography on matrix corrections.

If the surface of the specimen is irregular, it is clear that X-rays produced, within the X-ray production volume, will have to travel through different distances as they exit the specimen, thus suffering differing degrees of absorption. The dimensions of the roughness, in conjunction with the mean atomic number of the material and the accelerating voltage, will ultimately determine the errors in successfully correcting for matrix effects.

If the specimen is so rough, such that the line of sight to the detector is obstructed, then X-rays may be absorbed in the shadowing material and fail to reach the detector. Furthermore, remote excitation of X-rays by electrons scattered from the original beam position on a rough surface can occur and these remote X-rays may still reach the detector.
Even for a flat specimen, matrix corrections are affected if the surface is not normal to the incident beam. The volume from which X-rays are produced is closer to the surface of the specimen than for an untitled specimen.

When analysing particles, the position of the electron beam and detector relative to the curved surface of the particle may clearly have a profound effect on the degree of absorption. Furthermore, since electrons may escape from the sides and beneath the particle, the backscatter correction may be considerably different to that for a bulk specimen.

16.2 Topography.

Whilst a polished specimen makes it easy to calculate matrix corrections, it may not always be feasible to polish the specimen surface, and in doing so, the required detail, such as impurities exposed at facets of a fracture surface, may be destroyed. The consequence of analysing a specimen, with an undulating surface, is often an incorrect estimate of composition. In such cases, different approaches can be tried, which include normalising the results, applying analytical solutions for specific geometry’s, or using a peak to background method

16.3 Comparison of EDX and WDX.

Wavelength dispersive spectrometry and energy dispersive spectrometry is complementary techniques.

Historically, the WD spectrometer was the first type of elemental X-ray detector used on an electron microscope. As semiconductor technology improved in the 1960's and 70's, the faster ED spectrometer gained popularity making it today the first choice of a general purpose X-ray detector on electron optic columns.

However, despite its popularity, ED suffers from limitations, notably limit of detection and resolution, in this case its ability to separate small differences in energy. These weaknesses are compensated by the advantages of the WD spectrometer

17. SAMPLE PREPARATION FOR MICROANALYSIS (SEM).

17.1 Polishing and mounting techniques.

It is important that specimens for microanalysis are mounted correctly. This means that there must be a good electrical connection between the specimen end the microscope stage, to prevent charging. The type of mounting employed will depend on the specimen. It is also sometimes necessary that the sample is flat, if accurate information is to be obtained from the sample. Various polishing methods can be used to ensure a flat surface, depending on the type of sample.

Whatever sample mounting and polishing techniques are used, it is important that the information present in the sample is preserved, and not distorted.

17.1.1 Polishing.

Polishing techniques are varied, but similar in principle. The rough surface of the specimen is abraded through a series of polishing papers, starting with rough grit papers and progressively working through to finer papers. The polishing process will lubricate with water. Once the finest paper has been used, fine diamond polishing wheels may be used. These will be pads, with small amounts of diamond paste, with appropriate lubricants. The final finish will be to, usually, 1 or 0.5μm finish. The specimen must be thoroughly cleaned and dried after the final polish.

When polishing it is important to consider if the process will damage the sample. For example, will the water used to lubricate the wet and dry papers used in the first stages, damage the sample? Sometimes care must be taken when polishing specimens with small precipitates. Removing too much material at a time may dislodge the precipitates of interest. Different hardness materials will be polished at different rates, and will cause relief on the surface of the specimen. Similarly, specimens such as sections of coated components, e.g. tool bits, turbine blades, may need to be coated with another harder metal, to prevent the coating of interest from spalling away.

The mechanical action of polishing will mechanically strain the surface region, and if, for example, an imaging technique is being used, the relies on a contrast forming mechanism that takes place near the surface, then a more gentle polishing technique must be used. These will include final polishing with alumina rather than diamond or chemical and electro polishing. These latter two techniques will require additional equipment, but will result in a mechanically undamaged surface.

17.1.2 Mounting.

Specimens can be either mounted directly onto the sample stub, or are sometimes mounted in another medium, prior to, say, polishing. The specimens should also be cleaned, if possible,

to remove any hydrocarbon or other contaminants using mild solvents. If a low vacuum microscope is being used, these requirements are less stringent.

If the specimen is being mounted directly onto the stub, it needs to be fixed down, with either conductive tape or cement. Carbon, aluminium or copper double-sided conductive tape is widely available. Stick some tape to the stub, and then simply mount the specimen onto the tape. Sometimes it may also be necessary to coat the specimen with a conductive layer (carbon or gold/palladium). If the specimen is irregular, make sure that the cement is in contact with any conductive coating.

Metallographic specimens can also be mounted to make micro sections. A small amount of the material will be embedded in a resin mount. Conductive mounting resins are available. They are to be preferred if microanalysis is needed. Most mounting resins are hot press resins carried out under pressure using a mounting press. The resultant mount can then be polished and examined in the electron or optical microscope. If a non-conductive resin is used, a conductive path must be provided from the specimen to the microscope stage. This will usually be achieved with a thin evaporated or sputtered coating, or by a small trail of conductive paint, containing carbon or silver.

Geological type samples will be cut into thin slices, using diamond type saws, and then fixed onto glass slides using a strong adhesive. The mount will then be polished, and subsequently coated with a conductive layer.

Powder specimens can be sprinkled directly onto conductive tape, or almost dry paint. Take care to remove any excess unfixed particles in case they become loose inside the microscope chamber. Again a conductive coating may be necessary, depending on the materials involved.

Biological specimens need to be dried before they are fixed onto the stub. Alternatively, they can be examined using cryo-preparation techniques, or with a low vacuum microscope. These latter two techniques will preserve more information within the specimen.

18. COATING TECHNIQUES.

18.1 Coating.

In order to obtain a good image of most non-conductive specimens in the SEM the sample must first be covered with a thin coating. A coating serves a number of purposes including:

a) increased conductivity,
b) reduction of thermal damage,
c) increased secondary and backscattered electron emission, and
d) increased mechanical stability.

Conductivity is the single most important reason for coating a specimen. As the primary beam impinges on the specimen the increased electrical potential must be dissipated in some way. For a conductive specimen such as most metals this is not a problem and the charge is conducted through the specimen and eventually is grounded by contact with the specimen stage. On the other hand non-conductive specimens or “resistors” can not dissipate this excess negative charge and so localised build up charges cause a dielectric breakdown and gives rise to an artefact known as charging. Charging results in the deflection of the beam, deflection of some secondary electrons, periodic bursts of secondary electrons, and increased emission of

secondary electrons from crevices. All of these serve to degrade the image. In addition to coating the sample, the specimen should be mounted on the stub in such a way that a good electrical path is established. This is usually accomplished through the use of a conductive double-sided adhesive carbon tape or conductive adhesive silver or colloidal carbon paint.
A conductive coating can also be useful in dissipating the heating that can occur when the specimen is bombarded with electrons. By rapidly transferring the electrons of the beam away from the region being scanned, one avoids the build up of excessive heat.

Because secondary electrons are more readily produced by elements of a high atomic number than by those of a low atomic number a thin coating of specimen can result in a greatly improved image over what could be produced by the uncoated specimen. In cases where backscattered electrons or characteristic X- rays are of primary interest a coating of heavy metal such a gold or gold/palladium could obscure differences in atomic number that we might be trying to resolve. In this case a thin coating of a low atomic number element (eg. carbon) serves the purpose of increasing conductivity without sacrificing compositional information.
The fourth and final purpose of using conductive coatings is to increase mechanical stability. Although this is somewhat related to thermal protection, very delicate or beam sensitive specimens can benefit greatly from a thin layer of coating material that actually serves to hold the sample together. Fine particulates are a prime example of a case where a coating of carbon or heavy metal can add physical stability to the specimen.

Many of the negative effects of imaging an uncoated specimen can be reduced by using a lower energy primary beam (Kv) to scan the sample. Whereas this will tend to reduce such things as localised charge build up, thermal stress, and mechanical instability it has the distinct disadvantage of reducing overall signal. By carefully adjusting factors such as accelerating voltage and spot size, many of these same effects can be reduced but a fine coating of the specimen is still usually required.

Non-conductive specimens are coated with a conductive material prior to observation in the SEM, to prevent charge up, and sometimes to increase the emission of secondary electrons. Coatings are usually applied by using a vacuum evaporator, or a sputtering device. For microanalysis, carbon is the preferred coating material, which does not generally interfere with elements of interest in the specimen. Gold, usually used when a good secondary electron image is required, is not recommended for microanalysis, because of the large number of lines in the gold X-ray spectrum.

18.2 Sputtering.

Various types of sputtering machines are available. This technique is good for coating specimens that are not smooth, and coating will be obtained even where there is not a direct line of sight to the target. The target atoms suffer many collisions before they reach the specimen, and are travelling in all different directions. Complete coating can be achieved without rotating or tilting the specimen. Materials such as gold, platinum or gold/palladium are used for sputtering. These materials have a high secondary electron emission resulting in good imaging performance. Carbon cannot be deposited effectively with a sputter coater.

20. ORGANIC SAMPLE PREPARATION FOR ELECTRON MICROSCOPY.

Organic samples, such as biological materials and maybe foodstuffs, need special handling if they are to be observed in the electron microscope. These materials contain large amounts of water, and this will cause problems when the specimen is put under vacuum. The samples may be susceptible to beam damage.

The procedure of sample preparation depends on different factors, ea. the type and characteristics of the sample, the environment from which it comes the effect of chemicals on the sample and the purpose of the investigation.
There is a basic procedure which all biological material has to pass through: killing, fixing, dehydration, (for TEM embedding, sectioning and staining) and drying and coating for SEM. To keep damage to samples to a minimum, small sample holders are used. This also makes the handling of small samples easier.

20.1 Narcosis of samples.

To relax small organisms, the following can be used:

- In water, gradually heated till the organism is dead.
- Putting it in 10% methanol or ethanol, ether vapour, osmium tetroxide or osmium tetroxide vapour.

20.2. Fixation.

The fixation of organisms is to keep post-mortem changes or disintegration to a minimum and to have the condition as close as possible to that of the living organism.

The value of fixing is:

- To keep the condition of the organism as close as possible to the natural state.
- To prohibit structural damage.
- To preserve cytological elements.
- To harden the tissue.
- To enhance the penetration of the stain.
- To prohibit shrinkage during the preparation procedure.

It is important to fix the samples as quickly as possible. Fixing is done by $4^{o}C$ or at room temperature. If the samples float on or near the surface of the fixative, they must be put under vacuum to remove the air.

20.2.1. Primary fixatives.

This group includes glutaraldehyde (GA) and formaldehyde (FA).
GA is a di-aldehyde and the most effective aldehyde fixative for the preservation of ultrastructure and have the ability to cross-link proteins and retain lipids.
FA is a mono-aldehyde and is used in the fixation of compact tissue as its penetration ability is faster than that of GA.
Usually GA and FA are used in combination. Additives that can be used in low concentrations are picric acid and calcium chloride. Calcium chloride is used for the stabilisation of membranes.
Examples of fixative mixtures are Todd's, Bullock and Carnovski.

20.2.2 Secondary fixatives.

Osmium tetroxide is used as a secondary fixative to harden the tissue, as well as an electron dense stain. It stains tissue black and thus makes it more visible and easier to handle.
Osmium tetroxide vapour is used as fixative for fungi and bacteria and other samples where aquatic fixatives can not be used. Over-fixation with osmium tetroxide causes protein extraction.

20.2.3 Tertiary fixatives.

These fixatives react fast with proteins and stabilise structures before extraction takes place. ea. Uranyl acetate. It can also be used as a stain for thin sections in concentrations of 2-5%.

20.3 Buffers.

Buffers are used for dissolving fixatives and to carry the fixative through the tissue and for washing of samples between fixations.

To do this the buffer must have the correct:

- pH, usually between 7,2 - 7,6
- osmolarity to prohibit expansion or shrinkage.
- ion concentrations to prohibit extraction or precipitation of substances during fixation.

Examples of buffers are Phosphate -, Sodium cacodylate and Pipes.

When used in washing, it is usually done 3X for 10 - 15 min. depending on the size of the samples.

20.4 Dehydration.

Following chemical fixation the fixative must be removed from the tissue by way of rinsing. This is usually done be removing the fixative solution and replacing it with a pure buffer of the same concentration and pH. Two to three changes of buffer over a period of 10-20 min. is usually sufficient for removing most of the fixative. Additionally it is useful to rinse out the buffer with distilled water. This helps to eliminate the possibility that residual salts might affect polymerisation. The water is removed by a bringing the sample through a graded series of either ethanol or acetone. Since the shrinkage problems that often accompany dehydration are more pronounced with sudden changes in solvent concentration it is preferable to have a number of short exposures to gradually increasing concentrations of solvent.

Generally one of two procedures are followed prior to infiltration and embeddment with epoxy resins. The first involves bringing the sample through a graded acetone series from 70-100% up to 100% followed by another change in 100% acetone. There is some evidence that acetone causes less specimen shrinkage and lipid extraction than does ethanol. It is also non-reactive with osmium tetroxide and will not interfere with epoxy resin polymerisation. Phospholipids are particularly immune to extraction by acetone. Acetone is completely miscible with most epoxy resins and is not know to radically alter protein antigenicity.

The most significant problems caused by dehydration are those of shrinkage and extraction of cell constituents. For these reasons dehydration times should be kept to a minimum. As with fixation this process is aided by having small pieces of material in which the time it takes for

diffusion to occur is reduced. In some cases the dehydration and infiltration schedule can be shortened to a few hours however these procedures should not be applied in standard fixation protocols. Some resins can tolerate a small amount of water and one can thus begin the infiltration process before the dehydration is complete.
Related to dehydration is the problem of infiltration. Like dehydration infiltration involves the replacement of the solvent with the unpolymerised resin. Because this is a diffusion dependent event several factors can influence both dehydration and infiltration.
One of course is size of the sample. Another is the use of a slow rotator to keep the sample moving and always coming in contact with fresh fluid. Placing the sample under vacuum can also aid in this process.

20.5 Critical-point drying (CPD) for SEM.

After dehydration with 100% acetone or ethanol, the sample holders with samples are placed in the CPD-chamber filled with the same fluid as used for dehydration. The lid is screwed on and the dehydration fluid is replaced with the transitional fluid, liquid carbon dioxide. After repeating this (4-5X), the holder is heated with water to 45^{o}C for the liquid carbon dioxide to sublimate to carbon dioxide gas. The CO_2 gas is slowly released till the CPD-chamber reaches atmospheric pressure. The samples are then removed for mounting on SEM-stubs.

20.6 Embedding for TEM.

After the 100% dehydration step, the samples are transferred to a 1:1 mixture of resin and 100% acetone or ethanol, depending on which one was used during dehydration, and left for 2 - 4 hours. The samples are then transferred to 100% resin, left till they have sunk to the bottom of the vials, and left for a further 2-3 hours (this is done twice). This is to ensure that - the exchange of resin and the acetone is complete. During sample preparation the vials must be rotated continuously.

20.7 Sectioning for TEM.

The embedded sample blocks are trimmed in the laboratory and not on the microtome. First, semi-thin sections of ±0,5 µm are made on the microtome, placed on a drop of distilled water on a microscope slide, dried on a hotplate, stained with 0,5% toluidine blue in 1% boracs over moderate heat, washed, dried and viewed under a light microscope. This is done to determine the quality of the fixation and the orientation of the sample.
The sample block is the trimmed further with a scalpel under a stereo microscope. A new knife is placed in the microtome and ultra thin sections of ±100 nm are made. The sections are stretched with xilol vapour, oriented with an eyebrow-hair stick, picked up with a 200--mesh grid and dried on moist filter paper to remove the water.

20.8 Staining with heavy metals for TEM.

In order to visualise a specimen in the TEM one must have contrasting regions of electron transparency and electron opacity. Just as in light microscopy differences in contrast can be accentuated through the use of a stain. To be of use in a TEM a stain must have the ability to stop or strongly deflect the electrons of the electron beam so that they do not contribute to the final image. The most commonly used stains in electron microscopy are made up of heavy metal salts. These have atoms of high atomic weight which are especially good at deflecting electrons.

Electron staining falls into one of two categories:
1) positive staining in which contrast is imparted to the specimen itself and
2) negative staining in which the area surrounding the specimen is given increased electron opacity while the specimen itself remains more translucent. We will discuss positive staining here.

20.8.1 Positive Staining:

We have already discussed one type of positive stain, that being Osmium tetroxide. When OsO4 reacts with biomolecules in the specimen the Osmium atom serves as a bridge between the reacted sites. With an atomic weight of 190 it is of sufficient size to effectively deflect electrons. Because it reacts more readily with lipids than it does with proteins osmium tetroxide has the added of advantage of being somewhat structure specific positive stain.
The two most commonly used post-fixation positive stains are uranyl acetate (MW = 422) and lead citrate (MW = 1054) the two heavy metals being uranium and lead respectively. Both UA and lead citrate are heavy metal salt stains and are both categorised as general or non-specific stains. Because they are heavy metal salts they are quite toxic and should be handled and disposed of with great care. UA ions are believed to react with phosphate and amino groups (found in nucleic acids and certain proteins) while lead ions are thought to bind to negatively charged molecules such as hydroxyl groups. Because of this ability to stain different cellular components UA and lead citrate are often used in conjunction with one another though not simultaneously for reasons we will see in a few minutes.
Positive stains may be applied either prior to embedding or after sectioning. When applied to the specimen before dehydration this type of staining is referred to as en bloc staining meaning “on the block.” Because it is prone to forming image degrading precipitates lead citrate is not used as an en bloc stain. UA on the other hand is a very useful en bloc stain and is believed by some to actually act as a fixative in its ability to retain structural detail. When used as an en bloc stain UA is applied to the specimen as a 0.5% - 4.0% aqueous solution after the initial fixatives (glut and osmium) have been thoroughly rinsed from the specimen. After several hours in the stain the specimen is dehydrated and infiltrated as normally done. The dehydration step should not be long as UA is soluble in solvents and extended storage in a dehydrating agent will remove most of the UA. En bloc staining can greatly improve the contrast of membranous structures such as mitochondria, golgi, ER, as well as DNA and other fine filaments.

20.8.2 Post-embedding Staining:

Sections that have been picked up and dried can be stained on their grids. Usually this is done by floating the grids on a drop of 1%-4% UA for 15-30 minutes. The grids are then thoroughly rinsed, dried, and either stained with lead citrate or stored until they are examined in the TEM. Although grids can theoretically be stained any time after sectioning it is best to do so within 24 hours of having cut the sections. Grids that have been exposed to the energy of the electron beam will not absorb stain. Some resins are particularly difficult to penetrate and therefore do not stain well. In these cases one can try to either elevate the temperature of the stain or by staining in a methanolic UA solution. UA can be dissolved in 100% methanol and the grids placed into it. All of the steps are the same as for aqueous UA staining with the exception that the grids must be rehydrated through a graded methanol series before being

rinsed in dH_2O and finally dried. If this is not done the sections will wrinkle badly due to the temporary disassociation of the sections from the support film.
Lead citrate is often used to stain grids after they have been stained in UA. Because Lead citrate is very sensitive to CO_2 (it quickly reacts to form a precipitate that can ruin a section) every effort must be made to eliminate this gas from the staining procedure. For this reason very clean glassware, CO_2-free water, and other precautions must be followed in preparing lead citrate for use. Sections are stained by floating the grids on drops of lead citrate for 3-5 minutes at room temperature. After staining the sections are rinsed in a 1M NaOH solution (to wash off the lead citrate) and then thoroughly rinsed in dH_2O (to rinse off the NaOH). Grids are then blotted dry and stored until needed.

20.9 Negative staining for TEM.

In order to visualise a specimen in the TEM it is necessary to create what is known as image contrast. By this we mean that there must exist regions of varying electron opacity such that differences can be detected and therefore information about the structure of the specimen can be discerned. This is accomplished by the differential scattering, deflecting, and stopping of illuminating electrons. For an object to have a level of electron opacity there must be enough nuclear mass present to accomplish this deflection of electrons. A thick biological specimen meets this requirement by having a large number of relatively low atomic weight atoms aligned relative to the incident electron beam.

A second way of accomplishing this is to have a relatively thin layer of high atomic weight elements aligned relative to the incident beam. This same principle is utilised in the positive staining of biological specimens to selectively give electron contrast to different portions of the specimen. The primary distinction between positive and negative staining is that in positive staining the stain forms a complex with the specimen whereas in negative staining the stain and the specimen do not react with one another. Also as the name implies a positive stain will impart increased electron opacity to the specimen creating a darker specimen whereas in negative staining the specimen remains more electron translucent relative to the surrounding stain. By pooling up around the edges and crevices of the specimen and not as much on the top portions of the specimen an image of differential contrast of the specimen can be made. One of the limitations of negative staining is that only information about the microtopography of the specimen is produced. Little or nothing is learned about the internal structure.

A drop of the sample, in suspension, is placed on a Formvar coated grid, a drop of 2% phosphotungsten acid or uranyl acetate is added, the grid is dried on filter paper, left to air dry and examined under the TEM.

21. REFERENCES.

1. Oxford Instruments Plc, The Oxford guide to X-ray Microanalysis, CD-ROM Tutorial, (England, 1997).
2. Philips Electron Optics, All you wanted to know about electron microscopy, but didn't know what to ask., 1-17, Netherlands.
3. Philips Electron Optics, Environmental Scanning Electron Microscopy., 2nd Ed, (Robert Johnson Associates, California, 1996).
4. Microanalysis and Scanning Electron Microscopy, Maurice, F., Menly, L. & Tixtier, R., (1978), Summer School, St-Martin-d'Hères, France.